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Research Article

Association of the Gene *FTO* Single Nucleotide Polymorphism, rs9939609 with Type 2 Diabetes Mellitus in Pakistani Cohort

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Abstract: To date, inconclusive data is available about the insight of the *FTO* gene variant with type 2 diabetes mellitus. T2DM is a chronic disease and a rising problem worldwide. Its complications lead to an increase in the burden of mortality specifically in lower and medium-income countries. Genome-wide association studies have spotted many genetic loci that are related to T2DM and validate the complicated polygenic traits. Many variants of different genes including *FTO* are associated with T2DM hence, this study was designed to inspect and unfold obscure data in South Asians. The main objective of present study is to identify the relation of *FTO* intronic variant rs9939609 with T2DM in Karachi-based Sindhi population of Pakistan. Total recruited individuals were grouped as diabetic cases and controls. Out of the total recruited subjects, genotyping was done on 152 samples using T-ARMS PCR however, demographic and clinical data were recorded of all individuals. The results showed that the frequency of variant genotypes in the diabetic case group was 11 % for AA, 45 % for AT and 44 % for TT though, the frequency of the lethal allele (T) was 34 %. These outcomes concluded, rare T allele frequency is higher among diabetic cases as compared to controls and provides the contribution from the Pakistani population to support the previous controversial findings. This study concluded *FTO* gene-single nucleotide polymorphism, rs9939609 is associated with T2DM but still, it is a growing need to do further studies on T2DM susceptible genes with different polymorphisms to recognize targets in the field of pharmacogenomics for clinical implementation.

Keywords: Genome-Wide Association Studies, Type 2 Diabetes Mellitus, Single Nucleotide Polymorphism, Fat mass and obesity-associated gene

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is escalating enormously and is indicated by beta cell dysfunction and insulin resistance. Its complications proceed to enhance the burden of mortality globally according to World Health Organization [1]. A decade ago, it was estimated 3.96 million deaths in adults due to diabetes mellitus however, the number raised to million deaths later in five years which is almost equal to a single death in every second. In adults till 2030, a 20 percent rise in diabetes mellitus will occur in developed countries but an alarming 69 percent upsurge of diabetes mellitus is predicted in developing countries [2]. In accord with International Diabetes Federation (IDF) Diabetes Atlas, Asia is one of the major and highly prevalent area of T2DM epidemic and is propagating rapidly in low- as well as in middle-income countries [3]. After India and China, USA was listed as the third highest country of patients with T2DM thereby, 25 cents of health expenditure are utilized in its treatment. This global estimation focuses on the severity of the T2DM pandemic [4]. Moreover, T2DM also increases with the rise in obesity specifically in childhood however, complications initiate in adulthood [5].

The gene, Fat mass and obesity associated (*FTO*) is most commonly coupled alongside obesity, an excessive fat mass suffice to elevate the possibilities as well as hazards of mortality and morbidity [6]. It is recognized as the key vulnerable factor for various non-communicable illnesses including coronary

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heart disease, cardiovascular disease, hypertension and the T2DM. In addition, it predisposes to varied mental complications or physical infirmities. Forty-four percent of diabetic cases are accountable for the condition of overweight and obesity [7]. Among other non-communicable ailments, T2DM is most convincingly related to obesity. The occurrence of obesity-associated diabetes is likely to increase to three hundred million by the year 2025 [8]. Body mass index is a measurement for obesity and defines the anthropometric features of height and weight, though, not a sensitive metric to identify early fat deposits in adults and childhood [9].

Genome-wide association studies (GWAS) reported several genetic loci linked along obesity and T2DM that validate the complicated polygenic traits. Many variants of various genes are associated with T2DM and obesity. The gene *FTO* has been studied in numerous European and South Asian populations however, the results of studies are still unclear [10].

However, one recent study has been conducted among the people of Bangladesh and showed a positive association of *FTO* with T2DM [11]. On the other hand, another recent study on the Indian population showed no direct association of *FTO* with T2DM [12]. Moreover, no similar recent study has been done in Pakistan and to date, no previous data is available on Pakistani Sindhi origins of people. Hence, the present study was intended to further inspect and unfold obscure data of *FTO* in our targeted population which has not been studied before. The aim and objective of this analysis were to identify the involvement of *FTO* intronic variant rs9939609 with T2DM in the Karachi-based Sindhi population of Pakistan.

2. MATERIALS AND METHODS

2.1 Subject Recruitment

A total of 1666 samples representing adults from the largest metropolitan city, Karachi of Pakistan were analyzed. All recruited subjects belong to the Sindhi cohort of the Pakistani population, sum of 1504 people were enlisted in this study after gaining individual consent and ethical clearance from GC University, Pakistan and the remaining 162 samples were taken from a repository of the Department of Biological and Biomedical Sciences (DBBS), Aga Khan University. All the subjects were grouped into type 2 diabetic cases and diabetic controls. 152 samples from the recruited individuals were selected for variant genetic analysis.

2.2 Sample Collection

Blood sampling was done from the antecubital vein as per standard protocol. Participants' recruiting proforma was filled as per inclusion and exclusion criteria [13].

2.3 Physical and Biochemical Measurements

The quantitative anthropometric non-invasive measurements for the BMI of each recruited member were taken. The waist circumference (WC) was measured as per standard protocol with the tape. The tape was placed halfway in between the last bottom rib and the upper top of the hipbone. Later, the hip circumference (HC) was also measured with a measuring band positioned at the broadest area of the buttocks. In WC and HC measurements, the tape snugged around and was not constricting as per WHO steps. Another parameter, the waist-to-hip ratio (WHR) was computed by the formula as; WHR is waist circumference/hip circumference in centimeters [14, 15]. Systolic and diastolic blood pressure measurements were done by using a mercury sphygmomanometer and a stethoscope [16]. Biochemical profiling including fasting plasma sugar (FPS), total cholesterol (TC), as well as low/high density lipoprotein levels (HDL-C/ LDL-C), was done through commercial kits, Abcam USA [13].

2.4 DNA Extraction and Genotyping

Genomic DNA extraction of each sample was done as per the Promega kit protocol. A DNA purity check of each sample was done through Nanodrop-ND1000 by a company Thermo Fisher Scientific. All participants were genotyped for the *FTO* variant, rs9939609 by an economical inhouse polymerase chain reaction (PCR) technique, the tetraprimer - amplification refractory mutation system, T-ARMS PCR. The allele-specific fragments were generated by using specific primers as shown in Table 1. The primers were designed through the biocomputational tool, Primer3Plus.

This tool is freely available on the internet and can search the weblink by an online search engine.

PCR was performed in an absolute volume of 10 ul. The one reaction mixture contained 150 ng genomic DNA. Moreover, 1XPCR buffer, the 3.0 mM $MgCl_2$ besides, 1.0 mM dNTPs were added. In addition, outer forward or reverse (OF / OR) and inner forward or reverse allele-specific primers (IF / IR) were added with the concentration of 10 picomoles each. The enzyme, Taq polymerase was added with total units of 1.5 in each reaction (Promega, USA).

PCR was carried out by thermal cycler, Mastercycler Eppendorf program as per optimized conditions. Table 2 shows the allele (T/A) specific products generated by PCR reaction. Formerly, these products or amplicons were processed on one percent horizontal agarose gel electrophoresis and finally, spotted on the gel documentation system (Bio-Rad) under the ultraviolet light.

2.5 Statistical Analysis

All data was statistically investigated by using Statistical Package for Social Sciences, SPSS version IBM 20. Hardy Weinberg equilibrium (HWE) test was used to attain the deviation of population. Basic demographic and biochemical characteristics data were analyzed by mean ± standard deviation. In addition, association of SNPs with T2DM and obesity was statistically analyzed via logistic regression analysis. The association was also calculated after adjustment of BMI and obesity related trait, waist circumference. Linear regression was also used to test the factors effect size.

3. RESULTS AND DISCUSSION

3.1 Demographic and Biochemical Variables

Characteristics of study population, anthropometric and biochemical data were recorded and are showing in the table 3. All the controls were matched to T2DM cases on the basis of age and gender.

3.2 HWE, Genotype and Allele Frequencies of *FTO* Variant rs9939609

The genetic analyses were carried out by taking frequencies of all *FTO* variant genotypes and alleles in control subjects as well as in T2DM cases. All the individuals were tested and the data is provided in Table 4.

The genotype frequencies in control category individuals were 9 % for AA, 42 % for AT and 50 % for TT however, the frequency of lethal allele (T) in control category individuals was 29 %. HWE test was done only in controls to confirm the homogenous standardized population. Results indicate observed and expected value (χ^2 = 0.79; p-value = 0.07) of controls which is non-significant, specified control subjects are descending in consonance with HWE.

The occurrence of genotypes in the diabetic case

Table 1. Specific primer sequences for *FTO* variant, rs9939609

Primers abbreviations	Sequences 5'-3'	Len (bp)
OF	CAGTTCCAGTCATTTTTGACAGC	23
OR	TGTTCAAGTCACACTCAGCCTCT	23
IF	TCCTTGCGACTGCTGTGAATATA	23
IR	ACAGAGACTATCCAAGTGCATCTCA	25

Len (bp) = Length of sequence in base pairs outer forward or reverse (OF / OR) and inner forward or reverse allele-specific primers (IF / IR)

Table 2. Primer specific PCR products

Primers	Amplicon type	Amplicon size (bp)	Genotypes
OF and OR	non-specific	446bp	TA
OF and IR	for allele A	212bp	AA
IF and OR	for allele T	148bp	TT

Table 3. Demographic and biochemical characteristics of study participants

Variables	Diabetic Control subjects mean (SD, if specified)	T2DM case subjects mean (SD, if specified)
Total count	1281	385
Age in years	51.1 (10.7)	53.5 (10.7)
Percent male	46.3	40.0
Systolic blood pressure (mmHg)	135.6 (22.8)	143.4 (24.2)
Diastolic blood pressure (mmHg)	85.9 (12.8)	88.3 (12.6)
FPS (mmol/l)	5.3 (0.6)	10.6 (4.0)
Weight in Kgs	63.4 (13.8)	66.4 (15.0)
Height in cm	1.6 (0.1)	1.6 (0.1)
BMI Kg/m2	25.2 (5.2)	26.7 (5.6)
Waist-circumference in cm	88.1 (12.0)	93.2 (11.7)
T.C in mmol/L	4.8 (1.0)	5.0 (1.2)
T.G in mmol/L	3.0 (0.80)	3.1 (0.9)
LDL-C in mmol/L	3.0 (0.80)	3.1 (0.9)
HDL cholesterol in mmol/L	1.1 (0.3)	1.0 (0.3)

Values are presented as means (SD), where specified Total Cholesterol, T.C; Fasting plasma sugar, FPS; Triglycerides, T.G

Table 4. *FTO* SNP rs9939609 genotype and allele frequencies

Genotypes	Controls	Cases
AA	109(0.09)	43(0.11)
AT	536(0.42)	173(0.45)
TT	636(0.50)	169(0.44)
Total	1281	385
Minor allele frequency (A)	0.29	0.34
HWE (Chi-square, $\chi 2$)	0.07(degree of freedom df=1)	-
p-value	0.79	-

set individuals was 11 % for AA, 45 % for AT and 44 % for TT nevertheless, the frequency of lethal allele was 34 %.

Type 2 diabetes susceptible gene, *FTO* SNP rs9939609 genotyping of recruited subjects was done by T-ARMS PCR. Figure 1 is an electrophoretogram showing lanes from 1 to 12. The gel was prepared with agarose with a percentage of one. Subsequently, the prepared gel was assembled on gel electrophoresis apparatus (Sigma). The comb used in assembling the gel comprised of 12 wells. Fragments sizes are displayed on the right of Figure 1. The non-specific DNA fragment is of 446 base pairs, the wild type A-allele specific fragment is of 212 base pairs and the rare T-allele fragment is of 148 base pairs. All were visualized under the ultraviolet light through the Bio-rad gel documentation system.

3.3 Association of *FTO* rs9939609 with T2DM in Total Study Subjects

The results were evaluated by logistic regression analysis and showed the relationship of *FTO* genotype with T2DM, each copy of the A-allele expands the hazard of diabetes trait with an odds ratio, 1.21 with limit of 95 % confidence interval. These associations remained very similar although insignificant p-value once adjusting models for BMI (odds ratios of 1.17 with limit of 95 % confidence interval) or waist-circumference (odds ratios

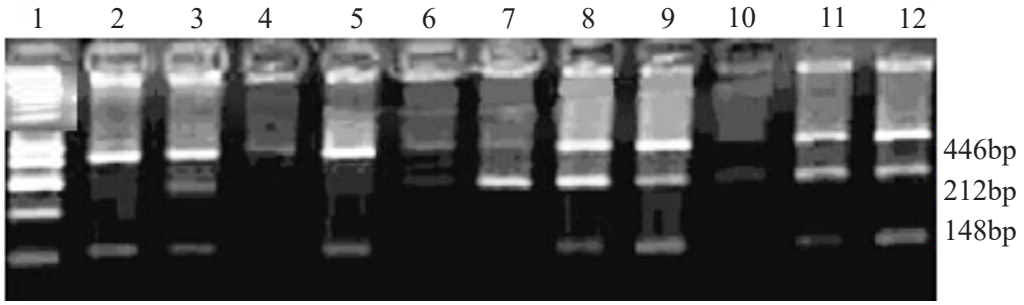

Fig.1. PCR results of *FTO* variant rs9939609 on type 2 diabetic cases in Pakistani population. The figure is an electrophoretogram showing lanes from 1 to 12. The gel was prepared by agarose with the percentage of one. Subsequently, the prepared gel was assembled on gel electrophoresis apparatus (Sigma). The comb used in assembling the gel comprised of 12 wells. Fragments sizes are displaying on the right of figure which was compared with the ladder fragments in lane 1. The non-specific DNA fragment is of 446 base pairs, the wild type A-allele specific fragment is of 212 base pairs and rare T-allele fragment is of 148 base pairs. All were visualized under the ultraviolet light through Bio-rad gel documentation system

of 1.15 with limit of 95 % confidence interval). In the study, results also indicated a connection between *FTO* genotype and FPS within individuals in the cohort. This also remained alike, even next adjustments for BMI or WC, though statistically insignificant (Table 5).

3.4 Association of *FTO* rs9939609 with BMI and Obesity in Total Study Subjects

In this study, a significant increase in BMI was observed with growing numbers of risk-allele in the study, 0.52 kg/m^2 (95 % CI; p value, 0.006). In addition, a similar outline was detected for waist-circumference, with a minor single allele effect of 1.20 cm (95 % CI; p value, 0.007). Moreover, when BMI was divided into two contrasting traits of abnormal weight (overweight or obesity) and normal weight as per WHO Asian criteria, the data indicated insignificant results in effect of normal BMI and with another measure of obesity, waist circumference (WC) however, abnormal BMI in the study showed a per allele effect with an odds ratio of 1.21 (95 % CI; p value, 0.02) as shown in Table 6.

The previous studies analyzed the involvement of rs9939609 variant with the disease T2DM in

Table 5. Association of *FTO* variant rs9939609 in accumulation with factors (age, gender, BMI, WC) with T2DM in total study subjects by linear regression analysis

Phenotypic variables	Models	beta Co-efficient	P-value
Diabetes* (odds ratio)	I (Age, gender)	1.21 (1.02–1.45)	0.03
	II (Age, gender, BMI)	1.17 (0.97–1.41)	0.09
	III (Age, gender, WC)	1.15 (0.94–1.41)	0.17
Fasting glucose (mmol/l) (β-coefficient)	I(Age and gender)	0.24 (0.02–0.46)	0.03
	II (Age, gender, BMI)	0.21 (−0.01 to 0.43)	0.06
	III (Age, gender, WC)	0.19 (−0.03 to 0.41)	0.09

Table 6. Odds ratio of *FTO* variant rs9939609 with adiposity in study subject by logistic regression analysis

Phenotypic Variables	Risk allele effect size 95%CI	P value
Normal BMI (kg/m2)	0.52 (0.15–0.89)	0.006
Waist circumference (cm)	1.20 (0.33–2.07)	0.007
Overweight/obesity	1.21 (1.03–1.41)	0.02

Asian populations. These studies have revealed results that are not consistent and still debatable [17, 18]. This study report is the contribution from Pakistan to explore the association of the *FTO* common variant with T2DM. The results of this study generate a main validation of *FTO* variant rs9939609, located on the chromosome 16. The wet-lab genetic analysis of *FTO* was done and the data showed promising results that *FTO* gene variant is strongly connected with type- 2 diabetes in our selected Sindhi cohort. Through statistical analysis, it was inferred that *FTO* variant in selected cohort from Karachi population is strongly associated with common disease T2DM. The results of this study are in parallel and similar to few other Asian and European studies [18-21].

T2DM is a chronic metabolic syndrome that has an impact on millions of individuals worldwide and is considered as a major public health concern in our society. There are several risk factors including the modifiable and non-modifiable, yet both are associated with diabetes. All the genetic factors that account for T2DM are non-modifiable however positive lifestyle incorporation such as exercise and controlled healthy diet can lead a way to either prevent or delay T2DM [22].

Another study showed this *FTO* rs9939609 risk allele is less recurring in East Asian individuals, 12.6 % [23] in comparison to the European individuals and West African individuals risk allele frequency, 45 % and 52 % respectively [24, 25]. Moreover, few studies showed significant association between *FTO* rs9939609 variant and T2DM [26] in contrary, other studies did not find any relationship between these two [27].

Another study has been conducted in North Indians to find out the common *FTO* variants association with T2DM but showed inconsistent results. Hence, *FTO* rs9939609 variant out of eight variants showed no association with T2DM [28]. The recent similar study was conducted among the Bangladesh people in the year 2023, their results revealed significant association with T2DM [11].

Another study in Vietnamese population was done in the year 2022 and reported that *FTO* variant is a predictor for future T2DM [29]. The present study is coinciding with these previous findings and could be used as a basis for future association research involving larger populations. In the present study, *FTO* polymorphism showed higher minor allele frequency in cases than controls. Moreover, the major power and strengths of this *FTO* polymorphism study is, the selected cohort was founded on similar population deprived of any genetic admixture. In addition, the diabetic cases were identified created on fasting glucose test. The *FTO* gene is popular for obesity and performs a polygenic effect as reported by genome wide association studies (GWAS). The selected gene variant has shown significant association of *FTO* risk-allele with BMI, a risk marker for obesity [30].

The limitations of the study are additional statistical tests have to be done by adjustments and making various models to evaluate the potential confounding variables including obesity-related traits as well as clinical patterns. Besides, more supportive studies in the Pakistani population are required with an increased sample size.

4. CONCLUSION

The current study uncovered significant associations of *FTO* gene SNP, rs9939609 and T2DM in the Karachi-based Sindhi population. Our results advocate that T2DM risk is especially raised in those with the risk A-allele of *FTO* variant rs9939609. The outcomes recommended that specific variants of the *FTO* gene could be used as a source to classify individuals who are more prone to the development of T2DM certainly in Asian individuals. In addition, this study also declares the *FTO* variant association with BMI and WC. However, further analysis with a larger sample size and confounding variables data, for instance, physical activity and diet intake, is needed to clarify the role of this variant as well as additional variants of the *FTO* gene on the predisposition to T2DM in Pakistani cohorts. It is a rising need to do more studies on T2DM susceptible genes with different polymorphisms to improve insight into the role of genes in predisposition to T2DM susceptibility. In future, this will generate a great target for drug discovery and development besides, the field of pharmacogenomics for clinical implementation.

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6. CONFLICT OF INTEREST

The authors declared that they have no competing interests.

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Research Article

Feeding Effect of Organic and Inorganic Zinc with Vitamin E on Growth Performance and Carcass Quality of Japanese Quail

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Abstract: The research facility of Avian Research & Training (ART) Center (Poultry Production), UVAS, Lahore was selected to investigate the effect of feeding organic and inorganic zinc in addition to vit-E on the growth performance of Japanese quail. for a period of 6 months. The diet formulated by the inclusion of Zn*O* + Zn*I* (mg)+VE(IU) as 0+0+0 (A), 25+0+0 (B), 0+15+0 (C), 0+0+12 (D), 25+15+0 (E), 25+0+12 (F), 0+15+12 (G) and 25+15+12 (H), respectively. The effect of zinc and vit-E on growth and slaughter traits of Japanese quail was significant ($P<0.05$) and it was perceived that in 6 weeks the broiler quails in groups H and F took higher feed (745.44 and 734.70 g/bird), with simultaneous effect on LBW of males (175.35 and 174.50 g), females (198.35 and 197.38 g), average (186.85 and 185.94 g), intestine filled (6.81 and 6.66 g) and empty weights (5.51 and 4.88 g), intestine length (44.87 and 44.75 g), cecal length (16.37 and 16.12 cm), the weight of carcass + giblets (142.01 and 141.48 g), carcass (130.88 and 130.25 g), heart (2.18 and 2.16 g), liver (5.02 and 5.06 g), gizzard (5.62 and 5.21 g), respectively. The dietary Zn + vit-E inclusion significantly ($P<0.05$) increased the relative length of gut segments, villi height, villus thickness, the villi height to crypt depth proportion in jejunum and ileum and the number of goblet cells in various parts of the intestine of quails. The meat production was significantly higher in female quails than the meat of male quails. This suggested that the organic Zn proved to be better than the inorganic Zn source in addition to vit-E to achieve physiology-related traits in quails.

Keywords: Japanese quail, organic/inorganic zinc, vit-E, growth performance

1. INTRODUCTION

Rural poultry farming has the potential to bridge the gap between animal protein supply and requirement of fast increasing human population. Japanese quail are not only fancy birds, but they produce high-quality meat that is most delicious in the avian species; it can produce up to four generations in a year, and rapid production of delicious meat-producing birds makes it the most effective and suitable poultry bird.

Japanese quail (*Coturnix coturnix japonica*) is a small game bird commercially raised for meat production [1]. The female quails' complete maximum egg production at the age of 50 days, whereas the males need around 6 weeks to reach sexual maturity. Quails are the most productive and appropriate poultry birds since they may generate up to four generations each year of excellent meat-producing birds. The adult female quail weighs between 120 and 160 g in live weight, and the mature male weighs between 110 and 140 g. The typical egg is 10 g. Quail laid eggs in the twilight, and they may take 17 to 18 days to hatch [2]. The Japanese quail is a member of the Phasianidae family and is a well-known migrant bird, moving often between Asia and Europe [3].

Quail has been gaining popularity as a meat bird due to its nutritional value, taste, and

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texture. Here are some citations that highlight the importance of quail as a meat bird. Quail meat is an excellent source of protein and contains essential amino acids, vitamins, and minerals. It has low-fat content, making it a healthy choice for meat consumption [4]. Quail meat has a high content of polyunsaturated fatty acids (PUFAs) and a low level of saturated fatty acids (SFAs), making it a healthier meat option compared to other poultry. Quail meat is also known for its tender and juicy texture, making it a popular choice among chefs and food enthusiasts [5]. Quail meat had a higher water-holding capacity compared to chicken meat, contributing to its unique texture. Quail is also a sustainable meat option, as it requires less space, feed, and time to raise compared to other livestock animals [6]. Quail farming has a low environmental impact and can provide a reliable source of protein for households in developing countries. Finally, quail meat is used in traditional medicine for its potential health benefits [7].

The poultry industry in Pakistan has overlooked quail farming, even though it has enormous potential, owing to a lack of study on the nutritional requirements of quails and general management in the local environment. This is true even though it can dramatically boost the economy of the country [8]. In addition, the use of antioxidants has risen due to the incorporation of polyunsaturated fat-rich components into the formulation of animal feeds which presents an additional advantage. Feeds that are rich in polyunsaturated fatty acids are extremely susceptible to lipid peroxidation [9]. It has been established that antioxidants perform a broad variety of biological roles, including several that are relevant to both animals and humans [10]. Oxidative stress and a subsequent decline in performance by a variety of factors, including those related to their nutrition, pathology, their physiology, and their environment [11]. As a consequence of this, it has been discovered that poultry birds can experience a reduction in the detrimental effects of oxidative stress when they consume antioxidants, and their growth is improved. In addition to the vitamins and minerals that may be found in food, the biochemical processes in the body require a diverse array of other nutrients. It has been discovered that the addition of antioxidants to animal-based foods improves oxidative stability, tenderness, storage properties, and colour [12].

Antioxidants like zinc and vitamin E are given to animal feed to enhance performance, promote immunity, improve the quality of meat and eggs, and increase the amount of vitamin E present in animal-derived foods so that humans may consume more of it [13]. Supplementing with zinc and vitamin E has antioxidant effects that may lessen certain physiological symptoms and boost thermotolerance [14]. Oxidative stress may occur in chicken production for a number of reasons, most often high temperatures, health issues, and poor feed quality [15]. Increasing the antioxidant capacity of the body can be ameliorated by consuming foods that include antioxidants, such as vitamin E, as well as antioxidant enzyme precursors, such as selenium, which is an essential component of Glutathione peroxidase [16-17]. The administration of Zn results in a significant rise in the blood concentrations of vitamins C and E, as well as zinc, in chickens [18]. Because it influences fat metabolism, zinc has a good antioxidant effect when combined with vitamin E to combat the negative effects of heat stress [19], combining vitamin E, a well-known antioxidant, with zinc, an antioxidant agent, may boost the activity of antioxidant enzymes. Zinc and vitamin E work together to promote the overall health and performance of birds by strengthening their immune systems and nutrition [20]. The spleen and bursa weight ratios of quails fed on zinc in either organic or inorganic form at a dose of 50 mg/kg meal were unaffected by the zinc supplementation [21]. Considering the above hypothesis, the study was conducted to determine the effects of Zn and vitamin E supplementation alone or in combination with the diet on the growth and production performance of Japanese quails.

2. MATERIALS AND METHODS

The experiment was conducted at the Avian Research & Training (ART) Center, Department of Poultry Production, University of Veterinary and Animal Sciences (UVAS), Lahore for a period of 6 months. Lahore has a hot semi-arid climate (Koppen climate classification BSh), with long, rainy, warm summers, and dry winters, as well as dust storms and a monsoon. During the months of May, June, and July, temperatures rise to between 40 and 48 °C. The monsoon season lasts from late June to August.

A total of 960-day old quail chicks were procured from the hatchery unit at the Avian Research and Training (ART) Center, University of Veterinary and Animal Sciences (UVAS) Lahore. Using CRD chicks were distributed in eight groups (4 replicates/ group) (Table 1). The quail chicks were reared in an open-sided house in cages and had free access to feed and water. Overall growth performance in terms of weight gain, FCR, Mortality, etc was investigated. At the age of 6 weeks, 02 (01 male and 01 female) birds from each replicate, were randomly taken, weighed, and slaughtered. The birds were reweighed and eviscerated. Carcass weight and weights of gizzard, liver, heart, alimentary canal, and abdominal fat were recorded.

The birds were given a starter basal diet containing 22 % crude protein (CP), and 2800 kcal/ kg metabolizable energy (ME). Seven experimental diets (B, C, D, E, F, G, and H) were formulated. The ration was made by Hi-Tech Feed Industries (Pvt.), Lahore, Pakistan, according to NRC standards (1994) (Table 2). The experiment was conducted in three phases i.e., growth, production, and hatching phase.

Day-old quail chicks were weighed individually when they arrived, and subsequent body weight measurements taken at weekly intervals for each experimental duplicate were subsequently recorded. After the sixth week, final body weight was also recorded. A sophisticated computerized digital balance was used to properly weigh the test birds. As a result, the measured weight was recorded for each bird separately by reading the scale. The quantity of feed supplied to each duplicate each day was weighed, and the residual was then quantified the next day. Each bird's feed consumption was recorded in order to determine weekly feed intake. The formula utilized was as follows: Feed intake is calculated as follows: number of birds = feed provided - feed residue during a certain time.

Two birds from each replicate—a male and a female—were chosen at random at the end of the experiment and kept off food for 5 to 6 hours before being slaughtered to maintain their intestines and crops clear of the undigested meal (feed withdrawal period). The birds were killed using a halal technique to guarantee full bleeding. Before killing, each bird was weighed individually on an advanced computerized digital balance, and all the organs were weighed as well.

Statistical Analysis

Data collected was analyzed through the one-way analysis of variance (ANOVA) technique under a Completely Randomized Design (CRD) as suggested by Hamid *et al* [22]. Means were compared through Duncan's Multiple Range (DMR) test using statistical analysis software (JMP 7.0).

3. RESULTS

3.1 Feed Intake (g/bird)

Table 3 shows that the feed intake of quails (1-6 weeks) varied significantly ($P<0.05$) due to the inclusion of Zn of diverse sources and vitamin E. The quails in groups H and F took more feed

Table 1. Treatment plan from day old to 6th weeks

Groups	Diets		
	Zinc (Organic) (mg/kg)	**Zinc (Inorganic) (mg/kg)**	**Vit. E (IU/kg)**
A (Control)	0	0	0
B	25	0	0
C	0	15	0
D	0	0	12
E	25	15	0
F	25	0	12
G	0	15	12
H	25	15	12

8 treatments * 4 Replicates * 30 Birds in each replicate = 960 birds

***1U** = 1U/kg = 0.67mg dl-ά-tocopherol acetate

Table 2. Feed formulation used in this experiment.

	Chemical Composition (%)							
Ingredient	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Corn (CP = 8 %)	54.985	54.96	54.97	54.972	54.945	54.947	54.957	54.932
Soybean meal (CP=42.78 %)	42.5	42.5	42.5	42.5	42.5	42.5	42.5	42.5
DCP (P=18 %, Ca=21 %)	0.71	0.71	0.71	0.71	0.71	0.71	0.71	0.71
Oyster shell (Ca = 38 %)	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
NaCl	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32
Vitamin supplement	0.012	0.012	0.012	0.025	0.012	0.025	0.025	0.025
Mineral supplement	0	0.025	0.015	0	0.04	0.025	0.015	0.04
DL-Methionine	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
L-Threonine	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
Washed sand	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013
Chemical composition								
ME (Kcal/kg)	2773	2773	2773	2773	2773	2773	2773	2773
Crude protein	22.68	22.68	22.68	22.68	22.68	22.68	22.68	22.68
Calcium	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.77
Available phosphorus	0.29	0.29	0.29	0.29	0.29	0.29	0.29	0.29
Sodium	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
Lysine	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28
Methionine	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48
Methionine + Cystine	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86
Threonine	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98
Arginine	1.54	1.54	1.54	1.54	1.54	1.54	1.54	1.54
Zinc (mg/kg)	0	0.0025	0.0015	0	0.004	0.0025	0.0015	0.004
Vitamin E	0.0012	0.0012	0.0012	0.0025	0.0012	0.0025	0.0025	0.0025

Table 3. Feed intake of Japanese quail (g/bird)

Groups	**1st week**	**2nd week**	**3rd week**	**4th week**	**5th week**	**6th week**	**Average**
A	22.45	66.17	99.08	152.13	177.1	173.04	689.97d
B	22.88	75.84	105.41	143.45	176.23	171.04	694.85cd
C	18.92	64.22	111.05	155.87	174.8	169.67	694.53cd
D	22.32	72.13	103.22	150.44	173.83	171.84	693.78bcd
E	24.55	72.29	117.83	158.47	175.47	171.75	720.36abc
F	23.7	76.47	115.53	157.23	186.32	175.45	734.7b
G	23.86	81.8	103.07	153.27	186.68	175.46	724.14ab
H	21.54	77.43	113.85	158.77	194.97	178.88	745.44a
	22.40f	72.45e	107.51d	153.95c	182.02b	174.77a	--

	Treatments (T)	**Weeks (W)**	**T × W**
S.E.±	8. 802	6.953	13.814
LSD 0.05	17.285	13.635	66.836
P-Value	0.0005	0.0000	0.0449

during six weeks' period (745.44 g and 734.7 g/bird), respectively; followed by the average feed consumption of 724.14 and 720.36 g/bird in groups G and group E, respectively. The average feed intake was relatively lower (700.78 g, 694.53, and 694.85 g/bird) in groups D, C and B, respectively; while the lowest average feed intake (689.97 g/bird) was observed in control group A. Statistically, the birds in groups H and F showed similar behavior regarding feed intake; while in lower feed intake groups C and B were similar (P>0.05). The average quail's feed consumption is influenced by the zinc source and the addition of vitamin E. However, the absence of zinc as picolinate in feed caused a significant decrease in average feed intake in the presence of $ZnSO_4$. Similarly, the quails' feed choice was significantly affected due to the presence or absence of vitamin E. This indicates that the inclusion of organic zinc (picolinate) was preferred by quails over $ZnSO_4$; while birds were pleased to take more feed when Vitamin E was added.

3.2 Live Body Weight of Slaughtered Birds

After 6 weeks, the live body weight (LBW) of quails were taken in different treatment groups comprised of a certain concentration of organic and inorganic zinc in addition to vitamin E. Table 4 exhibited that the LBW of birds designated in different treatment groups varied significantly (P<0.05). The male quails in group H possessed the highest LBW (175.35 g), followed by the quails of groups F, G, and E possessing LBW of 174.50, 173.45, and 172.0 g/bird, respectively. The LBW showed a decreasing trend (171.48, 171.23, and 170.13 g/bird) in groups D, B, and C, respectively. Whereas, the male quail LBW was the least (170.13 g/bird) in control group A. Statistically, the birds in groups H and F (P>0.05); groups G and E (P>0.05), and birds in groups B, C, and D showed similarity (P>0.05) for LBW.

The female LBW was also measured (Table 4) which exhibited female quails in group H possessed highest LBW (198.35 g), followed by the quails of group F, G, and E possessing LBW of 197.38, 196.5, and 196.45 g/bird, respectively. The LBW showed a decreasing trend (194.13,194.00 and 193.48 g/bird) in groups B, D, and C, respectively. Whereas the female quail LBW was least (192.23 g/bird) in control group A. Statistically, the birds in groups H and F (P>0.05); groups G and E (P>0.05), and birds in groups B, C, and D showed similarity (P>0.05) for LBW.

The feeding quails with a diet with zinc and vitamin E showed a positive impact on their LBW as indicated from the comparative analysis of treatments with control. The comparison of organic and inorganic zinc suggested that organic zinc

Table 4. Live body weight of slaughtered birds' male/female (g/bird)

Group	Sex		Average
	Male	Female	
A	169.38^{d}	192.23^{e}	180.81^{g}
B	171.23^{c}	194.13^{d}	182.68^{e}
C	170.13^{c}	193.48^{d}	181.81^{f}
D	171.48^{c}	194.00^{d}	182.74^{e}
E	172.00^{b}	196.45^{c}	184.23^{d}
F	174.50^{a}	197.38^{b}	185.94^{b}
G	173.45^{b}	196.5^{c}	184.98^{c}
H	175.35^{a}	198.35^{a}	186.85^{a}
172.19^{b}	**195.32^{a}**	**--**	

	Treatments (T)	Sex(S)	T × S
S.E.±	0.6407	0.9203	0.3403
LSD 0.05	1.4847	2.7424	0.6855
P-Value	0.0000	0000	1.0000

(picolinate) proved to have a better positive impact on male LBW as compared to those given inorganic Zn ($ZnSO_4$). Moreover, the addition of Vitamin E also showed an encouraging impact on male LBW even better than $ZnSO_4$. It can be concluded that for achieving healthy male quails, the organic zinc (picolinate) in addition to a certain level of Vitamin-E would be optimum supplementation, rather to add both organic (picolinate) and inorganic zinc ($ZnSO_4$).

3.3 Feed Conversion Ratio

The FCR of quails after 6 weeks were calculated to see the effect of dietary Zn + Vit-E and the results are given in Figure 1. The FCR was significantly influenced by the dietary Zn + Vit-E treatments ($P<0.05$). The birds kept in group F showed the highest FCR (3.59), followed by the birds in groups D, B, and C with average FCRs of 3.75, 3.75, and 3.76, respectively. The FCR in quail groups E, G, and H was not encouraging due to increased feed intake but not a simultaneous increase in weight gain. The quails in group F utilized feed more efficiently, which is clear from the nutrient digestibility results, and gain more weight as compared to the rest of the treatments.

3.4 Mortality (%)

The quail flocks were also monitored for mortality rate during 6 weeks of the experiment and the data are shown in table 5. It was noted that there was negligible effect of treatments based on organic and inorganic zinc with vitamin E in various combinations on the mortality rate ($P>0.05$). The overall mortality was higher (2.75 %) in control group A, followed by groups B and C, D with an

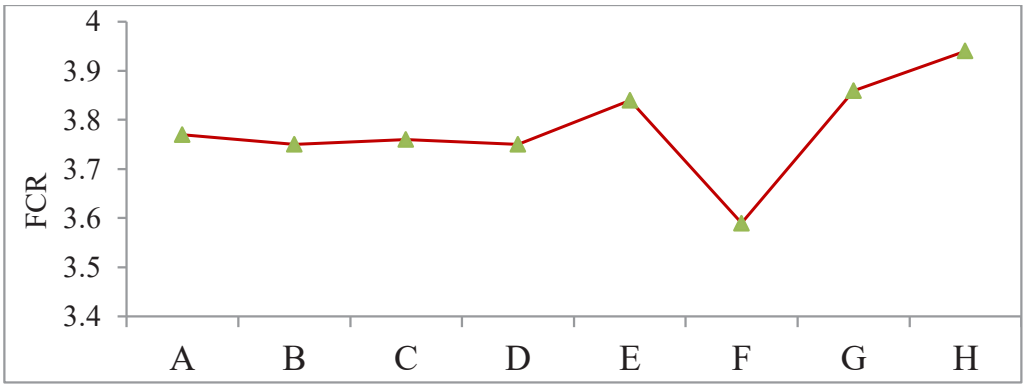

Fig. 1. Feed conversion ratio (FCR) of Japanese quail

Table 5. Mortality rate of Japanese quail

	Total
A	2.75[a]
B	2.50[ab]
C	2.50[ab]
D	2.25[b]
E	1.50[ab]
F	1.25[b]
G	1.50[ab]
H	1.25[b]

	Treatments (T)	Sex (S)	T × S
S.E.±	0.5121	0.2561	0.7242
LSD 0.05	1.0315	0.5157	1.4587
P-Value	0.7464	0.3935	0.0821

average mortality of 2.50, 2.50, and 2.25 percent, respectively. However, lower mortality rate (1.50 and 1.50 %) was recorded in groups E and G, respectively; while the equally lowest mortality rate of 1.25 % was seen in quails of groups H and F, respectively.

3.5 Slaughter Characteristics

3.5.1. Carcass + Giblets Weight of Quails (g/bird)

The carcass + giblets weight of slaughtered birds at 6 weeks of age were recorded to assess the treatment effect and the results are shown in Figure 2. The data demonstrated that the carcass + giblets weight of slaughtered birds was significantly affected (P>0.05) by organic and inorganic zinc in addition to vitamin E combinations. The carcass + giblets weight of male, and female birds and average in group H was higher (139.34, 144.68, avg: 142.01 g/bird) than the birds in groups F, G, and E with male, female carcass + giblets weights and the average of 138.31, 144.65, avg: 141.48 g/bird; 130.37, 143.23, avg: 136.80 g/bird, 131.76, 138.67, avg: 135.22 g/bird, respectively. Relatively lower carcass + giblets weight in male, female birds and average was recorded in group D; B and C, i.e., 134.73, 137.49, avg: 136.11 g/bird; 130.70, 134.64, avg: 132.67 g/bird; 128.87, 128.72, avg: 128.80 g/bird, respectively. However, male, female, and average carcass + giblets weight was least (126.95, 129.88, avg: 128.42 g/bird), respectively in control group A. The DMRT indicated that quails in groups H and F showed similar carcass + giblets weight suggesting that the inclusion of inorganic zinc in the presence of organic zinc was unnecessary. However, organic Zinc + Vit- E combination was more effective as compared to the inorganic Zinc + Vit-E combination. Moreover, the inclusion of Vit-E was also more effective than the inorganic zinc.

3.5.2. Carcass Weight of Quails (g/bird)

The results (Fig 3) illustrated that the effect of organic and inorganic zinc with vitamin E combinations on carcass weight was significant (P<0.05). The carcass weight of male, female birds and average in group H was maximum (127.75, 134, avg: 130.88 g/bird), followed by the birds in groups F, G, and E with male, female carcass + giblets weights and average of 130, 130.5, avg: 130.25 g/bird; 118.50, 131.78, avg: 125.14 g/bird, 126.25, 122.0, avg: 124.13 g/bird, respectively. The carcass weight was lesser for male, female birds and average in groups D; B and C, i.e., 114.50, 133.5, avg: 124 g/bird; 122.25, 123, avg: 122.63 g/bird; 117.25, 118.25, avg: 117.75 g/bird, respectively. The least male, female, and average carcass weight (118.25, 115.75, avg: 117 g/bird), respectively was recorded in control group A. The statistical analysis suggested that the carcass weight in birds of groups H and F were almost equal which clearly advocates that the addition of zinc in organic form (picolinate) with vitamin E combination was more effective as compared to combining both organic (picolinate) and inorganic zinc ($ZnSO_4$) with vitamin E. However, the inclusion of vitamin E

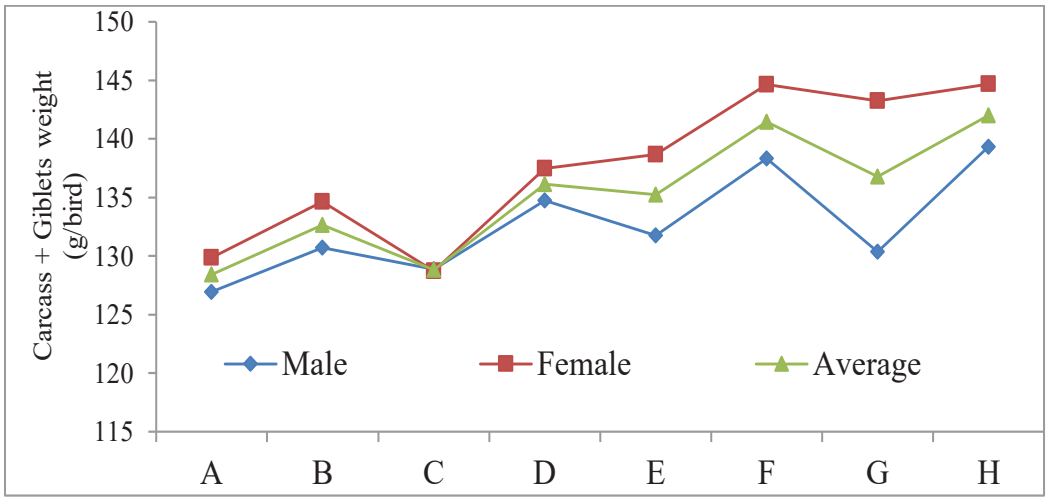

Fig. 2. Carcass + giblets weight of Japanese quail

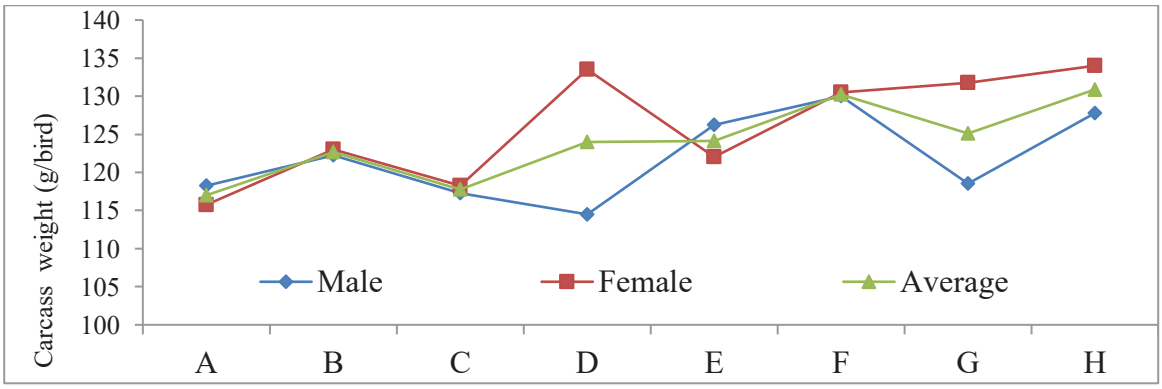

Fig. 3. Carcass weight of Japanese quail

resulted in better carcass weight when compared to the inclusion of inorganic zinc so far, the carcass weight is concerned.

3.5.3. Heart Weight of Quails (g/bird)

The results (Table 6) exhibit that the effect of organic and inorganic zinc with vitamin E combinations on heart weight was significant (P<0.05). The heart weight of male, female quails and their average in group H was highest (2.09, 2.26, avg: 2.18 g/bird), followed by the quails of groups F, G, and E with male, female heart weight and an average of 1.94, 2.38, avg: 2.16 g/bird; 1.81, 2.3, avg: 2.06 g/bird, 1.97, 2.06, avg: 2.02 g/bird, respectively. The heart weight was markedly lower for male, female birds and average in groups D; B, and C, i.e., 1.82, 1.87, avg: 1.85 g/bird; 1.78, 1.86, avg: 1.82 g/bird; 1.52, 1.94, avg: 1.73 g/bird, respectively. The lowest male, female, and average heart weight (1.49, 1.9, avg: 1.7 g/bird), respectively was recorded in control group A.

3.5.4. Liver Weight of Quails (g/bird)

The results (Table 7) revealed that different combinations of organic and inorganic zinc with vitamin E had an insignificant effect (P>0.05) on liver weight. The liver weight of male, female birds and their average in group H was maximum (4.37, 5.75, avg: 5.06 g/bird), followed by the quails of groups F, G, and E with male, female liver weight and average of 4.47, 5.56, avg: 5.02 g/bird; 4.41, 4.89, avg: 4.65 g/bird, 4.36, 4.60, avg: 4.48 g/bird,

Table 6. Heart weight (g/bird) of Japanese quail

Group	Sex		Average
	Male	Female	
A	1.49	1.9	1.70^{d}
B	1.78	1.86	1.82^{cd}
C	1.52	1.94	1.73^{cd}
D	1.82	1.87	1.85^{bc}
E	1.97	2.06	2.02^{ab}
F	1.94	2.38	2.16^{a}
G	1.81	2.3	2.06^{ab}
H	2.09	2.26	2.18^{a}
Average	1.80^{b}	2.07^{a}	--

	Treatments (T)	Sex (S)	T × S
S.E.±	0.1586	0.0793	0.2242
LSD 0.05	0.3194	0.1597	0.4516
P-Value	0.0160	0.0374	0.1355

Table 7. Liver weight (g/bird) of Japanese quail

Group	Sex		Total
	Male	Female	
A	4.02	4.42	4.22
B	4.29	4.41	4.35
C	4.19	4.47	4.33
D	4.18	4.64	4.41
E	4.36	4.6	4.48
F	4.47	5.56	5.06
G	4.41	4.89	4.65
H	4.37	5.75	5.02
Average	4.29	4.84	--

	Treatments (T)	Sex (S)	T × S
S.E.±	0.2468	0.1234	0.3491
LSD 0.05	0.4971	0.2486	0.7931
P-Value	0.7211	0.6045	0.4299

respectively. Liver weight was reduced in male, female birds and average in groups D; B, and C, i.e., 4.18, 4.64, avg: 4.33 g/bird; 4.29, 4.41, avg: 4.35 g/bird; 4.19, 4.47, avg: 4.33 g/bird, respectively. The least male, female, and average liver weight (4.02, 4.42, avg: 4.22 g/bird), respectively was recorded in control group A.

3.5.5 Gizzard Weight of Quails (g/bird)

The results showed that organic and inorganic zinc with vitamin E in various combinations had insignificant effect (P>0.05) on gizzard weight (Table 8). The gizzard weight of male, female birds and their average in group H was relatively higher (5.9, 5.34, avg: 5.62 g/bird), followed by the birds of groups F, G, and E with male, female gizzard weight and average of 5.52, 4.91, avg: 5.21 g/bird; 5.17, 5.11, avg: 5.14 g/bird, 4.27, 5.90, avg: 5.08 g/bird, respectively. Gizzard weight was reduced in groups D; B and C having male, female and average gizzard weight of 4.65, 5.25, avg: 4.95 g/bird; 5.31, 4.55, avg: 4.93 g/bird; 4.92, 4.72, avg: 4.82 g/bird, respectively. The lowest male, female

Table 8. Gizzard weight (g/bird) of Japanese quail

Group	Sex		Total
	Male	Female	
A	5.60	3.80	4.70
B	5.31	4.55	4.93
C	4.92	4.72	4.82
D	4.65	5.25	4.95
E	4.27	5.90	5.08
F	5.52	4.91	5.21
G	5.17	5.11	5.14
H	5.90	5.34	5.62
Weekly average	5.17	4.95	--

	Treatments (T)	Sex (S)	T × S
S.E.±	0.5121	0.2561	0.7242
LSD 0.05	1.0315	0.5157	1.4587
P-Value	0.7464	0.3935	0.0821

and average gizzard weight (5.60, 3.80, avg: 4.70 g/bird), respectively was recorded in control group A.

3.5.6 Morphology of the Reproductive Tract

The data in relation to the above parameters of Japanese quails-fed diets containing diverse sources and levels of zinc in addition to vit-E as compared to control are shown in Table 9. The effect of diet on oviduct weight, oviduct length, ovary weight, follicle number as well as left and right testes weight was significant (P<0.05) and values for all these reproductive traits of Japanese quail were greater for birds of Group E, followed by the birds in groups H, E and B. Both left and testis weight in diets weights were equally affected by the diets; and similarly, the oviduct weight and length were equally affected by the Zinc + Vit-E based quail diets. Similarly, the follicular number also varied significantly with the inclusion of zinc and Vitamin E in the quail diet. However, the effect of organic zinc was more pronounced as compared to inorganic zinc when added to the quail diet in combination with Vitamin E. In the absence of Vitamin E, the reproductive female reproductive tract, ovary weight, and left and right testes of male quails showed greater adverse effects, even in the presence of zinc in their diet. However, the combined effect of organic Zinc + Vit-E on the reproductive traits of male and female quails was positive and significant.

Table 9. Morphology of intestinal villi of Japanese quail

Group	Jejunum				Ileum			
	JVH (mm)	JCD (mm)	JVH: CD	JVT (mm)	IVH (mm)	ICD (mm)	IVH: CD	IVT (mm)
A	0.45^{d}	0.047^{c}	9.58^{b}	0.122^{b}	0.34^{c}	0.051	6.67^{c}	0.111^{b}
B	0.51^{ab}	0.050^{a}	10.20^{a}	0.130^{ab}	0.41^{b}	0.051	8.04^{a}	0.120^{ab}
C	0.48^{c}	0.048^{b}	10.00^{a}	0.125^{b}	0.37^{bc}	0.051	7.25^{b}	0.119^{ab}
D	0.47^{c}	0.048^{b}	9.79^{b}	0.123^{b}	0.36^{bc}	0.051	7.06^{b}	0.113^{ab}
E	0.52^{a}	0.051^{a}	10.20^{a}	0.130^{ab}	0.43^{a}	0.051	8.43^{a}	0.123^{a}
F	0.53^{a}	0.052^{a}	10.19^{a}	0.135^{a}	0.44^{a}	0.052	8.46^{a}	0.123^{a}
G	0.50^{b}	0.049^{b}	10.20^{a}	0.133^{a}	0.39^{b}	0.051	7.65^{b}	0.118^{ab}
H	0.53^{a}	0.050^{a}	10.60^{a}	0.135^{a}	0.43^{a}	0.051	8.43^{a}	0.123^{a}
P-Value	*0.047**	*0.051**	*0.005***	*0.042**	*0.008***	*0.089^{NS}*	*0.033**	*0.044**

JVH=Jejunum villus height (mm); JCD=Jejunum crypt depth (mm); JCD:CD=Jejunum villus height to crypt depth ratio; JVT=Jejunum villus thickness (mm); IVH=Ileum villus height (mm); ICD=Ileum crypt depth (mm); IVH:CD=Ileum villus height-to-crypt-depth ratio; IVT=Ileum villus thickness (mm)

Table 10. Morphology of the reproductive tract of Japanese quail

Group	Oviduct weight (g)	Oviduct length (cm)	Ovary weight (g)	Follicle number	Left testes weight (g)	Right testes weight (g)
A	6.94	34.33	5.48	4.19	2.69	2.62
B	7.89	35.63	5.81	4.62	3.05	3.19
C	7.63	35.33	5.67	4.36	2.96	2.94
D	7.41	34.78	5.58	4.28	2.87	2.77
E	8.63	35.52	6.11	4.73	3.51	3.31
F	8.71	36.13	6.21	4.86	3.76	3.42
G	8.19	35.81	5.66	4.55	3.18	3.08
H	8.61	36.02	6.08	4.85	3.72	3.37
P-Value	*0.0473**	*0.0514**	*0.0357**	*0.0249**	*0.0083***	*0.0218**

4. DISCUSSION

Japanese quail is the source of meat that is most flavorsome, delicious, and appetizing in the avian species. There is enormous potential for commercial quail farming, but this component of the poultry sector is identically neglected in Pakistan. Due to a lack of research focus, no advancement in quail breeding-related aspects (incubation, housing, nutritional requirements) and health management under local environmental conditions has been reported [23]. Generally, commercial poultry is formulated by adding antioxidants for prevention against lipid peroxidation and oxidative rancidity during production [24]; and dietary antioxidants and cofactors help counteract the negative effects of oxidative stress in poultry birds [25]. Some substances including zinc and vitamin E have strong antioxidant characteristics. Vit-E is one of the most powerful antioxidants that is added to poultry feed to improve performance, strengthen immunological status, improve the quality of meat and egg, and increase the vitamin E content of meat [26]. Similarly, Zn is an effective antioxidant and essential trace mineral in organisms having an important role in growth, bone development, enzyme structure and function, and eggshell formation in poultry. The study showed that Zinc in addition to Vitamin E (Zn + Vit-E) had a significant effect on feed intake, LBW, filled intestine weight, carcass, and giblets ($P<0.05$); while the treatment effect was insignificant ($P>0.05$) on intestine empty weight, cecal length, liver weight, gizzard weight, and mortality. The quail showed a preference to consume feed with organic zinc (picolinate) in addition to Vit-E over inorganic zinc ($ZnSO_4$) +Vit-E. Absence of zinc as picolinate in feed caused a significant decrease in average feed intake in the presence of $ZnSO_4$. The quails feed choice was affected significantly due to presence or absence of vitamin E. This indicates that inclusion of organic zinc (picolinate) was preferred by quails over $ZnSO_4$; while birds were pleased to take more feed when Vitamin E was added. In most cases, there was similarity ($P>0.05$) in the feed intake of quails in all groups with minute difference indicating that the growth and blood physiology of quails was improved by dietary Zn + Vit-E without additional consumption of feed. Combination of Zn*O* 50 + Zn*I* 30 + VE 25 resulted in better overall bird performance. Japanese quail is known for rapid growth and high body weight [27-28]. As an antioxidant, Vit-E has an effective role as an antioxidant in a biological system and individually explicates its antioxidant function in lipid phases by restoring antioxidant properties [29, 30-31] observed that Quail's body weight rose when dietary vitamin E levels were high; earlier research also came to the same conclusion that dietary vitamin e benefited quail development under heat stress conditions. The benefit of vitamin E on chicken performance only manifests under environmental stress conditions; it is undetectable under conditions of normal temperature [32].

5. CONCLUSION

The Zn + Vit-E significantly influenced the feed intake, LBW, filled intestine weight, intestine length, carcass weight and heart weight ($P<0.05$); while the treatment effect was insignificant ($P>0.05$) on liver weight, gizzard weight, and mortality. The quail showed a preference to consume feed with organic zinc (picolinate) in addition to Vit-E over inorganic zinc ($ZnSO_4$) +Vit-E. Absence of zinc as picolinate in feed caused a significant decrease in average feed intake in the presence of $ZnSO_4$. The quails feed choice is significantly affected due to the presence or absence of Vitamin E. This indicates that inclusion of organic zinc (picolinate) was preferred by quails over $ZnSO_4$; while birds were pleased to take more feed when Vitamin E was mixed. The dietary Zn + Vit-E inclusion significantly ($P<0.05$) increased the relative length of gut segments, villi height, villus thickness, the villi height to crypt depth proportion in jejunum and ileum and the number of goblet cells in different parts of the intestine of quails.

6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research Article

Diversity and Distribution of Endemic Flora in Pakistan

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Abstract: The objective of the current research is to provide a systematic account of the variety of endemic plant species found in Pakistan, with a focus on family, distribution, and life form status. The existing research effort, which is based on a survey of the literature, field observations, and herbarium records, has identified 306 endemic plant species among 50 genera and 40 families. In accordance with an analysis of the life form or status of these plant species, herbs are dominant (n= 243 species, 80 %), while shrubs (n= 33 species, 11 %), under shrubs (n= 13 species, 4 %), trees (n= 10 species, 4 %), and grasses (n= 7 species, 2 %). The study revealed that Asteraceae is the most dominant family (n= 38 species), while *Taraxacum* is a dominant genus (n= 23 species). Distribution analysis revealed that the majority of species are distributed in mountainous areas of Pakistan. Khyber Pakhtunkhwa province is rich in endemism (n= 142 species, 37 %). The current study sheds light on Pakistan's endemism situation. Further research that takes into consideration population levels and new risks is also required. The study will help policymakers in developing conservation strategies.

Keywords: Endemic, Species Distribution, Diversity, Conservation

1. INTRODUCTION

The word endemic was introduced by De Candolle in 1820, who adapted its meaning as a sickness that constantly occurs within an area, describing genre end'emiques as essentially analogous to a taxon that is limited to a particular area [1]. As an antonym to endemic taxa, species having a wide distribution were termed genres sporadiques [2]. Even though the phrase was first used in a biogeographic context, its regular use within the scientific community began in the early twentieth century, when it was used in books and journals to portray new species with limited transmission or to refer to threat classifications [3]. As the name implies, endemic plants are those with narrow distributions, low population sizes, and habitat specificity [3, 4].

An astonishing diversity of flora exists in Pakistan, a land of breath-taking beauty and rich culture. Throughout the country, over 7,000 vascular plants thrive, each as unique as the landscapes in which they are found, from the towering peaks of the Himalayas to the scorching sands of the Thar Desert [5]. A rich diversity of flora can be found in the Himalayan region, including conifers, alpine flowers, and rhododendron forests. [6, 7]. Many endemic and rare species are found in the mountainous areas in the north and west, which are significantly more numerous here than in other similar-sized countries [8, 9]. A wide variety of plant and animal life thrives in the fertile farmlands, lush forests, and river deltas of the Indus Valley. A unique and fascinating flora can also be found in the deserts of Pakistan, including hardy succulent species that have adapted to the harsh conditions of the desert. Additionally, the coastal areas of the country are rich in plant species that have adapted to the harsh coastal environment, including mangrove forests and salt-tolerant species [10].

Chaudhri & Qureshi [11] created a checklist of 707 uncommon and endangered plant taxa, which also includes some endemic plant species, based on the frequency of herbarium specimens and observations recorded in the flora of Pakistan. This list may undoubtedly be considered a great contribution, but it also strongly encourages researchers to gather more field data to back their findings. As a follow-up, some of the important studies that describe the assessments of the conservation status of different plant species are

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available in the literature [10, 12]. Many other researchers also reported endemics (*Pimpinella stewartii, Otostegia limbata, Aquilegia nivalis*) ranges [13-17]. All these reports merely represent 5 % of the endemic flora of Pakistan. But no systematic attempt has ever been made to understand a list of endemic species and the state of their dispersion.

Several projections predict that we are on the verge of experiencing the sixth major extinction. Plant species are disappearing at a rate of up to one every day [18]. A key contributor to the losses is anthropogenic activity that continuously alters the environment and fragments and destroys it. Climate change is another related factor [19].

To conserve species that are critically endangered, first, we must identify them. Endemic species require special care since they are in grave danger [20]. However, there aren't many studies that have mapped the spread of various plant species [21, 22]. Therefore, present study aimed to enumerate the diversity and occurrence of endemic flora of Pakistan. Additionally, this study will aid decision-makers in creating conservation and management strategies.

2. MATERIALS AND METHODS

2.1 Study area

Pakistan represents a number of the world's biological areas due to its large altitudinal range and broad latitudinal dispersion (Fig. 1), which spans around 1400 km from the seashore in the south to snow-capped mountains in the north. Pakistan is home to three of the world's eight biographic realms, including the Indo-Malayan, Palaearctic, and Afro-tropical, as well as four of the world's ten biomes, including the desert, temperate grassland, tropical seasonal forest, and mountains, all of

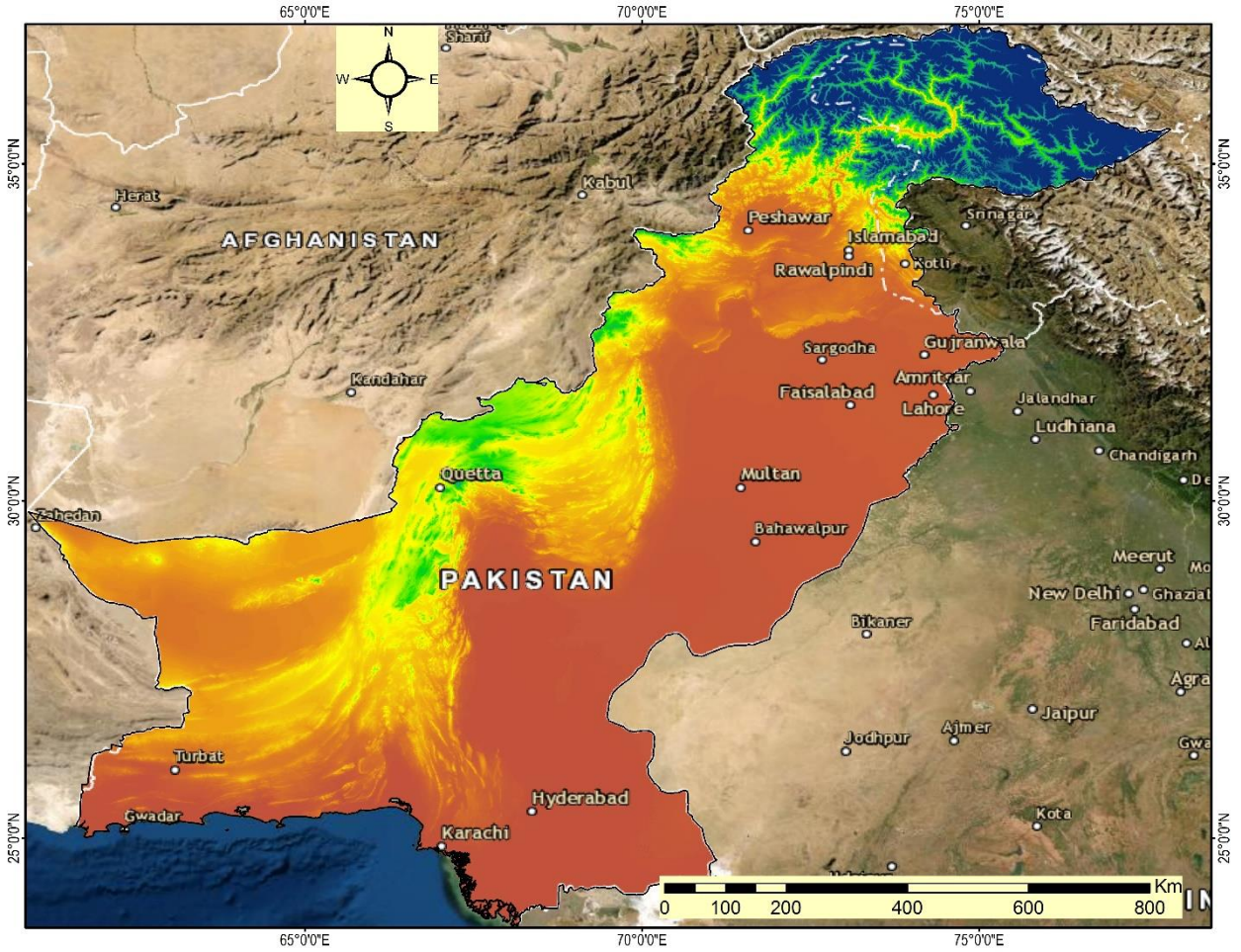

Fig. 1. Map illustrating the study area.

which are home to a variety of ecosystems [23]. There have been reports of about 6,000 flowering plant species, including both indigenous and alien varieties. The area has distinct seasons with notable temperature changes. Although there are occasional windy spells in the summer, the winds are often from the north or south-east throughout the year. In the area, there are two different seasons for rainfall: summer (July to September), and winter (December to March) [24].

2.2 Data Collection

The list of Pakistan's endemic plant species was compiled using Flora of Pakistan (www.efloras.org), Flora of Iranica, and the published literature [11, 25-28]. This resulted in 580 species that were endemic to Pakistan. After removing duplicates, the distribution ranges of each species were validated and cross-checked using online resources of www.theplantlist.com, www.powo.science.kew.org, and the Global Biodiversity Information Facility (GBIF). Synonyms were excluded and only species with accepted names and having occurrences records from Pakistan were included in the list resulting in finding 306 species to be endemic to Pakistan. Several sources were used to gather existing occurrence records (the latitude and longitude) of endemic plant species in Pakistan. These sources include our field surveys in 2018-2021, herbarium sheets, www.inaturalist.org, and www.gbif.org. The species were classified on the basis of herbs, shrubs, under-shrubs, trees and grasses. The accepted endemic plant species of Pakistan in the current study are arranged alphabetically in tabular form followed by family, distribution, habitat life form and flowering period shown in **(Table 1)**.

Table 1. A comprehensive list of Plant species endemic to Pakistan.

S. No.	Species	Family	Distribution	Life form / Status	Flowering period
1	*Abutilon alii*	Malvaceae	Lasbela	Shrub	July-Aug
2	*Abutilon ghafoorianum Abedin*	Malvaceae	Sahiwal	Undershrub	July-Aug
3	*Abutilon karachianum S.A. Husain & Baquar*	Malvaceae	Karachi	Herb	July-Aug
4	*Abutilon pakistanicum Jafri & Ali*	Malvaceae	Sindh	Undershrub	July-Aug
5	*Abutilon sepalum S.A.Husain & Baquar*	Malvaceae	Sindh	Herb	July-Aug
6	*Achillea millefolium subsp. Chitralensis*	Asteraceae	Chitral	Herb	May-June
7	*Aconitum curvipilum Riedl*	Ranunculaceae	Chitral	Herb	July-Aug
8	*Aconitum heterophyllum var. bracteatum*	Ranunculaceae	Hazara	Herb	July-Aug
9	*Aegopodium burttii E. Nasir*	Umbelliferae	Hazara	Herb	June-July
10	*Allium balochistanicum Wendelbo*	Alliaceae	Balochistan	Herb	May-June
11	*Anaphalis staintonii*	Asteraceae	Chitral, Kashmir	Undershrub	June-Sep
12	*Androsace hazarica R.R. Stewart*	Primulaceae	Hazara	Herb	July-Aug
13	*Androsace lowariensis Y. Nasir*	Primulaceae	Chitral	Herb	June-Sep
14	*Androsace ojhorensis Y. Nasir*	Primulaceae	Chitral	Herb	June
15	*Androsace staintonii Y. Nasir*	Primulaceae	Chitral	Herb	June
16	*Anemone falconeri Thoms*	Ranunculaceae	Pak & Kashmir	Herb	May-June
17	*Anemone obtusiloba var. potentilloides*	Ranunculaceae	Pak & Kashmir	Herb	May-June
18	*Anemone tetraSepala Royle*	Ranunculaceae	Pak & Kashmir	Herb	July-Aug
19	*Aquilegia fragrans var. fragrans*	Ranunculaceae	Chitral	Herb	July-Aug
20	*Aquilegia nivalis Falc. ex Baker*	Ranunculaceae	Pak & Kashmir	Herb	June-July
21	*Aralia cachemirica Dcne*	Araliaceae	Kashmir	Herb	June-Oct
22	*Artemisia amygdalina Decne*	Asteraceae	KP & Kashmir	Herb	July-Sep
23	*Asparagus dumosus Baker*	Asparagaceae	Sindh & Balochistan	Undershrub	March-Aug
24	*Asparagus gharoensis Blatter*	Asparagaceae	Sindh	Shrub	March-Aug
25	*Astragalus affghanus Boiss*	Papilionaceae	Chitral, Balochistan	Herb	Mar-April
26	*Astragalus auganus Bunge*	Papilionaceae	Balochistan	Herb	March
27	*Astragalus concretus Benth*	Papilionaceae	Kashmir	Herb	July-Aug

Table 1 Continued...

S. No.	Species	Family	Distribution	Life form / Status	Flowering period
28	*Astragalus falconeri Bunge*	Papilionaceae	Chitral	Herb	July-Sep
29	*Astragalus flemingii Ali*	Papilionaceae	Punjab	Herb	March
30	*Astragalus Gilgitensis.ensis Ali*	Papilionaceae	G.B.	Herb	July
31	*Astragalus hostilis Boiss*	Papilionaceae	Balochistan	Undershrub	April-June
32	*Astragalus lamondiae Deml*	Papilionaceae	Balochistan	Herb	April
33	*Astragalus maxwellii Royle*	Papilionaceae	Kashmir	Herb	June-July
34	*Astragalus nicharensis Bunge*	Papilionaceae	Balochistan	Herb	June-July
35	*Astragalus oihorensis Ali*	Papilionaceae	KP	Herb	June
36	*Astragalus sultani Ali*	Papilionaceae	Balochistan	Herb	April
37	*Astragalus toppinianus Ali*	Papilionaceae	Chitral	Herb	May-July
38	*Atriplex stocksii Boiss*	Amaranthaceae	Balochistan	Shrub	Dec-Jan
39	*Berberis balochistanica*	Berberidaceae	Balochistan	Shrub	Mar-May
40	*Berberis brevissima Jafri*	Berberidaceae	KP	Shrub	Mar-May
41	*Berberis huegeliana Schneid*	Berberidaceae	Kashmir	Shrub	April-July
42	*Berberis kashmirana Ahrendt*	Berberidaceae	Kashmir	Shrub	June-July
43	*Berberis parkeriana Schneid*	Berberidaceae	Hazara, Kashmir	Shrub	April-June
44	*Berberis pseudumbellata subsp. G.B.ica*	Berberidaceae	G.B.	Shrub	May-June
45	*Berberis royleana Ahrendt*	Berberidaceae	Pak & Kashmir	Shrub	May-June
46	*Berberis stewartiana Jafri*	Berberidaceae	Kashmir	Shrub	May-June
47	*Bongardia margalla R.R.Stewart*	Berberidaceae	Hazara, Margalla	Herb	June-July
48	*Bupleurum canaliculatum Diels*	Apiaceae	G.B.	Herb	Aug
49	*Bupleurum clarkeanum*	Apiaceae	Kashmir	Herb	July-Aug
50	*Bupleurum constancei Nasir*	Apiaceae	Swat	Herb	July-Aug
51	*Bupleurum jucundum Kurz*	Apiaceae	Kashmir	Herb	July-Aug
52	*Bupleurum kohistanicum Nasir*	Apiaceae	Swat	Herb	July-Aug
53	*Bupleurum nigrescens Nasir*	Apiaceae	Hazara	Herb	July-Aug
54	*Bupleurum stewartianum Nasir*	Apiaceae	Swat	Herb	July-Aug
55	*Bupleurum swatianum Nasir*	Apiaceae	Swat	Herb	July-Aug
56	*Buxus papillosa C.K.Schneid*	Buxaceae	Punjab, KP Balochistan,	Tree	Jan-May
57	*Calamagrostis decora Hook. F*	Poaceae	G.B. & Kashmir	Grass	April-July
58	*Calamintha hydaspidis (Falconer ex Benth.)*	Lamiaceae	Kashmir	Herb	June-Sep
59	*Campanula staintonii Rech.f. & Schiman-Czeika*	Campanulaceae	Chitral	Undershrub	April-may
60	*Campanula sulaimanii Nasir*	Campanulaceae	Sulaiman and Salt ranges	Herb	May-Aug
61	*Campanula tenuissima Dunn*	Campanulaceae	Jhelum, Kashmir	Herb	July-Aug
62	*Caragana ambigua Stocks*	Leguminosae	Balochistan	Shrub	April-Aug
63	*Caragana conferta Baker*	Leguminosae	Kashmir	Shrub	July
64	*Caragana ulicina Stocks*	Leguminosae	Balochistan	Shrub	April
65	*Caralluma tuberculata N.E.Br*	Apocynaceae	Punjab, KP, Balochistan	Herb	Jan-June
66	*Carex decaulescens subsp. alsia*	Cyperaceae	Chitral, G.B.	Herb	July
67	*Chesneya depressa (Oliv.)*	Leguminosae	G.B., Kashmir	Herb	May-July
68	*Clematis robertsiana Aitch. & Hemsl*	Ranunculaceae	Kurram Valley	Shrub	May-June
69	*Commiphora stocksiana (Engl.)*	Burseracea	Balochistan	Tree	April-July
70	*Consolida schlagintweitii (Huth) Munz*	Ranunculaceae	Kashmir	Herb	May-Aug
71	*Cortia depressa (D.Don)*	Apiaceae	G.B., Kashmir	Herb	June-Aug
72	*Cortia schmidii E. Nasir*	Apiaceae	Chitral	Herb	June-Aug
73	*Corydalis cashmeriana Royle*	Papaveraceae	Hazara, Kashmir	Herb	May-Aug
74	*Corydalis clarkei Prain*	Papaveraceae	Kashmir	Herb	June-Aug
75	*Corydalis clarkei Prain*	Papaveraceae	Kashmir	Herb	June-Aug

Table 1 Continued...

S. No.	Species	Family	Distribution	Life form / Status	Flowering period
76	*Corydalis diphylla subsp. murreeana (Jafri) Lidén*	Papaveraceae	Murree, Kashmir	Herb	June-Aug
77	*Corydalis govaniana var. swatensis Jafri*	Papaveraceae	Swat	Herb	June-Aug
78	*Corydalis pakistanica Jafri*	Papaveraceae	Hazara, Kashmir	Herb	June-Aug
79	*Corydalis stewartii*	Papaveraceae	Kashmir	Herb	May-June
80	*Corydalis thyrsiflora Prain*	Papaveraceae	Kashmir	Herb	June-Aug
81	*Cousinia bipinnata Boiss*	Compositae	Balochistan	Herb	April-July
82	*Cousinia chitralensis Rech. f.*	Compositae	Chitral	Herb	June-Aug
83	*Cousinia chitralensis Rech.f.*	Compositae	Chitral	Herb	June-Aug
84	*Cousinia mattfeldii Bornm*	Compositae	Chitral	Herb	Sep-Oct
85	*Cousinia quettensis Rech.f.*	Compositae	Balochistan	Shrub	June-July
86	*Delphinium bicarpellatum Qureshi & Ch*	Ranunculaceae	Chitral	Herb	May-July
87	*Delphinium lacostei Danguy*	Ranunculaceae	Chitral	Herb	May-July
88	*Delphinium nordhagenii Wendelbo*	Ranunculaceae	Chitral	Herb	July-Aug
89	*Delphinium roylei Munz*	Ranunculaceae	Chitral, Kashmir	Herb	July-Aug
90	*Delphinium vestitum Boiss*	Ranunculaceae	Hazara, murree	Herb	July-Aug
91	*Digitaria stewartiana Bor*	Poaceae	Kashmir	Herb	Aug-Sep
92	*Dionysia lacei (Hemsl. & Watt) Clay*	Primulaceae	Balochistan	Herb	Mar-April
93	*Draba pakistanica Jafri*	Brassicaceae	Chitral	Herb	May-June
94	*Draba tenerrima O.E.Schulz*	Brassicaceae	Kashmir	Herb	June-July
95	*Duthiea oligostachya (Munro ex Aitch.)Poa*	Poaceae	Kurram valley	Herb	May
96	*Echinops prionolepis Bornm. & Mattf*	Compositae	G.B.	Herb	July-Aug
97	*Echinops sulaimanii Rech.f.*	Compositae	Koh e Sulaiman	Herb	May-June
98	*Elymus borianus (Melderis) Cope*	Poaceae	KP	Herb	July-Aug
99	*Elymus dentatus (Hook.f.)*	Poaceae	G.B., KP, Kashmir	Herb	July-Aug
100	*Elymus dentatus (Hook.f.) Tzvelev*	Poaceae	KP, G.B., Kashmir	Herb	July-Aug
101	*Elymus jacquemontii (Hook. f.)*	Poaceae	Kashmir	Herb	July-Aug
102	*Elymus kuramensis (Meld.)*	Poaceae	Kurram valley	Herb	July-Aug
103	*Elymus russellii (Melderis) Cope*	Poaceae	G.B.	Herb	July-Aug
104	*Elymus stewartii (Meld)*	Poaceae	Kashmir	Herb	July-Aug
105	*Elymus stewartii (Melderis) Cope*	Poaceae	Kashmir	Herb	July-Aug
106	*Epilobium aitchisonii P.H.Raven*	Onagraceae	Kurram valley	Herb	Aug-Nov
107	*Epilobium chitralense P.H.Raven*	Onagraceae	Chitral	Herb	July-Sep
108	*Epilobium glaciale P.H.Raven*	Onagraceae	G.B., Kashmir	Herb	July-Sep
109	*Epilobium rhynchospermum Hausskn*	Onagraceae	Punjab, Kashmir	Herb	July-Sep
110	*Epimedium elatum C.Morren & Decne*	Berberidaceae	Pak & Kashmir	Herb	June-Aug
111	*Erigeron cedretorum Rech.f*	Compositae	G.B.	Herb	June-July
112	*Euphorbia micractina Boiss*	Euphorbiaceae	Kashmir	Herb	July-Aug
113	*Euphorbia talaina Radcl*	Euphorbiaceae	Balochistan	Herb	May-Aug
114	*Euphorbia thyrsoidea Boiss*	Euphorbiaceae	Swat	Herb	July-Oct
115	*Euphrasia aristulata Pennell*	Orobanchaceae	Kashmir	Herb	July-Sep
116	*Euphrasia densiflora Pennell*	Orobanchaceae	Swat, Kashmir	Herb	Aug-Sep
117	*Euphrasia flabellata Pennell*	Orobanchaceae	G.B., Kashmir	Herb	July-Aug
118	*Euphrasia foliosa Pennell*	Orobanchaceae	G.B., Kashmir	Herb	June-July
119	*Euphrasia incisa Pennell*	Orobanchaceae	G.B., Kashmir	Herb	July-Aug
120	*Euphrasia Kashmiriana Pugsley*	Orobanchaceae	G.B., Kashmir	Herb	June-Aug
121	*Euphrasia multiflora Pennell*	Orobanchaceae	Swat, G.B.	Herb	Aug-Sep
122	*Euphrasia omeri Qaiser & Siddiqui*	Orobanchaceae	Chitral, Kashmir	Herb	July-Aug

Table 1 Continued...

S. No.	Species	Family	Distribution	Life form / Status	Flowering period
123	*Euphrasia platyphylla Pennell*	Orobanchaceae	Naran, Kashmir	Herb	Aug
124	*Euphrasia qaiseri Siddiqui*	Orobanchaceae	Chitral, Kashmir	Herb	June-Aug
125	*Euphrasia remota Pennell*	Orobanchaceae	G.B., Kashmir	Herb	June-Aug
126	*Ferula stewartiana O.E.Schulz*	Apiaceae	Hassan abdal	Shrub	April-May
127	*Festuca debilis (Stapf)*	Poaceae	Kashmir	Grass	July-Aug
128	*Festuca hartmannii (Markgr.-Dann.)*	Poaceae	KP, Kashmir	Grass	July-Aug
129	*Festuca Kashmiriana Stapf*	Poaceae	Kashmir	Grass	July-Aug
130	*Festuca levingei Stapf*	Poaceae	Kashmir	Grass	July-Aug
131	*Gagea alii Levichev*	Liliaceae	Balochistan	Herb	March
132	*Gagea Balochistanica Levichev & Ali*	Liliaceae	Balochistan	Herb	Mar-April
133	*Gagea quettica Levichev & Ali*	Liliaceae	Balochistan	Herb	Feb-April
134	*Gagea rawalpindica Levichev & Ali*	Liliaceae	Hazara, Rawalpindi	Herb	March
135	*Gagea utriculosa Levichev*	Liliaceae	Punjab	Herb	March
136	*Galium Asperifolium Wall var. obovatum*	Rubiaceae	Hazara, Rawalpindi	Herb	July-Sep
137	*Galium ceratophylloides Hook.f*	Rubiaceae	Kahmir, murree	Herb	July-Sep
138	*Galium subfalcatum Nazim. & Ehrend*	Rubiaceae	Hazara	Herb	July-Aug
139	*Galium tetraphyllum Nazim. & Ehrend*	Rubiaceae	Hazara	Herb	July-Aug
140	*Gaultheria trichophylla Royle*	Ericaceae	Hazara, Kashmir	Shrub	May-July
141	*Gentiana kurroo Royle*	Gentianaceae	Hazara, Murree, Kashmir	Herb	Sep-Nov
142	*Gentianodes cachemirica (Decne.)*	Gentianaceae	Chitral, Kashmir	Herb	Sep-Nov
143	*Gentianodes lowndesii (Blatt.)*	Gentianaceae	Waziristan	Herb	Sep-Oct
144	*Geranium swatense Schönb*	Geraniaceae	Swat, G.B.	Herb	June-Aug
145	*Graellsia chitralensis O.E.Schulz*	Brassicaceae	Chitral	Herb	June-July
146	*Habenaria aitchisonii Rchb.f.*	Orchidaceae	Kurram valley	Herb	July-Aug
147	*Hackelia macrophylla I.M. Johnst.*	Boraginaceae	Kashmir	Herb	June-July
148	*Heliotropium Balochistanicum Kazmi*	Boraginaceae	Balochistan	Undershrub	May
149	*Heliotropium dasycarpum var. gymnostomum Kazmi*	Boraginaceae	Waziristan	Undershrub	April-May
150	*Heliotropium lamondiae Kazmi*	Boraginaceae	Balochistan	Undershrub	Mar-April
151	*Heliotropium ophioglossum C.B. Clarke*	Boraginaceae	Sindh	Herb	Dec-Jan
152	*Heliotropium remotiflorum Rech. f. & Riedl*	Boraginaceae	Makran	Herb	April-May
153	*Heliotropium ulophyllum Rech. f. & Riedl*	Boraginaceae	Loralai	Herb	May
154	*Hylotelephium pakistanicum*	Crassulaceae	G.B., Kashmir	Herb	Aug
155	*Impatiens edgeworthii Hook. f.*	Balsaminaceae	Hazara, Kashmir	Herb	July-Sep
156	*Impatiens meeboldii Hook. F*	Balsaminaceae	G.B., Kashmir	Herb	Mar-April
157	*Indigofera nephrocarpa Balf. f.*	Leguminosae	Makran	Herb	Mar-April
158	*Iris crocea Jacquem. ex R.C.Foster*	Iridaceae	Kashmir	Herb	June
159	*Iris Kashmiriana Baker*	Iridaceae	Kashmir	Herb	May
160	*Lagotis blatteri O.E.Schulz*	Plantaginaceae	Waziristan	Herb	March
161	*Launaea quettaënsis N.Kilian*	Compositae	Sindh, Balochistan	Herb	May-dec
162	*Lepechiniella microcarpa (Boiss.) Riedl*	Boraginaceae	Chitral, Kashmir	Herb	May
163	*Lespedeza elegans Cambess*	Leguminosae	KP, Kashmir	Shrub	Aug-Oct
164	*Lycium makranicum Schonebeck*	Solanaceae	Makran	Shrub	Sep-April
165	*Mattiastrum karakoricum Podlech & Sadat*	Boraginaceae	Hunza	Herb	July-Aug
166	*Megacarpaea polyandra Benth. ex Maden*	Brassicaceae	G.B., Kashmir	Herb	May-July
167	*Melanoseris decipiens var. pakistanica*	Asteraceae	G.B., Kashmir	Herb	July-Sep
168	*Melanoseris gilgitensisnsis (Bano, Roohi & Qaiser)*	Asteraceae	Haramosh, G.B.	Herb	Aug

Table 1 Continued...

S. No.	Species	Family	Distribution	Life form / Status	Flowering period
169	*Melanoseris Kashmiriana (Mamgain & R.R. Rao)*	Asteraceae	Kashmir	Herb	Sep-Nov
170	*Melanoseris stewartii (Roohi & Qaiser)*	Asteraceae	Kashmir	Herb	June-July
171	*Moluccella otostegioides Prain*	Lamiaceae	Sulaiman range	Undershrub	July-Sep
172	*Impatiens flemingii Hook. f.*	Balsaminaceae	Hazara, Kashmir	Herb	July-Sep
173	*Muhlenbergia duthieana Hack*	Poaceae	Punjab, KP	Herb	Aug-Oct
174	*Nanorrhinum ramosissimum subsp. pakistanicum G.R.Sarwar*	Plantaginaceae	Muzaffarbad, Swat Abbottabad, Makran Mansehra, Larkana,	Herb	March-Oct
175	*Nepeta adenophyta Hedge*	Lamiaceae	G.B.	Herb	Aug
176	*Nepeta glechomifolia (Dunn) Hedge*	Lamiaceae	Chitral	Herb	April-Oct
177	*Nepeta griffithii Hedge*	Lamiaceae	Malakhand	Herb	April-Oct
178	*Nepeta schmidii Rech.f.*	Lamiaceae	Chitral	Herb	April-Oct
179	*Olgaea thomsonii (Hook.f.)*	Compositae	Kashmir, G.B.	Herb	June-Aug
180	*Onobrychis stewartii Baker*	Leguminosae	Rawalpindi, hazara	Herb	June-Sep
181	*Orobanche clarkei Hook. f.*	Orobanchaceae	G.B., Kashmir	Herb	June-Aug
182	*Oxytropis birirensis Ali*	Leguminosae	KP	Herb	May
183	*Oxytropis chitralensis Ali*	Leguminosae	Chitral	Herb	June-July
184	*Oxytropis gloriosa Ali*	Leguminosae	Chitral	Herb	June-July
185	*Oxytropis sikaramensis (*Širj. *& Rech.f.)*	Fabaceae	Kurram valley	Herb	June-July
186	*Oxytropis staintoniana Ali*	Leguminosae	Chitral	Herb	May
187	*Paracaryum intermedium var. calathicarpum*	Boraginaceae	Balochistan	Herb	April
188	*Pedicularis elephantoides Benth.*	Orobanchaceae	Hazara, Kashmir	Herb	June-Sep
189	*Pedicularis Kashmiriana Pennell*	Orobanchaceae	Hazara, Kurram, G.B	Herb	July-Sep
190	*Pedicularis multiflora Pennell*	Orobanchaceae	Kashmir	Herb	July-Sep
191	*Pedicularis murreeana R.R. Mill*	Orobanchaceae	Rawalpindi, Murree	Herb	July-Oct
192	*Pedicularis numeniicephala T.Yamaz*	Orobanchaceae	Kashmir	Herb	June-Aug
193	*Pedicularis staintonii R.R.Mill*	Orobanchaceae	Chitral, G.B.	Herb	June-Aug
194	*Pimpinella hazariensis H. Wolff*	Apiaceae	Hazara	Herb	June-Aug
195	*Pimpinella stewartii Nasir*	Apiaceae	Chitral, Hazara,	Herb	June-Aug
196	*Poa stewartiana Bor*	Poaceae	Hazara, Kashmir	Grass	July
197	*Polygonum cashmiriense H.Gross*	Polygonaceae	Kashmir	Herb	June-Aug
198	*Polygonum cognatum subsp. Chitralicum*	Polygonaceae	Chitral	Herb	June-Aug
199	*Primula clarkei G.Watt*	Primulaceae	Kashmir	Herb	May-June
200	*Primula duthieana Balf. f. & W.W. Sm.*	Primulaceae	Hazara, Kashmir	Herb	July-Aug
201	*Primula obtusifolia Royle*	Primulaceae	Kashmir	Herb	June-July
202	*Psammogeton stocksii (Boiss.) Nasir*	Apiaceae	Balochistan	Herb	Mar-April
203	*Pseudomertensia chitralensis Riedl*	Boraginaceae	Chitral	Herb	May
204	*Pseudomertensia drummondii Kazmi*	Boraginaceae	G.B.	Herb	June-July
205	*Pseudomertensia efornicata (Rech. f. & Riedl)*	Boraginaceae	Chitral	Herb	June-July
206	*Pseudomertensia efornicata (Rech. f. & Riedl) Riedl*	Boraginaceae	Chitral	Herb	June-July
207	*Pseudomertensia elongata (Decne.) Riedl*	Boraginaceae	Hazara, Kashmir	Herb	June-Aug
208	*Pseudomertensia moltkioides (Royle ex Benth.)*	Boraginaceae	Kashmir	Herb	July-Aug
209	*Pseudomertensia nemorosa (A. DC.) Stewart & Kazmi*	Boraginaceae	Kashmir	Herb	April-May
210	*Pseudomertensia sericophylla (Riedl) Y.J. Nasir*	Boraginaceae	Kurram valley, Hazara	Herb	Aug
211	*Pseudomertensia trollii Stewart & Kazmi var. trollii*	Boraginaceae	Kashmir	Herb	May-June
212	*PseudPseudomertensia trollii var. harrissii Kazmiomertensia trollii Stewart & Kazmi var. trollii*	Boraginaceae	Chitral	Herb	June-July

Table 1 Continued...

S. No.	Species	Family	Distribution	Life form / Status	Flowering period
213	*Psudomertensia moltikioides var. primuloides*	Boraginaceae	Kashmir	Herb	July-Aug
214	*Psudomertensia moltikioides var. tanneri*	Boraginaceae	G.B.	Herb	May
215	*Psychrogeton chitralicus Grierson*	Asteraceae	Chitral	Herb	June-Aug
216	*Puccinellia minuta Bor*	Poaceae	Chitral	Herb	May-June
217	*Puccinellia stapfiana R.R.Stewart*	Poaceae	Kashmir	Herb	May-June
218	*Puccinellia thomsonii (Stapf) R.R.Stewart*	Poaceae	Kashmir	Herb	May-June
219	*Pulicaria balochistanica Qaiser & Abid*	Asteraceae	Quetta	Herb	Aug-Sep
220	*Pulsatilla wallichiana (Royle)*	Ranunculaceae	G.B., Kashmir	Herb	May-June
221	*Ranunculus karakoramicola Tamura*	Ranunculaceae	Baltistan	Herb	June-July
222	*Ranunculus membranaceus Royl*	Ranunculaceae	Kashmir	Herb	June-July
223	*Ranunculus munroanus J.R.Drumm. ex Dunn*	Ranunculaceae	Kashmir	Herb	April-June
224	*Ranunculus palmatifidus Riedl*	Ranunculaceae	Kashmir	Herb	June-July
225	*Ranunculus stewartii Riedl*	Ranunculaceae	Baltistan	Herb	May-June
226	*Rhodiola saxifragoides (Fröd.)*	Crassulaceae	GB, Kashmir	Herb	April-Sep
227	*Rhododendron afghanicum Aitch. & Hemsl*	Ericaceae	Kurram valley	Shrub	May-June
228	*Rostraria clarkeana (Domin) Holub*	Poaceae	Kashmir	Grass	June-July
229	*Rubia infundibularis Hemsl. & Lace*	Rubiaceae	Balochistan	Undershrub	May-Sep
230	*Ruellia sindica Ghafoor & Heine*	Acanthaceae	Sindh	Herb	Aug-Oct
231	*Rumex crispellus Rech. F*	Polygonaceae	Chitral, Hazara, Kurram	Herb	
232	*Rydingia limbata (Benth.)*	Lamiaceae	Jhelum, Rawalpindi, Kashmir	Shrub	April-May
233	*Saponaria subrosularis Rech. F*	Caryophyllaceae	Quetta	Herb	May
234	*Saxifraga afghanica Aitch. & Hemsl*	Saxifragaceae	Kurram valley	Herb	May-July
235	*Scaligeria stewartiana (Nasir) Nasir*	Apiaceae	Rawalpindi, Swat, Margallah	Herb	April-Oct
236	*Schoenoplectus rechingeri Kukkonen*	Cyperaceae	swat	Herb	June
237	*Scorzonera gageoides Boiss*	Compositae	Balochistan	Herb	April-July
238	*Scorzonera hondae Kitam*	Compositae	Hunza	Herb	June-Aug
239	*Scrophularia edelbergii subsp. pseudodeserti Grau*	Scrophulariaceae	Kurram valley	Shrub	July-Oct
240	*Scrophularia jafrii Khatoon & Qaiser*	Scrophulariaceae	G.B.	Herb	May-Sep
241	*Scrophularia nudata Pennell*	Scrophulariaceae	G.B.	Herb	June-Aug
242	*Scrophularia omeri Khatoon & Qaiser*	Scrophulariaceae	G.B.	Herb	Mar-June
243	*Scrophularia rodinii Hamidullah*	Scrophulariaceae	Landi kotil,	Herb	Mar-June
244	*Scrophularia scabiosifolia subsp. stewartii (Pennell) Qaiser & Khatoon*	Scrophulariaceae	Baltistan	Herb	May-Aug
245	*Scutellaria chamaedrifolia Hedge & A.J.Paton*	Lamiaceae	Chitral	Herb	April-June
246	*Scutellaria swatensis Murata*	Lamiaceae	Chitral	Herb	June-Oct
247	*Seriphidium quettense (Podlech) Y.R.Ling*	Compositae	Quetta	Shrub	May-Nov
248	*Sida spinosa L*	Malvaceae	Jhelum, Thatta, Pind dadankhan	Undershrub	May-June
249	*Silene kunawarensis Benth*	Caryophyllaceae	Kashmir, G.B.	Herb	July-Sep
250	*Silene longiSepala Nasir*	Caryophyllaceae	Chitral	Herb	May
251	*Silene staintonii Ghaz*	Caryophyllaceae	Chitral	Herb	May
252	*Sorbaria tomentosa (Lindl.) Rehder*	Rosaceae	Chitral	Shrub	July-Nov
253	*Sorbus cashmiriana Hedl*	Rosaceae	Kashmir	Herb	May-June
254	*Sorbus G.B.ana McAll*	Rosaceae	G.B.	Tree	Oct
255	*Sorbus rosea McAll*	Rosaceae	G.B.	Tree	Oct
256	*Spiraea brahuica Boiss.*	Rosaceae	Loralai, Quetta, Ziarat	Shrub	July
257	*Spiroseris phyllocephala Rech.f.*	Compositae	Kohat	Herb	May-June

Table 1 Continued...

S. No.	Species	Family	Distribution	Life form / Status	Flowering period
258	*Stipa chitralensis Bor*	Poaceae	Chitral	Herb	May
259	*Syringa emodi Wall. ex Royle*	Oleaceae	Hazar, Changla gali, Ghora gali,	Shrub	May-July
260	*Syzygium cumini (L.) Skeels*	Myrtaceae	Rawalpindi, sub Himalayan	Tree	March-may
261	*Tamarix pakistanica Qaiser*	Tamaricaceae	Thatta, Hyderabad	Shrub	Jan-Oct
262	*Tamarix salina Dyer*	Tamaricaceae	Sindh, Punjab	Shrub	January
263	*Tanacetum baltistanicum Podlech*	Asteraceae	G.B., Hunza	Shrub	Aug-Sep
264	*Tanacetum chitralense (Podlech) K.Bremer & Humphries*	Asteraceae	Chitral	Shrub	July-Aug
265	*Tanacetum chitralense (Podlech) K.Bremer & Humphries*	Asteraceae	Chitral	Herb	July-Aug
266	*Tanacetum pakistanicum Podlech*	Asteraceae	Swat	Herb	July-Aug
267	*Tanacetum stoliczkae (C.B.Clarke) R.Khan*	Asteraceae	Kashmir	Herb	July-Aug
268	*Taraxacum baltistanicum Soest*	Asteraceae	Baltistan	Herb	May-July
269	*Taraxacum canum Soest*	Asteraceae	G.B., Hazara	Herb	April-June
270	*Taraxacum gilgitensis Abedin*	Asteraceae	G.B, Hunza	Herb	June
271	*Taraxacum gulmargense Soest*	Asteraceae	Kashmir	Herb	June-Aug
272	*Taraxacum ladakense Soest*	Asteraceae	Chitral, Kashmir	Herb	July-Sep
273	*Taraxacum longirostre Schischk.*	Asteraceae	Chitral	Herb	July-Aug
274	*Taraxacum mansehracum Abedin*	Asteraceae	Mansehra	Herb	May-June
275	*Taraxacum melleum Soest*	Asteraceae	Baltistan	Herb	July
276	*Taraxacum nagaricum Soest*	Asteraceae	G.B.	Herb	July-Sep
277	*Taraxacum nasiri Soest*	Asteraceae	Chitral	Herb	July-Aug
278	*Taraxacum nigrum Soest*	Asteraceae	G.B.	Herb	July-Aug
279	*Taraxacum obtusum (Soest) R.Doll*	Asteraceae	Chitral	Herb	June-July
280	*Taraxacum pakistanicum Soest*	Asteraceae	Kurram Valley	Herb	April-May
281	*Taraxacum pseudotenebristylum Soest*	Asteraceae	Chitral	Herb	June July
282	*Taraxacum pubens Soest*	Asteraceae	G.B.	Herb	July-Aug
283	*Taraxacum qaiseri Abedin*	Asteraceae	G.B.	Herb	July-Aug
284	*Taraxacum quettacum Abedin*	Asteraceae	Quetta	Herb	May-June
285	*Taraxacum rawalpindicum Abedin*	Asteraceae	Rawalpindi	Herb	May-June
286	*Taraxacum stewartii Soest*	Asteraceae	Kashmir	Herb	July-Aug
287	*Taraxacum tricolor Soest*	Asteraceae	Chitral	Herb	June-Aug
288	*Taraxacum tricolor Soest*	Asteraceae	Kashmir	Herb	Aug
289	*Taraxacum wendelboanum Soest*	Asteraceae	Chitral	Herb	June-Aug
290	*Taraxacum xanthophyllum G.E.Haglund*	Asteraceae	G.B.	Herb	July
291	*Tephrosia rechingeri Ali*	Fabaceae	Quetta	Herb	May
292	*Tephrosia shamimii Ali*	Fabaceae	Quetta	Herb	Sep
293	*Teucrium stocksianum subsp. patulum (Hedge & Lamond) Rech.f.*	Lamiaceae	Quetta	Herb	May-Aug
294	*Teucrium stocksianum var. patulum Hedge & Lamond*	Lamiaceae	Quetta	Herb	May-Aug
295	*Thalictrum secundum Edgew*	Ranunculaceae	Hazara	Herb	July-Aug
296	*Thalictrum secundum var. hazaricum*	Ranunculaceae	Hazara, Dunga Gali	Herb	July-Aug
297	*Thesium himalense Royle*	Santalaceae	Hazara, Kashmir	Herb	April-June
298	*Tricholepis infundibuliformis Dittrich*	Asteraceae	Basham, Patan	Undershrub	July-Aug
299	*Trigonella podperae (Sirj.) Vassilcz*	Leguminosae	Kashmir	Herb	July-Aug
300	*Vincetoxicum arnottianum (Wight) Wight*	Apocynaceae	Hazara, Kashmir	Herb	April-July
301	*Vincetoxicum arnottianum (Wight) Wight*	Rubiaceae	Hazara, Rawalpindi, Kashmir	Tree	May-June

S. No.	Species	Family	Distribution	Life form / Status	Flowering period
302	*Wendlandia puberula DC.*	Rubiaceae	Hazara, Rawalpindi, Kashmir	Tree	May-June
303	*Xylanthemum macropodum (Hemsl. & Lace) K.Bremer & Humphries*	Compositae	Balochistan	Shrub	May-June
304	*Pinus gerardiana Wall. ex D. Don*	Pinaceae	Chitral, Kalam, Balochistan	Tree	May-June
305	*Sorbus G.B.ana McAll.*	Rosaceae	G.B.	Tree	Oct
306	*Sorbus rosea McAll.*	Rosaceae	G.B.	Tree	Oct

Source: Flora of China Editorial Committee. (n.d.). eFloras.org. Retrieved March 20, 2021, from http://www.efloras.org/; The Plant List, "The Plant List - A Working List of All Plant Species," Accessed: May. 29, 2021. [Online]. Available: http://www.theplantlist.com/.; Plants of the World Online, "Plants of the World Online - Kew Science," Accessed: Jun. 12, 2021. [Online]. Available: http://www.powo.science.kew.org/.)

3. RESULTS

During present investigation, a total of 306 plant species categorized into 126 genera and 50 families were found endemic to Pakistan **(Fig. 2)**. The most dominant family is Astereaceae (n= 38 species), followed by Boraginaceae, Poaceae and Ranunculaceae (n = 22, 22 and 22 species respectively). The family with least number of species is Lamiaceae (n = 11 species) (**Fig. 3**).

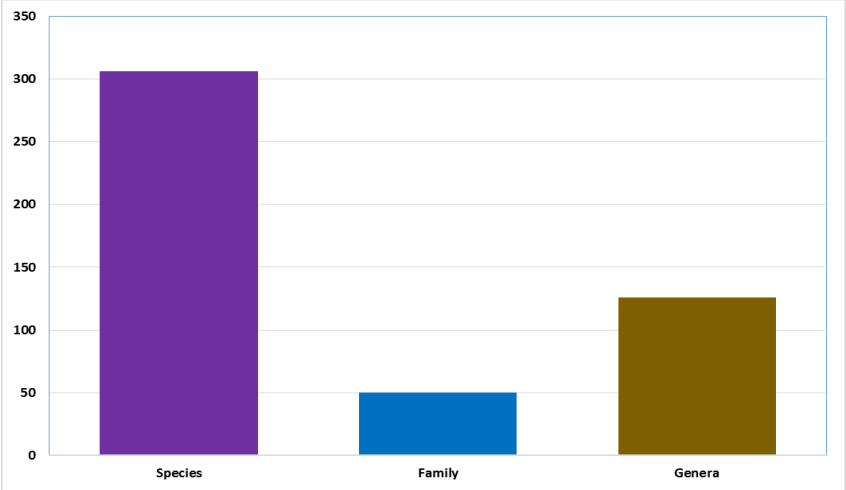

Fig. 2. Total number of endemic plant species of Pakistan and their distribution in genera and families.

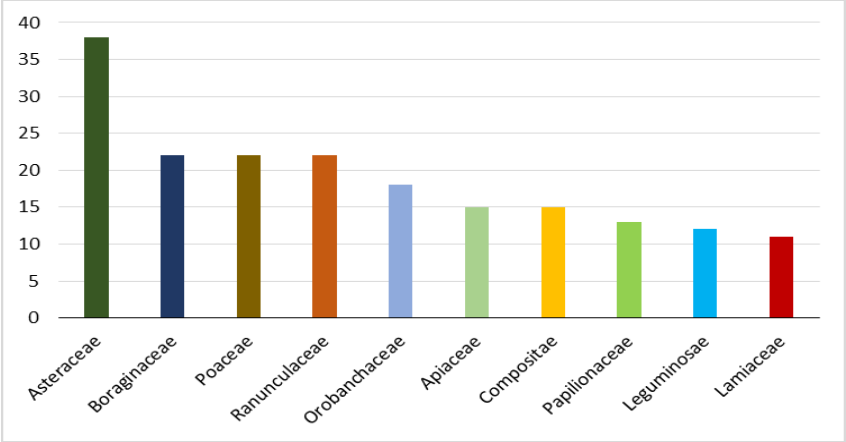

Fig. 3. Dominant families presenting the number of endemic plant species in Pakistan.

Data analysis of life-form indicates that herbs constitutes 80 % (n = 243 species), followed by shrubs 11 % (n = 32) and tree 3 % (n = 10). While, undershrub constitutes 4 % (n = 13) and grasses 2 % (n = 6) **(Fig. 4)**. The study reveals that, the most dominant genus is *Taraxacum* (n = 23 species), followed by *Astragalus* (n = 13 species), *Pseudomertensia* (n =12 species), and *Euphrasia* (n = 11 species). The genera *Berberis, Bupleurum, Corydalis* and *Elymus* represents (n =8 species) each **(Fig. 5)**. While seventy eight genera represents only single species and fourteen genera represents two species each.

The study revealed that most endemic plant species were from Khyber Pakhtunkhwa (KP) Province (n = 148 species, 37 %), followed by Azad Jammu and Kashmir (AJK) (n = 110 species, 28 %). The GB represents (n=59 species, 15 %), Balochistan (n=42 species, 11 %), and Punjab (n=26 species, 7 %). The least number of endemic species were recorded from Sindh (n=10, 3 %)

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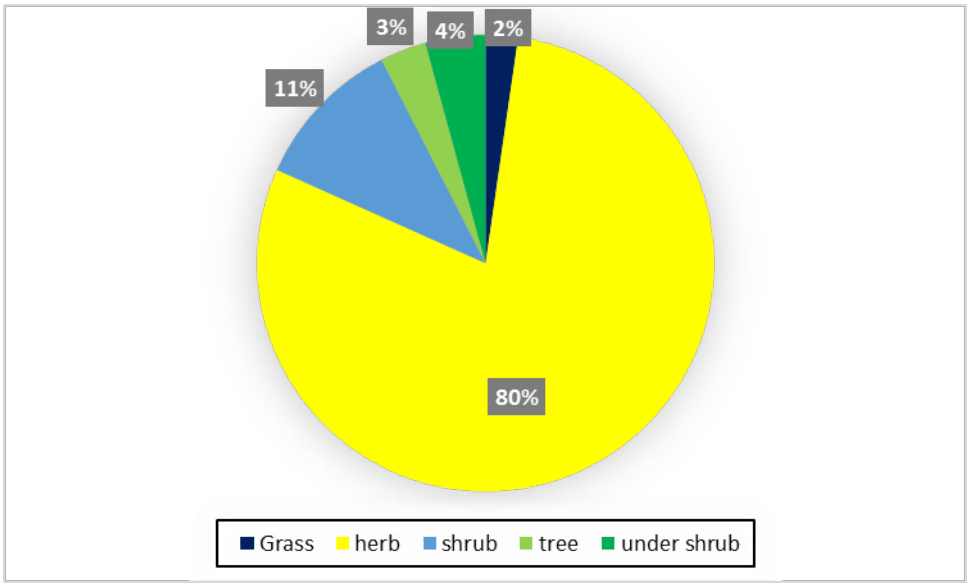

Fig. 4. Life forms / habitat status of endemic plant species of Pakistan

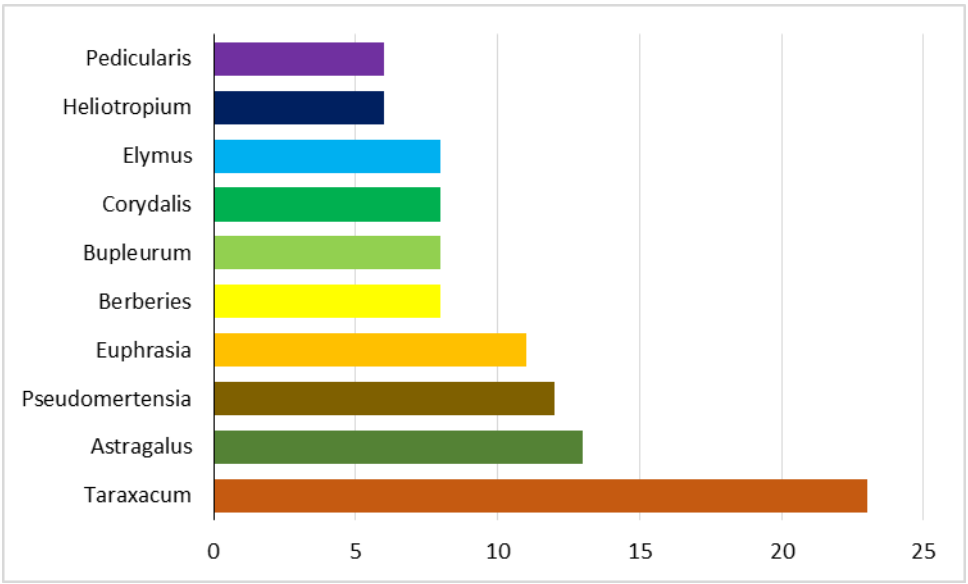

Fig. 5. Dominant genera showing number of endemic species in the study area.

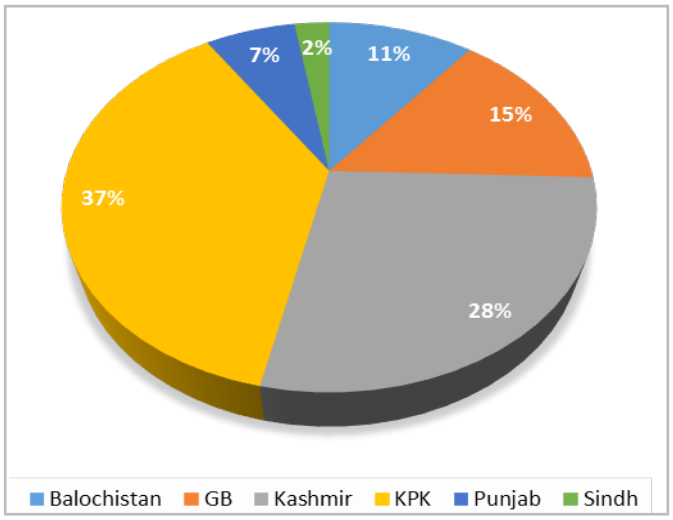

Fig. 6. Endemic richness in different areas of Pakistan.

(Fig. 6).

4. DISCUSSION

The catalogue of endemic plant species and datasets is not available to capture the comprehensive information regarding the endemic plant species in Pakistan. Previously some studies [13-17, 29] reported the endemics ranges. These studies represent only 5 % of Pakistan's endemic flora. In this scenario, this study conducted to focus on the documentation of endemic flora of Pakistan. This study of plant species endemism will benefit groundwork for future research into the protection of endemic, rare, indigenous and natural forest vegetation in Pakistan. The study found that northern Pakistan is rich in endemism, and majority of endemic plant species are distributing in mountainous areas of Chitral, Gilgit and Kashmir. The results also revealed that most of the endemic plant species are located above 1000 m, which indicated that altitude is one of the important factor in endemism **(Fig. 7)**.

Three hundred and six endemic species were found in the research region, the major genera being *Taraxacum, Astragalus,* and *Pseudomertensia*, with the largest families being *Asteraceae* and *Boraginaceae*. Previous study conducted by Majid *et al* [10] also reported Boraginaceae as largest family in Himalayan region in terms of endemism. The study area's distribution of more than 300 endemic species indicates the area's richness and value as a source of biodiversity. The complex genus *Pseudomertensia* has roughly 2000 species worldwide [30]. Herbs substantially outnumbered shrubs and trees among endemic taxa. Richness of herbaceous flora over trees and shrubs demonstrates that herbaceous flora has undergone more speciation than woody species [31, 32]. Our findings concur with several other findings [33-36]. Ten trees are said to be endemic when looking at endemism at the national level.

Berberis parkeriana, Otostegia limbata, Aegopodium burttii, Caltha alba var. alba, Scaligeria indica, Clinopodium hydaspidis, Pimpinella stewatii, and *Alchemilla cashmeriana* were the species with the greatest geographic distribution. Many endemics can have enormous populations inside their distributional zones, but they are unable to expand outside of such zones [37]. The *Otostegia limbata* is widely found throughout Pakistan and even at the borders with Afghanistan and India, however it has never been described from these two nearby nations [38, 39]. The fact that the environment and rock strata are suitable and exclusive to this country may be one factor.

5. CONCLUSION

The current study sheds light on Pakistan's endemism situation. An essential component of conservation strategy is accurate cataloguing of endemic plant species. In comparison to neighboring nations, Pakistan has a notably high number of endemic plants. However, Pakistan has a heavy burden for

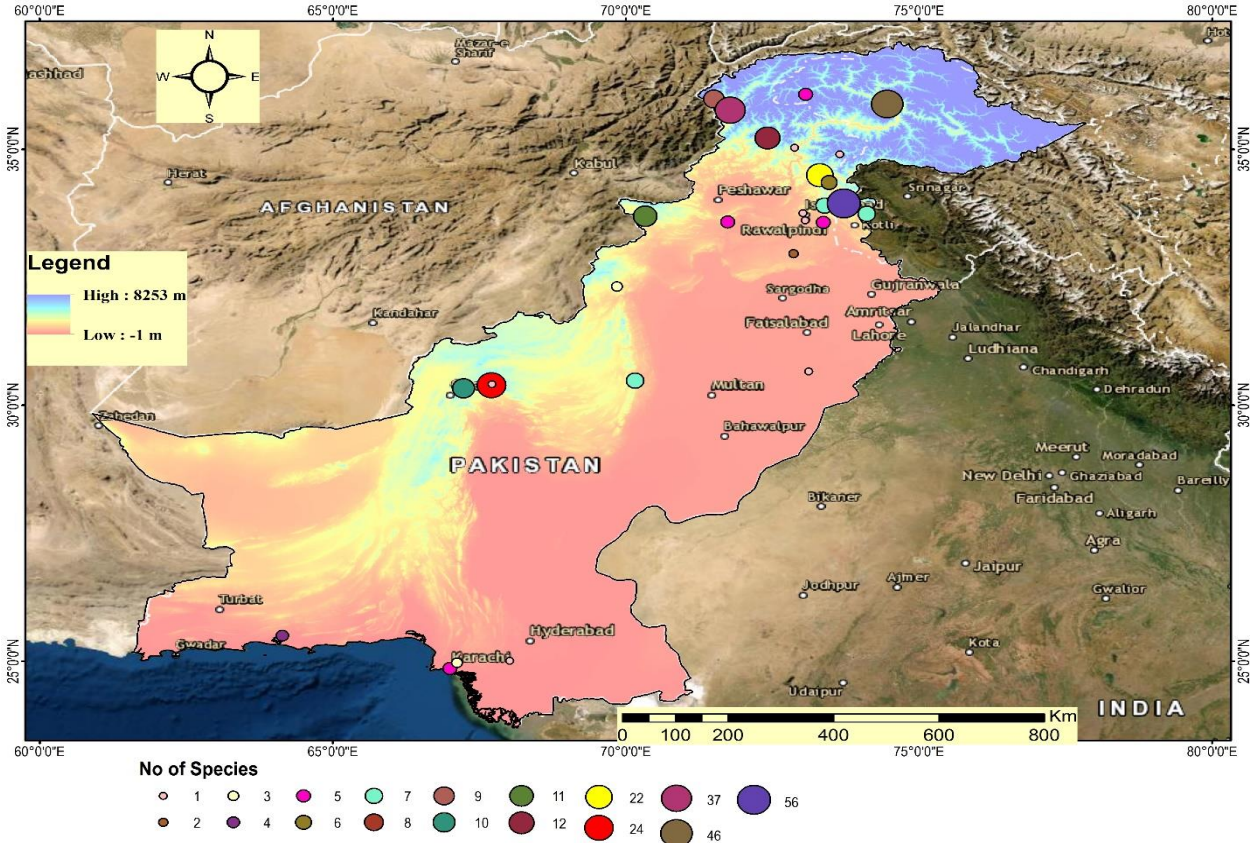

Fig. 7. Map illustrating the occurrence of three hundred and six endemic plant species in Pakistan. The size and colour of bubble represents the number of species at a particular area.

their preservation since these unique taxa support regional biodiversity. Unfortunately, there is still a huge gap in our understanding and evaluation of the taxa's conservation status. Furthermore, the current study can provide as a springboard for the systematic answer to this issue. Our findings show that mountainous regions in Pakistan should be given priority for conservation because they are home to the majority of Pakistan's endemic plant species. To help guide conservation efforts and the creation of protected areas. The current study sheds light on Pakistan's endemism situation. Further research that takes into consideration population levels and threats is also required.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest concerning research, authorship, and/or publication of this article.

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Research Article

Antibacterial Activities and Chemical Characterization of the Secondary Metabolites of *Aspergillus terreus*

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Abstract: The present study aims to assess the biological impact of secondary metabolites isolated from *Aspergillus terreus* that have been isolated from the soil, the fungus was grown on a fermentation medium to produce secondary metabolism, and the fungal extract extracted from the secondary metabolite was purified and chemically characterized. Antimicrobial activities of bioactive compounds extracted from the secondary metabolite of *Aspergillus terreus* isolate were tested against five types of human pathogenic bacteria*, Escherichia coli, Klebsiella* spp*., Staphylococcus aureus, Pseudomonas* spp*.,* and *Proteus* spp*.* The cytotoxicity was tested against a human blood solution. purification and chemical identification were carried out on a crude extract of *A. terreus* using TLC, GC–MS, and NMR data (1H proton and 13C carbon) analysis. The *A. terreus* secondary metabolite extract was effective against all isolated bacterial strains. The biocompatibility test showed no cytotoxic effect against a human blood solution used in different concentrations. One fraction was purified and identified as a novel compound: 2-(4-hydroxyphenyl) tetrahydro-3,4-furan diol. The results from a GC–MS analysis showed 18 peaks of the ethyl acetate extract of *A. terreus* metabolites, and the major compounds were bis(2-ethylhexyl) phthalate (47.60 %), n-hexadecanoic acid (16.41 %) and dodecamethyl-cyclohexasiloxane (9.79 %). According to the outcomes of this study, *A. terreus* can produce secondary metabolites that show potent activity against human pathogenic bacteria and are dependent sources for new secondary metabolites.

Keywords: *Aspergillus terreus* Secondary Metabolites, Antibacterial Activities, Chemical Identification

1. INTRODUCTION

Soil is a huge home of microbial types. Most of the bioactive secondary metabolites used today, including antimicrobial compounds, were isolated from soil microorganisms. Fungi are the most biotechnologically useful organisms. They are a critical source for producing enzymes, organic acids, and food additives besides antibiotics [1].

The genus *Aspergillus* is saprophytes spread in the natural environment [2]. Mycologists emphasize discovering *Aspergillus* species due to its simplicity of cultivation in media and economic application in addition to the notable ability in producing unique metabolites, recently about 315 compounds were documented [3, 4].

A. terreus highly contributes to the production of many novel chemical compounds as secondary metabolites, and these compounds are characterized by promising bioactivities, such as the anti-tumor compound beauvericin [5], and lovastatin, which has been found to lower cholesterol in humans [6]. Besides lovastatin, A. terreus produces many bioactive compounds, such as sulochrins, terretonines, asterriquinones, and butyrolactones, some of which have antimicrobial activities [7]. Also recently recorded the fungus production of new secondary metabolites including "terrein, aspulvinone E, flavipesolide C, butyrolactone II, and butyrolactone I, have been reported to exhibit a variety of bioactivities including anti-proliferative, anti-inflammatory, and antioxidant activities" the identified terrain.

The compound showed high antifungal activity against the yeast *Cryptococcus neoformancysteine* [8]. Secondary metabolites are necessary for the development of new pharmaceuticals. This is

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particularly urgent in the case of antibiotics due to the rapid spread of drug-resistant pathogenic bacteria, which pose dangerous clinical challenges in the treatment of infectious diseases [2, 9].

In 2019, more than 5 million deaths were recorded due to the generation of resistant bacterial strains [10]. This frightening number encouraged the discovery of a new antibiotic with a novel mode of action so the main purpose of this study is to evaluate the antibacterial activity of secondary metabolites extract of *A. terreus*, and isolate and identify the chemical compounds of crude extracts of *A. terreus.*

2. MATERIALS AND METHODS

2.1 Isolation of Fungus

Soil samples were collected in sterile polyethylene bags from different places in Basrah City, South Iraq. Samples were then transformed into the laboratories at Basrah University. A serial dilution method was used to isolate fungi. Potato carrot agar medium (PCA, 20 g potatoes, 20 g carrots, and 20 g Bacto agar (Difco) per L of distilled water containing 25 ppm chloramphenicol) was used for pre-isolation. Three serial dilutions of soil were used 10^{-1},10^{-2} and $10^{-3.}$ PCA was poured into petri dishes containing 1 ml of each dilution. All cultured petri dishes were incubated at 25 °C for 7 days with periodic examination [11].

2.2 Identification of Fungus

Morphological characteristics of *Aspergillus* spp., including colony colour, conidial shape, length, width, and conidiophore shape and length were examined on malt extract agar medium (MEA, from Oxoid LTD company, the country producer in England), where isolates were grown for 5-7 days at 12 h photoperiod; identification carry out under dissecting and compound microscopes [12]. Isolates were then cultured in a slant culture of MEA for 7 days at 25 °C and maintained at 4 °C for further experiments.

2.3 Fermentation Medium

A potato dextrose broth (PDB) from Oxoid LTD company, the country producer in England was used as a fermentation medium. The medium was sterilized in an autoclave for 15 min. Three discs of growing mycelia measuring 5 mm from activated 5 days ago *A. terreus* isolate colony were taken from the MEA medium and placed in PDB, the broth was incubated at 25 °C for 21 days [13].

2.4 Extraction

The fungal growth in PDB was filtered with Whatman Grade 1 filter papers from the supernatant remaining after centrifugation (6000 rpm/min for 10 min). Hydrochloric Acid (HCl) of 2N was used to regulate the pH to 3 to convert soluble compounds to crystallized form and facilitate the extraction of compounds The filtrate of the fermentation medium was extracted by organic solvent ethyl acetate with a ratio (1:1 vol). The organic layer was gathered and dried at room temperature. The dried extract was kept in a glass vial at 4 °C until use [13].

2.5 Antibacterial Activity

The antibacterial action of the extract was tested on 10 clinical bacterial strains: three strains of *Staphylococcus aureus*; two strains of *Klebsiella*; two strains of *Pseudomonas aeruginosa;* two strains of *Proteus*; and one strain of *Escherichia coli*. These strains were gained from several sources (stool, wound infections, urine, skin lesions) of patients admitted to Al-Fayhaa Hospital in Basrah. All the collected samples were processed upon receipt in the Al-Fayhaa laboratory and cultured in appropriate media [14].

The separated bacteria were classified into Gram-negative and Gram-positive classes, and biochemical tests were carried out by using VITEK2 automated system. A 100 µg/ml bacterial suspension for all isolates was set and balanced to 0.5 McFarland standard. The Mueller–Hinton agar was used to measure the activity of the fungal extract. A nine-millimeter diameter pore was made using a cork borer with triplicate and control plates. All petri dishes were incubated at 37 °C overnight. The inhibition zone was measured in millimeters. The dishes of each extract were examined for the minimum inhibitory concentration (MIC) [14, 15].

2.6 Determination of Minimum Inhibitory Concentration(MIC)

The susceptibility of five bacterial isolates *S. aureus* III, *Klebsiella* spp, *E. coli, P. aeruginosa* II, and *Proteus* spp. II was investigated (MIC) against fungal extract using four different concentrations 500, 250, 100, and 50 µg/ml. The results showed different MIC accounts (Table 2; Figure 3) explain MIC values.

2.7 Biocompatibility test

A. terreus secondary metabolite extract was applied to conduct a biocompatibility test with fresh human blood according to Wang *et al.* [16] four concentrations of *A. terreus* metabolite extract were prepared (10, 50, 100, and 200) µg/ml, and 100 µl was added from the studying concentrations to all tubes that contain blood solution. Test tubes were kept at weather temperature and monitored to notice the development of turbidity.

2.8 Isolation, Purification, and Identification of Bioactive Secondary Metabolite

2.8.1. GC–MS Analysis

GC–MS analysis was carried out on *A. terreus* secondary metabolite extract at Basrah Oil Company, Nahr Bin Omar Laboratory. An Agilent Technologies 7890B GC system was used coupled to an Agilent Technologies 5977A MSD with an EI ion source, using HP-5MS 5 % phenyl methyl siloxane (30 m × 250 um × 0.25 mm). The carrier gas was helium at a 1 ml/min constant flow mode and a purge flow of 3 ml/min. The oven temperature was set at 40 ºC, held for 5 min, then the temperature was held at 280 ºC for the remaining 20 min. The mass spectrometer applied an ion source temperature of 230 ºC with a 1,562 (N2) scan speed. The electron ionization was obtained over the mass range of 35–650 m/z. Data were run through the NIST 2014 library database [17].

2.8.2. Purification

Preparative thin-layer chromatography, PTLC, (silica gel, aluminum-backed plates, Merck Art 5554) was carried out for the *A. terreus* secondary metabolite extract. The plates were then developed in the selected solvent system ethyl acetate hexane 4:1, and viewed under UV light and marked. The plates were also observed using an anisaldehyde spray reagent (97 % cold methanol; 2 % sulphuric acid; 1 % p-anisaldehyde) following heating. The marked portion of the TLC plate was then scratched off and dissolved in a similar solvent used for the plate [18].

2.8.3. Nuclear Magnetic Resonance Spectroscopy

For purified compounds of *A. terreus* secondary metabolite extract, 1H, ^{13}C, and 2D NMR spectra were performed on a Bruker AVANCE III NMR spectrometer operating at 125.76 MHz for ^{13}C and 500.13 MHz for 1H at the University of Surrey, Department of Chemistry, UK. The spectra were recorded in deuterated methanol (CD_3OD). The CD_3OD was referenced at δC 49.15 in the ^{13}C NMR spectrum and at δH 4.87 in the ^{1}H NMR spectrum

3. RESULTS

3.1 Fungal Appearance

Macroscopic features for one isolate of *A. terreus* showed a brown colony with dense growth and sporulation on MEA medium at 25 °C (Figure 1b). Microscopic examination for 100 spores of *A. terreus* showed a founding of a hyaline conidiophore carry vesicle (conidial head) with biseriate of stigmata that held small and globose yellow conidia (Figure 1a).

3.2 Biological Activity

The results of the antimicrobial activity of *A. terreus* secondary metabolite extract against 10 different bacterial isolates under study were very encouraging. Both gram-positive and gram-negative bacteria exhibited altitude inhibition rates all results were significantly different except with *Staphylococcus aureus* II and *Klebsiella* spp. I, *Pseudomonas aeruginosa II* and *Escherichia coli*, *Proteus* spp I and *Proteus* spp II (Table 1; Figure 2)

3.3 Determination of Minimum Inhibitory Concentration

The susceptibility of five bacterial isolates *Staphylococcus aureus* III, *Klebsiella* spp.,

Table 1. Antimicrobial activity of *A. terreus* secondary metabolite extract against bacterial test organism.

Bacteria	**Zone of inhibition in (mm)**
Staphylococcus aureus I	12
Staphylococcus aureus II	16
Staphylococcus aureus III	25
Klebsiella spp I	16
Klebsiella spp II	28
Pseudomonas aeruginosa I	7
Pseudomonas aeruginosa II	12.5
Proteus spp I	2
Proteus spp II	2
Escherichia coli	12.5

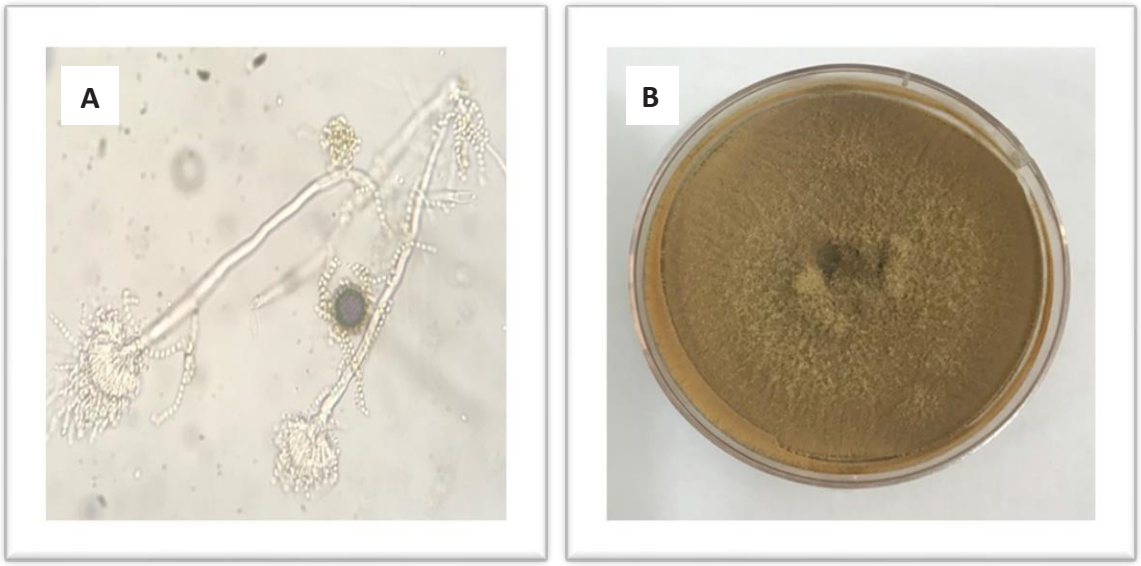


Fig. 1. (A) Conidia of *A. terreus,* (B) Colony

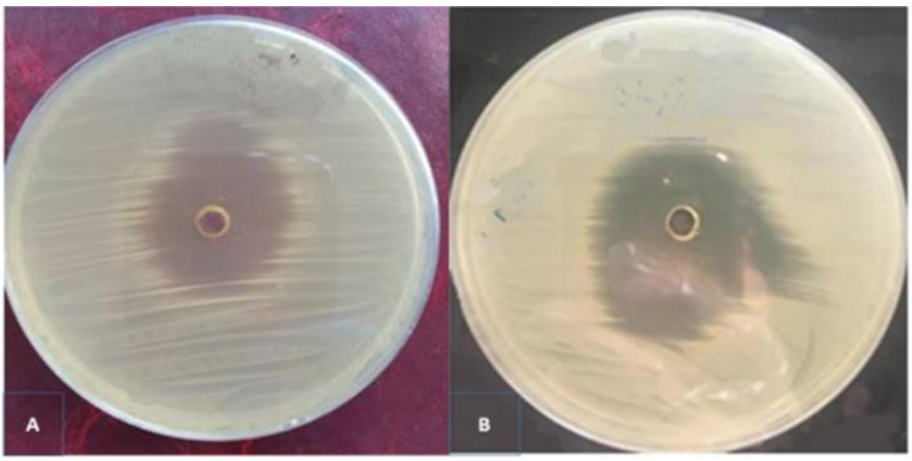


Fig. 2. The bacterial inhibitory zone by *A. terreus* extract. (A) *Staphylococcus aureus* (B) *Klebsiella* spp.

Escherichia coli, Pseudomonas aeruginosa II, *Proteus* sp. II was investigated against fungal extract using four different concentrations (500, 250, 100, 50). The results show different MIC values (Table 2; Figure 3) explain MIC values.

3.3 Biocompatibility test

Biocompatibility test explained there was no turbidity creation in all studying extract concentrations found in blood solution tubes this indicates the fungal extract is not toxic (Figure 4).

3.4 GC–MS analysis

GC–MS analysis of *A. terreus* secondary metabolite extract showed 18 peaks (Figure 5; Table 3). The major compounds were bis(2-ethylhexyl) phthalate (47.60 %), n-hexadecanoic acid (16.41 %), dodecamethyl-cyclohexasiloxane (9.79 %), in addition to other fatty acid and amino acid compounds, such as octadec-9-enoic acid (1.14 %) and cysteine (1.62 %).

3.5 Purification and NMR Spectra

PTLC of *A. terreus* extract yielded four fractions (A, B, C, D). Only fraction C was identified using NMR to be unreported compounds 2-(4-hydroxyphenyl) tetrahydro-3,4-furandiol was obtained as a yellow oil (Figure 6). The ^{13}C NMR and DEPT spectrum together with the analysis of HSQC, COSY, and HMBC experiments showed the presence of one methylene, 7 methines, and 2 quaternary carbons, allowing the molecular formula $C_{10}H_{12}O_4$ to be assigned to compound 2-(4-hydroxyphenyl) tetrahydro-3,4-furandiol (Table 4). The ^{1}H NMR spectrum of the compound showed signals attributed to para phenyl moiety at δ_H 7.64 (2H, d, J = 8.0Hz, H 2", H-6"), δ_H 8.18 (2H, J= 2.0, 7.0

Fig. 3. Different inhibition zones according to different concentrations of *A. terreus extract*. MIC for *Klebsiella* spp

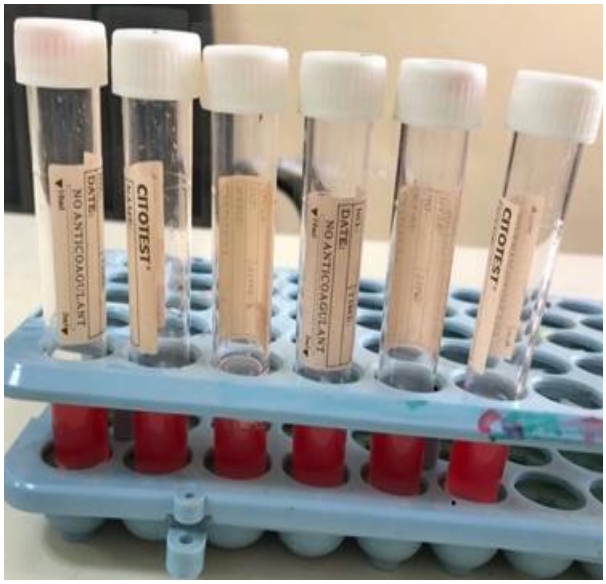


Fig. 4. Biocompatibility test display that has no formation of turbidity in blood solution tubes with different concentrations of the used extract.

Table 2. Minimum Inhibitory Concentration rate of *A. terreus* secondary metabolite extract against bacterial test organism mg/ml. (Yellow color refers to MIC values)

Bacteria	500 µg/ml	250 µg/ml	100 µg/ml	50 µg/ml
Staphylococcus aureus III	+	+	-	-
Klebsiella spp I	+	+	+	-
Escherichia coli	+	+	-	-
Pseudomonas aeruginosa II	+	-	-	-
Proteus spp II	-	-	-	-

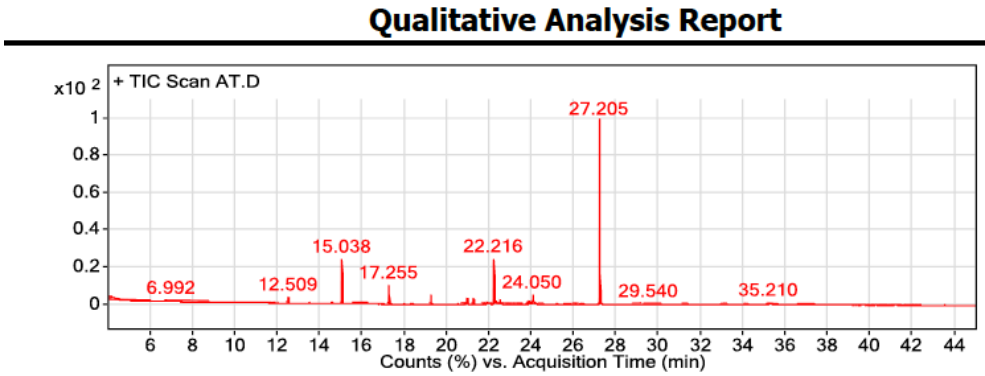
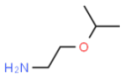
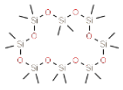
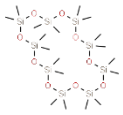
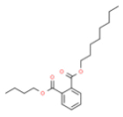
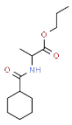
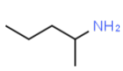
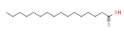
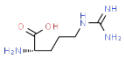
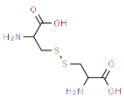
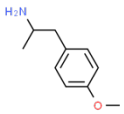
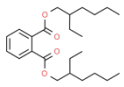
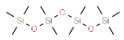
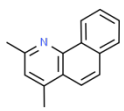


Fig. 5. GC–MS chromatogram of A. terreus secondary metabolite extract.

Table 3. Chemical Composition of *A. terreus* secondary metabolite extract by GC–MS.

S. No.	RT	Area%	Library/ID	CAS	Formula	Synonyms	M.W	Chemical structure
1.	12.509	1.64	2-Propan-2-yloxyetha-namine	81731-43-3	$C_5H_{13}NO$	2-Isopropoxyethana-mine	103.16	
2.	15.038	9.79	Dodecamethylcyclo-hexasiloxane	540-97-6	$C_{12}H_{36}O_6Si_6$	Cyclohexasiloxane	444.92	
3.	17.255	4.18	Tetradecamethylcyclo-heptasiloxane	107-50-6	$C_{14}H_{42}O_7Si_7$	Cyclomethicone 7	519.078	
4.	19.235	1.98	Hexadecamethyl cy-clooctasiloxane	556-68-3	$C_{16}H_{48}O_8Si_8$		593.24	
5.	20.958	1.51	Octadecamethyl cy-clononasiloxane	556-71-8	$C_{18}H_{54}O_9Si_9$		667.4	
6.	21.243	1.91	1,2-Benzenedicarbox-ylic acid, butyl octyl ester	84-69-5	$C_{20}H_{30}O_4$	Phthalic acid, diiso-butyl ester	334.44	
7.	21.716	1.30	propyl 2-(cyclohex-anecarbonylamino) propanoate	1000314-17-4	$C_{13}H_{23}NO_3$	Propyl N-(cyclohexylcarbonyl) alaninate	241.33	
8.	21.896	2.10	2-Pentanamine	63493-28-7	$C_5H_{13}N$	1-methyl- butylamine	87.16	

S. No.	RT	Area%	Library/ID	CAS	Formula	Synonyms	M.W	Chemical structure
9.	22.216	16.41	n-Hexadecanoic acid	57-10-3	$C_{16}H_{32}O_2$	palmitic acid	256.429	
10.	22.487	1.04	L-arginine	74-79-3	$C_6H_{14}N_4O_2$	(2S)-2-amino-5-(diaminomethylideneamino)pentanoic acid	174.20	
11.	23.842	1.14	octadec-9-enoic acid	112-80-1	$C_{18}H_{34}O_2$	Oleic Acid	282.5	
12.	23.898	1.62	cystine	56-89-3	$C_6H_{12}N_2O_4S_2$	2R)-2-amino-3-[[(2R)-2-amino-2-carboxyethyl]disulfanyl]propanoic acid	240.3	
13.	24.05	4.48	Octadecanoic acid	57-11-4	$C_{18}H_{36}O_2$	Stearic acid	84.483	
14.	26.038	1.11	1-(4-methoxyphenyl) propan-2-amine	23239-32-9	$C_{10}H_{15}NO$	4-Methoxyamphetamine	165.23	
15.	27.205	47.60	Bis(2-ethylhexyl) phthalate	117-81-7	$C_{24}H_{38}O_4$	Dioctylpftalat	390.56	
16.	33.035	0.84	hexamethylcyclotrisiloxane	541-05-9	$C_6H_{18}O_3Si_3$		222.46	
17.	34.119	0.49	Decamethytetrasiloxane	141-62-8	$C_{10}H_{30}O_3Si_4$		310.68	
18.	35.196	0.85	2,4-dimethylbenzo[h] quinoline	605-67-4	$C_{15}H_{13}N$		207.27	

Hz, dd H-3", H-5").

An oxymethine proton resonance at δH 5.15 (J=0.01) revealed correlations with the C-2' (δ_C 128.16) resonance, in the HMBC spectrum, thus it was assigned as H-2. The placement was confirmed by its correlation C-2 (δ_C 71.16) with one of the oxymethylene proton resonance at δ_H 3.60 (H-4) and δ_H 3.56 (H-5) in the HMBC spectrum. Also, the H-3 oxymethine proton resonance at (δ_H 4.13) displayed a correlation with the C-5 (δ_C 62.06) resonance in the HMBC spectrum.

The resonance at (δ_C 150.26) was assigned as C-4' due to correlations seen in the HMBC spectrum with the H-2' (δ_H 7.64) and H-3' (δ_H 8.18)

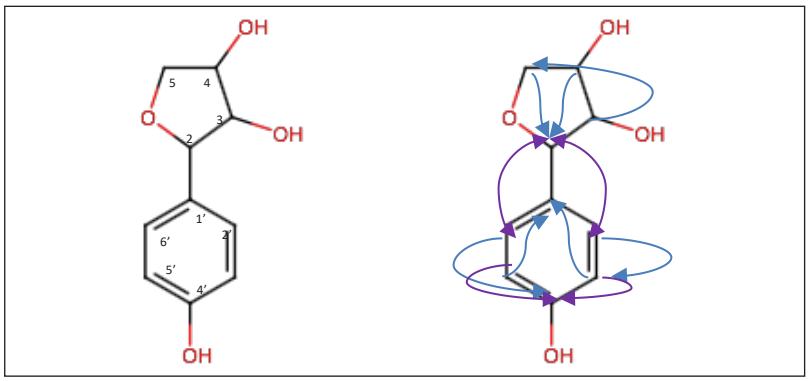


Fig. 6. 2-(4-hydroxyphenyl)tetrahydro-3,4-furandiol compound isolated from A.terreus and correlation structure of the compound in the HMBC spectrum

Table 4. NMR Data for the 2-(4-hydroxyphenyl)tetrahydro-3,4-furandiol (1) (CD_3OD, J in Hz).

Position	∂_C type	∂_H (J in Hz)	HMBC	COSY
2	71.16 CH	5.15, d (2.6)	2', 6'	
3	58.36 CH	4.13, dd (2.6, 7.0)	5	4
4	67.26 CH	3.60, dd (7.0, 10.0)	2	3
5	62.06 CH^2	3.80, dd (8.4, 10.0)		
		3.65, tri (8.6)	2	
1'	151.4628 C			
2', 6'	128.16 CH	7.64 d (8.0)	2', 4'	3', 5'
3', 5'	124.06 CH	8.18 dd (2.0, 7.0)	2', 4'	2', 6'
4'	150.26 C			

resonances. The fully substituted aromatic carbon resonance C-1' (δ_C 151.46) revealed correlations with the H-3' (δ_H 8.18) resonance in the HMBC. Coupling was seen in the COSY spectrum between the H-2 (δ_H 7.64) and H-3 (δ_H 8.18) and between H-4 (δ_H 3.60) and H-3 (δ_H 4.13) resonances.

In the NOESY spectrum, The H-3 resonance revealed correlations with the H-4 resonances. Therefore, they were assigned to the same face of the molecule. However, the relative configuration at C-2 could not be set from the NOESY spectrum

4. DISCUSSION

Recently, there has been a necessity for the discovery of new antibiotics nearly every day due to the emergence of drug-resistant bacteria, The death of 3.57 million cases associated with resistance of *K. pneumoniae, E.coli, S. pneumoniae, A. baumannii, P. aeruginosa*, and *S. aureus*.[10] Despite the major importance of medicinal plants in producing antibiotics, the soil is still an important source of unique antibiotics [14]. More than 50 % of the annual production of antibiotics comes from microorganisms isolated from soil [15].

Aspergillus genus has a strong biosynthetic ability for the production of most of the necessary pharmacological products such as Asperchondols H, butenolide, aspergivones, and Aurasperone [16-17]. The fungus *A. terreus* is one of the important species that is isolated from the soil, especially agricultural soil since it is very rich in organic compounds and inorganic elements that support the fungal community [18]. The literature reported that isolated Terrein a compound from *A. terreus*, this compound showed antimicrobial activity against *P. aerogenes* by reducing virulence factor expression and the formation of biofilm without

effect on cell growth [19]. In response to various growth conditions, *A. terries* produces different secondary metabolites through biosynthesis [20], So, its natural compounds were the target of this study and the phenolic part in particular.

The study identified to be 2-(4-Hydroxyphenyl) tetrahydro-3,4-furandiol based on its ^{1}H-NMR, ^{13}C-NMR and 2D-NMR. The findings of the study could be useful in exploring novel bioactive compounds from fungi. The compound 2-(4-Hydroxyphenyl)tetrahydro-3,4-furandiol has not been reported previously. However, a derivative of the compound -2-(3',4'dihydroxyphenyl) tetrahydrofuran-3,4-diol, was isolated from the phytopathogenic fungus *Colletotrichum gloeosporioides* and is known as gloeosporiol [21]. The isolated compound 2-(3',4'Dihydroxyphenyl) tetrahydrofuran-3,4 diol was synthesized previously and displayed a radical scavenging activity at the efficient concentration (EC50) of 0.14 mol/L [21-22].

There was considerable antibacterial activity against 10 bacterial isolates. The growth of both gram-positive and gram-negative was inhibited at a substantial rate due to the treatment with the fungal extract [11, 12]. *A. terreus* extract showed strong antibacterial against *Klebsiella* spp II and *Staphylococcus aureus* III, while it displayed exhibited a very limited impact on *Proteus* spp I and II growth. The results come consistent with the conclusions of many studies [23-25]. The antibacterial effect of *A. terreus* extract against bacteria suggests the products of this species as a prospective antibiotic in the future. In terms of toxicity test, all concentrations of the species extracts showed no turbidity in blood aliquots which suggests that it lacks toxicity [19].

The GC–MS analysis of *A. terreus* extract revealed phthalate comprises (47.60%), polysiloxane compounds (18.79 %), and n-hexadecanoic acid (16.41 %) and these were predominant constituents [22, 26-27]. The results agreed with Previous study also detected cyclooctasiloxane, hexadecamethyl in the GC–MS analysis of *A. terreus* and other derivative compounds, such as octadec-9-enoic acid and 1,2-benzene dicarboxylic acid, butyl octyl ester, with different percentages [28]. Phthalic acid esters (PAEs), which were found to be the main constituent, is commonly known as a synthesized plasticizer. Over 50 diverse derivatives of PAEs have been described as a product of different organisms, including plants, animals, fungi, actinomycetes, and bacteria [29].

Detection of Cysteine in the extract of *A. terreus* belongs to that cystine is the element sulphur in filamentous fungi. *A. nidulans* and *Neurospora crassa* employ organic and inorganic S compound pathways for cysteine biosynthesis [30]. Previous studies also reported that cis-9-octadecenoic acid was the predominant compound in crude methanol extract of *Trichoderma* sp. by GC–MS [27].

The biological activity of the crude extract of *A. terreus* might belong to n-hexadecanoic acid (palmitic acid), which was found to have a high percentage (16.41 %) in the present study. Palmitic acid showed a strong antifungal effect against scedosporium apiospermum in soap, antibiofilm, and antivirulence potency against *Candida tropicalis* and antibacterial activity [31-33]. In addition, the activity of Amyl-1–18, an antimicrobial peptide, increased by replacing aspartic acid with arginine without a major rise in hemolytic activity [34-35]. Arginine detection in the extract at 1.04 % proposes a potential antibacterial activity since a study by Sepahi *et al*. [32] showed that arginine-rich peptides had antibacterial activity against *E. coli* and *S. aureus*.

5. CONCLUSION

It has been concluded from the current study that the fungus *A. terreus* can produce secondary metabolite under laboratory conditions, this metabolite had potent power against clinical bacterial isolates. The extract contained a significant percentage of the antibacterial, antifungal, and anti-virulence n-hexadecanoic. As far as the authors know that the phenolic compound 2-(4-Hydroxyphenyl) tetrahydro-3,4-furandiol is isolated for the first time from *A. terreus* species. The study suggests that the extract of *A. terreus* could be a potentially effective antibiotic against resistant bacteria which is a big challenge these days over all the world.

6. CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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Research Article

Antimicrobial and Antioxidant Activity of Secondary Metabolites Isolated from *Citrullus colocynthis* (L.) Schrad.

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Abstract: Available antibiotics have lost their efficiency against several multidrug-resistant (MDR) microbes. Phytochemicals possess great antimicrobial activity and can be an alternative to available antibiotics for MDR microbes. *Citrullus colocynthis* (L.) Schrad is reported as an antimicrobial and anticancer herb in traditional medicinal cultures. Column chromatography was used to isolate secondary metabolites from ethanolic extracts of *C. colocynthis* whole fruits. Agar well diffusion method was used to determine antimicrobial activity. Antioxidant activity was measured by the DPPH (1,1-Diphenyl-2-Picrylhydrazine) radical scavenging assay. In this research, the informant consensus factor (ICF) and fidelity level (FL) were calculated on the basis of data collected from local herbalists and elderly villagers of age groups 51–60 who had knowledge of ethnomedicinal uses of plants. It was found that mostly the fruit and its parts (rind, pulp, and seeds) of *C. colocynthis* were used for the treatment of cancer and microbial infections. Alkaloids showed significant antibacterial activity against *Micrococcus luteus* (activity index 1.11; zone of inhibition (29.1±0.3 mm) *and Pseudomonas pickettii* (activity index 1.14; zone of inhibition 32.4±1.7 mm) as compared to streptomycin. It was noticed that flavonoids and phenolics at a concentration of 2000 μLmL^{-1} showed significant inhibition of free radicals, i.e., 91.57 % and 92.31 %, respectively. It was slightly higher than that of standard butylated hydroxytoluene (BHT), which was 89.07 %. It was found that with the increase in the concentration of phytochemicals, their radical scavenging potential also increased. It can be concluded that alkaloids are the main antimicrobial agents. Flavonoids and phenolics have great potential for free radical scavenging.

Keywords: Antibacterial, Antifungal, Antimicrobial, Antioxidant, *Citrullus colocynthis,* Fidelity Level, Informant's Consensus Factor, Plant Secondary Metabolites.

1. INTRODUCTION

Numerous antimicrobial formulae have lost their effectiveness against a number of pathogenic bacteria. These multidrug-resistant pathogenic bacteria are wreaking havoc on people's health and credit. Natural medicines are increasingly popular these days [1]. Because of multi-drug resistance, the death rate and loss of credit have increased [2]. Multidrug resistance is now a global hazard to public health. Several microbes, including *Clostridium difficile, Neisseria gonorrhoeae*, *Candida albicans*, *Pseudomonas aeruginosa, Salmonella typhimurium, Streptococcus pneumonia*, and *Staphylococcus aureus,* have become resistant to available antibiotics [3]. The threat is greater in underdeveloped nations, where the prevalence of contagious diseases is considerable and the emergence of antibiotic resistance is also present [4]. Treatment of bacterial infections has become more expensive and less effective due to these resistant strains of bacteria [5]. The presence of multidrug-resistant microbes in humans and domestic animals has been documented in research [6]. The majority of foodborne pathogens are resistant to antibiotics, and the fundamentals of this resistance are not understood. There is a need to find new ways of treating multi drug resistant bacteria [7].

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Plants have long been used as herbal remedies for a large number of diseases and disorders. All the traditional medicinal cultures have greatly relied on plants for safe, easy, and affordable medical remedies. Therapeutic agents obtained from medicinal plants are thought to be a possible alternative to available antibiotics [8]. These alternative medicines can be used to overcome the problems of antibiotic resistance and loss of credit [9]. Plant-oriented medicines are getting more popular with increasing drug resistance in common human pathogens [10]. *C. colocynthis* seed extracts possess antimicrobial, radical scavenging, and antiproliferative potential [11]. Secondary metabolites obtained from plants can be possible alternatives to antibiotics. Plant-oriented medicines have fewer side effects and greater efficiency [12]. In the past, people used to rely on natural herbs and plant parts as medicines [13].

Citrullus colocynthis L. Schrad is a medicinal plant that belongs to the Cucurbitaceae family. It is a xerophytic herb [14]. It possesses antimicrobial, antioxidant, anticancer, and hypolipidemic potential [15]. *C. colocynthis* possesses sufficient amounts of antioxidants and phenolic contents in fruit, peel, pulp, and seeds [16]. Triterpenoid spinasterol and 22,23-dihydrospinasterol isolated from *C. colocynthis* leaf extracts showed great antifungal, antioxidant, and aphicidal activity [17]. Cucurbitacin B from *Ecballium elaterium* L. improved the antibacterial and antiviral activity of antibiotics used against some isolates of multidrug-resistant human pathogens [18]. Fernonol and 22,23-dihydrospinasterol isolated from *C. colocynthis* have shown significant pesticidal activity [19].

Natural substances are found in various types of organisms and exhibit a wide range of structures. Metabolites like tannins, anthocyanins, and alkaloids are unquestionably substances of great interest to the pharmaceutical industries for manufacturing different medicines [20]. Whole fruits and vegetables contain a variety of antioxidants in the form of secondary metabolites. These plant-oriented metabolites should be preferred to synthetic antioxidants. Regular consumption of synthetic metabolites causes additional complications in cardiovascular disease and cancer [21].

The goal of the current study was to identify plant secondary metabolites from *C. colocynthis* ethanolic fruit extracts and determine their bioactivity for possible antibacterial activity and antioxidant activity. Three gram-positive and three gram-negative bacterial strains were used to assess the antibacterial activity. Streptomycin and Griseofulvin two common antibiotics, were used to compare the effectiveness of antimicrobial action.

2. MATERIALS AND METHODS

2.1 Analysis of Traditional Ethno-Medicinal Importance

Data on the ethnomedicinal importance of *C. colocynthis* were collected from 118 elderly people from villages in district Bhimber, Azad Jammu, and Kashmir (AJ&K), Pakistan. Older people were selected because they have better knowledge of the traditional uses of medicinal plants. A questionnaire was prepared about the traditional importance of *C. colocynthis* and its parts being used traditionally for different ailments or disorders. From the gathered data, Fidelity Level was calculated by following Friedman *et al.* [22].

$$FL = N_p/N \times 100$$

Here "N = Total number of respondents" and "N_p = Number of respondents who reported specific use of particular plant".

For the measurement of Informant's Consensus Factor, following formula was followed [23].

$$ICF = n_{ur} - n_t/n_{ur} - 1$$

Where n_{ur} = Number of Uses of a particular plant against a particular disease reported by respondents.

n_t = Total number of plants used for treatment of this particular disease.

2.2 Sample Collection

Plants along with fruits were collected from Bhimber, 32° 28′ 0″ N (latitude) and 75° 6′ 0″ E (longitude), AJ&K, Pakistan. Plants were identified by a taxonomist. Identified *C. colocynthis* was submitted to the herbarium of the Department of

Botany under reference no. MUST-Bot.-MUH-517. Fruits were separated and shade-dried for 2 months.

2.3 Crude Extraction

Fruits were ground into a fine powder. The cold soaking method was used for crude extraction, following Preethi *et al.* [24]. Fruit powder (100 g) was soaked in 500 mL of ethanol (BDH, Poole, England Cat# 101077Y). Solute was kept soaking for 7 days. After 7 days of soaking, Whatman filter paper (no. 42) was used for filtration. A rotary evaporator (EYELA N1100, China) was used for the evaporation of the solvent. After evaporation of the solvent, the remaining crude extracts were stored at 4 ˚C for further experimentation.

2.4 Phytochemistry

2.4.1. Column chromatography (CC)

Separation of individual classes of compounds from crude fruit extracts was done by column chromatography following the published method of Ahmad *et al.* [19]. The stationary phase in CC was a silica gel (200–300 mesh) column. The mobile phase consisted of ethyl acetate (AE) and petroleum ether (PE) in four different proportions. These fractions were AE:PE, i.e., 25:100, 50:100, 75:100, and 100:100 mL. The fractions obtained through column chromatography were confirmed by the following procedure.

2.4.2. Confirmation of phytochemicals

Isolated fractions were identified by confirmation tests following Harborne *et al.* [25] for the confirmation of alkaloids, flavonoids, glycosides, saponins, and tannins. For the confirmation of phenolics and terpenoids method of Harith *et al.* [26] was followed.

2.5 Antimicrobial Activity

2.5.1. Pathogenic strains

Micrococcus luteus, Staphylococcus epidermitis, and *Listeria monocytogenes* were gram-positive pathogenic bacterial strains, *Pseudomonas pickettii*, *Vibrio cholera*, and *Vibrio parahaemolyticus* were gram-negative pathogenic bacterial strains and *Alternaria alternata, Botyritis cinera,* and *Curvularia lunata* were the fungal strains that were selected for the experiments. Test bacterial strains and fungal taxa were obtained from the Department of Botany, Mirpur University of Science and Technology (MUST), Mirpur, AJ&K, Pakistan.

2.5.2. Culture medium

Potato Dextrose Agar (PDA) medium with pH 5.6 was prepared for the growth of microbial strains following Mazher *et al.* [27]. PDA powder weighing 39 g was dissolved in 900 mL of distilled water and boiled until a uniform mixture of yellowish colour was obtained. After that volume of the solution was raised to 1L. PDA medium and all glassware were autoclaved for 15 min at 121 °C before using as culture medium for inoculation of bacterial or fungal strains.

2.5.3. Zone of inhibition

Agar well diffusion method was used for measurement of zone of inhibition following Perez *et al,* [28]. Petri plates were placed in laminar flow to avoid contamination. In each plate wells measuring 5 mm were made through cork borer. Streptomycin and isolated phytochemicals were poured into these wells with the help of a micropipette. Streak method was used for inoculation of pathogenic strains. Streaked petri plates were placed in an incubator at 37 °C for 48 h. After that zone of inhibition (clear zone with no bacterial/fungal growth) was measured. Each experiment was performed in triplicate.

2.5.4. Determination of activity index (AI)

Antimicrobial activity index of extracts was measured by the following formula [29].

Activity Index = ZI (Sample) / ZI (Standard)

Here, ZI (Sample) means Zone of Inhibition shown by a particular sample ZI (Standard) means Zone of Inhibition shown by standard antibacterial/ antifungal drug.

2.6 Measurement of Antioxidant Activity

Antioxidant potential of phytochemicals isolated

from *C. colocynthis* fruit extracts was determined following Brand-Williams *et al.* [30]. For radical scavenging assay, 1,1- Diphenyl-Picrylhydrazine (DPPH) was used and its result were validated with standard antioxidants i.e. α-tocopherol, Ascorbic acid and BHT (butylated hydroxytoulene).

2.6.1. Preparation of stock solution and serial dilutions

Each isolated phytochemical weighing 0.02 g was dissolved in 10 mL of methanol. Final volume of the solution was raised to 20 mL. Solutions of 250 μLmL^{-1}, 500 μLmL^{-1}, 1000 μLmL^{-1} and 2000 μLmL^{-1} concentration were prepared from stock solution.

2.6.2. Preparation of DPPH solution

For DDPH Radical Scavenging Assay (DSRA) 33 mL of 0.01 mM solution of DPPH was dissolved in 1L of methanol.

2.6.3. DPPH radical scavenging assay (DSRA)

Free radical scavenging activity of different phytochemicals isolated from *C. colocynthis* was checked through DSRA following Shekhar and Anju [31]. For DSRA 1 mL of each stock solution was taken in different cuvettes and 5 mL of DPPH was added in each cuvette. These cuvettes were kept for 30 min at room temperature and then absorbance was determined at 515 nm. Results were compared with standard antioxidants. Free radical scavenging activity was determined by percentage inhibition by the following formula;

$$\% \text{ inhibition} = (A_C - A_P) / A_B \times 100$$

A_C = Absorbance of Control
A_P = Absorbance of Phytochemical
A_B = Absorbance of Blank

2.7 Statistical Analysis

All the data were analyzed through the statistical package for social sciences (SPSS 16.0) and results are presented as arithmetic mean ± standard deviation (SD). One-way ANOVA (analysis of Variances) was carried out for comparing means. Values $P < 0.05$ were deliberated statistically significant. Duncan multiple range test (DMRT) was carried out for the values that were significant

3. RESULTS

3.1 Ethnobotanical Importance of *C. colocynthis*

Result of ICF indicated that *C. colocynthis* has been in use traditionally for the treatment of several diseases or disorders. However, it is mostly used for antidiabetic and antilipidemic activities as per ICF of 13 %. ICF also indicates that it has been used for antimicrobial and antioxidant activities with ICF citation of 12 % and 11 % respectively as shown in figure 1.

The FL% results show that all parts of *C. colocynthis* have traditionally been used to treat various ailments (Figure 2). With an FL value of 23 %, whole fruit and seeds are the most important medicinal part of *C. colocynthis*. Fruit rind is the second-most important plant part, with a 21 % FL value. With an FL value of 20 % fruit pulp is the third most important parts of *C. colocynthis*. Most citations of using *C. colocynthis* fruits and parts were reported by local herbalists and elderly villagers, we studied the effectiveness of *C. colocynthis* fruit and validated the use of *C. colocynthis* fruit parts for antimicrobial and antioxidant activities in this study.

3.2 Column Chromatography for the Isolation of Different Class of Phytochemicals

Different confirmatory tests were performed for the identification of individual classes of compounds. Results of confirmatory tests are presented in Table 1. Nine different fractions were obtained by column chromatography. Different colored fractions were taken in different test tubes. Test tubes were labeled with capital alphabets from A to I as shown in Figure 3. For the confirmation of phytochemicals in each fraction, different phytochemical tests were performed.

Confirmatory experiments were performed three times, and finally the fractions obtained by column chromatography were confirmed as A and B being alkaloids, C being phelonics, D being glycosides, E being saponins, F being flavonoids, and H and I being terpenoids. Details of the

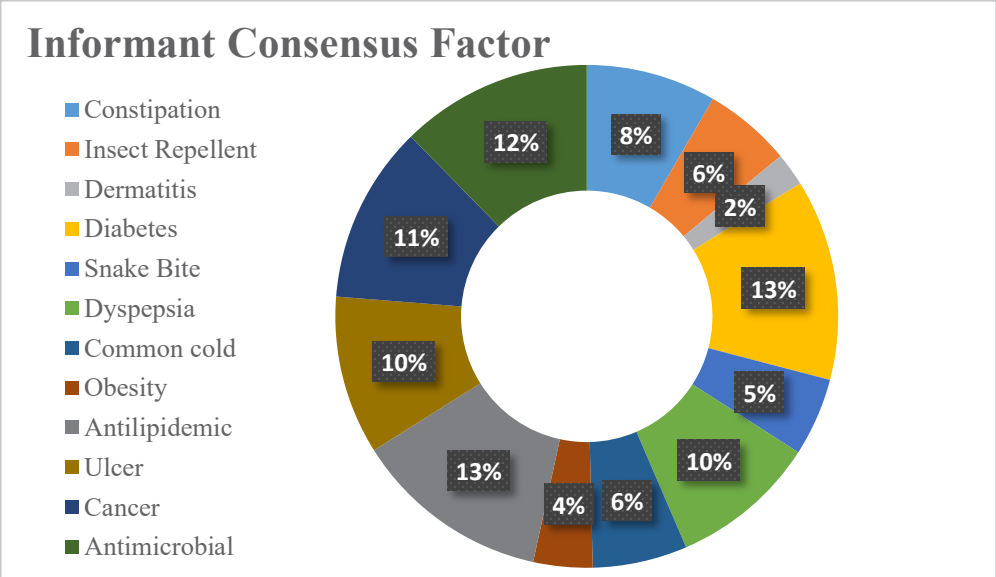

Fig. 1. Informant's Consensus Factor of *C. colocynthis* calculated on the basis of data gathered from local herbalists and elderly villagers

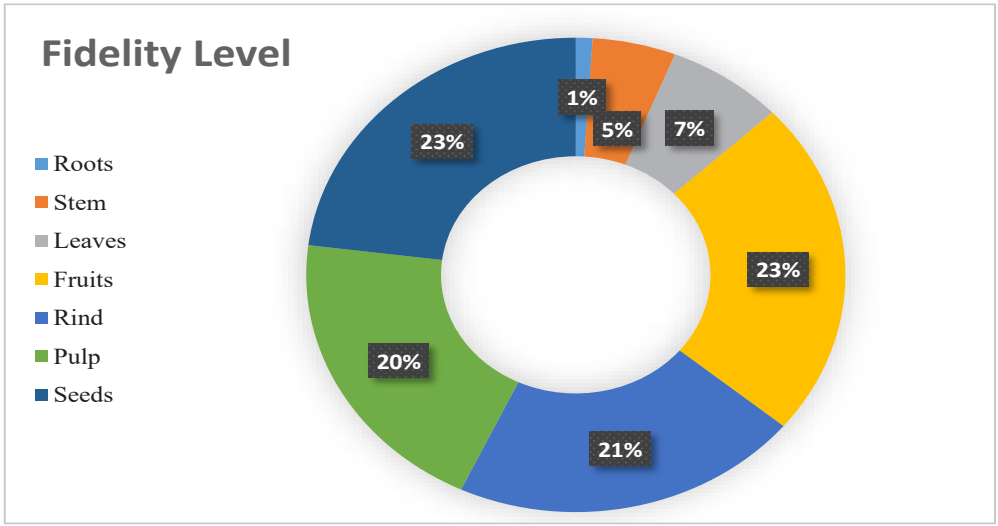

Fig. 2. Fidelity Levels of different parts of *C. colocynthis*

confirmative aspects are given in Table 1.

3.3. Antimicrobial Activity

3.3.1. Antibacterial activity of individual class of phytochemicals against gram-positive bacterial strains

Antibacterial activity of different phytochemicals was tested against three gram-positive bacterial strains, including *Listeria monocytogenes, Micrococcus luteus,* and *Staphylococcus epidermitis.* The activity of phytochemicals was compared with that of the standard antibiotic streptomycin. Alkaloids, flavonoids, phenolics, and terpenoids showed antibacterial activity; however, only alkaloids' antibacterial activity was significant as compared to the standard drug. Alkaloids have the highest zone of inhibition of 29.1 ± 0.3^{a} mm against Micrococcus luteus, which is significantly higher than streptomycin 26.3 ± 0.3^{b}, with an activity index of 1.11 (Table 2).

In one petri dish, only streptomycin was poured into a 5 mm agar well. Whereas in all other petri plates except the control, three wells were bored, and in

Fig. 3. Fractions obtained by column chromatography.

Table 1. Confirmatory tests for different fractions isolated through column chromatography

Phytochemicals	Tests Performed	Confirmative Aspect	Fraction(s)
Alkaloids	Dragandroff Test	Red precipitate formation	A, B
	Hager Test	Yellow colour formation	
	Mayer Test	Yellow colored precipitate	
	Wagner Test	Brown reddish precipitate	
Flavonoids	Ferric Chloride	Becomes colorless	F
	Alkaline Reagent	Yellow colour precipitate	
	Lead acetate Test	Orange to red coloration	
Glycoside	Bromine water Test	Brownish coloration	D
	Keller Test	Dark brown ring formation	
Phenolics	Ferric Chloride Test	Blue and green coloration	C
Saponins	Foam test	Appearance of froth	E
	Bromine water test	Blue-black coloration	
Tannins	Ferric chloride Test	Brown ring at the junction	G
Terpenoids	Salkowaski Test	Reddish brown coloration	H, I
	Liebermann Test	Appearance of froth	

each well, a different phytochemical was poured. Zones of inhibition were measured after 48 h. The zone of inhibition of alkaloids against *M. luteus* was significantly greater than that of streptomycin (Fig. 4). However, flavonoids and glycosides showed little antibacterial activity.

3.3.2. Antibacterial activity of individual class of phytochemicals against gram-negative bacterial strains

Glycosides, tannins, and saponins showed negligible antibacterial activity whereas flavonoids, phenolics, and terpenoids showed antibacterial activity but it was not significant as compared to standard antibiotic streptomycin (Table 3). Alkaloids showed significant antimicrobial activity as compared to streptomycin.

Alkaloids showed 32.4 ± 1.7^{a} mm zone of inhibition (ZI) against *Pseudomonas pickettii,* it was significantly greater than ZI of streptomycin i.e. 28.3 ± 1.3^{b} mm (Fig. 5). An activity Index of 1.14 was calculated for alkaloids against *P. pickettii.*

Table 2. Zone of Inhibition (ZI) and Activity Index (AI) of phytochemicals isolated from *C. colocynthis* crude extracts against gram positive bacterial strains

Phytochemical	*Listeria monocytogenes*		*Micrococcus luteus*		*Staphylococcus epidermitis*	
	ZI (mm)	**AI**	**ZI (mm)**	**AI**	**ZI (mm)**	**AI**
Alkaloids	24.5±0.7^{b}	0.89	29.1±0.3^{a}	1.11	22.9±1.3^{b}	0.89
Flavonoids	17.4±1.1^{c}	0.63	12.4±1.7^{e}	0.47	16.2±0.6^{c}	0.63
Glycosides	07.2±0.5^{f}	0.26	9.3±0.3^{f}	0.32	09.2±0.5^{e}	0.36
Phenolics	13.3±0.4^{d}	0.49	16.1±0.6^{c}	0.61	11.3±0.3^{d}	0.44
Saponins	NG	NG	NG	NG	NG	NG
Tannins	NG	NG	NG	NG	08.1±0.3^{f}	0.32
Terpenoids	11.7±2.0^{e}	0.43	14.4±2.8^{d}	0.55	16.0±1.0^{c}	0.62
Streptomycin	27.4±0.7^{a}		26.3±0.3^{b}		25.7±0.7^{a}	

Values are expressed as Mean±SD (n = 3). Level of significance 95% (P<0.05). Different superscripts indicate significant differences; NG = Not Given.

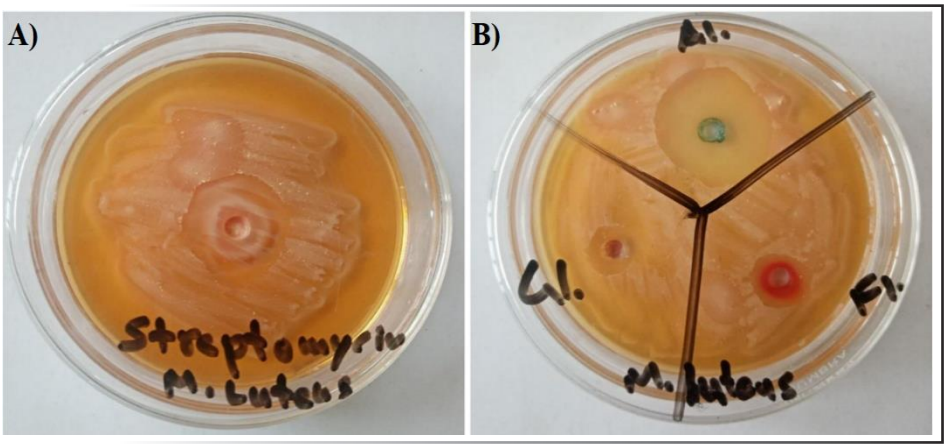

Fig. 4. Zone of inhibition against *Micrococcus luteus* shown by streptomycin **(A)** and different phytochemicals **(B)**. Al. = Alkaloids; Fl. = Flavonoids; Gl. = Glycosides

Table 3. Zone of Inhibition (ZI) and Activity Index (AI) of phytochemicals isolated from *C. colocynthis* crude extracts against gram-negative bacterial strains

Phytochemical	**Pseudomonas pickettii**		**Vibrio cholera**		**Vibrio parahaemolyticus**	
	ZI (mm)	**AI**	**ZI (mm)**	**AI**	**ZI (mm)**	**AI**
Alkaloids	32.4±1.7^{a}	1.14	30.1±0.7^{a}	0.98	25.3±2.7^{b}	0.92
Flavonoids	17.6±1.2^{c}	0.62	16.7±0.3^{c}	0.54	14.2±0.5^{c}	0.52
Glycosides	NG	NG	10.2±1.4^{e}	0.30	09.0±1.1^{e}	0.33
Phenolics	14.1±0.7^{d}	0.50	17.3±0.7^{b}	0.56	14.2±0.8^{c}	0.52
Saponins	08.7±3.3^{e}	0.31	NG	NG	11.4±2.3	0.41
Tannins	NG	NG	NG	NG	NG	NG
Terpenoids	14.3±0.7^{d}	0.51	12.5±0.5^{d}	0.41	13.6±1.3^{d}	0.49
Streptomycin	28.3±1.3^{b}		30.7±0.6^{a}		27.5±0.6^{a}	

Values are expressed as Mean±SD (n = 3). Level of significance 95% (P<0.05). Different superscripts indicate significant difference; NG = Not Given

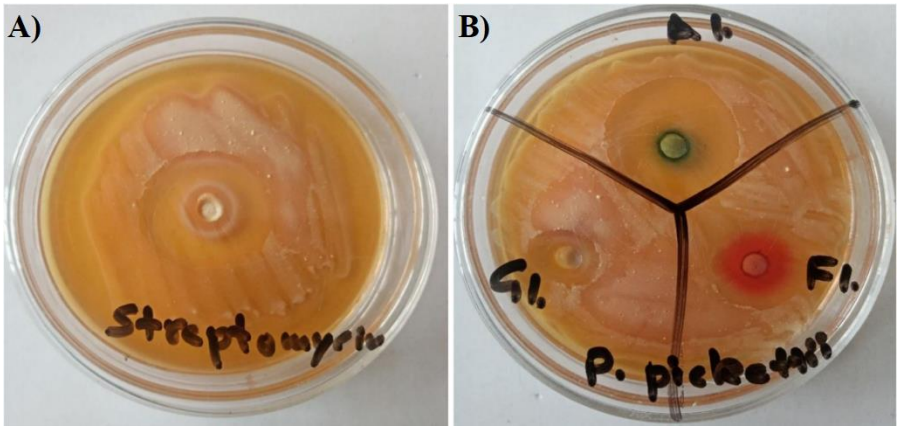

Fig. 5. Zone of inhibition against *Pseudomonas pickettii* shown by streptomycin **(A)** and different phytochemicals **(B)**. Al.= Alkaloids; Fl.= Flavonoids; Gl.= Glycosides

3.3.3. Antifungal activity of individual class of phytochemicals

It was noted that tannins showed no antifungal activity, whereas saponins and glycosides showed negligible antifungal activity. Flavonoids, phenolics, and terpenoids showed antifungal activity, but it was not significant as compared to standard Griseofulvin. The antifungal activity of alkaloids was comparable to that of griseofulvin but not higher (Table 4).

3.4 Antioxidant Activity of Individual Class of Phytochemicals

It was shown that as phytochemical concentrations increased, so did their radical scavenging potential. It was noted that flavonoids and phenolics at a concentration of 2000 μLmL^{-1} showed significant inhibition of free radicals, i.e., 91.57 % and 92.31 %, respectively. It was slightly higher than the industry standard of 89.07 %. Alkaloids, glycosides, and saponins also showed comparable antioxidant activity, whereas tannins and terpenoids showed negligible antioxidant activity (Table 5). Values are expressed as Mean±SD (n = 3). Level of significance 95 % (P<0.05). Star as a superscript indicates higher antioxidant activity as compared to standard antioxidants

4. DISCUSSION

Present study has found that *C. colocynthis* is mostly employed for antidiabetic, antilipidemic,

Table 4. Zone of Inhibition (ZI) and Activity Index (AI) of phytochemicals isolated from *C. colocynthis* crude extracts against pathogenic fungal strains

Phytochemical	*Alternaria alternata*		*Botyritis cinera*		*Curvularia lunata*	
	ZI (mm)	**AI**	**ZI (mm)**	**AI**	**ZI (mm)**	**AI**
Alkaloids	29.0±1.3^{b}	0.86	28.7±1.6^{b}	0.93	22.4±0.6^{b}	0.77
Flavonoids	20.3±3.7^{c}	0.61	16.2±0.7^{c}	0.52	17.7±1.3^{c}	0.61
Glycosides	11.0±1.3^{f}	0.33	09.7±3.3^{e}	0.31	NG	NG
Phenolics	11.2±0.1^{f}	0.33	10.3±0.5^{e}	0.33	12.4±1.1^{e}	0.42
Saponins	13.6±1.6^{e}	0.41	NG	NG	NG	NG
Tannins	NG	NG	NG	NG	NG	NG
Terpenoids	17.5±1.3^{d}	0.52	12.3±0.3^{d}	0.40	13.9±1.3^{d}	0.48
Griseofulvin	33.7±0.7^{a}		30.9±1.3^{a}		29.2±0.6^{a}	

Values are expressed as Mean±SD (n = 3). Level of significance 95 % (P<0.05). Different superscripts indicate significant difference; NG = Not Given

Table 5. DPPH Radical Scavenging Activity (% inhibition) of phytochemicals from *C. colocynthis* at different concentrations

Phytochemical	**Concentration**			
	250 µl/ml	500 µl/ml	1000 µl/ml	2000 µl/ml
Alkaloids	56.83±2.7	61.62±1.1	65.41±3.6	66.88±4.5
Flavonoids	60.23±3.5	77.93±2.7	89.74±0.4*	91.57±4.9*
Glycosides	55.71±2.2	59.13±5.4	66.87±4.5	70.03±5.5
Phenolics	73.03±7.4	84.55±3.4	86.63±7.1	92.31±1.7*
Saponins	70.13±4.2	75.44±5.1	81.20±3.2	84.34±1.6
Tannins	23.11±4.4	24.55±3.4	29.60±2.3	37.41±1.1
Terpenoids	20.24±3.6	26.57±6.3	34.30±0.3	41.12±3.7
Standards			2000 µl/ml	
BHT			89.07±4.7	
Ascorbic Acid			81.21±1.7	
α-tocopherol			87.59±5.2	

antimicrobial, and antioxidant activity. All parts of the *C. colocynthis* have historically been used to treat a variety of diseases, according to the fidelity level (FL) percentage, but the fruit and all of its components, including the rind, pulp, and seeds, receive the most mentions. FL and ICF for *C. colocynthis* have not been documented yet. The current work has used ethanolic fruit extracts to separate several phytochemicals for researching antibacterial and antioxidant activity based on the findings of the ethnobotanical surveys (ICF and FL).

Many antibiotics no longer work as well against a variety of pathogens [32]. A large number of bioactive alkaloids and flavonoids are constituents of *C. colocynthis* and have antibiotic potential. Recently, a pesticide formulation (NNRC-82) has been developed from *C. colocynthis,* and its patent has been registered [33]. Alkaloids, flavonoids, phenolics, and terpenoids all exhibited antibacterial action, but alkaloids' antibacterial activity was comparable to that of prescription drugs. Alkaloids demonstrated higher zones of inhibition than streptomycin against *pickettii* and *Micrococcus luteus*. The findings of this study are consistent with those of earlier investigations by [34] in which ethanolic extracts of whole *C. colocynthis* were found to be highly antibiotic. The results of this study are also in accordance with [35-36], which revealed that alkaloids have strong antibacterial properties.

Numerous illnesses in humans and animals are brought on by fungus-borne infections. A few biochemically active plant chemicals that can thwart fungus growth have been identified by scientists [37]. This study examined the antifungal efficacy of isolated phytochemicals against *Botyritis cinera*, *Curvularia lunata*, and *Alternaria alternata* and compared it to the activity of Griseofulvin, a common antifungal medication. It was revealed that saponins and glycosides had minimal antifungal action compared to tannins, which exhibited no activity. The antifungal activity of flavonoids, phenolics, and terpenoids was not significant. Alkaloids demonstrated antifungal efficacy that was comparable to that of Griseofulvin. Previous studies [38, 39] investigated the antifungal activity of *C. colocynthis* extracts and found similar results. They have found that *C. colocynthis* seed extracts are significantly antifungal against *Aspergillus niger* and *A. flavus* but none of the zones of inhibition were greater than those of standard.

DNA damage and malignancies are mostly caused by reactive oxygen species (ROS). Antioxidants are consumed in various ways to treat these issues. Plant phytochemicals are confirmed by research to be very powerful antioxidants. In addition to removing ROS, phytochemicals are also less expensive and have fewer adverse side effects [19, 40]. In the present study, antioxidant activity of isolated phytochemicals was determined by the DPPH free radical scavenging assay (DSRA). It was depicted that with the increase in concentration

of phytochemicals, the percentage of inhibition of free radicals also increased. It was noticed that flavonoids and phenolics showed significant inhibition of free radicals. The antioxidant activity of these phytochemicals was higher than that of standard antioxidants. Alkaloids, glycosides, and saponins also showed comparable antioxidant activity; however, tannins and terpenoids show negligible antioxidant activity. The results of the study have similar findings as those previously reported [41, 42].

5. CONCLUSION

It can be concluded that the primary antibacterial components of *C. colocynthis* fruit extracts, which account for the majority of their antimicrobial action, are alkaloids. The flavonoids and phenolics in *C. colocynthis* fruit extracts have excellent potential as free radical scavengers. Thus, it can be said that the primary components of *C. colocynthis* for antioxidant activity are flavonoids and phenolics.

6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research Article

Phenotypic Analysis and Growth Performance of Rhode Island Red, Barred Plymouth Rock and their Hybrid at Jaba Mansehra, Pakistan

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Abstract: Poultry farming is a big business in Pakistan. Poultry farming is a substantial supply of eggs and meat in rural areas, as well as a reliable source of income. Therefore the goal of this study is to compare the Rhode Island red, barred plymouth rock, and their hybrids' phenotypic traits and egg-laying capacities. The 100-day-old Rhode Island Red, Barred Plymouth Rock, and cross chicks were used in the study at Jaba District Mansehra, KP, Pakistan. The whole flock was sheltered, fed, ventilated, and immunized. Breed 3 (Jabba 21) was reported to have the longest shanks (3.78 inches SL), followed by breed 1 (Rhode Island Red) and breed 2 (Barred Plymouth Rock) (3.47 inches SL). Breed 2 is the broadest shank at 0.47 SW, followed by Breed 1 at 0.43 SW and Breed 3 at 0.30 SW (0.43 Inches SW). The breadth of the shinbone did not change substantially from week 8 to week 13 (p=0.05), although body weight changed from week 1 to week 18. Breed 1 consumed the most feed (2,112 g), followed by Breed 3 (1,452 g) and Breed 2. (990 g). Breed 2 acquired the greatest amount of weight (1,638.57 g), followed by Breed 1 (1,557.07 g) and Breed 3. (1530.3 g). From the first to the eighteenth week of observations, there was a significant (p0.05) difference between the three chicken breeds in weekly body weight gain. Breeds 1, 2, and 3 saw growth rates of 1st 63.59, 2nd 64.02 and 3rd 64.03 percent. This research gave us a fresh perspective on several products that might come to market and contend with one another. This study provides insight into what will work best for Pakistan's poultry sector going forward in the future.

Keywords: Phenotypic, Poultry, Barred Plymouth Rock, Breed.

1. INTRODUCTION

Poultry farming is a big business in Pakistan. This firm employs more than 1.5 million people (directly and indirectly). To keep the price of beef and lamb in control, the market today incorporated chicken as a counterweight. Currently, the poultry sector spends more than 700 billion rupees each year. In 2020-21, about 1.39 million tonnes of chicken meat were produced, representing 32.7 % of the nation's total meat output (4.3 million tonnes). This year, poultry contributed 1.4 % of the gross domestic product, while agricultural and livestock value added contributed 7.5 % and 12.7 %, respectively. The value of poultry added increased by 7.8 percent over the previous year, reaching Rs 175.5 billion in 2017-18 at the current labor cost. During 2016-2017, commercial, jam, and meat breeders reported increases of 7 %, 5 %, and 10 %, respectively, while rural poultry breeders witnessed a rise of 1.5 % [1]. People have grown chickens in their backyards since the beginning of time. About 80% of rural households in Pakistan engage in backyard poultry farming, which is a substantial source of income [2]. Eighty to ninety-nine percent of hens raised in developing countries are local poultry genotypes [3]. The majority of small flocks in Pakistan are comprised of indigenous 2 breeds such as Desi (non-described indigenous chicken), Aseel, naked neck (NN), Lyallpur silver black (LSB) (A breed developed several centuries ago at the University of Agriculture, Faisalabad), and other foreign breeds such as Fayoumi (Fay) and Rhode Island Red [4].

Cross-breeding may improve bird growth, feed conversion efficiency, and reproductive capacity

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without sacrificing local adaptability [5]. As a consequence of a major economic boom, both the efficiency and productivity of domestic chicken production will rise. According to Kiani-Manesh, the financial efficiency of rural chickens may be increased by increasing their sexual maturity, egg output, and egg and body weight at eight weeks of age [6]. Poultry farming is a substantial supply of eggs and meat in rural areas, as well as a reliable source of income. The majority of Pakistani households maintain small flocks of backyard chickens (11-13 birds) for consumption inside the household [6]. Poultry farming is gaining popularity in small-scale agricultural systems that need minimum in-house production. Utilizing local free nutritional resources and the ruins of a kitchen, using rural animals that are adapted to their environment, and preserving the old system's inherent ability to incubate and reproduce are all advantageous [7]. Low reproductive output, poor development, sickness, mortality, predation, and insufficient farmer education are obstacles to smallholder chicken production [8]. Egg production and associated parameters, such as age and body weight at sexual maturity, have been thoroughly investigated and shown to differ considerably across species and/or chicken breeds [9].

Several physical traits may be utilized to determine the genetic resources of a location [10]. Rhode Island Reed, which has been successfully maintained in rural and agricultural settings throughout the nation, may deliver higher financial returns than golf courses [11]. Due to its high egg production (178 eggs each production cycle), Rhode Island Red (RIR) is the most popular chicken breed in Pakistan [12]. Due to its extended stay in Pakistan, it has successfully acclimated to the local climate. Known as "Golden birds" in rural regions, it is a dual-purpose American breed [13]. The levels of glucose, cholesterol, calcium, total protein, alkaline phosphate, and uric acid may be used to determine the energy state of a bird.

In addition, knowing how illness affects the body's metabolic activity and electrolyte levels is crucial for repairing bird anomalies. The number of native birds is extremely low. Even though rural Pakistanis produce a broad array of poultry breeds, including Desi, Fayoumi, and RIR, very little is known about their growth, production capacity, and egg quality features [14]. The purpose of this research was to compare Rhode Island Red, Plymouth Rock, and their hybrids. To compare egg-laying ability, the phenotypic characteristics of both species and their hybrids were examined.

2. MATERIALS AND METHODS

2.1 Study Area

The present study was conducted in Jaba, which is found in the Mansehra District of Khyber Pakhtunkhwa. This district has a total land area of 1632 square kilometers and is situated in the northwestern part of the province. The population of the district as a whole is 2.34 million people; this number accounts for people living in both urban and rural regions. District Mansehra has a summer climate that is typical of the region, with an average temperature of 20.2 degrees Celsius during the summer months and a minimum temperature of 2.0 degrees Celsius during the winter months, with temperatures averaging 20.2 degrees Celsius during the summer months and 2.0 degrees Celsius during the winter months. Agriculture takes up the great majority of the available land, and the majority of the people are dependent on the agricultural industry for their livelihood (Figure 1).

Along with a vaccine, Vernier calipers, a screw gauge, and a digital weighing balance, the Poultry Research Institute of Jaba Mansehra will provide day-old chickens for purchase. These days-old chickens will include Rhode Island Reds, Barred Plymouth Rocks, and the offspring of these two breeds, which will be designated as Jaba 21. Additionally included will be leg tags in a variety of sizes.

2.2 Study Duration

According to the history of PRI Jaba for RIR and PMRT, it was anticipated that the chickens would lay their first egg in the 18th week of age; thus, the overall research period would be up to 25 weeks for comparison of egg production characteristics.

2.2.1. Housing

On the day that the eggs are laid, each of the Rhode Island red-barred Plymouth rock, and PRI21

Fig. 1. Map of Jaba District Masnehra, KP, Pakistan.

chicks will each be given a tag so that they may be recognized later on. Up to the age at which they begin producing eggs, the birds of each breed will be kept in a home with a litter floor that is three inches thick, and the weight of each bird will be recorded separately. It is planned that all of the chicks will be vaccinated at the same time, under the same conditions in terms of management, sanitation, and the environment at the same time. In line with the requirements, it will be carried out manually to ensure that food, floor space, and water are provided.

2.2.2. Feeding management

During the period of brooding, the beginning ratio had a crude protein content of 21 percent and an energy content of 2,950 kcal/kg. During the era of development, there should be 16 percent protein and 2800 kilocalories per kilogram of body weight; however, during the period of producing eggs, there should be 18 percent protein and 2700 kilocalories per kilogram

2.2.3. Lighting program

Lights are on for 24 hours the first week, then reduced by an hour a day until they are only needed for 8 hours a day throughout the growing stage. In production, lighting will run for 16 hours each day (16 hours light and 08 hours dark).

2.2.4. Measurements of body weight

The chicks will be individually weighed at the start of the experiment (when they are one day old), and then they will be weighed once a week until the end of the trial. The amount of weight gained each week will be noted.

2.2.5. Feed intake

The difference between the weight of the feed that was offered and the rest of the feed will be used to figure out how much food each animal needs each day. It will be necessary to divide the total amount of food each group eats each day by the number of birds in each group to get the average amount of food each bird eats each day. The feed intake varies with three different types of feed being used in this study. The first feed given to birds in this study was Starter, 14, afterward, the second feed with the name Ghol 12 was given to birds for experiment and the final feed is named finisher. The feed intake in this study was given a proper systematic approach to have proper empirical and accurate findings. The above format was followed throughout the experiment.

2.2.5.1 Feed conversion ratio

The feed conversion ratio (FCR) of all the selected breeds will be recorded according to the formula given as under.

FCR= Total Feed Intake ÷ Weight gain

2.2.6. Age at first lay

Age at the first lay egg in each group will be recorded.

2.2.7. The weight of the Egg first lay

The weight of the first laying egg will be recorded.

2.2.8. Morphological traits

Individual birds of both sexes will be examined until they lay their first egg (at an estimated age of 18 years) for feather/plumage features, skin color, shank, earlobe, comb, comb type, wattle, and the color of the egg they lay.

3. RESULTS AND DISCUSSION

From the eighth to the eighteenth week, the initial shank lengths for breeds 1, 2, and 3 were 2.92 cm, 2.94 cm, and 3.04 cm, respectively, in the eighth week. As of the ninth week, the shank lengths of breeds 1, 2, and 3 had grown to 2.95 percent, 2.96 percent, and 3.06 percent. Although there was no statistically significant variation in shank length across chicken breeds from weeks 8 to 12, all chicken breeds had an increase in shank length from weeks 13 to 17. (p 0.05). During the 17th and 18th weeks of observation, the shank length did not grow (Table 1; Figure 2)

A variety of chicken breeds were measured weekly for their shank width, which varied from 8 to 18 millimeters. The eighth week of the experiment revealed that breeds 1, 2, and 3 had shank widths of 0.38, 0.38, and 0.36 cm, respectively, at the beginning of the study. Between the ninth and eighteenth weeks of the trial, there was only a little rise in the hock widths of breeds 1, 2, and 3. From the eighth to the thirteenth week of observations, there were no significant variations in shank width among the 28 chicken breeds (p 0.05); however, there was a significant difference (p 0.05) from the fourteenth to the eighteenth week of observations (Table 2; Figure 3).

Table 1. Shank length of the three selected breeds from the 8th to 18th week of trail at Jaba District Mansehra, KP, Pakistan

Week	Breed 1 Shank Length	Breed 2 Shank Length	Hybrid	P-value
8th	2.92	2.94	3.04	0.1413
9th	2.95	2.96	3.06	0.3797
10th	3.06	3.04	3.17	0.3898
11th	3.26	3.25	3.28	0.3998
12th	3.38	3.36	3.38	0.4080
13th	3.47	3.45	2.47	0.4260
14th	3.56	3.55	3.59	0.4376

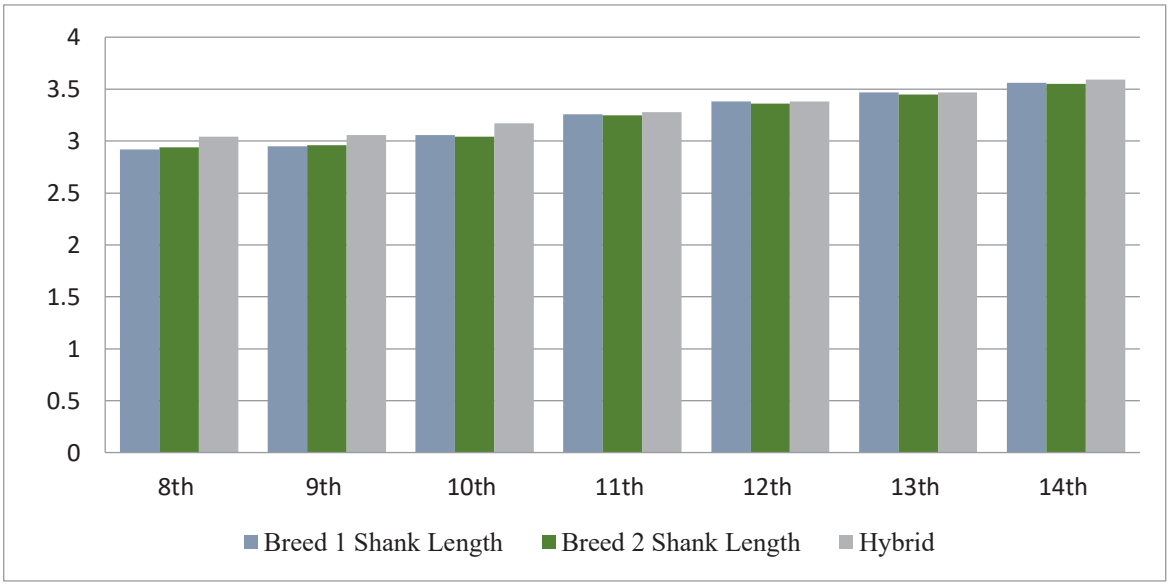

Fig. 2. Graphical representation of the variation in the shank length of the chicken from 3 selected breeds at Jaba District Mansehra, KP, Pakistan

Table 2. Shank width of the 3 selected breeds from the 8th to 18th week of the trail at Jaba District Mansehra, KP, Pakistan

Week	Breed 1 Shank Width	Breed 2 Shank Width	Hybrid Width	P-value
8th	0.38	0.38	0.36	0.1124
9th	0.40	0.39	0.40	0.1326
10th	0.41	0.40	0.41	0.1531
11th	0.41	0.41	0.42	0.1726
12th	0.42	0.41	0.43	0.1943
13th	0.43	0.42	0.44	0.1988
14th	0.43	0.43	0.45	0.2010
15th	0.44	0.44	0.46	0.2102
16th	0.45	0.44	0.47	0.2245
17th	0.46	0.45	0.48	0.2316
18th	0.48	0.47	0.48	0.2437

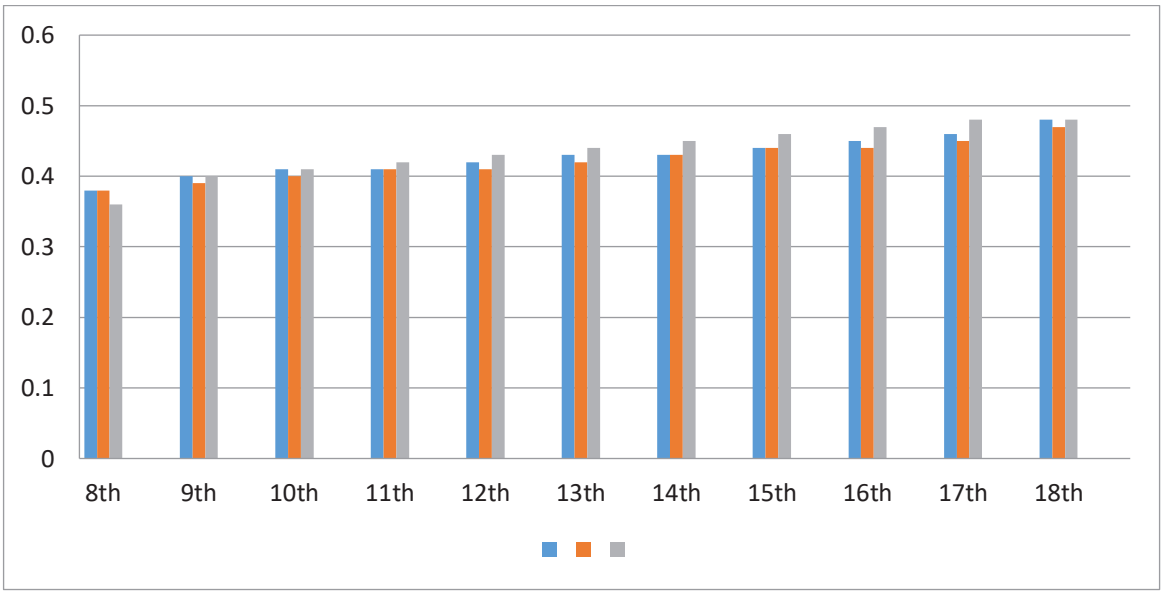

Fig. 3. Graphical representation of the variation in shank width of the chicken from 3 selected breeds at Jaba District Mansehra, KP, Pakistan

From the first to the eighteenth week of life, the body weight of chickens of different breeds was measured. At one week, the starting weights of chicken breeds 1, 2, and 3 were, respectively, 32.93 g, 34.53 g, and 37.00 g. Breed 1 weighed 45.06 g, barred 2 weighed 40.26 g, and Breed 3 weighed 37.73 g after the first week. From the beginning of the final eighteen weeks of the trial, body mass increased. Breed 2 was the biggest at 1590.0 pounds, followed by breeds 1 and 3 at 1673.1 pounds apiece. The weight of the three breeds varied greatly between the 0th and 18th weeks of monitoring as shown in (Table 3; Figure 4).

The chickens of three separate breeds were fed between 0 and 18 weeks of age. During the trial, which spanned 0 to 18 weeks, the feed consumption of the three chosen chicken breeds varied significantly (p 0.05). Breed 1 ingested no less than 25 g of feed in week zero and no more than 108 g of feed in week seventeen. During week 0, only 28 g of feed were ingested, the highest intake (114 g) happened during week 16, the lowest intake (109 g) occurred during week 16, and the highest

Table 3. Body Weight of the 3 selected breeds from the 0th to 18th week of trial at Jaba District Mansehra, KP, Pakistan

Week	Breed 1 Body Weight (g)	Breed 2 Body Weight (g)	Hybrid Body Weight (g)	P-value
0	32.93	34.53	37.00	0.0001
1st	45.06	40.26	37.73	0.0002
2nd	76.20	86.46	81.73	0.0163
3rd	128.53	133.07	135.33	0.5809
4th	175.47	175.07	192.53	0.0457
5th	253.00	248.33	290.20	0.0005
6th	380.67	367.33	420.93	0.0161
7th	471.10	495.00	541.13	0.0285
8th	625.27	661.93	680.93	0.2761
9th	735.73	729.27	811.87	0.1088
10th	874.53	870.13	920.27	0.5264
11th	924.73	943.33	968.27	0.7533
12th	1083.1	1075.5	1116.2	0.7901
13th	1176.1	1168.1	1184.1	0.8004
14th	1258.1	1446.4	1396.4	0.8025
15th	1399.7	1486.9	1498.5	0.8045
16th	1497.9	1496.3	1528.4	0.8156
17th	1500.7	1509.1	1539.8	0.8239
18th	1590.0	1580.4	1608.9	0.8246

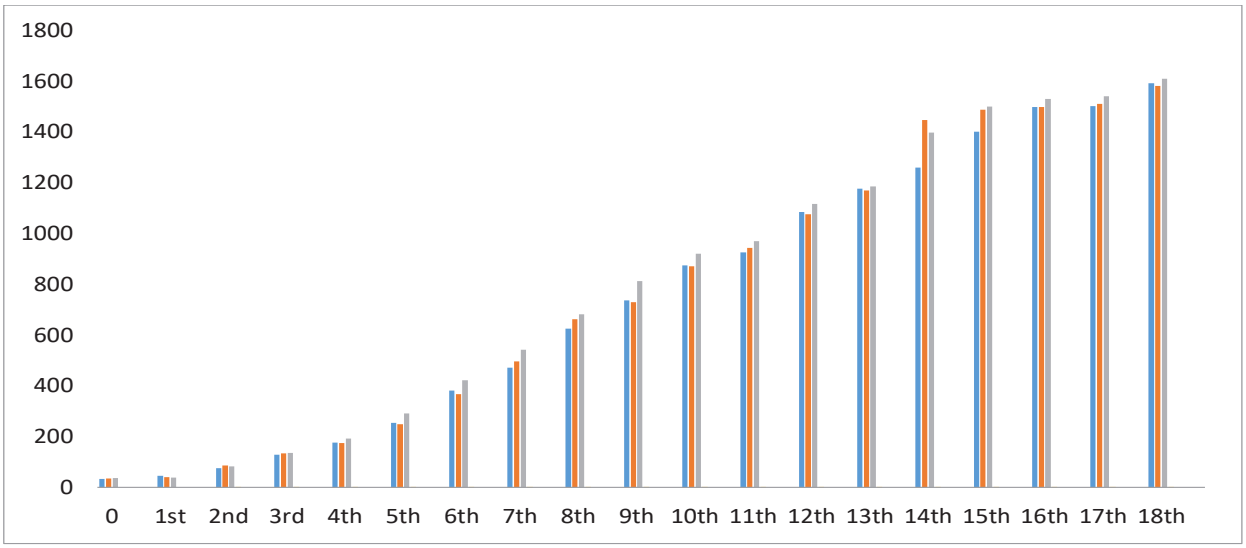

Fig. 4. Graphical representation of the variation in Body Weight of the chicken from 3 selected breeds at Jaba District Mansehra, KP, Pakistan.

intake (114 g) occurred during week 18. Breed 3 saw a minimum of 24 g in week zero, a maximum of 103 g in week sixteen, and a minimum of 99 g in week 18. During the first week, the body weight of breeds 1, 2, and 3 increased by 12.13 g each week. Breeds 1, 2, and 3 gained 43.27, 51.93, and 44.73 g body weight during the second week, but by the 18th week, their weights had reached a maximum of 1557.07, 1638.57, and 1530.3 g, respectively. It was established that Jabba 18, RIR, and Barred

reached sexual maturity at 120, 122, and 124 days, respectively. Before Jabba's eighteenth birthday, RIR and Barred had reached sexual maturity. The hybrid of Barred Plymouth Rock and Rhode Island red, RIR, and Barred weighed 1673.1, 1590.0, and 1567.3 g, respectively, at sexual maturity. The Hybrid of Barred Plymouth Rock and Rhode Island red and two other breeds have the heaviest maximum body weight during sexual maturity. The hybrid of Barred Plymouth Rock and Rhode Island red, Rhode Island Red, and Barred eggs, at sexual maturity, weighed 38, 36, and 34 g, respectively. A hybrid of breeding Plymouth Rock and Rhode Island Red had the second-heaviest eggs after Rhode Island Red and Barred Plymouth Rock.

4. DISCUSSION

In rising nations, there have been efforts to replace old breeds or establish new ones since the turn of the 20th century. Advancement has been inconsistent and depends on regional factors. Concerns about the long-term viability of cross-breeding in some regions or for specific breeding techniques have been raised due to the lack of crossbreed adaptation to challenging production settings (such as climate, illnesses, and feed availability) as well as insufficient supplementary socio-economic support. Completely contrary, crossbreeding, when done correctly in a given location, has significantly improved both animal performance and farmers' income. It is necessary to achieve increased native poultry output [15]. According to the study of Haunshi *et al*. (2009), the production performance of indigenous chicken of northeastern region and improved varieties developed for backyard farming [16]. According to the study of Choo *et al*. (2014), the comparison of Growth Performance, Carcass Characteristics and Meat Quality of Korean Local Chickens and Silky Fowl. Nacked neck and Aseel day-old, chicks weighed higher in form circumstances, however, Nicobari fowl weighed less at four weeks of age in intensive and backyard systems [14].

According to the study by Council in 2003 [18], the Kadaknath chicken breed had a greater body weight at 0, 1, 2, 3, and 4 weeks of age under farm circumstances (290.26 g, 370.38 g, 581.00 g, 831.69 g, and 1232.47 g, respectively). At 6, 8, 10, and 12 weeks of age, Kadaknath chickens weighed 2494.03 g, 3975.23 g, 5556.96 g, and 7544.72 g, respectively, compared to prior reports by [14, 16, 17] and 2494.03 g, 397.523 g, 5556.96 g and 7544.72 g as the average body weights. According to Singh, at eight weeks of age, the Kadaknath breed has a lower body weight of 250 g. Cyril *et al.* (2018), estimate that Aseel weighed 552 g at 10 weeks of age [18]. According to the study by Cyril *et al.* (2010), at six, eight, and ten weeks of age, Nicobari hens weighed 1122.45, 1173.64, 1835.11, 22212.60, and 2308.54 g, respectively [19]. The Kadaknath breed weighs less than the Aseel Bareed at various ages [17]. The genetic differences between the Aseel and Kadaknath breeds may account for the Aseel's larger body size group at various ages [20]. Several examples are reported by Chatterjee et al. [12], Mohammed et al. (2005) [21], and Devi and Reddy [22]. From 0 to 18 weeks of age, the average weight gain (in grams) of the three chicken breeds investigated varied significantly (p 0.05). There were parallels between this investigation's findings [22]. The Kadaknath breed acquired the highest weight between 8 and 12 weeks of age, according to Singh. This demonstrated that the Aseel breed gained much more weight as they aged than other kinds. At the 15-week milestone, Aseel and Kadaknath both surpassed their maximum weight growth were astounded to discover that the Rhode Island Red and White Leghorn chicken breed had the greatest growth in body weight compared to the Desi chicken breed. Variable chicken breeds emerge at different rates due to selective breeding [23].

Genes associated with faster growth rates or the strongest breed selection may be to blame. The average body weight (g) of three distinct chicken species declined considerably (P 0.05) from week zero to week eighteen observation, according to the results of this research. As found according to the study of Duncan in 1955 the crossbreed BPRxRIR generated the maximum body weight in chickens of all breeds and ages, correlating with the findings of this research. There were significant differences in BW among the different hybrids. The egg weights of various hybrids vary considerably (P 0.05) [24]. These researchers discovered that the average weights of hens at six, eight, ten, and twelve weeks of age were greater than those reported by El-Safty (2012) earlier this year (2494.03 g, 3975.23 g, 5556.96 g, and 7544.72 g, respectively [25].

Additional investigations have shown that genetic group significantly affects the weight of chickens of different ages [26]. Similar to the study of Gunawardana *et al*, (2009), the results of this study indicate that the body weights of broilers of the Aseel breed varied (p 0.05) at four weeks of age, which is consistent with the findings of this study [27]. Cyril *et al*. 2010, monthly weighed RIRBPR and other pure chicken breeds, including barred Plymouth Rock (BPR) and Rhode Island Red (RIR) [19]. At 18 weeks of age, RIRBPR had the heaviest live weight (P 0.05), whereas BPR had the heaviest live weight (P 0.05). RIRBPR had the greatest average day-old weight, RIR was in the middle, and BPR had the least. Farooq *et al*. Found that RIR chickens (34.53 g) and BPR chickens (37.00 g) had higher day-old chick weights than RIRBPR chicks (which were lighter) (2001) [28]. (32.93 g). RIRBPR chicks may be heavier than RIR and BPR chicks due to the bigger egg size. Between the ages of 14 and 18, RIR and BPR breeds consumed the most food and gained the highest weight, which may be attributed to genetic variation. However, there was no statistically significant difference in feed intake and weight increase between the RIR and BPR breeds (p 0.05).According to the study of Hassan *et al*. (1962), demonstrated that BPR chicks grew less body weight than RIRBPR and RIR chicks, confirming our findings [29]. According to the reported result of Iqbal *et al*. (2012), RIRBPR's day-old weight, ultimate body weight, and body weight growth were respectively 37.00, 1673, and 1638 g. The day-old weight of BPR was less than in the prior trial, but his ultimate body weight and weight increase were more than in the previous experiment [30].

This research discovered that BPR hens have a slow development rate, which may be attributable to their genetics.Throughout the experiment, the mean weekly feed consumption (mg) of three diverse chicken breeds varied greatly (p 0.05) Under the findings of the study by Islam and Dutta (2010), they discovered that the Fayoumi bird's feed performance was poorer and its feed consumption was much greater than that of the Lyallpur Silver Blackbird [31]. Iqbal et al. [30] noticed a substantial variation in feed consumption between four species of local Aseel hens between weeks 3 and 4 (p 0.081 and 0.0336, respectively) [31-32]. Mushki, Peshawari, and Mianwali Aseel birds consumed an average of 68.57.5 g per day, whereas Peshawari birds consumed an average of 81.810.9 g per day (Mean S.D).

5. CONCLUSION

This study concludes that poultry has a significant role in human life because it has reduced the deficiency of protein by providing meat and eggs to humans. Where the researchers give the idea that daily intake is lesser and not all people included eggs in their daily routine in different areas because every person has a different taste and different preferences unless they are unaware of the energy that their body is missing because intake of protein is very much needed in every age cycle. On the other hand, there are many problems in the poultry industry of Pakistan they are facing so many issues regarding chickens, their breeding process as well as their feeding process too. Costing and money are other issues that are hitting the poultry industry. This research has emphasized that in this manner to read the production of the egg and other related traits because this research is very much helpful for those who are new in this poultry industry. This study also concluded that different breeds give high-quality products and to evaluate this process different genes have been tested in this regard Rhode Island Red chickens have been used in rural farming Mansehra regions of the country because these chickens can produce high-quality breeding processes. Their body weight is significantly high economic component specifically barred Plymouth Rock hens because it reflects the degree of production in the economic strength of the farming operation. Poultry farming is one of the fastest growing and most profitable agricultural businesses in the current situation if all the safety measures and all the requirements are fulfilled by one who wants to start this business it is a huge industry and it can give the opportunity of jobs in rural areas to poor people so they can provide food to their homes by working on the poultry farms.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research Article

Gradient-based LASER Land Leveling Increases the Water Use Efficiency, Growth, and Yield of Cotton Crop under Changing Climate

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Abstract: Increasing water shortage has compelled farmers to develop plans for efficient use of water resources. The improvement in water use efficiency at the field level is very important and can redress water scarcity. LASER land leveling is increasing quickly in the world to increase water use efficiency. However, in developing countries, the practice of LASER leveling is to level land or field with zero (0 %) gradient due to unawareness of gradient-based land leveling while a small gradient (e.g., 0.1 %) is usually kept during land leveling in developed countries of the world. But farmers of developing countries are not well, therefore, an experiment was conducted in farmers' fields covering an area of 3 acres in south Punjab of Pakistan to assess the LASER leveling with a 0 % and 0.05 % grade and general farmer's practice of leveling. Land leveling with LASER using a 0.05 % gradient considerably decreased the amount of irrigation water and/or enhanced water use efficiency by increasing crop yield followed by LASER leveling with a 0 % gradient. Similarly, with a 0.05 % gradient, bolls per plant and final cotton yield increased considerably followed by a 0 % gradient while minimum bolls per plant and cotton yield were obtained from the farmer's practice of leveling. LASER land leveling with a 0.05 % gradient resulted in higher net benefit due to increased yield and a considerable decrease in irrigation amount that significantly improved use efficiency. The outcomes suggest that benefits from land leveling with LASER keeping a 0.05 % gradient are significantly higher when compared with 0 % gradient and/or farmers' practice of leveling.

Keywords: Land LASER Leveling, Gradient, Cotton, Yield, Water Use Efficiency

1. INTRODUCTION

On the eve of climate change, a rapid decrease in irrigation water is causing significant threats to agriculture in different parts of the world. The growing water scarcity as a result of climate change has brought us together on this point that the available water resources must be used judiciously. It is a dire need of time to encourage growers and farmers to use and adopt different water-saving technologies [1,2]. Increasing the water use efficiency at the field level is one of the most suitable solutions to address the water shortage [3]. LASER leveling is a mechanical process of leveling soil. With LASER leveling, the slope of the soil can be leveled up to zero grade. The practice of LASER leveling is increasing rapidly in South Asia, and by 2015, 1.5 million hectares were LASER leveled [4]. LASER leveling improves the uniformity of water application in the field and thus results in

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better crop yield with less amount of irrigation [4-6]. It also increases cultivable cropping soils through the reduction of unnecessary field channels and bunds in the field, decreases weed density, and improvement in input-use efficiencies such as fertilizers and pesticides [7-9].

LASER land leveling is also increasing in the Indo-Gangetic Plains of India and Pakistan. However, the current practice of leveling in these areas is to level the land to a zero (0 %) grade while in developed countries, a slight slope or grade (e.g., 0.1 %) is given during leveling of the agricultural land, but it is not so common in developing countries and nor our farmers have much knowledge about gradient-based LASER leveling [10]. Although, benefits of common LASER land leveling have been well-established in South Asian farmers [4] whereas land LASER leveling using a slight gradient such as 1 % or 0.05 % would further confer remunerations in terms of irrigation water use efficiency and/or yield improvement [10]. For example, in some parts of Australia, a small gradient of 0.08–0.2 % in 100–700-meter-long fields is common to irrigate crops in both beds and flat [10-12] Field layout with a small gradient i.e. 0.4–0.5% are generally kept to ease the drainage. A small gradient from the head (front) of field to the tail (end) increases surface water movement from the waterfront to the field, thus decreasing the time required for irrigation, and preventing excessive water accumulation (waterlogging) in the root zone of crops [10]. Previous studies showed that suitable field gradients decreased the amount of irrigation water in different crops by up to 20 % [10, 13-15]. Thus, gradient-based LASER leveling has the potential to save irrigation water amount and improve crop yields. However, there is no study exists in Pakistan. Therefore, the present study was conducted at farmer's field to evaluate the impact of gradient-based LASER land leveling on water use efficiency, growth, yield, and quality of cotton crops.

2. MATERIALS AND METHODS

The experiment was conducted in a farmer field at Chak-109/1L in 2021, district Rahim Yar Khan, Pakistan (Fig. 1). District Rahim Yar is located at 28◦ 41′ N latitude and 70◦ 30′ E longitude and falls within an extensive alluvial plain located adjacent to the Indus River. The experimental soil texture is clay loam (Table 2) and the climate of district Rahim Yar Khan is sub-tropical according to weather indicators (Table 1). During crop season 2021, the maximum average temperature prevailed from 38.7 ℃ to 43.1 ℃ during the last week of April to June (Table 1). While a minimum temperature of 23 ℃ to 30.5 ℃ was observed in the crop season from April to November. Rainfall occurred in July (18 mm) August (43 mm) and September (11 mm) during crop season 2021. It can be figured out that overall crop season was dry and hot. Rainy season started from July to the end of September during which more than 85 % of rainfall was received during August.

2.1 Design and Treatments

Experimental treatments were land LASER leveled with 0.0 % and 0.05 % gradient and farmers' practice of leveling. Total area of the experiment was 3 acres from which 1 acre was leveled with LASER with 0 % gradient, 1 acre with 0.05 % gradient, and 1 acre with farmers' practice of leveling.

2.2 Land Preparation and Crop Husbandry

The fields to be LASER leveled were ploughed 3 times with tractor mounted cultivator. After cultivation, the field was leveled with tractor mounted LASER land leveler. The LASER

Table 1. Weather data of experimental trial during 2021

	April	May	June	July	August	September	October	November
Avg. min. Temp. ˚C	23.0	27.8	30.5	30.5	29.1	27.3	23	16.4
Avg. Max. Temp. ˚C	38.7	42.1	43.1	40.8	38.6	38	34	30.1
Rainfall (mm)	-	-	-	18	43	11	-	-
Humidity (%)	28	23	34	48	57	50	43	46

Fig. 1. Map of Pakistan indicating study area in District Rahim Yar Khan, Punjab province.

transmitter sends a LASER beam that is caught by the signal receiver attached to a leveling blade mounted to the tractor. The control panel attached to the tractor reads the signal from the receiver and closes or opens the hydraulic control valve that downs or raises leveling blade. The gradient of 0.05 % was created and then it was confirmed using a LASER land leveler receiver and transmitter.

Plot size of each treatment was 88 meters long, and 46 meters wide, so in the case of gradient-based LASER leveling, 0.05 % gradient resulted in 4.0±0.02 cm alteration in elevation between irrigation inlet side (head) and tail ends of the plot. When the soil was leveled, samples from different sites of each plot were drawn to a depth of 30 cm (0-15 cm and 15-30 cm) with the help of an auger to analyze the physicochemical properties of the soil. The collected samples of soil were numbered for identification and then sent to the soil and water testing laboratory of the district Rahim Yar Khan, and the report is given in Table 2. After sampling in

Table 2. Physiochemical properties of the experimental soil

Soil Properties	**Soil depth**	
	0-15 cm	**15-30 cm**
Texture	clay loam	clay loam
pH	8.1–8.3	8.4–8.6
EC (mS-cm)	1.4–2.3	1.2–1.7
Organic matter (%)	0.62–0.73	0.51–0.59
Bulk density (g cm^{-3})	1.10	1.10
Saturation (%	52–60	49–54
Available N (%)	0.048	0.026
Available P (mg kg^{-1})	4–6.5	3.5–5.8
Available K (mg kg^{-1})	115–125	100–118

each plot, beds were developed through bed shaper and the distance between the centers of adjacent beds was 75 cm while furrow width and depth were 30 and 15 cm, respectively.

Seed of cotton variety CKC-3 @ 20 kg ha^{-1} was used. Seeds were sown with manual labor keeping plant to plant distance 30 cm. Recommended doses of fertilizers such as nitrogen (200 kg ha^{-1}), phosphorus (120 kg ha^{-1}) and potash (100 kg ha^{-1}) were used. All the amount of potash and phosphorus while 1/3rd of N were used during seed bed making. The remaining nitrogen was applied at squaring formation and at the flowering stage. The phosphorus was applied as DAP, and nitrogen was applied as urea and potassium as potassium chloride. Weeds such as purple nutsedge (*Cyperus rotundus*), Bermuda grass (*Cynodon dactylon)*, horse purslane (*Trianthema portulacastrum*), common purslane (*Portulaca oleracea*), wild rice (*Echinochloa colonum*), green amaranth (*Amaranthus viridis*), puncture vine (*Tribulus terrestris*) and false amaranth (*Digera muricata*) were control through manually by hand weeding whereas pesticides were used to control the pests of cotton such as whitefly (*Aleurodicus dispersus*), jassid (*Amrasca biguttula*), thrips (*Frankliniella schultzei*), pink bollworm (*Pectinophora gossypiella*), American bollworm (*Helicoverpa armigera*) and spotted bollworm (*Earias vittella*).

2.3 Irrigation Water and its Measurement

The crop was irrigated by canal water. The inflow rate of irrigation water was monitored through a cutthroat flume (Fig. 2) (122 cm length × 72 mm width), fixed at several meters upstream from inlet of the field trial. The inflow rate readings of the flume and time were recorded periodically until the flow cut-off (stopped). Each field plot was irrigated independently to estimate the correct amount of irrigation water. Soil moisture was noted daily from each plot through a digital soil moisture meter (Misol WH0291, China). The crop was irrigated when average soil moisture reached 25 % on the basis of a soil moisture meter. In gradient-based land leveled plot, irrigation water was stopped ("cut-off") as soon the water flowing over the soil surface reached the mid-point of the bottom edge of the plot.

The quantity of irrigation water was measured using the float method through a cutthroat flume [16]. The cutthroat flume was fixed in water-channel and cemented so that water can only pass through the flume. When water depth in the cutthroat flume was constant then the downstream flow depth (*h b*) and upstream flow depth (*h a*) were recorded in meters using scale. The flow condition was determined through the following equation:

Flow condition = *h b* /*h a*

If the value of *h b* /*h a* is less than 0.65 then it will be free flow. In our experiment, flow condition was free flow (*h b* /*h a* = *<0.65*). After determining the condition for upstream flow, the discharge was measured with the value of *h* a and *h b* for a flume size of 122 × 92 cm. Stop-watch was used to record time taken to fill each plot.

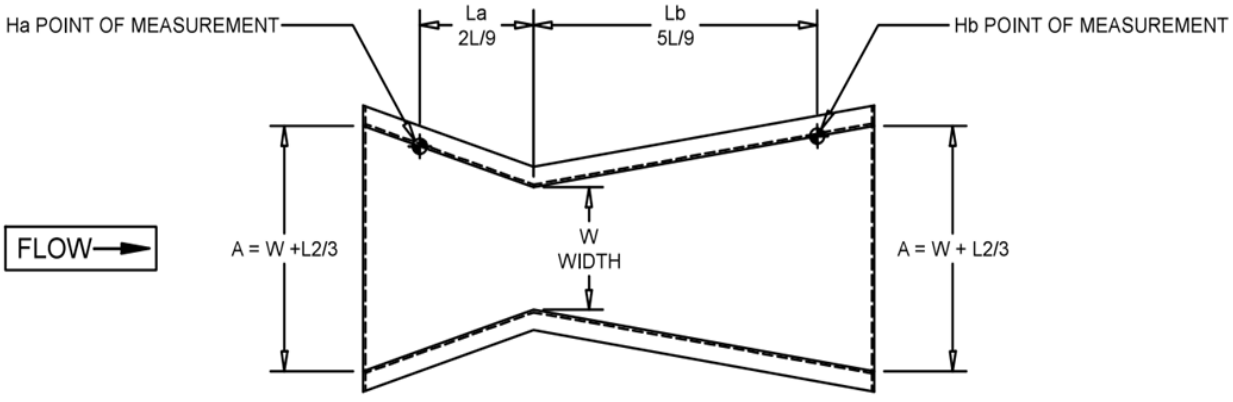

Fig. 2. Plain view of a cutthroat flume

Then discharge (Q) and amount of irrigation water were measured using the following formula:

Discharge (Q) = 2.85 × Hu × 1.82 ..... (1)

Where;

Q is discharge (m^3 Sec^{-1}) and Hu shows the upstream head reading of the cutthroat flume.

Volume of irrigation water applied (V) = Q × T...(2)

Where;

V is the volume (m^3), Q is discharge (m^3 Sec^{-1}) and T is the time taken in seconds to fill the field.

2.4 Observations

Three sites, front, middle and tail, from each plot, were selected for data collection. From each site, 10 plants were tagged for measurements of leaf area index, plant height, bolls per plant and average boll weight. At the end, total seed-cotton yield was measured and samples were taken for ginning out turn (%), staple length (mm), fiber uniformity index (%), micronaire or fiber fineness (µg/inch), and fiber strength (tppsi). Plant height and intermodal distance were recorded using a meter rod and scale, respectively, and then the average was calculated. Similarly, the number of bolls from each plant was counted and then averaged, and these bolls were picked from plants and their weight was noted in grams with the help of digital balance and then averaged. For the leaf area index, three plants were cut from the front, middle and tail sites of each plot. The leaves from harvested plants were removed from the stem and then the leaves and stems were weighed separately. A sub-sample of leaves (5 g) was brought to the laboratory and their leaf area was noted through a digital leaf area meter and then the leaf area index was derived through a formula [17].

Leaf area index (LAI) = Leaf area/Ground area

For the number of days taken to squaring, flowering and boll splitting, an area of 3 m^2 was selected and when 50 % of plants had squared, flowered and boll opened then the date was noted. Seed cotton yield was noted during each picking from each plot. After the last picking, the total seed-cotton yield was calculated from each plot. With the help of the following formula, ginning out turn was measured.

Ginning out turn (GOT) % =

$$\frac{\text{Lint weight}}{\text{Weight of seed cotton}} \times 100$$

For fiber uniformity index, staple length, micronaire or fiber fineness, and fiber strength, 40 grams of lint from each plot (front, middle, and tail end) was taken and sealed in envelopes that were brought to Central Cotton Research Institute, Multan for analysis.

2.4.1. Water Use Efficiency (WUE)

Crop WUE was calculated according to the procedure followed by Neal *et al.* [18].

Crop water use efficiency (kg m^{-3}) = Economic yield / total amount of water supplied.

2.4.2. Economic Analysis

Economic analysis shows the cost and income of the system. It was calculated by following the method of Byerlee [19]:

Cost of production (USD/ha) = Permanent cost (USD/ha) + variable cost (USD/ha)

2.5 Statistical Analysis

All the obtained data of parameters were analyzed statistically using Statistix 8.1 [17] and the difference between treatments was assessed through the least significant difference test (LSD) at a 5 % probability level [20].

3. RESULTS

Results showed that there was a consistent trend for higher plant height (131 cm) and intermodal distance (4.75 cm) in the farmer's practice of leveling followed by LASER leveling with 0 % gradient (Table 3). Minimum plant height (110 cm) and intermodal distance (4.10 cm) were measured in LASER leveling with a 0.05 % gradient. Similar

trend was recorded for leaf area index, cotton grown on the field with the farmer's practice of leveling had a leaf area index of 3.75 followed by LASER leveling with a 0 % gradient (3.47) whereas less leaf area index (3.19) was recorded for LASER leveling with 0.05% gradient (Table 3). However, the maximum number of bolls per plant (32 bolls per plant) was recorded in 0.05 % gradient LASER leveling than in the 0 % gradient LASER leveling 7 bolls per plant) while the minimum number of bolls (21 bolls per plant) was recorded for farmers' practice of leveling (Table 3).

Cotton took more days to reach squaring, flowering, and boll splits when planted on a field leveled with farmers' practice followed by soil leveled with a 0 % gradient (Table 4) might be due to higher soil moisture content. Cotton planted on soil level with a 0.05 % gradient through LASER leveling took a minimum number of days for squaring, flowering, and boll splitting.

Boll weight increased in cotton (2.89 g) planted on 0.05% gradient LASER leveling soil (Table 5) but the difference was non-significant with 0 % gradient (2.82 g) whereas cotton planted on farmers' practice of leveling had minimum boll weight (2.63 g). Due to more bolls per plant, yield of cotton (seed-cotton) was higher (2267 kg ha^{-1}) when cotton was planted on 0.05 % gradient soil as compared to those planted on 0.0 % gradient soil (2019 kg ha^{-1}) whereas minimum cotton yield (1715 kg ha^{-1}) was recorded for cotton planted on farmers' practice of land leveling. Similarly, ginning out turn (GOT) was maximum (39.73 %) in cotton grown on soil LASER leveled with 0.05 % gradient as compared to those grown on soil LASER leveled with 0 % gradient (38.26 %) while minimum GOT (35.14 %) was recorded for cotton planted on soil with farmers' practice of leveling (Table 5).

Lint quality parameters such as staple length, fiber uniformity index and were affected by gradient-based land leveling while no effect on fiber strength. Minimum value of staple length (27.16 mm), fiber uniformity index (85 %), and high micronaire (4.93

Table 3. Effect of land gradient on plant height, intermodal distance, leaf area index, and number of bolls per plant in cotton

Treatments	Plant height (cm)	Intermodal distance (cm)	Leaf area index	Bolls per plant
Farmers' practice of leveling	131 a	4.75 a	3.75 a	21 c
LASER leveling with 0.0% gradient	125 b	4.31 b	3.47 b	27 b
LASER leveling with 0.05% gradient	110 c	4.10 c	3.19 c	32 a
LSD value	4.89	0.12	0.17	1.68

Table 4. Effect of land gradient on days to squaring, flowering, and boll splits in cotton

Treatments	Days to squaring	Days to flowering	Days to boll splits
Farmers' practice of leveling	38 a	58 a	132
LASER leveling with 0.0 % gradient	33 b	52 b	124
LASER leveling with 0.05 % gradient	30 c	50 c	120
LSD value	1.03	0.85	2.12

Table 5. Effect of land gradient on boll weight, seed-cotton yield, ginning out turn and staple length in cotton

Treatments	Boll weight (g)	Seed-cotton yield (kg ha^{-1})	Ginning out turn (%)	Staple length (mm)
Farmers' practice of leveling	2.63 b	1715 c	35.14 c	27.16 c
LASER leveling with 0.0% gradient	2.82 a	2019 b	38.16 b	27.90 b
LASER leveling with 0.05% gradient	2.89 a	2267 a	39.73 a	28.14 a
LSD value	0.12	149	0.45	0.11

Table 6. Effect of land gradient on fiber uniformity index, micronaire, irrigation amount and crop water use efficiency in cotton

Treatments	Fiber uniformity index (%)	Micronaire (µg/inch)	Fiber strength (tppsi)	Irrigation amount (mm)	Crop water use efficiency (kg m^{-3})
Farmers' practice of leveling	85 c	4.93 a	94 a	546 a	0.41
LASER leveling with 0.0 % gradient	86 b	4.43 b	94 a	364 b	0.91
LASER leveling with 0.05 % gradient	87 a	4.17 c	94 a	254 c	1.12
LSD value	0.44	0.12	0.25	0.34	0.10

µg/inch) were noted for cotton grown on farmers' practice of leveling followed by LASER leveling with 0.0%. Cotton grown on soil with a 0.05 % gradient through LASER leveling showed better lint quality (Tables 5 and 6). Land gradient significantly affected the amount of irrigation in cotton crops (Table 6). Land LASER leveling 0.050 % gradient reduced the irrigation amount in cotton by about 26 % (254 mm) compared with a 0 % gradient (384 mm) and 53 % compared with farmers' practice of leveling (546 mm). Similarly, land gradient greatly affected the water use efficiency (Table 6). Water use efficiency was significantly lower in farmers' practice of leveling (0.41 kg m^{-3}) than in gradient-based leveling. Within the land gradient, there was a trend for higher water use efficiency with a 0.05 % gradient (1.12 kg m^{-3}) than a 0 % gradient (0.91 kg m^{-3}).

Economic analysis showed that with farmers' practice of leveling, the total expenses of cotton production was USD 508 ha^{-1} followed by LASER leveling with 0 % gradient (477) while the minimum was in LASER leveling with 0.050 % gradient (430) (Table 7). The high cost of cotton was probably due to the high irrigation cost in farmers' practice of leveling. LASER leveling significantly reduced the cost of production of cotton over farmers'= practice of leveling due to the reduced cost of irrigation water. LASER leveling with a 0.05 % gradient enhanced cotton production by USD 1570 ha^{-1} and reduced the cost of production (USD 430 ha^{-1}). LASER leveling with 0 % and 0.05 % gradients significantly enhanced the net returns from cotton in comparison with the farmer's practice of leveling. Beds with a 0.05 % gradient further increased returns of cotton crops relative to farmers' practice of leveling (Table 7).

4. DISCUSSION

Results indicated that plant height, intermodal distance and leaf area index were maximum in the farmer's practice of leveling followed by a 0 %

Table 7. Effect of land gradient on the economic return of cotton crop

Treatments	Land LASER leveling cost (US$)	Irrigation cost (US$)	Fertilizer cost + pesticides (US$)	Total cost (US$)	Total income (US$)	Net income (US$)	BCR
Farmers' practice of leveling	32	155	321	508	1511	1005	1.98
LASER leveling with 0.0 % gradient	51	105	321	477	1781	1304	2.73
LASER leveling with 0.05 % gradient	51	58	321	430	2000	1570	3.65

gradient. The increase in plant height, intermodal distance and leaf area index might be due to high moisture content that promoted the vegetative growth of plants. A planned moisture regime is very important because both the high moisture content in the soil and/or very low moisture are harmful to cotton crops. Under high moisture, more vegetative growth occurs which can interfere negatively with productivity [20]. During the vegetative phase, if the cotton crop is subjected to higher moisture content, the plant produces excessive vegetation and results in less number of bolls, reduced boll weight, and final seed-cotton yield. Opening of bolls was delayed and the time needed for maturity increased. On the other hand, cotton planted on LASER leveled soil with a 0.05 % gradient-based had ideal plant height, intermodal distance and leaf area index that resulted in higher seed-cotton yield. There was less number of bolls in cotton growing on the field with the farmer's practice of leveling and 0% gradient field might be due to higher vegetative growth and other common sub-optimal practices of farmers other than leveling [10] while cotton planted in fields with LASER leveled soil with 0.05 % gradient had a maximum number of bolls that resulted in higher seed-cotton yield. Ginning out turn was maximum in cotton that was planted in fields leveled with LASER with 0.05 % gradient probably due to higher seed-cotton yield. Lint quality such as staple length, fiber uniformity index, and micronaire was better in cotton planted on soil LASER leveled with 0.05 % while farmers' practice of land leveling reduced the lint quality might be b due to high soil moisture content, which delayed the maturity of cotton. Micronaire shows the thickness of the cell wall of cotton fibers and is usually used as an indicator of fiber maturity and fineness. Fiber fineness decreases when a cotton plant produces more quantity of carbohydrates than required to support the plant's development. Excess carbohydrates accumulate or are available to fiber cells wall and thicken the cell wall of fiber [22]. Fiber length generally depends on the growth environment, varietal interaction with the environment, and crop management. High temperature and moisture stress during boll development significantly affect the fiber length. Similarly, nutrient deficiencies and insect pressure can reduce the staple length. Previous research showed that frequent irrigation definitely delayed the maturity of crop. Higher water use efficiency of cotton planted on LASER land leveled with 0.05 % was higher due to higher yield and less amount of irrigation during the growing season. It seems that soil leveled by LASER with 0.05 % gradient provided suitable soil-moisture content to cotton crops and crop reached maturity by 6-8 days earlier (Table 2) and higher yields were obtained as compared to farmer's practice of irrigation or land leveled by LASER with 0% gradient. Cotton sown on soil with farmers of leveling delayed the maturing might be due to the high moisture content of the soil that promoted vegetative growth (Table 1).

The lower irrigation amounts and high water use efficiency of the LASER leveling with 0.05 % gradient in comparison with farmers' practice of leveling and also with 0 % gradient and are consistent with the findings of Devkota *et al.* [10] who documented high water productivity and lower irrigation amount in LASER leveling with 0.01 % gradient. Other studies by Aryal [6], Ferrari *et al.* [21], and Jat *et al.* [23] reported that LASER leveling increased water productivity in small farmers' fields in South Asia. Previous studies showed that a small gradient has the potential to decrease the irrigation amount as the length of the field or plot increases particularly for permeable soil [14]. For example, in the Philippines, a 0.1 % gradient decreased 22 % irrigation water amount for dry seeded rice on highly permeable clay loam soils rather than a 0 % gradient [24]. Alike, Gonzalez *et al.* [14] reported that a gradient of 0.04 % in 200 m × 50 meters plots resulted in a 20 % decrease in irrigation water for rice crops as compared to a 0 % gradient. In Brazil, Winkler *et al.* [25] compared land leveling using different gradients such as 0 %, 0.20 %, 0.25 %, 0.28 %, and 0.40 % in rice and reported that 0.1 % gradient enhanced the yield of rice by 10 % (0.5 t ha^{-1}) when compared with 0 % gradient. Maximum net income was obtained when the soil was leveled with LASER with a 0.05 % gradient while the farmer's practice of leveling resulted in minimum profit. The minimum profit in Farmers' practice is probably due to lower yield and maximum irrigation cost, but LASER leveling with 0.05 gave a high due to high yield and lower irrigation cost.

5. CONCLUSION

Under severe water scarcity due to climate change, there is a dire need to shift from traditional land leveling to an innovative gradient-based LASER land leveling technique that not only uses less irrigation water but also increases productivity and net profit in the context of future climate shifts.

6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research Article

Catalytic Conversion of Castor Oil into Biodiesel by Tri-organotin(IV) Catalysts: Chromatographic and Spectroscopic Characterization with Theoretical Support

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Abstract: Economic concern over fossil fuel reserves, extensive increase in CO_2 emission, and change in the world climate due to the combustion of carbon sources have been driving the attention of both commercial and academic researchers towards new sustainable fuel routes to encounter rapidly growing worldwide population demands. In the present study, we have focused on catalytic transesterification towards the environmentally friendly biodiesel synthesis which is low cost, easily implemented and best alternative source of fossil fuels. Herein three triorganotin(IV) carboxylates derivatives namely trimethylstannyl cyclopentanecarboxylate, tributylstannyl cyclopentanecarboxylate and triphenylstannyl cyclopentanecarboxylate were resynthesized according to our reported procedure and theoretically investigated through DFT by applying LANL2DZ as functional with B3LYP basis set (level of theory) to calculate Molecular Electrostatic Potential (MEP) to determine electrophilic center of complexes and Lewis acidity. Owing to the Lewis acid character, the synthesized complexes were then used as catalysts in the transesterification reaction of castor oil. Different reaction parameters were also optimized to obtain maximum biodiesel yield. Synthesized castor oil biodiesel (COB) was characterized and confirmed by employing multitude spectroscopic techniques. The present study evaluated that these complexes can be potential candidates for biodiesel conversion from non-edible and cheap feedstock.

Keywords: Triorganotin(IV) Carboxylates, Theoretical Studies, Castor Oil Biodiesel (COB), Spectroscopic Studies, GCMS

1. INTRODUCTION

Energy demand in the world is continuously increasing as it is one of the important resources for sustainable development. Fossil fuel contributes up to 80 % of total energy and is the main source of mankind's energy demand. According to World Energy Outlook (WEO) 2007, fossil fuels are the major source of producing energy till 2030 and will contribute up to 84 % to meet energy demand [1]. But due to rapid population growth, high demand for energy than total production, environmental issues related to harmful emissions from fossil fuel consumption, reduction of fossil fuels and high rates of fossil fuels world is facing energy crises. Major conventional energy resources petroleum, natural gas and coal are on the verge of extinction and unable to meet future energy needs for a long [2]. So, due to uncertainty about the future supply of fossil fuels, researchers are trying to replace fossil fuels with sustainable, renewable, and trustable energy resources.

Biofuel or biorenewable fuel refers to biogas, bioalcohols [3, 4], vegetable oils [5], biodiesel [6], biohydrogen and pyrolysis oils [7, 8]. Two major important biomass-based liquids are bioethanol and biodiesel which might replace diesel fuel and gasoline in liquid transportation fuels. Biodiesel is methyl- or ethyl ester of fatty acids (FAME) acquired from renewable lipid feedstock through catalytic transesterification reaction in the presence

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of organic solvents like methanol or ethanol. Feedstock for biodiesel includes first-generation edible oil plants, non-edible oil crops as second-generation, third-generation algal sources and animal fats [9]. Non-edible oils are preferred as these are not included in human food because of toxic compounds and could be grown in non-cultivated lands [10].

In transesterification reactions, generally, two types of catalysts are employed. In alkali-catalyzed transesterification alkoxide or hydroxides (Bronsted bases) are used but drawbacks of soap formation, as well as wastage of free fatty acid content (10 to 25 %), are associated with this type of catalyst. Alternatively, Bronsted acids are used with the drawback of slow conversion rate and corrosion of the reactor [11]. Recent studies showed that organotin(IV) complexes as catalysts can be a potential candidate in transesterification reactions to produce biodiesel.

These compounds are used in transesterification reactions as catalysts due to their ability to accelerate the reaction between ester and alcohol (by activating the carbonyl carbon of ester for the electrophilic attack of alcohol) because of the Lewis acidity of tin atom and capability of tin(IV) is the expansion of its coordination number. These are mild acids than Bronsted acids so safe in handling and efficient to catalyze a broad range of homogeneous liquid phase reactions. Lewis acidity of tin(IV) increases due to the presence of electron-withdrawing carboxylate ligand and also depends upon the nature of the organic group bonded with tin. Lewis acidity of organotin(IV) carboxylates is higher than their respective chlorides and increases with an increase in the acidic strength of carboxylic acid [12]. The density functional theory (DFT) method is a common method used for theoretical calculations of complexes [13]. Various spectral, structural and Lewis acidity details of the complexes were obtained through DFT calculations [14].

Because of the above-mentioned attractive features of organotin(IV) carboxylates, they were theoretically studied via the DFT method to calculate Molecular Electrostatic Potential (MEP) (to evaluate Lewis character) and tested as catalysts for conversion of castor oil into biodiesel. For the high yield of biodiesel, various reaction conditions were optimized i.e. (time, temperature, oil-methanol ratio and catalyst concentration). The synthesized biodiesel was confirmed by employing FT-IR (ATR), ^{1}HNMR and GC-MS techniques.

2. MATERIALS AND METHODS

Reagents i.e., $NaHCO_3$, Me_3SnCl, Ph_3SnCl, Bu_3SnCl and cyclopentanecarboxylic acid were attained from Aldrich (USA) and were used as such. Castor oil was purchased commercially from the nearest market of Islamabad, Pakistan. The solvents used in the experiments were purchased from Merck (Germany) and dried accordingly [15]. The melting points were checked in a capillary tube, using Gallen Kamp (UK) apparatus. Bruker Tensor II (ATR) FT-IR spectrophotometer was employed to record the spectra (4000 – 400 cm^{-1}). ^{1}H NMR spectra were recorded on FT-NMR (Burker-300 MHz) spectrometer with DMSO-d6 as solvent [^{1}H (DMSO) = 2.50 ppm]. For GC-MS analysis the GC (GC-6890 N) coupled with MS 5973 MSD fitted with DB-5MS capillary column (30-0.32 mm and 0.25 μm of film thickness) was used.

2.1 Synthesis

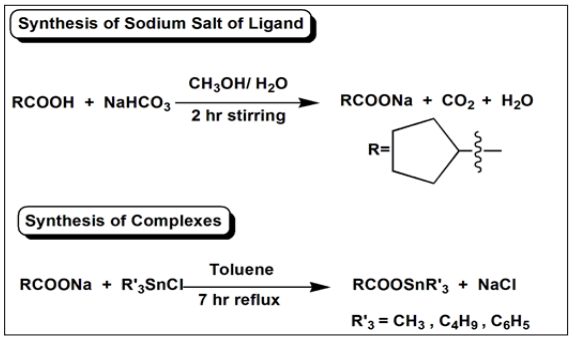
2.1.1. Synthesis of the Sodium Salt of Ligand

Sodium salt of cyclopentane carboxylate was synthesized according to the reported literature [16] (Scheme 1). Equimolar aqueous solution of $NaHCO_3$ (1.6 g, 20 mmol) was added slowly into the stirring solution of cyclocarboxylic acid-dissolved in methanol (2.16 mL, 20 mmol). The stirring was continued for 2 h at room temperature. Solid product of Sodium Salt of Ligand (NaL) was isolated on a rotary evaporator and then dried. The FT-IR spectroscopy was employed to confirm NaL formation because the OH vibration frequency which is present in the carboxylic acid ligand disappeared. This NaL was then further used as such for complex formation.

2.1.2. Synthesis of Complexes

Trimethylstannyl carboxylate (Me_3SnL), tributylstannyl carboxylate (Bu_3SnL) and triphenylstannyl carboxylate (Ph_3SnL) were resynthesized according to our recently reported method [16] (Scheme 1). Briefly, all the complexes

were synthesized in stoichiometric amounts, sodium salt of the ligand (5 mmol, 0.68 g, 25 mL toluene) in two necks RBF and were refluxed for 7 hours with a solution of trimethyltin chloride in toluene (5 mmol 0.99 g, 25 mL toluene). The reaction mixture was cooled, filtered and transferred to a rotary flask to evaporate toluene under reduced pressure. The crystals of (R_3SnL) were isolated from hexanes: chloroform (3:1). All the complexes were characterized and the data are reported elsewhere [16].



Scheme 1. Synthesis of NaL and its organotin (IV) complexes.

2.2 Computational Studies

Synthesized complexes have been studied theoretically through the density functional theory (DFT) method. The gaussian 09 with LANL2DZ functional with B3LYP as basis set (Lee, Yang and Parr) was used for calculation [17, 18]. Molecular Electrostatic Potential (MEP) was executed to determine Lewis acidity and the electrophilic center of synthesized complexes, respectively.

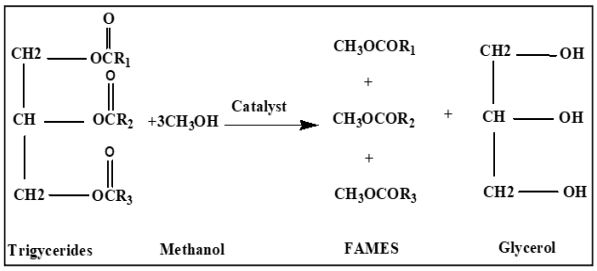
2.3 Transesterification

Owing to Lewis acidity character as determined from the DFT study (NBO analysis) the synthesized organotin(IV) complexes were checked for their catalytic activity towards transesterification of castor oil into biodiesel (Scheme 2) according to reported literature [12]. Before starting the reaction, castor oil was preheated up to 100 °C to remove the moisture. Reactions of castor oil with methanol were carried out following the same protocol (2.1.2.). For maximum yield different parameters of catalytic transesterification reaction i.e., oil to methanol ratio, time, catalyst concentration and temperature were investigated by performing a series of reactions. Once the reaction is completed, the reaction mixture is kept in a separating funnel overnight for phase separation. The glycerol layer being denser settled down at the bottom and is removed, while the above layer which contains biodiesel was isolated from methanol on a rotary evaporator. The conversion yield was calculated using given equation 1 and through ^{1}H NMR spectroscopy by employing equation 2 [19, 20].

$$\%\ yield = \frac{\text{Grams of methyl ester produced}}{\text{Grams of oil used for reaction}} \times 100 \quad (1)$$

$$\%\ \text{Conversion} = \frac{2A_{Me}}{3A_{CH_2}} \times 100 \quad (2)$$

Where A_{CH2} is integration value for α-methylene peak and A_{Me} is integration value for methoxy peak [21].



Scheme 2. General transesterification reaction.

3. RESULTS AND DISCUSSION

3.1 Molecular Electrostatic Potential (MEP)

MEP of complexes was analyzed by utilizing LANL2DZ functional with B3LYP level of theory. MEP map is used to recognize electrophilic centres in molecules. MEP map results revealed positive charge density on Sn(IV) atoms recognizing them as nucleophilic attack sites (see Figure 1). Red lines surrounding oxygen atoms showed negative charge density on atoms of ligand in complexes. MEP analysis supports that Sn(IV) atoms in complexes have Lewis acid character and hence these complexes become potential candidates for acid catalysts used in transesterification reactions.

3.2 Transesterification

The synthesized organotin(IV) complexes have been used in the conversion of castor oil into

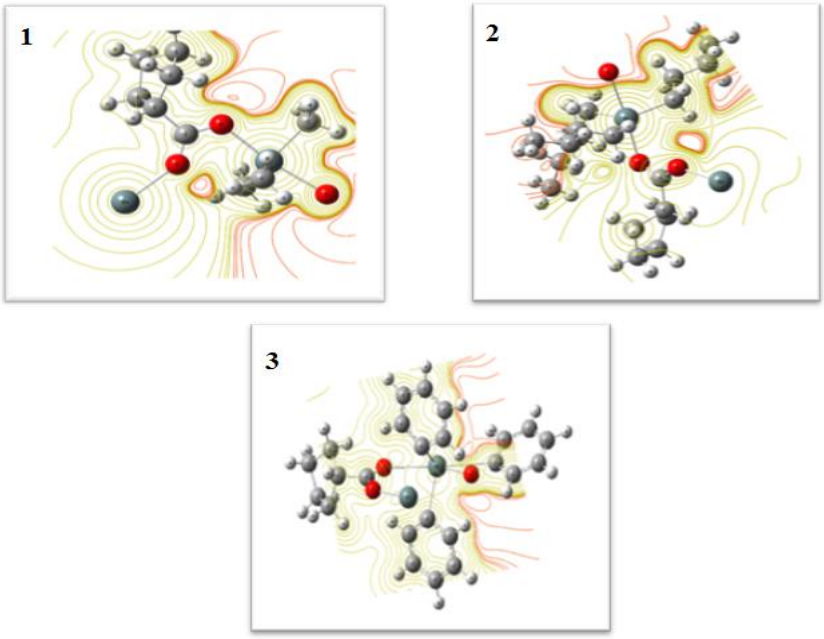


Fig. 1. MEP of complexes 1, 2 and 3.

biodiesel as a catalyst being an eco-friendly and low-cost fuel. To obtain maximum biodiesel yield different parameters have been optimized. These parameters were reaction time, temperature, catalyst concentration and oil-to-methanol molar ratio. Optimization of reaction conditions and comparing their results showed which catalyst is more efficient. The quantification of synthesized biodiesel was checked by using equation 1 (See experimental section).

3.2.1. Optimization parameters for biodiesel synthesis

3.2.1.1 Effect of oil-methanol ratio

For biodiesel synthesis, oil to methanol ratio plays an important role. According to stoichiometry, for every 1 mole of triglyceride, 3 moles of methanol are needed to form 3 ester molecules, so a higher amount of methanol is needed. To obtain optimum oil: methanol, transesterification reaction was carried out by using 1:8, 1:10, 1:12 and 1:14 oil to methanol ratios, keeping catalyst loading 1 % and time 14h at 65°C temperature. It was noticed that initially reaction yield was increased with an increase in methanol ratio which is supported by stoichiometry and a decrease in viscosity favors effective interaction between reactants and catalysts molecules by lowering the mass transfer problem but after 1:12 oil to methanol ratio, the yield decreases gradually. This decrease in yield at a higher oil-to-methanol ratio is due to the dilution effect. Best yield for complexes 1, 2 and 3 were 95 %, 85 % and 82 % respectively at a 1:12 oil-to-methanol ratio (see Figure 2).

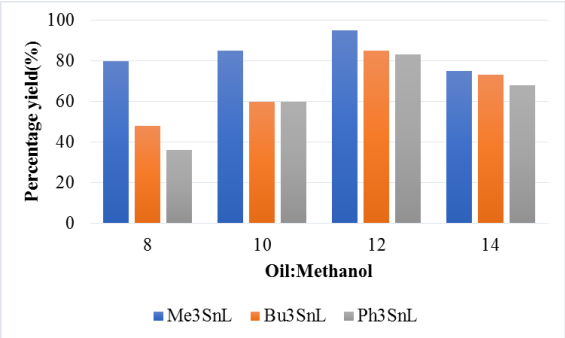


Fig. 2. Effect of oil: methanol on transesterification reaction.

3.2.1.2 Effect of catalyst concentration

Yield in the transesterification reaction was also affected by variations in catalyst concentration. In the present study, four different concentrations (0.5-2 %) of each catalyst have been used at an optimized 1:12 oil to methanol ratio and 14h reaction time. Investigation of obtained results showed the maximum % yield for each complex at 1 % of catalyst concentration (see Figure 3). Initially, biodiesel yield increase with the increase

in catalyst amount indicating an increase in active sites but after a specific concentration decrease in yield occurs which may be possibly due to the coagulation of catalyst in reaction mixture and an increase in viscosity.

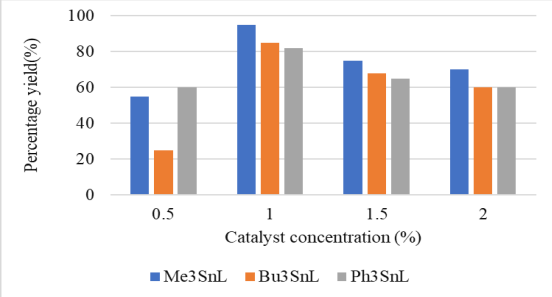


Fig. 3. Effect of catalyst amount on transesterification reaction.

3.2.1.3 Effect of time

In order to understand the effect of time the biodiesel conversion reaction was performed by varying time intervals (1h, 6h, 14h and 24h) by keeping the catalyst and oil to methanol concentration as 1 wt. % and 1:12, respectively. The maximum conversion rate was achieved at 14 hours (see Figure 4). Initially, reaction yield was increased with increasing time but after a certain time, yield decreased. This decrease in yield may be due to blockage of active sites with glycerol molecules which is the byproduct of the reaction.

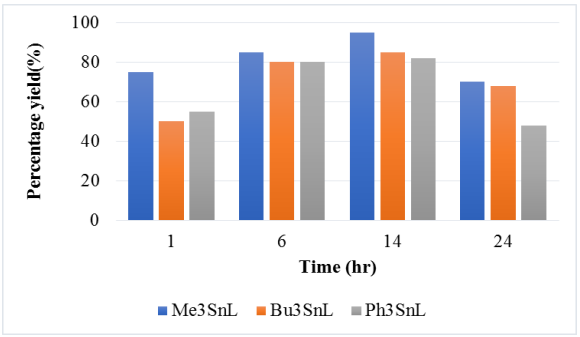


Fig. 4. Effect of time on transesterification reaction.

3.2.1.4 Effect of temperature

The effect of temperature was tested by changing the temperature (45-75 °C) for repeated reactions by maintaining other parameters constant. Initially, biodiesel yield increased with increasing temperature and a high yield was attained at 65 °C (see figure 5) After that gradual decrease occurs which may be Possibly due to the gasification of methanol.

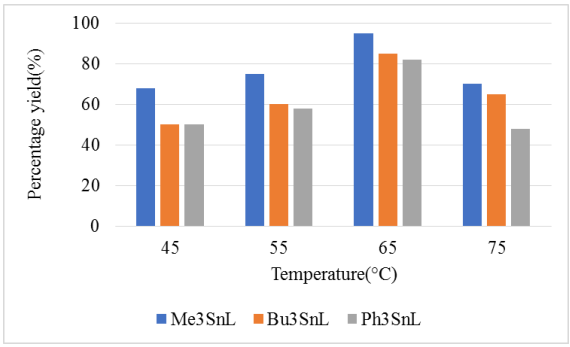


Fig. 5. Effect of temperature on transesterification reaction.

3.3 Spectroscopic Studies of COB

3.3.1. Infrared spectroscopy

FT-IR spectroscopy was employed for synthesized biodiesel to analyze the disappearance and appearance of specific vibrational bands in castor oil and biodiesel spectra. In biodiesel spectrum broad absorption vibration observed in region of 3500-3000 cm^{-1} indicate presence of hydroxyl group in ricinoleic acid [22]. At 1740 cm^{-1} sharp high intensity methoxy peak was observed. Absorption vibration at 3007 cm^{-1} appeared due to sp^2 CH stretch. High intensity absorption vibrations at 2923 cm^{-1} and 2853 cm^{-1} can be ascribed to methyl group. Methyl ester group shows absorption vibration at 1438 cm^{-1}. Other peaks at 1164 cm^{-1}, 1052 cm^{-1} is attributed to O-CH_3 stretching vibrations [23]. FT-IR spectrum of COB obtained by using representative catalyst is depicted in figure 6.

3.3.2. ^1H NMR spectroscopy

The ^1H NMR spectroscopy was used to characterize castor oil biodiesel and the spectrum is depicted in Figure 7. Due to the presence of FAMEs appearance of two distinct peaks in spectrum confirm the synthesis of biodiesel. One characteristic peak is for methoxy protons as singlets at 3.65 ppm and second peak is for α-methylene (α-CH_2) protons as triplets at 2.21 ppm. Other peaks in the spectrum are of terminal methyl protons at 0.87 ppm,

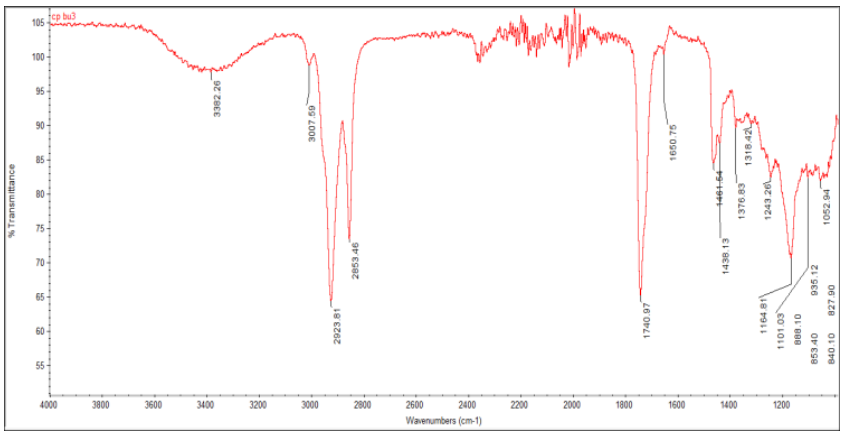


Fig. 6. FT-IR spectrum of COB obtained by using the representative complex as catalyst.

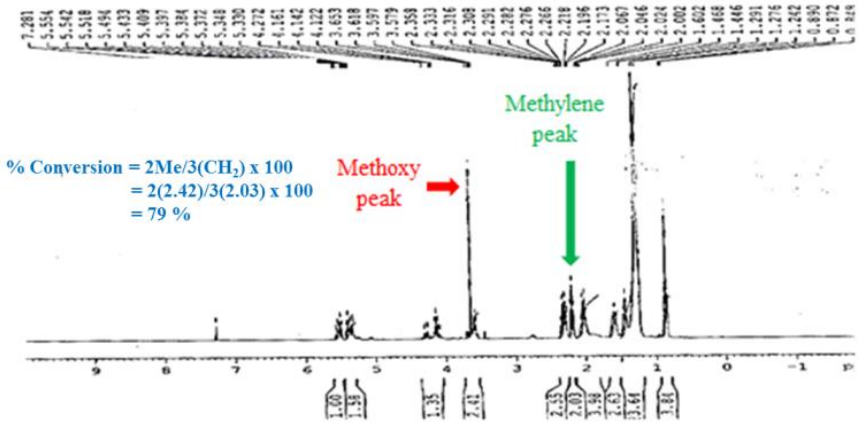


Fig. 7. ^{1}H NMR spectrum of COB obtained by using representative complex as catalyst.

sharp peak at 1.14 ppm for methylene protons of the carbon chain, β-carbonyl methylene peak at 1.60 ppm, allylic protons peak at 2.0 ppm and olefinic protons signal at 5.3 ppm. Multiplets at 4.31 ppm indicate the presence of glyceridic protons[21]. From ^{1}H NMR data % is calculated by using equation 2 (See experimental section).

3.3.3. Gas chromatography and mass spectrometry

The chemical composition of castor oil biodiesel and different fragments of FAMEs were confirmed by GC-MS and results are matched with library data software (NO. NIST II). Different fragments have specific retention time. In GC non-polar column was used and thus unsaturated FAMEs eluted in front of saturated ones. Trans-isomers elute after cis-isomer as cis-isomers elute rapidly. A single sharp peak at 14 min in spectra corresponds to Methyl-12-hydroxyoctadeca-9-enoate which is main component of castor oil biodiesel and comprises up to 90 %. GC-MS data depicted that methyl hexadecanoate, methyl 12-hydroxyoctadeca-9-enoate, methyl octadeca-9-enoate and methyloctadeca-9,12-dienoate were the main fatty acid fragments present in COB. The results obtained were in good agreement with the expected composition. The main components of the obtained biodiesel were found to be Ricinoleic acid, linoleic acid and oleic acid methyl esters [24, 25]. GC-MS chromatogram of COB using representative complex as catalyst is depicted in figure 8.

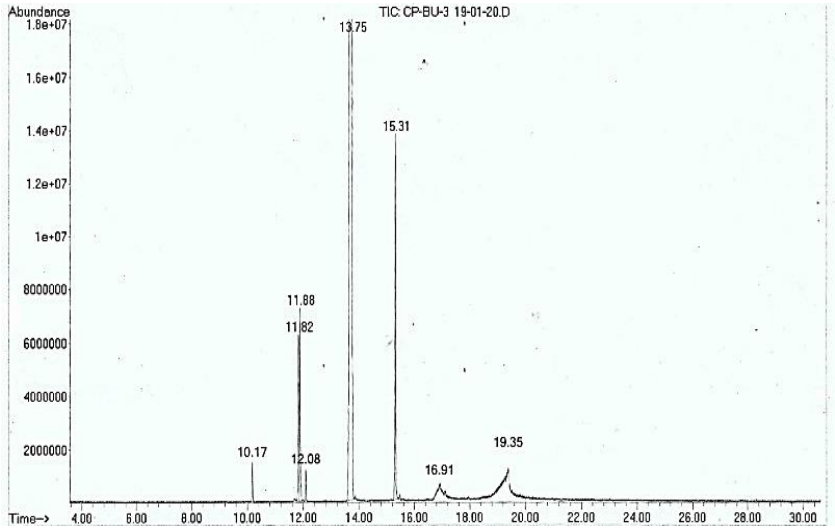


Fig. 8. GC-MS chromatogram of COB using representative complex as catalyst.

4. CONCLUSION

In the present study, three triorganotin(IV) based catalysts were resynthesized and employed for biodiesel synthesis from castor oil through a transesterification process. These catalysts were theoretically studied by MEP map to investigate the Lewis acid character of complexes. These complexes were successfully utilized for the conversion of biodiesel using non-edible castor oil feedstock. Synthesized biodiesel was characterized by FT-IR, ^{1}H NMR and GC-MS. The catalytic application of the assessed compounds was found as follows: Me3SnL > Bu3SnL > Ph3SnL with a maximum conversion efficiency of 95 % under the optimized conditions (oil to methanol ratio 1:12, at 65 °C, for 14 h with catalyst concentrations 1 wt %). From this study it is expected that it will open a new chapter for researchers and biodiesel can be synthesized on a large scale from low-cost castor oil.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

The authers declared that there is no conflict of interest.

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Pakistan Academy of Sciences

Research Article

Effects of Plant Extracts on Bacterial Isolates from Infections of the Female Genital Tract

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Abstract: Fifty samples of female genital secretions were collected from a group of women whose age ranges from 15 to 50 years old at Al-Batool Hospital for Maternity and Children. The isolates *Escherichia coli, Staphylococcus aureus, Candida albicans*, and *Staphylococcus epidermidis* were found in the fungal and bacterial cultures of the samples, with percentages of (45, 31, 13, 11), respectively. Positive samples were isolated at the highest rate of 87 percent for samples isolated from people aged 30 to 40. In the case of pregnancy, there is an increase in the infection rate with fungal and bacterial species, with a percentage of 60 % compared to women. The results of testing the effect of plant extracts (marjoram, chamomile, thyme, peat, and thuja) on isolated bacterial and fungal species showed inhibition diameters (20, 16, 15.5, 13, 10, 10) mm at concentrations (25 %, 50 %, 75 %, 100 %). We discovered that different concentrations of Marjoram, chamomile, thyme, peat, and thuja extracts (25 %, 50 %, 75 %, 100 %) were associated with increased inhibition rates of fungal and bacterial infection with different diameters (10, 12, 15, 16, 20 mm).

Keywords: *Candida albicans*, Bacterial species, Plant extracts, Infectious diseases.

1. INTRODUCTION

The recurrence of urinary tract infection (UTI) after antibiotic treatment is an indication that the bacteria have developed resistance to the medicine. This necessitates the investigation of an alternate treatment. It is known that a certain proportion of people are now using alternative medications, as a significant number of women are using herbal therapies to boost their immunity, drinking cranberry juice, or consuming probiotics in order to restore their normal vaginal flora. That is occasionally distressed following antibiotic medication [1]. Plants have an essential role in the treatment of fungal and bacterial illnesses. Several studies show that plant extracts (Chamomile and Thyme, Marjoram, Peat, Thuja) have antifungal and antibacterial activity against *Candida albicans* and bacterial infection (*Staphylococcus aureus, Escherichia coli, and Staphylococcus epidermidis*) [2-6].

Candida species are generally known as fungal pathogens in humans. These species are typically commensal yeasts that are a component of the normal microbiota. They settle lightly on human skin, vagina, oral cavity, and gut, but they may change into opportunistic pathogens if they have insufficient host immunity. [7]. The global distribution, versatility in adjusting to novel difficult environments, and variety of *Candida* genus species and straining to dictate that such yeast-like fungi lead to a wide spectrum of infections in susceptible persons [8]. These might include diseases with significant illness but without lethal consequences, like excruciating and scratchy mucocutaneous candidiasis such as candidal vulvovaginitis, oropharyngeal candidiasis, gastrointestinal tract overgrowth, keratitis, or skin and nail mycoses [9]. The *Candida* genus has about two hundred species, with the most common being *C. albicans*, which represent almost half of all identified shallow and systematic candidiasis in people [10]. Commonly, *C. albicans* can be the furthermost cause of Vulvovaginal candidiasis (VVC), followed by *C. glabrata*, *C. krusei, C. lusitaniae,* and *C. parapsilosis* [11]. Vulvovaginal candidiasis is common among

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women who have had antibiotic treatment, are pregnant, or are on hormone replacement therapy [12]. The current study attempts to apply alternative medicines (plant extracts) in the treatment of fungal and bacterial infections due to fungi and resistance of bacteria to antibiotics.

2. MATERIALS AND METHODS

2.1 Sample Collection

Fifty swabs were collected using a sterile cotton swab from different locations of the female genital tract of women with inflammation of the female genital tract during visits to Al-Batool Hospital advisory clinics as well as Al-Zahra in Muqdadiya from 12/20/2019 to 8/20/20. The patient was chosen based on the existence of clinical symptoms identified by the specialist and including; the presence of vaginal fluids, odour, ulceration, itching, burning, and fever. The ages of the ladies varied anywhere from 15 to 50.

2.2 Isolation and Diagnosis

The swabs taken from the patients were planted on the culture media (Blood agar, MacConkey agar, Sabouroud dextrose, and Mannitol salt). Dishes were incubated in the incubator at a temperature of 37 °C for 24-28 hours, then growth was checked after that period and then a secondary culture was made of the dishes that showed a positive result. As for the direct examination, the samples were examined directly by making two slides. The first was stained with gram dye, while the second slide was placed on it a drop of physiological solution and was examined under 40v after placing the slide cover on it. All bacterial and fungal isolates were subjected to some biochemical diagnostic tests as well as the gram dye test, studying shapes, colours and edges of colonies growing on nutrient medium, growth on the blood agar medium and hemolysin production [41].

2.3 Preparation of the Hot Aqueous Extract

Extract is prepared by dissolving 50 g vegetable powder in 500 ml of distilled water. Then, boil at a temperature of 100 °C and leave for 10 minutes. Then, filtering through the Whatman No. 1 filter papers that were poured into glass dishes and left in the incubator at a temperature of 37 °C. When power develops, it is kept in the refrigerator until use [42].

2.4 Effect of Plant Extracts on the Growth of Bacteria and Fungi

Agar well diffusion process was employed to observe the effect of aqueous extracts of marjoram, chamomile, thyme, peat, and thuja at concentrations (25, 50, 75, 100) mg/ml on bacterial growth and isolate *C. albicans* yeast. From the female genital tract, Mueller–Hinton agar medium was inoculated with a sterile cotton wick of bacterium containing 108 YS ml and SDA medium that was inoculated with Mycobacterium. Drills were made in the middle of the cultivated culture media using a flint drill and the prepared concentrations of each extract were placed in the amount of (50 milliliters per hole) with the use of distilled water as a control. The diameter of the damping area was measured in mm for each hole, and the average of the three replicates was calculated [43].

2.5 Statistical Analysis

The parameters were expressed as percentage frequencies, and the Pearson-Chi-square test was used to determine if there were significant differences between frequencies. The statistical package SPSS version 25.0 and Excel 2013 were employed to carry out these analyses. A p-value $\leq$ 0.05 was considered significant.

3. RESULTS

3.1 Age Groups and bacterial infections

Data from the current study show significant differences among age groups related to bacterial infections, where the age groups 21-2 and 31-0 years scored the highest percentage (81 % and 87 %) respectively, compared to other groups (table 1).

3.2 Microbial Infections and Women Status

Data from the current study showed a significant difference between expectant and non-pregnant women related to bacterial and fungal infections, where pregnant women scored the highest

Table 1. Frequency and percentage of bacterial infections according to age groups

Age groups (Years)	Total Number	Positive Number	%	p-value
15-20	6	3	50	1
21-25	16	13	81	0.001***
26-30	8	4	50	1
31-40	16	14	87	0.001***
41-50	4	2	50	1
p-value	0.001***			

percentage (60 %) with bacterial and fungal infections than non-pregnant (40 %). Similarly, there is a significant difference between those females who are pregnant and those who are non-pregnant related to *E. coli* infection. Finally, there is no significant difference between pregnant and non-pregnant women related to *S. aureus, S. epidermidis, and C. albcans* infections (table 2).

3.3. Effect of Plant Extracts on the Growth of Bacteria and Fungi

The study's findings showed that there was no statistically significant difference between amounts of Chamomile and Thyme extracts and their ability to inhibit *C. albicans*, *E. coli*, *S. epidermidis*, and *S. aureus* (Figures 1 and 2).

Table 2. Frequency and percentage of bacterial and fungal infections according to woman's status

Woman status	*S. aureus*	*S. epidermidis*	*E. coli*	*C. albicans*	*%*	*p-value*
Pregnant	3	13	4	7	60	0.03*
Non-pregnant	3	7	1	7	40	0.13
Total	6	20	5	14	100	0.003**
p-value	1	0.18	0.002**	1	0.52	

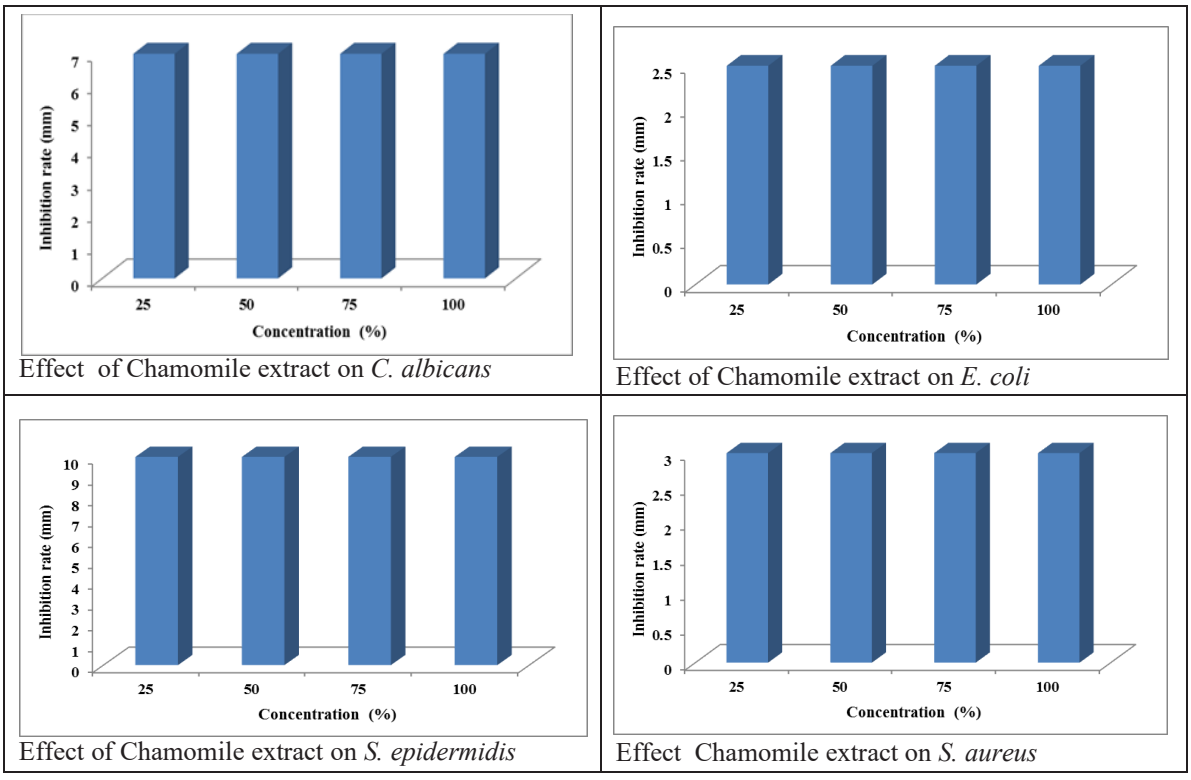
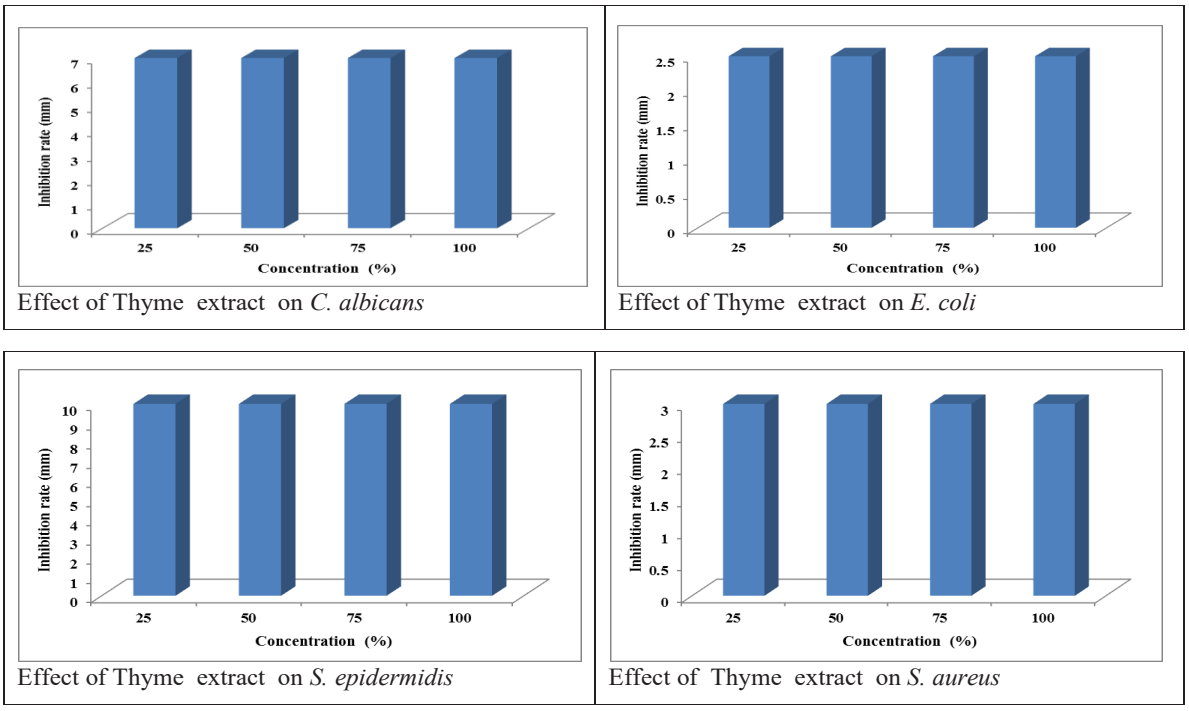


Fig. 1. Effect of Thuja extract on *C. albicans, E. coli, S. epidermidis,* and *S. aureus*

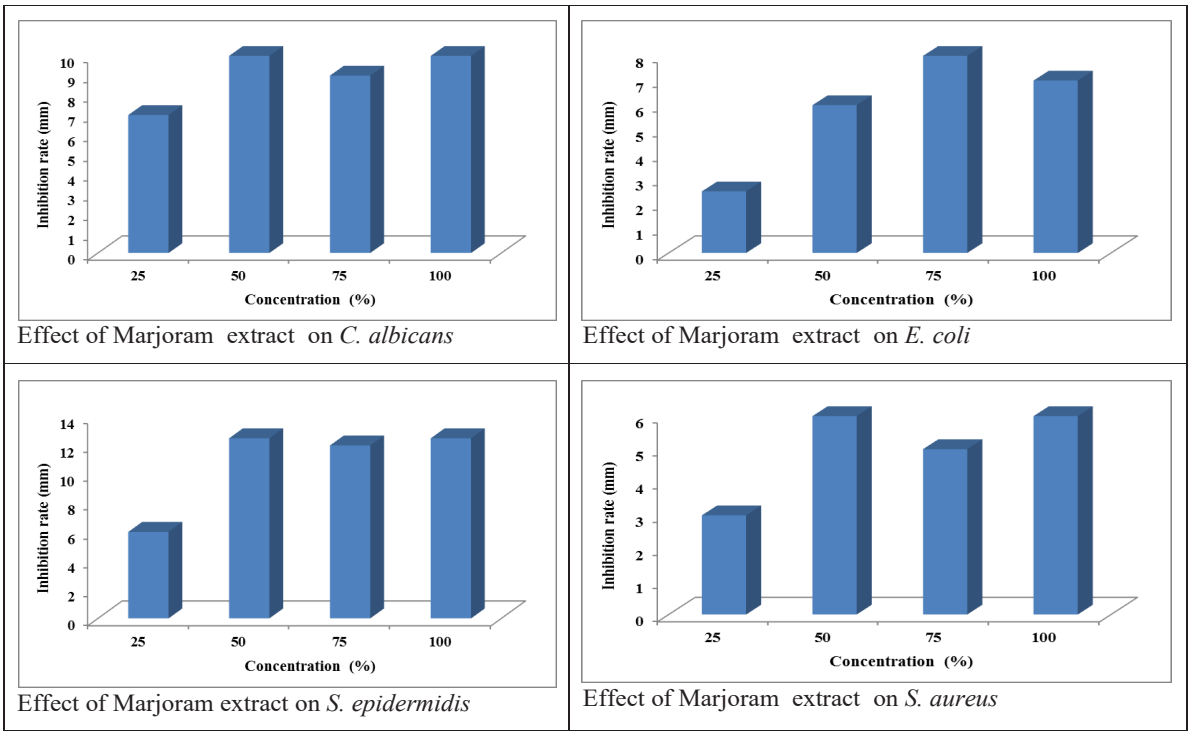


Effect of Thyme extract on *C. albicans* | Effect of Thyme extract on *E. coli*

Effect of Thyme extract on *S. epidermidis* | Effect of Thyme extract on *S. aureus*

Fig. 2. Effect of Thyme extract on *C. albicans, E. coli, S. epidermidis*, and *S. aureus*

The current investigation demonstrated that the inhibition rates of *E. coli*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* differed significantly depending on the concentration of marjoram extract and that the inhibition rate increased with concentration. Contrarily, our study found no correlation between the amounts of marjoram extract and the pace at which *Candida albicans* were inhibited *(*Figure 3). Similarly, our study showed there is a significant difference among Peat extract concentrations and inhibition rate of *Candida albicans, E. coli, and Staphylococcus*



Effect of Marjoram extract on *C. albicans* | Effect of Marjoram extract on *E. coli*

Effect of Marjoram extract on *S. epidermidis* | Effect of Marjoram extract on *S. aureus*

Fig. 3. Effect of Marjoram extract on *C. albicans, E. coli, S. epidermidis,* and *S. aureus*

epidermidis.

It was discovered that as concentration rose, the inhibition rate rose as well. On the other hand, our research did not discover a connection between Peat extract concentrations and how quickly *Staphylococcus aureus* was suppressed (Figure 4). The study concluded by showing a significant relationship between Thuja extract concentration and inhibition rates of *C. albicans*, *E. coli, S. epidermidis*, and *S. aureus*, finding that the inhibition rate increases as the concentration does (Figure 5).

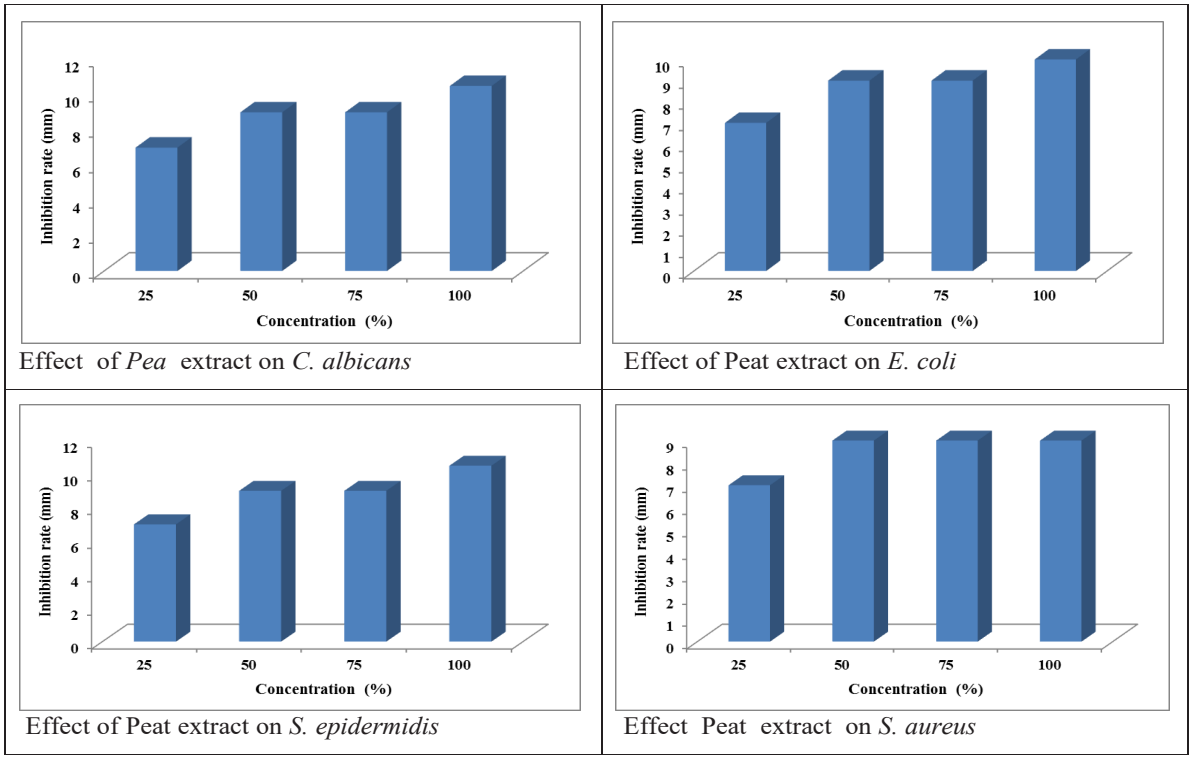


Fig. 4. Effect of Peat extract on *C. albicans, E. coli, S. epidermidis,* and *S. aureus*

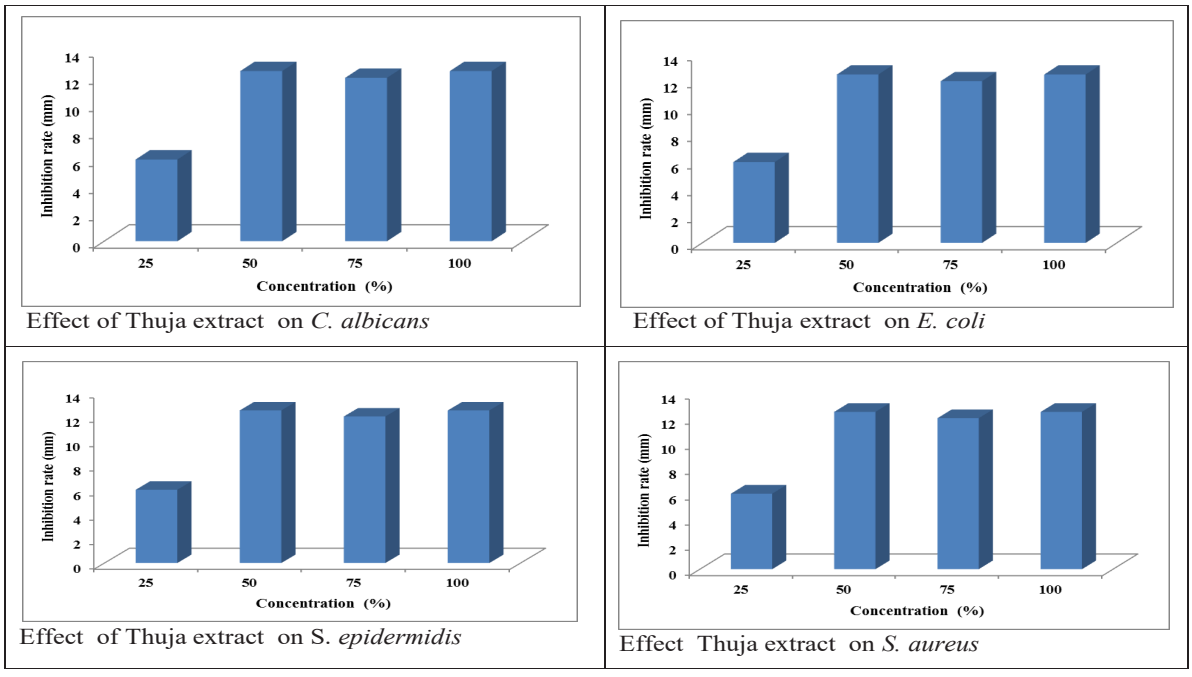


Fig. 5. Effect of Thuja extract on *C. albicans, E. coli, S. epidermidis,* and *S. aureus*

According to the findings, women aged 21-52 and 31-40 years had the highest percentage of bacterial infections (81 percent and 87 percent, respectively) compared to other age groups, and the reason for this could be due to age progression, impaired immune status, multiple births, and sample collection. The high incidence of bacterial infection in women was linked to the pH of the vaginal fluid (pH=4.5), participant age, pregnancy, and HIV infection history. To avoid mother and infant morbidity and mortality, early detection of conditions contributing to bacterial overgrowth on the vaginal wall is critical [13]. Protecting mother and child from morbidity and death is critical [13].

4. DISCUSSION

Bacterial vaginosis is a global public health risk for women, particularly throughout their reproductive years. The age and gender of the patient are important variables in establishing the etiology of UTIs; they can improve the accuracy of identifying the causative uropathogen and provide good information for empiric therapy [14]. Bitew, Abebaw, et al. found that the prevalence of bacteria was linked to a variety of variables, which is consistent with our findings [13]. A total of 413 research participants were tested for bacterial vaginosis, with a 39.5 percent overall prevalence of bacteria that cause bacterial vaginosis. This result is similar to studies carried out in Kenya (43.1 %), Nigeria (33 %), Addis Ababa (41.5 %), and Cameroon (38 %) [15]. Mucci *et al.* found *C. albicans* 4+ positive in pregnant women, which is consistent with our findings [16]. Researchers in Beirut, Lebanon, found a considerable incidence of *C. albicans* and non-*Candida albicans*. Vulvovaginitis is an infection that may be caused by certain Candida strains, and it is more common in pregnant women. *Candida* testing as part of prenatal care is recommended to reduce the risk of a negative neonatal outcome or gestational problems. [17]. *Candida* species were found in much larger numbers in symptomatic pregnant women than in asymptomatic pregnant women. *Candida* infection was substantially linked to those between the ages of 26 and 40. The antifungal medicine amphotericin B was the most sensitive. Multiple drug-resistant *Candida* species were found in high numbers. As a result, symptomatic women should be examined and treated on a regular basis [18]. The prevalence of vulvovaginal candidiasis (VVC) was observed to be higher among symptomatic non-pregnant women of reproductive age in Vietnam in comparison to various other nations. Non-albicans *Candida* species were found in high numbers, and they were typically resistant to antifungal therapies. Antifungal resistance was low in vaginal *C. albicans* isolates. The findings suggest that changes in species distribution and antifungal sensitivity should be monitored and treated on a regular basis [19]. Pregnant and postpartum women are increasingly susceptible to this virus. *S. aureus*, including MRSA, is a prevalent source of healthcare-associated illnesses and community-acquired infections [20]. The carriage of *S. aureus* in mothers is associated with an increased likelihood of *S. aureus* infection in neonates. The prevalence data indicate that there is a higher number of MRSA isolates in comparison to *S. aureus* isolates. This phenomenon may be attributed to the inappropriate use of antibiotics during pregnancy in China, and warrants further investigation. The greater incidence of vaginal isolates in pregnant women compared to nasal isolates implies that pregnant women should prioritize vaginal hygiene. [21]. The study's results indicate a high prevalence of *E. coli* and extended-spectrum-lactamase-producing (ESBL) *E. coli* in pregnant women, which aligns with our own findings. Furthermore, it was observed that the misuse of antibiotics was more prevalent among pregnant females in Europe and Asia as compared to other continents [22].

The findings indicate that certain virulent clones are implicated in *E. coli* bacteremia among pregnant women, however, the severity of the condition, as evidenced by fetal mortality, is primarily linked to bacteremia originating from the genital region [23]. The study findings indicate that there exists a noteworthy prevalence of ESBL carriage among expectant women in Lebanon. In order to accurately assess the potential for transmission, further investigation is warranted, such as conducting longitudinal radiographic studies on expectant mothers and monitoring the postnatal health status of their offspring [17]. Antibiotic-resistant strains can survive in the mother's vaginal tract and be passed on to the baby after birth. Preterm infants in the neonatal care unit are at a notable risk of sepsis caused by Enterobacteriaceae that produce extended-spectrum beta-lactamase (ESBL). In

the case of maternal-neonatal transmission, it was found that identical strains were transmitted vertically from the mother to the newborn [24]. The study's findings indicate that the ethanol extracts of chamomile did not exhibit any antifungal activity against *C. albicans* in both the control and test groups. However, the hydroalcoholic extract of chamomile demonstrated inhibitory and fungicidal effects that were attributed to 70 % ethanol rather than the chamomile itself [2]. In comparison to the untreated biofilm, the utilization of chamomile extract exhibited a significant reduction in the quantity of *Enterobacter cloacae* colony-forming units per milliliter in biofilm. However, it did not demonstrate any impact on the amount of viable DNA in *C. albicans* biofilm. [3]. By using the agar diffusion method, the antimicrobial activity of chamomile essential oil was examined, and it was discovered to have a considerable antibacterial impact, with inhibition zones of 13.33 mm to 40.00 mm in diameter (on *Listeria monocytogenes*) (on *S. aureus*). Chamomile oil has little antibacterial effect against *P. aeruginosa* bacteria. The chemical composition, antibacterial activity, and antioxidant properties of the studied chamomile essential oil, which was extracted from plant material from the Republic of Srpska's northern region, show that it has great phytomedical potential [25]. Chamomile has antibacterial action against a wide range of microorganisms, including *Enterococcus faecalis*, *P.aeruginosa, S. aureus, K. pneumoniae,* and *E. coli* [26]. The ethanolic extract of chamomile leaves showed antibacterial action against MDR *P. aeruginosa* isolates; however, the extract of flowers demonstrated superior activity against MRSA isolates [4]. Chamomile ethanolic flower extract has shown considerable antibacterial action against MRSA isolates. Accordingly, this extract might be a viable alternative to antibiotic therapy and a practical choice for handling infections produced by MRSA and detrimental bacteria [27]. Essential oils utilized at sub-inhibitory concentrations were sequestered in yeast vacuoles, indicating that cell detoxification activated *Candida* defensive systems. Due to their anti-biofilm action, clove and thyme essential oils can effectively inhibit *Candida* sp. colonization of the studied abiotic surfaces [5]. The findings of this study give a wealth of experimental data on the therapeutic effectiveness of thyme essential oils against drug-resistant clinical isolates of *C. albicans*, which might be utilized to build a novel antifungal medication [28]. Some Thyme essential oils exhibit high effectiveness against both developing and stationary phase *S. aureus*, according to the research [6].

Data results are shown. Thyme has a greater antimicrobial effect at the 30 and 40 percent concentrations. These findings demonstrate that these essential oils have powerful antibacterial activities, implying that they might be useful in treating *S. epidermidis* [29]. The essential oils of marjoram were shown to have potent antifungal properties against the *C. albicans* fungus strain [30]. Another study found that Marjoram essential oils are effective against *C. albicans* [31]. The findings will allow for more research into oregano essential oil (*Origanum heracleoticum* L.) as an alternative antibacterial remedy for boosting the treating process in bacterial infections and as an efficient technique of averting the formation of antibiotic-resistant strains [32]. Data results showed the use of Marjoram essential oil to control *E. coli* in salad dressing might be seen as promising and enable lower salt levels in meals to be assimilated [33]. The findings demonstrate the bioactive potential of decoctions of *Satureja montana* and *Origanum majorana* in inhibiting *E. coli* and encourage the creation of innovative formulations with broad antibacterial capabilities based on these medicinal and fragrant plants [34]. The antibacterial concentrations of Marjoram essential oils are utilized to inhibit *S. aureus*. Because of the potential for inductive biofilm formation, aureus biofilms should be approached with caution [35]. Data results showed antimicrobial activities of peat extract against *S. aureus*, *E. coli*, and *Pseudomonas* spp. [36]. Study results showed the most efficient composites against pathogenic microorganisms in peat are those containing a 70 % ethanol solvent. [37]. Except for *E. coli* and *S. typhi*, fresh leaf juice showed strong effectiveness against Gram-negative pathogens. Except for *S. aureus* and *S. epidermidis*, it is similarly potent against Gram-positive bacteria. Only Gram-positive bacteria were inhibited by acetone, methanol, chloroform, and petroleum ether extracts of the leaves [38]. The results of the inhibition zone measurement revealed that the antibacterial activity of Thuja occidentalis extract gets to 29 and 38 mm against *S. aureus* and 23 and 31 mm against *C. albicans* at concentrations of 40 and 50 %, respectively [39]. The most resistant

strain was discovered to be *C. albicans* (MTCC 3018). Except for *C. albicans*, Thuja essential oil displayed remarkable antifungal efficacy against all pathogens [40].

These findings contradict ours, which demonstrated the impact of *C. albicans* inhibition. According to the findings, extracts made from the leaves and seeds of *Thuja occidentalis* can be utilized as a natural remedy for the treatment of different bacterial infections (e.g., *E. coli* and *S. aureus*).

5. CONCLUSION

We conclude that women in age groups 21-25 and 31-40 years scored the high prevalence with UTIs. Bacterial and fungal infections are more incidence in pregnant than non-pregnant women. Chamomile and Thyme extracts have no significant effects on their ability to inhibit *C. albicans*, *E. coli*, *S. epidermidis*, and *S. aureus*. Marjoram extract is preferred in inhibition rates of *E. coli*, *S. epidermidis*, and *S. aureus*, but it does not have significant activity in the inhibition of *C. albicans*. Peat extract concentrations have a significant inhibition rate against *C. albicans*, *E. coli*, and *S. epidermidis*. Finally, Thuja extract concentration and inhibition rates of *C. albicans, E. coli, S. epidermidis,* and *S. aureus*, found that the inhibition rate increases as the concentration does.

6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research Article

Application of Morphometric Ranking Approach using Geospatial Techniques for Flash Flood Susceptibility Modelling in District Shangla, Pakistan

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Abstract: Every year, disaster strikes, and led to thousands of casualties and deaths around the world. A meteorological disaster such as a flash flood is a multifaceted hydro-meteorological phenomenon that can cause a huge loss of human life and can create severe economic problems. In this study, techniques based on Geographic information systems and Remote sensing were used to get the flood susceptibility map for District Shangla, Pakistan. For the susceptibility of flash floods, geo-morphometric ranking model was used. Various causative factors were considered including; topography, river pattern, and flow accumulation. ALOS PALSAR digital elevation model was used for calculating the required causative factors. Eleven different sub-basins were delineated in the Shangla basin. A total of eighteen morphometric parameters were studied. The morphometric ranking approach (MRA) score was determined with a range of 1 to 5. Rank 5 represents high risk while rank 1 exhibits low risk. The results of the model were categorized into five flood vulnerability classes; very low, low, moderate, high and very high. The total population of Shangla district is 757,810 with a population density of 480 persons per sq km^2, and results from this study revealed that 23% of the total geographic area (364.11 km^2) of the district is vulnerable to high flash floods.

Keywords: Geo-morphometric, GIS, Remote Sensing, Susceptibility, Vulnerability, Flash flood

1. INTRODUCTION

Every year, disaster strikes, and led to thousands of casualties and deaths around the world [1, 2]. According to estimates, hydrological and climatological disasters have produced extensive damage to individuals and infrastructure [3, 4]. One of the most devastating hydrometeorological disasters is flooding [5-7]. More than one-third of the earth's land surface inhabited by over 70 % of the world's population is prone to floods [8]. It has been observed that floods are mainly caused by heavy rainfall, changes in terrain and glaciers melting [9]. It is obvious that there is a possibility that the rainfall will increase, which may lead to an increase in destructive flooding in the future [10, 11]. A meteorological disaster such as a flash flood is a multifaceted hydro-meteorological phenomenon which can cause huge losses to human lives and can create severe economic problems [12]. Flash floods constantly occur without any early warning system or form of forecasting [13], especially in Pakistan. Heavy and persistent rain increases river and stream discharge, which leads to severe flash floods [14]. Flash floods are local wonders which always occur in basins having area of a few square kilometers with a very short reaction time [15]. Uneven and unstable land surfaces can also increase surface flow and abruptly decrease the reaction time to a flash flood event [16]. Hu discovered that the geomorphometric characteristics of the basin and climatic conditions are the main triggering factors for flash floods [17]. The high peak discharge from flash floods bring the human life and urban

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structure at more risk [18]. Different factors which lead to flash floods are high and heavy rainfall, the topography of watershed, land cover/land use, permeability of soil, soil texture and natural susceptibility [19-20]. Flash floods are considered as the most devastating hydrological hazard because of their abrupt and unpredictable character, the extensive harm they wreak, and the hazards they bring to physical infrastructure and livelihoods [21-24]. Two of the key factors that determine the likelihood of flash floods are volume and intensity of precipitation [25].

Additional interconnected factors responsible for overall intensity of flash floods include evaporation, the characteristics of the river system, catchment size, natural and anthropogenic activities within the basins. [26]. Watershed's geomorphic structures are of utmost importance for evaluating and controlling extreme hydrological phenomena like flash floods. The structure and shape of a watershed are measured mathematically using morphometry. Stream order, stream number, stream length, stream density, drainage frequency, watershed size, perimeter, shape factor, and circulatory ratio are some of the basin variables that are numerically analyzed in morphometry [27-30].

Many researchers performed significant research on basin characterization morphometry [31]. Mapping the earth's surface morphology is helpful to analyze geological, hydrological/ groundwater conditions to prevent soil erosion [32-35] and modeling of flash flood vulnerability [36-39]. Geological, geomorphological, and hydrometeorological characteristics of a basin/ watershed are responsible factors for controlling the drainage geometry and density [40, 41]. Until recently, fieldwork, topographical maps and aerial photography were employed to determine the boundaries of drainage networks. Most recently, geographic information systems and remote sensing are often employed for morphometry and drainage system delineation [42-46]. Flash flood susceptibility modelling uses geospatial approaches, which are robust, time- and capital-saving tools for processing, mapping, and evaluating of watersheds [47-50]. Various studies (especially those cited in this manuscript), have applied Morphometric analysis for watershed assessment and flash flood susceptibility modelling [51, 52].

In morphometric analysis, mathematical and quantitative analysis is carried out in order to understand the correlation of flow patterns and terrain characteristics with the geohydrological features of a hydrological domain. Morphometric parameters (MPs) obtained from remotely sensed data collected are effective, precise, and cost- and time-effective input data for forecasting flash flood susceptibility. Numerous hydrological disasters, such as flash floods, can either be mitigated or avoided with the use of geospatial approaches, which can evaluate the hydrological response at the watershed level [53, 54]. To lessen the danger posed by the flash flood, it is crucial to identify watershed flash flood potentiality [55]. Mohamed and El-Raey [56] employed MPs to assess the flash flood vulnerability in Southeast Bangladesh. The study found that remote sensing data in integration with GIS considerably improved comprehension of flash floods, and assisted in reducing their consequences on property destruction and financial losses. Researchers have found that using the GIS ArcHydro tool to extract properties from a DEM and automatically demarcating topographic and morphometric features is a suitable alternative to manually reviewing topographic maps and conducting field research [57-58].

The primary goal of the current study is to locate the most vulnerable places to flash floods as well as the key flash flood-prone zones in the study area.. Like the rest of Pakistan, the Shangla basin's physiography and climate make it particularly susceptible to flash floods. In monsoon, due to intense rainfall and heavy melting of snow on surrounding mountains, the area is facing frequent flash floods. The current study will offer an avenue for research and knowledge to lessen the devastating consequences of flash floods in the study area.

2. MATERIALS AND METHODS

2.1. Study Area

This research focused on the district Shangla, Khyber Pakhtunkhwa, Pakistan. District Shangla lies between 34^{o} 31 to 35^{o} 01 north latitude and 72^{o} 33 to 73^{o} 01 east Longitude. The total geographical area is 1586 square kilometers with 36 % cultivated and 64 % of forest area. The district's landscape is dominated by tall mountains and small valleys

in the western Himalayas. The district is generally between 2000 and 3500 meters above mean sea level. Figure 1 shows the study area.

2.2 Data Collection

Primary sources in addition to secondary data sources were utilized for achieving the mentioned aims. A physical visit to district Shangla was arranged to collect all the records of damages that occurred due to severe flood phenomena occurred in the past. The precipitation statistics were obtained from the Pakistan Meteorological Department (PMD), Islamabad. The irrigation department of Khyber Pakhtunkhwa facilitated net cashiering information on the river. Lithological layers of the study area were acquired from the regional office of the Geological Survey of Pakistan (GSP). The ground particles consistency layer was extracted from the Directorate of Soil Survey, Khyber Pakhtunkhwa. Topographical information on the ground surface was extracted from high spatial resolution ALOS PALSAR DEM. Land use/cover patterns of the study area were derived for the year 2021 using freely available Landsat 8 satellite. Figure 2 shows the input data maps, while definitions and mathematical calculations of various linear and aerial morphometric parameters are displayed in Table 1.

Identification of maximum (X-Max) and Minimum (X-MIN) risk values for the morphometric parameters is shown in Table 2. The formula below was used to calculate the minimum and maximum risk values.

$$Risk\ value = 4x\ X - X\ min\ /\ Xmax - Xmin$$
$$Risk\ value = 4x\ X - X\ max\ /\ Xmin - Xmax$$

X shows the variable value, Xmax denotes the extreme value and Xmin denotes the slightest value in the variables group.

Systemized variables were considered which contemplate the measured risk for each Morphometric variable in contrast with the equal variable lying in each sub-vessel. MRA grade estimated ranked between 1 to 5. Score 5 indicates a high flash flood risk and a score 1 indicates the least risk. The calculated ranking scores are shown in Table 3. In the final step, all the variables of each sub-vessel were added, followed by categorizing

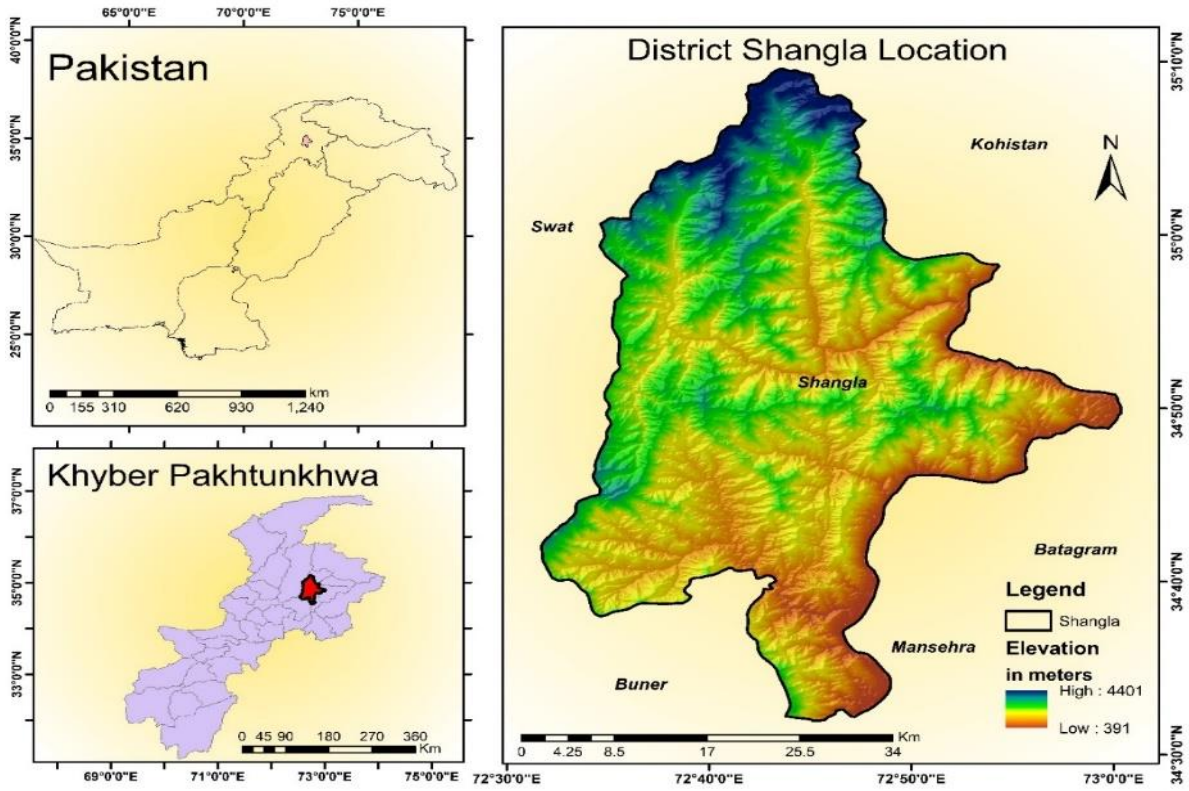

Fig. 1. The Study Area Map

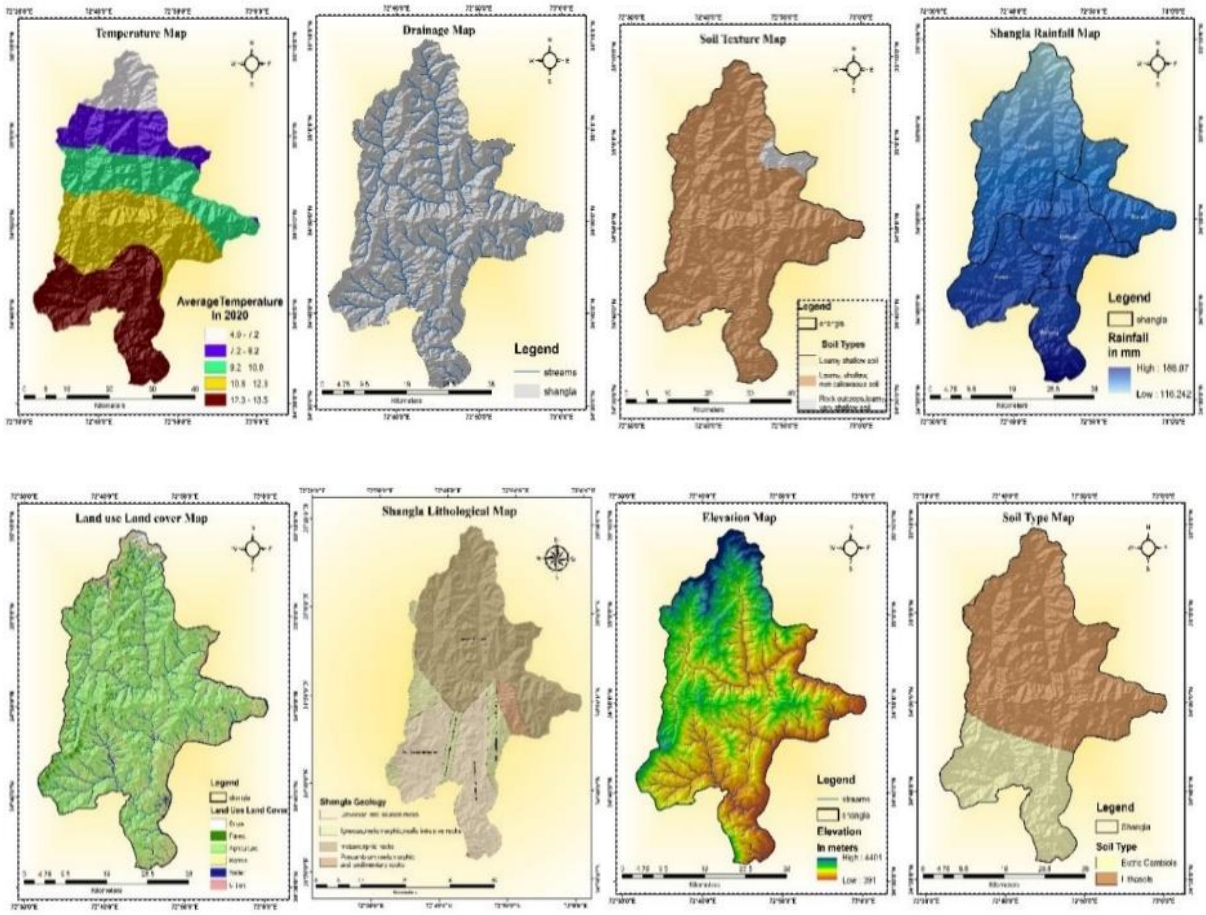

Fig. 2. District Shangla Input Data Maps

them into five groups. i.e. very high, high, moderate, low, and very low flooding susceptibility. Figure 3 shows the methodology flow chart.

3. RESULTS

Eleven (11) sub-basins were delineated in the study area, where eighteen geo-morphometric parameters for each sub-basin were analyzed and computed. For linear, aerial, and shape aspects of all sub-basins, a quantitative geo-morphometric analysis was carried out. The morphometric features reveal that there are some variations in different characteristics of each sub-basin (Table 2). Results reveal that area-wise, Sb-2 (sub-basin) is the smallest sub-basin covering an area of 30.64 km² and Sb-4 is the largest by area of 153.88 km² (Table 2).

According to geomorphology, the relief, gradient, type of rocks, and geologic formations in a drainage region, all influence the drainage flow pattern in a particular basin. Stream order 4th was found to be the highest and stream order 2nd was the lowest in stream order (Table 2). Maximum of the sub-basins was delineated using stream order 3rd and stream order 4th. Figure 4a shows the stream order map of the sub-basins of the study area. The numbers represent the total number of streams that drain the basin. The lithology, soil properties, and rainfall patterns of the basin affect the number of streams. The immediate discharge can be greatly increased by a higher stream number (S_n). The Sb-4 is the sub-basin with the most streams (48), while Sb-2 has the fewest streams (9) (Table 2). Figure 4b shows the number of streams in sub-basins of the study area. Stream length (L_u) is considered one of the basin's most important hydrological qualities as it exposes information about surface runoff. The Sb-4 has the highest length of stream (79.27 km) and Sb-2 has the lowest (14.71 km) (Figure 4c & Table 2). The stream frequency (F_s) values are from 0.25 (Sb-1) to 0.38 (Sb-9) (Table 2). High relief and an impervious surface are indicated by the larger number of streams and stream frequency. The

Table 1. Morphometric Parameters and their Mathematical Formulas

Factor	Formula	Reference
Area (A) (km^2)	A=area of basin	[59]
Length (L_b)	L_b= length of basin	[59]
Perimeter (P) (km)	P = Parameter of basin	[59]
Stream order (S_o)	Ranking of stream	[60]
Number of stream (S_n)	S_n= N1 + N2 +.. ……. + Nn	[59]
Stream length (L_u)	L_u = L1 + L2. . ……. + Lu	[60]
Stream frequency (F_s)	$F_s = S_n / A$	[59]
Drainage density (D_d)	$D_d = L_u / A$	[59]
Relief (B_h) (m)	$B_h = h_{max} - h_{min}$	[61]
Relief ratio (R_r)	$R_r = B_h / L_b$	[61]
Gradient (G)	$G = B_h / L_b X 60$	[62]
Circulatory ratio (C_r)	$C_r = 4\pi A / P^2$	[60]
Elongation ratio (E_r)	$E_r = 1.128 A^{(1/4)} / L_b$	[61]
Shape factor (B_s)	$B_s = L_b^2 / A$	[59]
Length of overland flow (L_o)	L_o=0.5 X $1/D_d$	[59]
Ruggedness number (R_n)	$R_n = D_d X (B_h/1000)$	[60]
Geomatery number (G_n)	$G_n = B_h X D_d / G$	[60]
Compactness coefficient (C_c)	C_c=0.2812 X $P/A^{0.5}$	[59]

frequency of the sub-basins is shown in Figure 4d. Drainage density (D_d) values are between 0.44 km/km^2 (Sb-3) and 0.53 km/km^2 (Sb-11). The drainage density in all sub-basins is more than 0.44 km/km^2 (Table 2 & Figure 5e). Sub-basin relief (B_h) ranges from 1.6 meters (Sb-11) to 2.68 meters (Sb-1) (Table 2 & Figure 5f). The relief ratio (R_r) of sub-basins is ranging from 0.10. (Sb-5) to 0.25 (Sb-2) (Table 2 & Figure 5g). The other important parameter is gradient which is derived from relief divided by the length of the basin (L_b) multiplied by 60. The gradient values range from 6.44 (Sb-10) to 15.27 (Sb-2) (Table 2 & Figure 5h).

All sub-basins had elongation ratio (E_r) values that were less than one, with the highest and lowest values ranging from 0.29 for Sb-2 to 0.17 for Sb-4 (Table 2 & Figure 6i). The values of shape factor (B_s) ranges from 2.74 to 3.41 (Table 2 & Figure 6j). The geometry number (G_n) of the sub-basins ranges from 0.07 to 0.20 (Table 2 & Figure 6k). The ratio of the basin's size to its perimeter is known as the compactness coefficient (C_c). The Sb-2 got the lowest C_c value of 1.19 and Sb-11 got highest C_c value of 1.66 (Table 2 & Figure 6l). The perimeter (P) of Sb-2 is showing lowest value equal to 23.36, and Sb-4 is having the highest value equal to 58.93 (Table 2 & Figure 7m). Circularity ratios (R_c) with a value of more than 0.5 are present in more than half of the sub-basins (Table 2 & Figure 7n). Sb-2 had the highest R_c score (0.70). As flood hazard levels are growing in proportion to the circularity ratio's magnitude, more than half of the sub-basin are at high risk from flood hazard. Ruggedness number (R_n) is lowest in Sb-7 (0.0007) and highest in Sb-4 (0.0013) (Table 2 & Figure 7o). Length

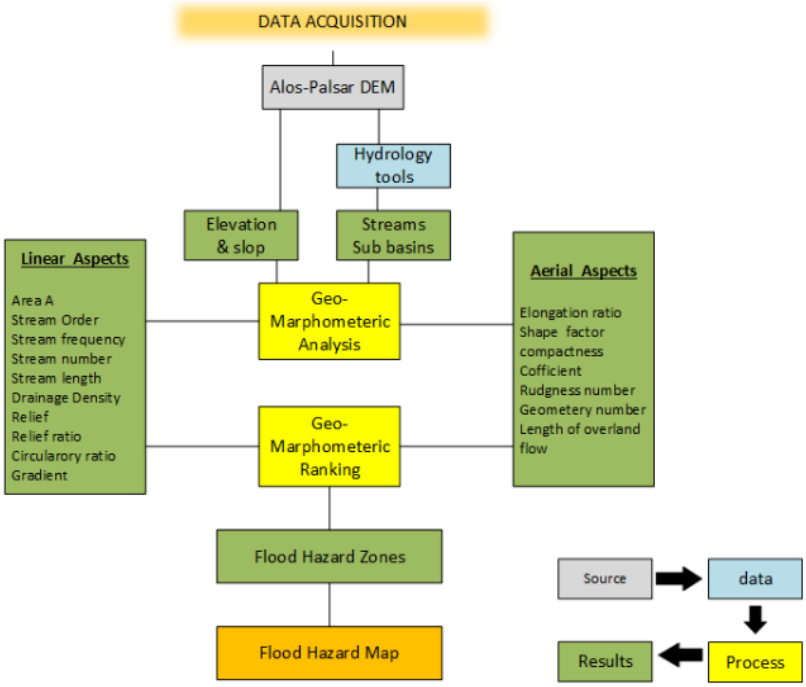

Fig. 3. Flow chart.

of the overland flow (L_o) is the reciprocal of the drainage density. Sb-3 exhibits a high L_o value while Sb-11 has the lowest L_o value (Table 2 & Figure 8p). The period of concentration and size of the peak discharge at the basin outflow is directly impacted by the basin shape factor (B_s). In comparison to an elongated basin with the same surface area, a circular basin will produce high discharge more quickly. Low values of the basin shape factor show a circular basin, whereas large values show an extended basin form. The lowest B_s value were observed for Sb-2 (2.74) (Table 2 & Figure 8q).

4. DISCUSSION

According to the current study, Shangla is divided into 11 sub-basins with various geo-morphometric and physical properties. The upper range of the sub-basins represents snow-covered peaks and narrow valleys. Usually, a flash flood is caused by the monsoon rainfall. The melting of snow is accelerated in these seasons due to the heavy rainfall and high temperature above 32 °C, this results in flash floods in the upper ranges and riverine floods without prior warning in the lower ranges, and cause damages and disastrous effects throughout the basin.

5.1 Flash Flood Hazard Zonation

The zones are further divided into very low, low, moderate, high, and very high zones and apply the geo-morphometric ranking approach. The nasscore of 1 to 5 were assigned to each geo-morphometric parameter of each basin. The geo-morphometric ranking number (GRN) was aggregated for each sub-basin ranked score to represent the hazards degree of flash flood hazard. The higher GRN score will represent a higher degree of flash flood hazard and vice versa. The range of GRN for all sub-basins is 39.18 to 55.14 (Table 3).

4.1.1. Very High Flash Flood Hazard Zone

Geo-morphometric ranking model analysis in the study region reveals that 23 % (364.11 km²) of the region is marked as a very high hazard zone with respect to flash flood hazards in the Shangla basin (Figure 9). The very high hazard zone is categorized by the highest GRN (>51.41) which is shown by Sb-4, Sb-9, and Sb-10 (Table 3, & Figure 10). This zone remains highly vulnerable for the local people where the frequency of flash flood is very high. The dry channels are activated by the heavy rainfall in the months of June to August and cause flash floods. Steep slope of the channel causes high-speed flash

Table 2. Geo-Morphometric Characteristics of Sub Basins

zSub basin	A	P	L_b	S_o	S_n	L_u	F_s	D_d	B_h	R_r	G	C_r	E_r	B_s	L_o	R_n	G_n	C_c
Sb-1	71.38	38.12	14.82	2	18	34.28	0.25	0.48	2.683	0.18	10.86	0.62	0.22	3.08	1.04	0.001288686	0.12	1.27
Sb-2	30.64	23.36	9.16	3	9	14.71	0.29	0.48	2.333	0.25	15.27	0.70	0.29	2.74	1.04	0.001120007	0.07	1.19
Sb-3	132.86	56.72	21.09	3	37	59.04	0.28	0.44	2.622	0.12	7.46	0.52	0.18	3.35	1.13	0.001165205	0.16	1.38
Sb-4	153.88	58.93	22.92	3	48	79.27	0.31	0.52	2.563	0.11	6.71	0.56	0.17	3.41	0.97	0.001320378	0.20	1.34
Sb-5	113.65	54.70	19.30	3	33	55.52	0.29	0.49	1.866	0.10	5.80	0.48	0.19	3.28	1.02	0.000911539	0.16	1.44
Sb-6	100.48	58.80	17.99	4	27	48.64	0.27	0.48	2.439	0.14	8.13	0.36	0.20	3.22	1.03	0.001180559	0.15	1.65
Sb-7	64.84	45.02	14.03	2	23	31.58	0.35	0.49	1.512	0.11	6.47	0.40	0.23	3.04	1.03	0.000736318	0.11	1.57
Sb-8	115.27	52.91	19.45	4	33	57.85	0.29	0.50	2.206	0.11	6.80	0.52	0.19	3.28	1.00	0.001107048	0.16	1.39
Sb-9	91.62	44.46	17.07	3	35	46.87	0.38	0.51	1.9	0.11	6.68	0.58	0.20	3.18	0.98	0.000971982	0.15	1.31
Sb-10	118.61	47.00	19.77	4	35	56.85	0.30	0.48	2.123	0.11	6.44	0.67	0.19	3.30	1.04	0.00101754	0.16	1.21
Sb-11	64.73	47.53	14.02	3	19	34.61	0.29	0.53	1.672	0.12	7.16	0.36	0.23	3.04	0.94	0.000893967	0.12	1.66

Table 3. Geo-morphometric Ranking

Sub basin	A	S_o	S_n	L_b	F_s	D_d	B_h	R_r	G	C_r	E_r	B_s	L_o	R_u	G_n	C_c	Ranking
Sb-1	2.32	1	1.92	2.25	1.00	2.78	5.00	3.00	3.14	4.06	3.33	2.97	1.32	1.22	4.99	4.32	44.62
Sb-2	1.00	3	1.00	1.03	2.23	2.78	3.80	5.00	5.00	5.00	1.00	5.00	1.32	2.37	5.00	5.00	49.53
Sb-3	4.32	3	3.87	3.78	1.92	1.00	4.79	1.29	1.70	2.88	4.67	1.36	1.00	2.06	4.98	3.38	46.00
Sb-4	5.00	3	5.00	5.03	2.85	4.56	4.59	1.00	1.38	3.35	5.00	1.00	3.75	1	4.91	3.72	55.14
Sb-5	3.69	3	3.46	3.56	2.23	3.22	2.21	0.71	1.00	2.41	4.33	1.78	4.29	3.8	4.87	2.87	47.43
Sb-6	3.27	5	2.85	3.14	1.62	2.78	4.17	1.86	1.98	1.00	4.00	2.13	4.61	1.96	4.77	1.09	46.23
Sb-7	2.11	1	2.44	2.08	4.08	3.22	1.00	1.00	1.28	1.47	3.00	3.21	4.82	5	4.66	1.77	42.14
Sb-8	3.75	5	3.46	3.71	2.23	3.67	3.37	1.00	1.42	2.88	4.33	1.78	4.93	2.46	4.12	3.30	51.41
Sb-9	2.98	3	3.67	3.03	5.00	4.11	2.33	1.00	1.37	3.59	4.00	2.37	4.96	3.39	3.57	3.98	52.35
Sb-10	3.86	5	3.67	3.64	2.54	2.78	3.09	1.00	1.27	4.65	4.33	1.66	5.00	3.07	1.93	4.83	52.32
Sb-11	2.11	3	2.03	2.27	2.23	5.00	1.55	1.29	1.57	1.00	3.00	3.21	5.00	3.92	1.00	1.00	39.18

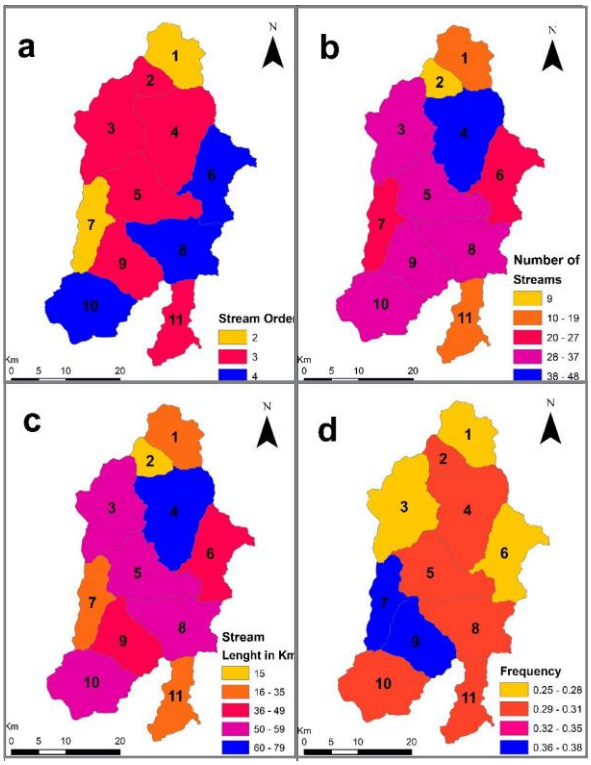

Fig. 4. District Shangla showing a. Stream order, b. Stream No, c. Stream Length, d. frequency.

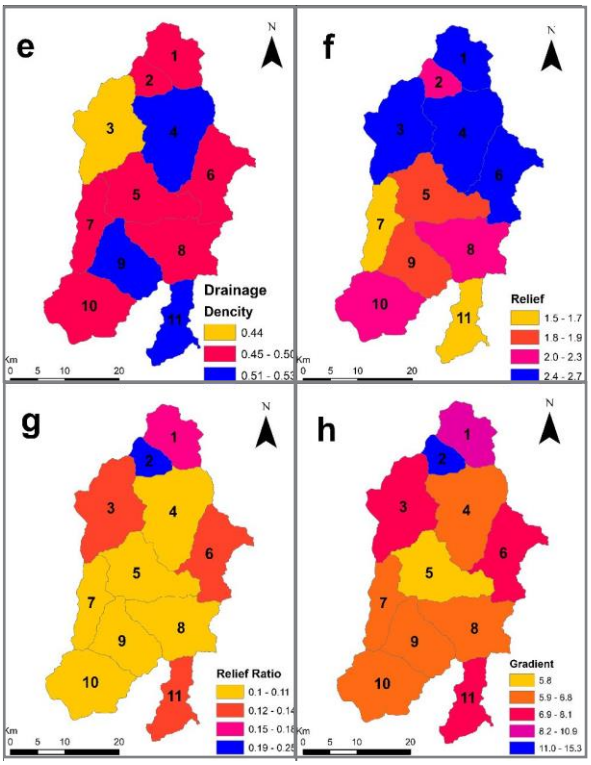

Fig. 5. District Shangla showing e. Drainage density, f. Relief, g. Relief ratio, h. Gradient.

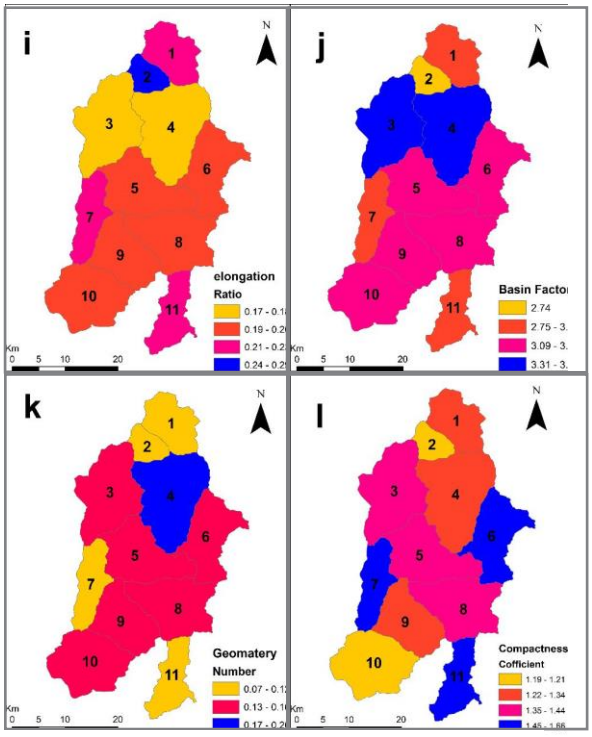

Fig. 6. District Shangla showing i. elongation ratio, j. Basin factor, k. Geometry Number, l. Compactness coefficient.

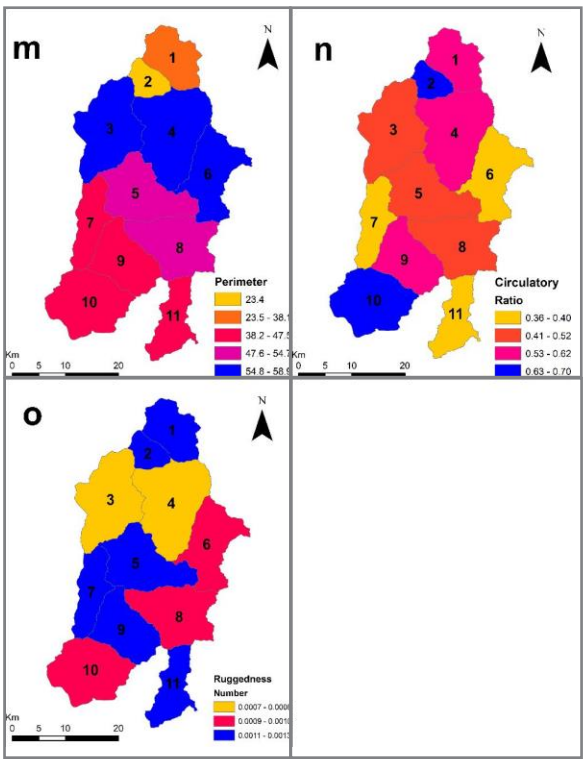

Fig. 7. District Shangla showing m. perimeter, n. circulatory ratio, o. Ruggedness Number.

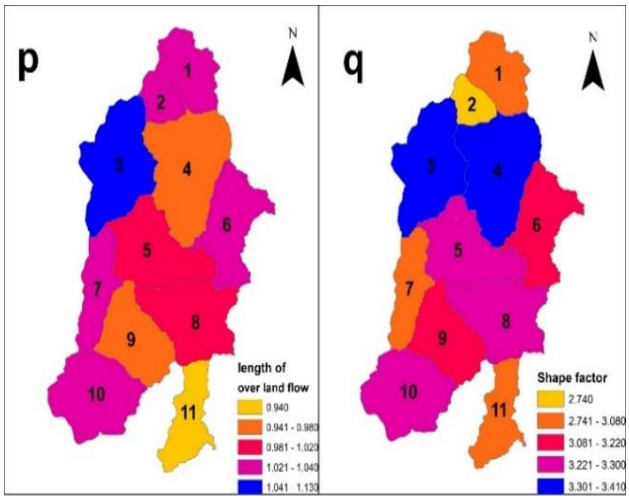

Fig. 8. District Shangla showing p. length of overland flow, q. Shape factor

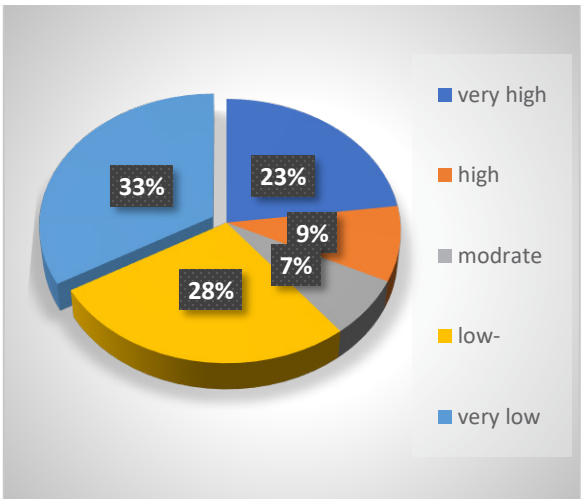

Fig. 9. Percentage of the area covered by flash flood hazard zones

flood flow.

4.1.2. High Flash Flood Hazard Zone

Results from the current study revealed that 9 % (145.89 km²) of the study area is mapped as a high-hazard zone for flash floods (Figure 9). Thus collectively, the total area under high to very-high hazard zone for flash floods is about 32 % of the study region. The high hazard zone was marked by ranking number (GRN) value ranges from 47.4 to 51.4 which are exhibited by Sb-2 and Sb-8 (Table 3, & Figure 10).

4.1.3. Moderate Flash Flood Hazard Zone

The moderate flash flood hazard zone of the study region accounts for 7 % (113.65 km²) of the region (Figure 9). The moderate hazard zone was categorized by the geo-morphometric ranking number (GRN) value ranging from 46.23 to 47.43 as shown by Sb-5 (Table 3, & Figure 10). This basin has 3rd number of stream order and hence flood frequency is considered as a medium.

4.1.4. Very Low to Low Flash Flood Hazard Zone

In the Shangla Basin, 61 % (1026.27 km²) has very low to low hazard of flash floods. It was also verified in the field that this zone is not experiencing a flood.

5. CONCLUSION

This study concludes that the morphometric analysis is a crucial tool for understanding flow patterns and its effects on the surrounding area. Such studies could be used for better planning, designing, and management of floodwaters (dams, embankments, protective walls, spurs etc.). Flood risks can be mitigated by designing a flood defence system based on the results derived from morphometric analysis. The morphometric analysis carried out in this study are helpful for risk modeling in order to establish link between flow pattern and terrain characteristics. Such modeling is an important factor in determining how risk will shift over time in response to varying pattern of different environmental variables. The engineering design of dams, bridges, culverts, and flood control structures are only a few examples of the various engineering applications for which the flood morphometric analysis can be utilized. Additionally, it can be used to delineate floodplains, determine human activity within floodplains, and estimate the financial benefits of flood mitigation schemes. Presently, the process of flood risk modeling and management has been significantly strengthened by the collection and accessibility of high-resolution spatial information, high performing computing systems, and development in hydrological modeling methodologies. Flood danger is very dynamic with respect to space and time. The probability, size, regional extent, depth levels, and frequency of changes in the type of flood danger may be determined using the present scenarios of global climate variability. Flood risk is directly related to the geo-morphometric ranking number, and in this study it ranges from 39.18 to 55.14. Sb-4 was found to be at high flood risk with a ranking value of 55.14, whereas sb-11 was marked

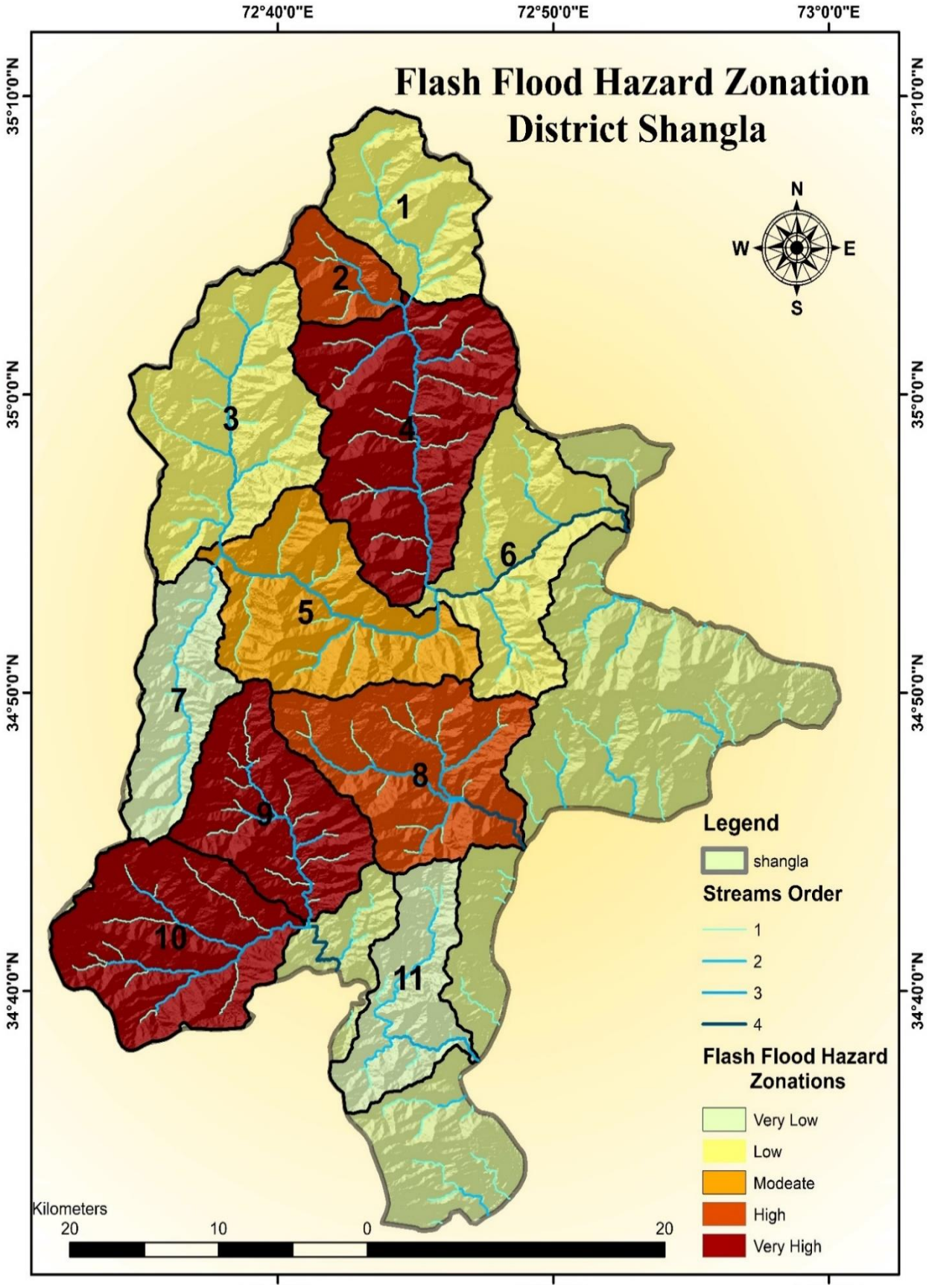

Fig. 10. Flood Hazard Zonation Map

at a very low flood risk with ranking value of 39.18. Sb-11 and sb-7 having the ranking value from 39.18 to 42.14, respectively, fall in the very low flood risk sub basins. Sb-1, sb-3 and sb-6 having ranking value 42.14 to 46.23 are in low flood risk zone. Sb-5 having ranking value 46.23 to 47.43 is in moderate flood risk. Sb-2 and sb-8 having the high flood risk with rankingvalue of 47.43 to 51.41, respectively. Sb-4, sb-9 and sb-10 are in the very high flood risk zones in the study area with ranking value ranging from 51.41 to 55.14. This model identifies sub-basins with very high to very low flood potential and gives spatial evaluation of hydrologic responsiveness of all sub-basins based on geo-morphometric parameters. The study concludes that primary causes of a high degree of flood hazard are the dense network of streams, high relief ratio, steep gradient, and the impermeable character of the surface rocks. The National, provincial Disaster Management Authorities and the District administration should use the outcome of this study and extend the technique to other regions.

6. RECOMMENDATIONS

On the basis of results derived from this study, the following are recommended;

- A long term policy should be adopted to mitigate the flash floods.
- Conservation Structures should be constructed to conserve the soil and reduce the impact of flash floods on agriculture land.
- Disaster risk reduction activties shoul be started on priorty basis in order to reduce the human loss and livestocks, and to reduce the damage of floods to physical infrastructure.

7. ACKNOWLEDGEMENTS

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8. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Distribution, Morphometrics and DNA barcoding of *Archotermopsis wroughtoni* Desneux (Termopsidae: Blattodea) in District Mansehra, Pakistan

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Abstract: *Archotermopsis wroughtoni* is a primitive termite species with distinct biological and behavioral features. Despite its presence in temperate regions worldwide, including the Oriental region, there is a lack of data on the current distribution of this species in the Mansehra district of Pakistan. Samples were collected from forested areas, including the Kaghan, Naran, Mahandri, and Shogran valleys. The distribution of *A. wroughtoni* was determined by observing decayed and fallen wooden logs, and host plants were identified to assess the species' host preference. Morphometric identification was performed following relevant literature, and the barcoding technique of mtDNA *COII* was used to authenticate the species. Phylogenetic analyses were conducted using the neighbor-joining and maximum parsimony methods. The study revealed the presence of *A. wroughtoni* in the forests of northern Mansehra, where it preferred tree species such as *Cedrus deodara* and *Pinus excelsa* for nest construction and foraging. The findings of this research will contribute to future studies on the biology and ecology of *A. wroughtoni* and aid in developing conservation strategies for this species and other social insects.

Keywords: Termites, Termopsidea, *Archotermopsis wroughtoni*, distribution, morphometric, DNA barcoding, Mansehra

1. INTRODUCTION

Termopsidea family is distinguished as a small and primitive group comprised of three surviving genera: *Archotermopsis*, *Hodotermopsis*, and *Zootermopsis* [1]. These termites are geographically restricted to the Oriental and Nearctic regions [2]. Oriental region covers Asia's tropical territories, including India, Pakistan, Sri Lanka, Indonesia, and the Philippines. In contrast, the Neartic region encompasses North America, extending southward to the middle part of Mexico [3]. Among the three genera, *Archotermopsis* is the sole genus found dwelling in the foothills of the Himalayas and Vietnam [1]. On the other hand, *Hodotermopsis* and *Zootermopsis* inhabit Vietnam, South China, Japan, and the western region of the United States [1, 2].

Archotermopsis wroughtoni, commonly called the Himalayan termite, is a primitive and extant species that fall under the subfamily Termopsinae within the Termopsidae family. It inhabits the northern regions of Pakistan, the northwestern Himalayas in India, and the eastern parts of Afghanistan [2]. Termites can be classified into two distinct groups, namely lower termites and higher termite species, based on the specific nature of their symbiotic partners residing within their gastrointestinal tracts [4]. Lower termites exhibit a symbiotic relationship with prokaryotes and flagellated protozoans inhabiting their intestinal tracts [5]. In contrast, higher termites do not possess flagellated protozoa but rather harbor symbiotic prokaryotes within their intestinal tracts [4]. *A. wroughtoni* is categorized as a member of the lower termite group [6].

Archotermopsis wroughtoni serves as a keystone species in the ecosystem, playing a vital role in maintaining the balance of the forest. Through its

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breakdown of dead wood and subsequent return of nutrients to the soil, this species helps to sustain the forest's vitality [7-8]. Furthermore, *A. wroughtoni* is a crucial food source for other organisms, including birds and insects. The termite's role in the carbon cycle is also noteworthy, as it facilitates the decomposition and recycling of carbon-rich plant material. Additionally, its activity improves soil structure, thus contributing to the growth of new vegetation [8].

A. wroughtoni termite species in Pakistan is experiencing a decline and is presently considered endangered [9]. This species breeds in decaying logs commonly found in pine tree forests [2]. However, due to the escalating human population in these areas, these logs are rapidly being harvested for fuel, which results in *A. wroughtoni* losing suitable breeding sites. This human activity significantly threatens the species' survival in the wild [2, 9].

The existing body of literature regarding the historical distribution of *A. wroughtoni* documents its occurrence in Pakistan. Specifically, this species has been recorded in various northern regions of Pakistan, including Kumrat, Kalam, Roringar, Murree, Hazara, Kaghan, Naran, and Shogran [7, 10-12]. However, there is limited knowledge regarding its recent distribution, host preferences, and molecular characterization. Moreover, the phylogenetic relationships among different populations of *A. wroughtoni* still need to be adequately understood.

Therefore, this research aimed to conduct DNA barcoding and phylogenetic analysis, in addition to morphometric characterization and distribution, of *A. wroughtoni* in the Mansehra district of Pakistan. To achieve this, partial sequences of mitochondrial *COII* genes were analyzed to explore the genetic diversity and phylogenetic relationships among the regional populations of *A. wroughtoni*.

2. MATERIALS AND METHODS

2.1 Sampling

This study was conducted within the geographical boundaries of the Mansehra district in Pakistan, which can be located by coordinates situated in the northern latitudes between 34° 14' and 35° 11' and the eastern longitudes between 72° 49' and 74° 08' [13].

A survey of the district Mansehra was conducted between March and November 2020 and 2022. The belt transect method was utilized to collect samples from fallen logs [14]. Visible galleries were identified, and on-site samples were collected and preserved in vials containing 80 % ethanol for morphometric analysis and 99 % ethanol for DNA extraction [15]. Additionally, the coordinates of the sampling sites were recorded using a handheld GPS device (Garmin, GPSMAP 64sx), and the forage substrate for all locations was documented.

2.2 Morphometric Identification

We conducted morphometric identification of soldiers using available literature on keys, illustrations, pictures, characters, and indices. Measurements were taken using a binocular microscope equipped with built-in magnification, and statistical measures were calculated, including means [16]. A digital camera-equipped stereo zoom trinocular microscope (Olympus, SZX7) was employed to capture photographs. We documented and noted down ten distinct features/metrics, comprising the distance between the head and the lateral base of mandibles, the widest point of the head, the longest measurement of the labrum, the broadest point of the labrum, the length of the left mandible, the shortest median length of the postmentum, the middle point width of the postmentum, the median length of the pronotum, the median width of the pronotum, and the count of segments in the antennae [17].

2.3 Distribution and Mapping

The sampling sites' coordinates were recorded using a GPS device and projected onto a map utilizing ArcGIS 10.7 [18].

2.4 DNA Extraction and Amplification

In order to extract DNA, a single soldier termite specimen was selected as a representative and identified from available specimens. The specimen was washed with distilled water and air-dried, after which its legs were placed in a 2.5 mL eppendorf tube [19]. Liquid nitrogen was added to the tube to

freeze the specimen, which was then crushed with a pestle. The CTAB method was employed to extract DNA from the crushed specimen [20]. To assess the amount and purity of the genomic DNA extracted, an agarose gel containing 1 % concentration was utilized, which was subsequently stained with ethidium bromide. The samples were preserved at -20 °C for future experimentation after this analysis. For the PCR amplification, a 2 µL volume of the upper phase of the extracted DNA was combined with 23 µL of a master mix. The target fragment was a 684 bp segment of the COXII gene, amplified using forward and reverse primers 5′-TCTAATATGGCAGATTAGTGC-3′ and 5′-GAGACCAGTACTTGCTTTCAGTCATC-3′ [21, 22].

The PCR master mix (Thermo Fisher Scientific, Waltham, MA) included several compounds at specific concentrations: 2.5 µL of PCR buffer (10X), 1 unit of Taq polymerase (3U/µL), 2.5 µL of Bovine Serum Albumin (BSA) (100 µg/mL), 1.5-2.0 µL of Magnesium Chloride (25 mM), 0.5 µL of dNTPs Mix (10 mM), 1.25 µL of Primer (F) 10 pM, 1.25 µL of Primer (R) 10 pM, and 1 µL of Genomic DNA Template (20-50 ng). PCR-grade water was added to adjust the final volume of the mixture to 24 µL [23].

The PCR reaction was subjected to thermal cycling, utilizing the following conditions: an initial denaturation step was conducted at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 54 °C for 30 seconds, and extension at 72 °C for 45 seconds. A final extension step was performed at 72 °C for 10 minutes. The amplified PCR products were subsequently analyzed through 1 % agarose gel electrophoresis [24].

2.5 DNA Sequencing and Phylogenetic Analysis

To sequence the PCR products, 20 µL of each sample was transferred to an eppendorf tube, which was subsequently dispatched to Celemics (Celemics, Korea) for Sanger sequencing. The obtained *COII* sequences were trimmed to an approximate length of 684 bp with the elimination of specific starting and ending fragments to ensure uniform sequence length and minimize interference [21, 25]. In order to assess the nucleotide sequence similarity, only the 30 most pertinent GenBank sequences (selected based on % query coverage, E-value, % identity, and relevant taxon matching using BLASTn) were scrutinized [26].

To maintain the precision of species identification for sequences forwarded to GenBank through BLASTn, a process of aligning the matching sequences of the target sequence with the reference sequence was conducted. The top ten sequences most closely related to *Archotermopsis* species were chosen to construct neighbour-joining and maximum likelihood trees and alignments of sequences using ClustalW in MEGA 11 [27-30]. The outcome of this process was a total of 12 *COII* sequences with accession numbers OQ753771, OQ753772, EU253892.1 (*Archotermopsis wroughtoni*), MF477197.1 (*Zootermopsis* laticeps), DQ442267.1 (*Zootermopsis* angusticollis), GQ922444.1:1-708 (*Zootermopsis* nevadensis), MZ058037.1 (*Cryptocercus matilei*), OM991373.1 (*Postelectrotermes* sp.), OM991347.1 (*Glyptotermes* sp. 11), OM991330.1 (*Comatermes perfectus*), KY224587.1 (*Mirocapritermes* sp.), and KY224622.1 (*Postsubulitermes parviconstrictus*) retrieved from GenBank. Furthermore, two newly generated sequences were deposited into the GenBank database and identified as OQ753771 and OQ753772.

3. RESULTS

3.1 Recorded Species of *Archotermopsis wroughtoni*

The head exhibits a posterior margin that is bilobed, while the cerci consist of 6 to 7 segments, and the antenna displays 22 to 27 articles (*Archotermopsis wroughtoni* Desneux) (Figure 1). The specimens gathered were identified for their taxonomic classification following the guidelines outlined by Roonwal *et al.* [2] and Imms and Hickson [16] and were found to conform to the published descriptions. The measurement of the soldier caste was conducted, considering several characteristics, and then compared to the previously reported range (Table 1).

Based on the statistical analysis, it can be inferred that all means fall within the established ranges, thus suggesting that the samples are a

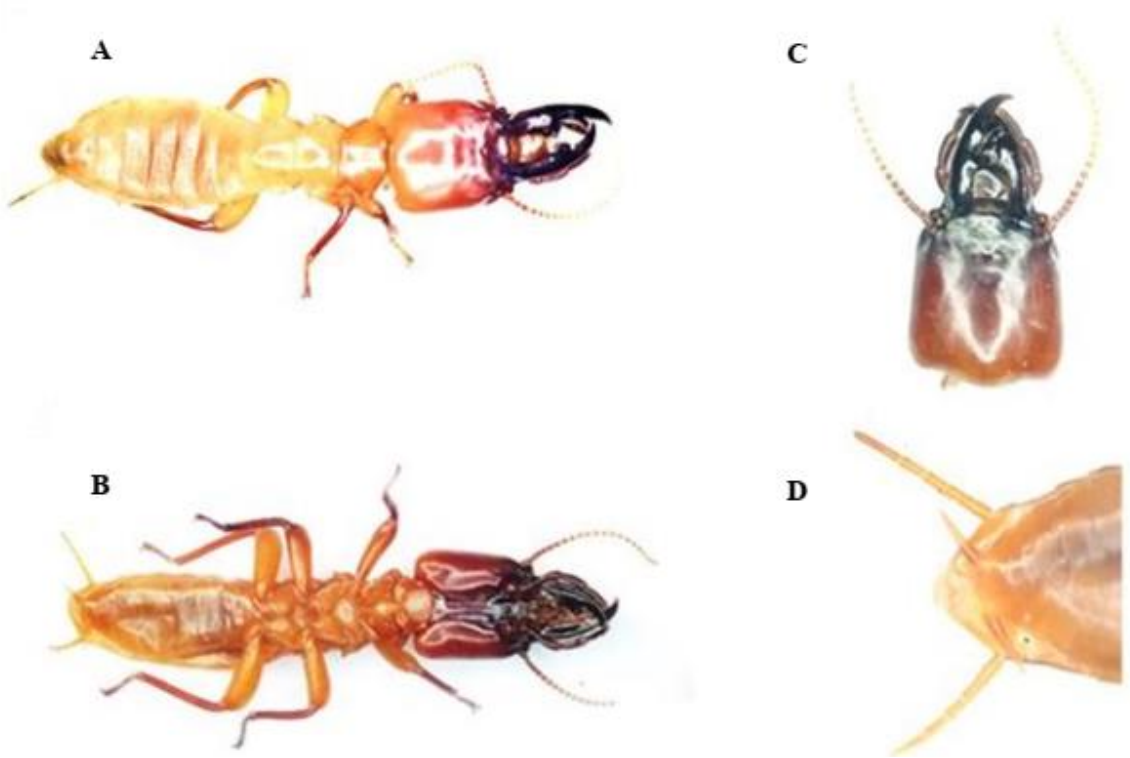

Fig. 1. Different morphometric indices of the Soldier caste of *Archotermopsis wroughtoni* **A**. *A. wroughtoni* soldier dorsal view, **B**. Ventral view of the soldier **C**. Head dorsal view, **D**. Cerci

probable reflection of the known population or that the pre-established range encompasses the population means.

3.2 Habitat Description and Wood Preference

Archotermopsis wroughtoni has been observed to inhabit high-altitude coniferous forests between 900 and 3000 meters. This particular species has demonstrated the ability to flourish by consuming decayed wood particularly that of *Cedrus deodara*, *Pinus roxburghii*, and *Pinus wallichiana* without causing harm to living trees. Its colonies, which their extended, vertical galleries characterize, are generally modest, typically consisting of 30-40 individuals.

Despite its wood consumption, no visible external signs of damage are evident. Furthermore, it has been observed that this species reproduces

Table 1. Comparison of index measurements (in mm) in the *Archotermopsis wroughtoni* soldier caste

S. No.	Indices	Measurements in (mm) of *A. wroughtoni*
1	Head length up to the lateral base of the mandibles	3.5*, 2.80-5.20**
2	Maximum width of the head	3.3*, 2.55-4.55**
3	Maximum labrum length	0.625*, 0.33-0.80**
4	Maximum labrum width	0.825*, 0.63-1.10**
5	Left mandible length	3.5*, 2.10-4.80**
6	Median length of postmentum	3.475*, 2.23-4.43**
7	Maximum postmentum width	2.315*, 1.53-3.03**
8	Maximum pronotum length	1.465*, 0.95-1.95**
9	Maximum pronotum width	2.315*, 1.53-3.03**
10	Number of segments in the antenna	23*, 22-27**

*Designates measurements recorded in the current study, ** denotes reference range [2]

within the wood, and during the monsoon season, particularly from June to August, swarming behaviour has been documented.

3.3 Local Distribution and Mapping

The distribution of *Archotermopsis wroughtoni* species was found to be restricted to specific localities within the Mansehra district, namely Kaghan Valley, Naran, Kiwai, Hangrai, Mahandri, Shogran, Pae, Paras, Garlat, and Ghannol. The species was not observed at lower elevations in the district. The coordinates of the collected and analyzed specimens were systematically plotted on a geographical map. The black dots on the map indicate the locations where the *Archotermopsis* species were encountered during the sampling process (Figure 2).

3.4 Remarks

The present study pertains to observations on *A. wroughtoni*, a termite species whose unique soldier caste morphology allows for straightforward identification. Notably, the species exhibits an uneven distribution within the Mansehra district and is confined to the upper mountainous regions characterized by the prevalence of pine forests. The species primarily occupies decaying logs as its preferred habitat, posing challenges to its detection owing to the propensity of local inhabitants to collect windfall trees.

3.5 DNA Barcoding

3.5.1. Sequences Alignment and Similarity Validation

The sequences of *Archotermopsis wroughtoni* corresponded to 97.49 % similarity with EU253892.1, as indicated by the results of the BLASTn search.

3.5.2. Neighbor-Joining Method Tree

The study employed the neighbour-joining [26]

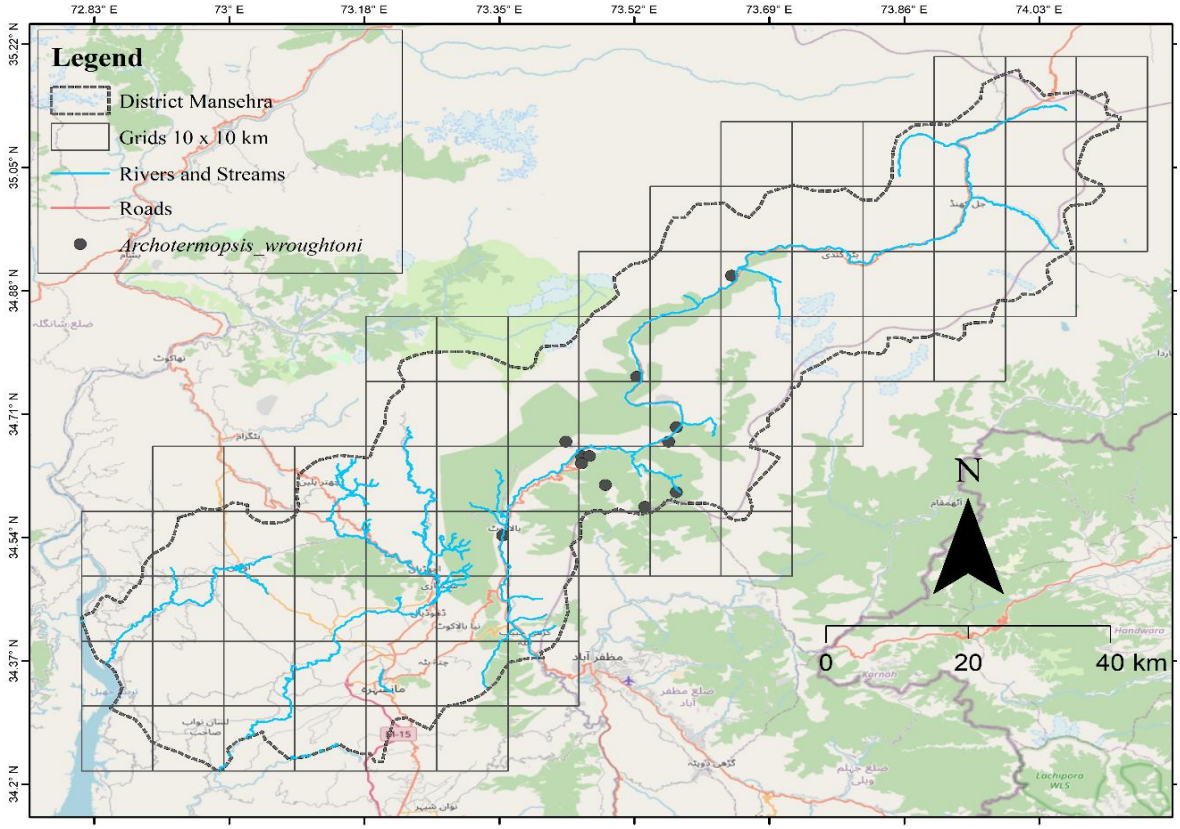

Fig. 2. Distribution of *Archotermopsis wroughtoni* in district Mansehra, Pakistan. The map was created using ArcGIS 10.7.

method to derive the evolutionary relationships, and the resulting optimal tree is presented herein (Figure 3). The bootstrap test (1000 replicates) illustrates the proportion of trees in which the associated taxa clustered with the branches.

The tree is drawn to scale, with the branch lengths representing the same units used for calculating the evolutionary distances, which were determined using the Maximum Composite Likelihood method and expressed as base substitutions per site [24, 25]. The analysis involved 12 nucleotide sequences, with all ambiguous positions removed for each sequence pair using the pairwise deletion option. The final dataset consisted of 736 positions. The evolutionary analyses were performed using MEGA11 [23]. The phylogenetic tree of the *Archotermopsis* sequence successfully matched the top sequence obtained from the BLASTn searches and isolated the *Archotermopsis* clades from other termite species (Figure 3).

3.5.3. Maximum Parsimony Method Tree

The Maximum Parsimony method was used to infer the evolutionary history, and Tree #1 out of the two most parsimonious trees (with a length of 0) is presented. The consistency index (0.644979) and the retention index (0.639885) indicate the degree of homoplasy in the dataset.

In contrast, the composite index (0.412712) measures the overall fit of the data to the phylogenetic tree. The tree branches are annotated with the percentage of replicate trees in which the associated taxa clustered together, as determined by the bootstrap test (1000 replicates) [24].

The MP tree was generated using the Subtree-Pruning-Regrafting (SPR) algorithm [26], with search level 1, and 10 initial tree additions were performed. The final dataset consisted of 12 nucleotide sequences with 736 positions. The phylogenetic tree, which includes the top 10 sequences from BLASTn searches, effectively distinguished the clades of *Archotermopsis wroughtoni* from other species, as illustrated in Figure 4. Evolutionary analyses were performed using MEGA11 [23].

4. DISCUSSION

The present investigation aimed to assess the distribution of *Archotermopsis wroughtoni* and

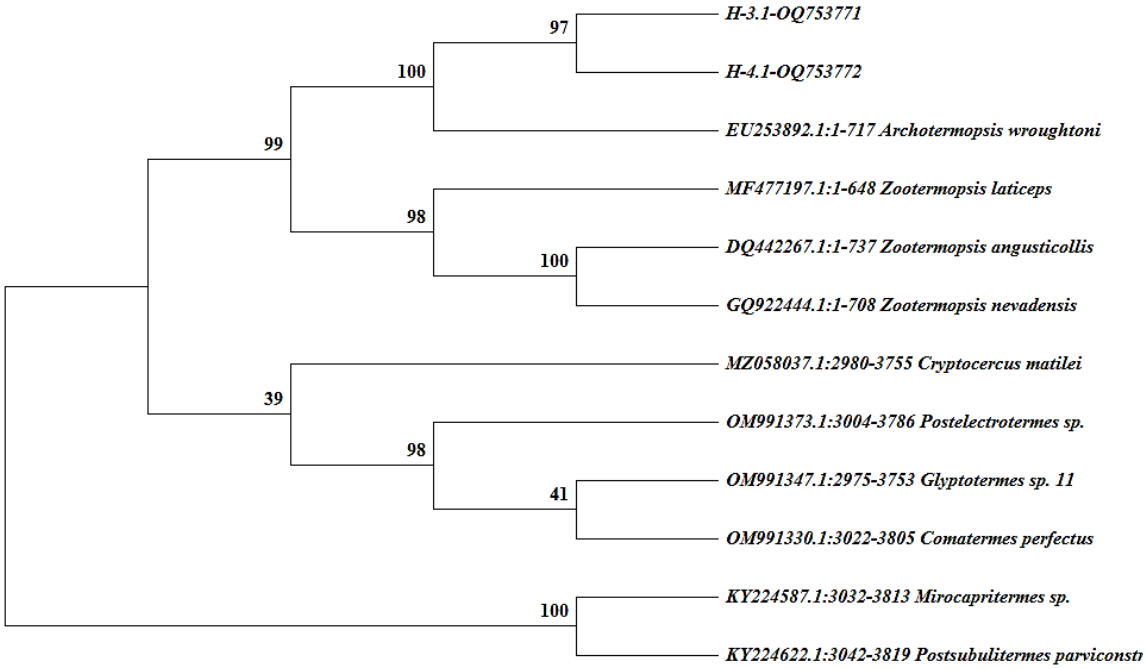

Fig. 3. Phylogenetic analysis of Archotermopsis species gathered from different regions of the Mansehra district using the Unrooted Neighbor-Joining method. The type specimens submitted by the sequence uploader are represented by sequences H-3.1-OQ753771 and H-4.1-OQ753772.

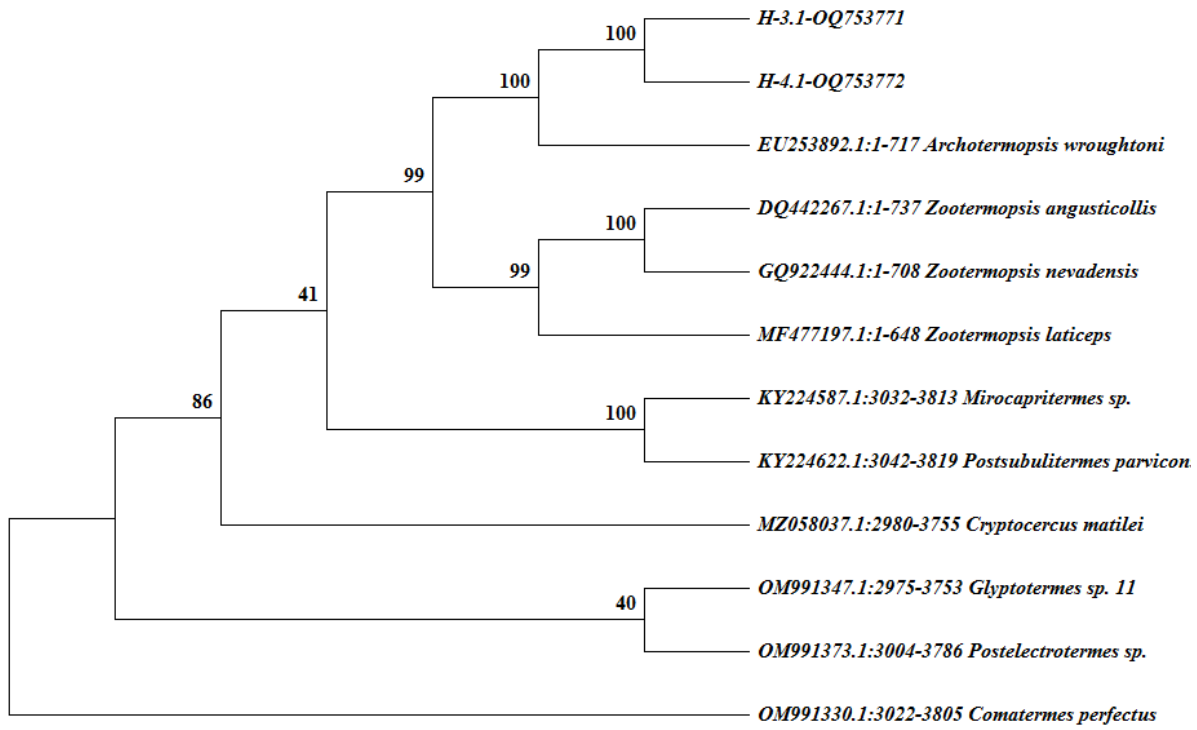

Fig. 4. Unrooted MP phylogeny of Archotermopsis species collected from the various areas of district Mansehra, Pakistan. The type specimens submitted by the sequence uploader are represented by sequences H-3.1-OQ753771 and H-4.1-OQ753772.

perform DNA barcoding and morphometric characterization in the Mansehra district. This species was found to be sparsely distributed across the district [2]. The lesser Himalayan region, ranging from 750–1700 meters in elevation, was found to be the primary distribution area for *Cedrus deodara*, *Pinus roxburghii*, and *Pinus wallichiana* [31], and it was observed that *A. wroughtoni* inhabits the decaying and fallen logs of these tree species. The range of *A. wroughtoni* was restricted to specific locations such as Kaghan Valley, Naran, Kiwai, Hangrai, Mahandri, Shogran, Pae, Paras, Garlat, and Ghannol within the district, with no presence observed at lower elevations or other areas.

The current research is consistent with previous investigations on the geographic range of *Archotermopsis wroughtoni* in the Himalayan region. This termite species has been well-documented in various regions of Pakistan, including Kumrat, Kalam, Hazara, Kaghan, Naran, and Shogran [2]. Ahmad [32] was the first to report the presence of it in West Pakistan, and later it was discovered in the Murree Hills of Punjab, Pakistan [12]. Chaudhry and Akhtar reported the species in their technical reports on the termites of Pakistan [11] and the zoogeography of the termites of Pakistan [10], respectively. Akhtar's most recent report on the species' distribution in Pakistan, published in 2000, recorded its presence in Roringar, Swat, consistent with the current study's results [9]. *A. wroughtoni* was the least commonly found termite species in the present study, which aligns with Akhtar's findings [9]. He classified the species as endangered due to human activities leading to the removal of felled logs used as breeding sites for *A. wroughtoni*.

Archotermopsis wroughtoni is found in the Himalayan regions of Pakistan, India, and Afghanistan. It has been observed in Himachal Pradesh, Jammu and Kashmir, and various districts of Uttar Pradesh in India, as well as in Nangarhar Province's Barikot in Afghanistan [1, 2].

The study included an analysis of *Archotermopsis* using DNA barcoding and phylogenetic methods, focusing on a specific segment of the mitochondrial *COII* DNA sequence. The resulting relationships between taxa were well-supported, as indicated by the bootstrap analysis. Interestingly, the relationships inferred through

both parsimony and distance analyses were highly similar, with a maximum support of 100 in MP and 97 in neighbour-joining. The phylogenies obtained from molecular data and morphological characters exhibited no significant differences in species-level relationships.

DNA barcoding, which utilizes short, standardized DNA sequences from genes like *COI*, has revolutionized the identification and classification of biological specimens [33-34]. Compared to traditional morphological identification methods, DNA barcoding offers faster, more accurate, and more objective species identification [35-40]. Its applications span conservation biology, wildlife forensics, and food safety, contributing to the understanding and management of invasive and endangered species while enriching our knowledge of biodiversity [41].

DNA barcoding employs genetic markers such as *COI* to identify and differentiate species [39]. However, for several reasons, *COII* (cytochrome c oxidase subunit II) has emerged as the most effective marker for DNA barcoding. *COII* exhibits higher variability and evolutionary rates than other markers, making it a suitable candidate for differentiating closely related species and improving the resolution of DNA barcoding analysis. Additionally, *COII* is a universally conserved region of the mitochondrial genome. It can be applied to diverse taxa and easily amplified and sequenced using standard laboratory methods [40].

Moreover, the accuracy and effectiveness of *COII* as a DNA barcoding marker have been demonstrated by large-scale projects such as the Barcode of Life Initiative (BOLD). *COII* is thus considered the best option for DNA barcoding due to its high variability, universality, and accuracy [41]. In this study, the amplified *COII* region of *Archotermopsis wroughtoni* was subjected to sequence cleaning and BLAST analysis to retrieve similar sequences. Subsequently, phylogenetic trees were constructed using the neighbour-joining and maximum parsimony methods.

The neighbor-joining algorithm is commonly used to construct phylogenetic trees based on genetic distances, connecting taxa iteratively [39-40]. This distance-based method is efficient for analyzing large datasets. However, its accuracy relies on suitable distance metrics and either homoplasy or conflicting signals. To mitigate uncertainties, we also employed the Maximum Parsimony method for phylogenetic inferences [40]. Maximum parsimony seeks to identify the tree with the fewest evolutionary changes needed to explain the observed data using a heuristic approach.

In our investigation, we observed that the clade most closely related to our sequences following *Archotermopsis*, as indicated by both the neighbour joining and maximum parsimony trees, was that of *Zootermopsis*. Notably, the family Archotermopsidae was determined to be monophyletic, despite previous research [36] highlighting a close relationship between two of the three genera within this family, namely *Archotermopsis* and *Zootermopsis* [1]. Living *Zootermopsis* occurred in western North America and was introduced in Japan [42].

As previously documented, the base composition of insect mitochondrial DNA exhibits a marked preference for adenine and thymine [43]. The present study examined the *Archotermopsis* species of termites and found a similar bias, with an average of 66.20 % adenine and thymine and 33.79 % guanine and cytosine.

5. CONCLUSION

The present study has significantly contributed to our understanding of the distribution, morphology, and DNA barcoding of *Archotermopsis wroughtoni* in the Mansehra district of Pakistan. We have confirmed the existence of this primitive termite species in the region and gained insight into its preferred host plants for nesting and foraging. Our findings provide a foundation for future research on the biology and ecology of *A. wroughtoni*.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest regarding the publication of this manuscript.

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Pakistan Academy of Sciences

Research Article

Pharmacognostic Study of *Ehretia acuminata* R.Br.

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Abstract: *Ehretia acuminata*, commonly known as "Puna" in Pakistan, is from the Boraginaceae family and is used in ecological, medicinal, and agricultural sectors. The current study was conducted to assess the pharmacognostic potency of bark extracts from *E. acuminata*. The crude distilled water, ethanol, and chloroform extracts signified a concentration-dependent increase in intestinal mobility of the experimented animal, and the plant delivered methodical proof for its pharmacological usage as an antispasmodic drug. The bark distilled water, bark ethanol, and bark chloroform extracts revealed antispasmodic potential (11±1, 9±1, and 11±1) at 300 mg/kg. The distilled water, ethanol, and chloroform extracts also showed analgesic and muscle relaxant potential in the present study and the results were concentration dependent. The bark distilled water, bark ethanol, and bark chloroform extracts revealed the analgesic potential (10±1, 16±1, and 11±1) at 300 mg/kg. The Bark distilled water, Bark ethanol, and Bark chloroform extracts revealed the muscle relaxant potential (6±1, 5±1, and 5±1) at 300 mg/kg. While the distilled water, ethanol and chloroform extracts did not show acute toxic effects against the tested animal mice. In this study, bark extracts of *E. acuminata* showed pharmacological potency in experimental animals. The plant delivered scientific proof for its pharmacological usage as an antispasmodic, acutely toxic, muscle relaxant, as well as an analgesic drug.

Keywords: Analgesic, Antispasmodic, Bark extracts, Boraginaceae, *Ehretia acuminata,* Pharmacological potential

1. INTRODUCTION

The genus *Ehretia* comprises about 150 species, predominantly dispersed in torrid regions of tropical Asia, Australia, Africa, and North America [1]. The genus includes pergolas and shrubs. All parts of the plant, including the leaves, stem, offshoots, fruits, roots, and duramen, are used as herbal medicines separately [2]. *Ehretia acuminata*, a native plant of Pakistan vernacularly known as Puna, has a variety of uses; the wood of the plant is widely used for fuel purposes, while the leaves are used as fodder for livestock. Moreover, the tree is also used as an erosion controller in farm forestry and ~~for~~ gunstock purposes, whereas; the unripe fruit is used as pickles in food [3, 4].

In southern China, various parts of the genus, including the leaves, root, fruit, bark, and duramen, are widely used as traditional medicines for inflammation, cough, itches, diarrhea, dysentery, swellings, cachexia, fever, and syphilis [2]. Many species such as *E. acuminata*, *Ehretia laevis*, as well as *Ehretia microphylla,* have been described to be used in numerous traditional and herbal remedies in China as well as India due to satisfactory feedback in various tests such as anti-inflammatory, anti-diabetic, and antibacterial activity [1, 5].

In Zimbabwe, different parts of *Ehretia obtusefolia* are used for the treatment of sore throat, toothaches in infants, menstrual cramps, abdominal spasms and infertility in women [6]. In India,

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E. laevis is used to treat headaches and ulcers. It also possesses potent anthelmintic, diuretic, demulcent, expectorant, and astringent properties. The duramen of *E. laevis* has been used as nosh [7]. The plants of the genus *Ehretia* are renowned for possessing the rich traditional medicinal uses, including the treatment of chest pains, abdominal cramps [8], toothaches, cachexia, cough, diarrhea, syphilis, stomach diseases, and eczema [9, 10]. Moreover, they have also been widely used in the treatment of asthma, tonsils, dry cough, pneumonia, malaria, typhoid, epilepsy, wounds, mental problems, and venereal diseases [11], as well as nervous disorders and kidney inflammations [12].

Ehretia genus has also been described to as possessing some of the important secondary metabolites, including flavonoids, lignans, phenolic acids, nitrile glycosides, steroids, triterpenoids, quinonoids, and pyrrolizidine alkaloids [1]. Ehretianone, a novel quinonoid xanthene extracted from the bark of the roots of *Ehretia buxifolia* is reported to hold antisnake venom potential [13]. The main objective of this study is to assess the pharmacognostic potency of bark extracts of *Ehretia acuminata and* to discover their use as an antispasmodic drug, an acute toxic drug, a muscle relaxant drug as well as an analgesic drug for future.

2. MATERIALS AND METHODS

2.1 Accumulation of Plant Material and Identification

The undried bark of *E. acuminata was* taken from the Pakistan Forest Institute, Peshawar. The act of taxonomical identification was done by Ghulam Jelani, the curator of the herbarium at the Botany Department, University of Peshawar, Pakistan. The specimen sample (Voucher No. 59) is preserved in the herbarium of the Botany Department Government Superior Science College Peshawar, Pakistan.

2.2 Extraction Preparation of Plant Material

The collected bark was kept in the shade for 3 weeks at room temperature to be completely dried. After the drying process, grinding was done with the help of a grinder machine. The bark was grounded to be used as extracts in distilled water, chloroform, and ethanol. The powder materials were weighted by Electrical balances (Gottingen, Germany), and 50 mg powder was taken and dissolved in 500 ml of solvents followed by the measurement of the solvents using graduated cylinders. Each of the given solutions was then prepared in a 1000 ml beaker (Borosilicate glass). All of the solutions were kept in reserve for three days (72 h) in a stirrer (DAIHAN SRICO). After stirring for 3 days, the solutions were then sieved through filter paper (WHATMAN NO. 1), The filtrates were accumulated and poured into respective beakers. Distilled water, chloroform, and ethanol extracts were then evaporated with the help of a Rotatory vacuum machine (HAHNSHIN S/Co, South Korea). The distilled water, chloroform, and ethanol extracts were then poured into the china dishes, which appeared to be in crude form. They were placed in the Water Bath (DAIHAN Scientific, Germany) at 55 °C for 120 minutes to completely purify the extracts. Protected sterile vials were used in order to prevent any impurities. When all of the extracts had completely dried out and got ready, they were then collected from the china dishes and placed in the aforementioned three separate vials with the help of a stirrer, followed by placing them in a refrigerator at 40 ^{0}C in order to protect them from bacteria, fungi, or any other contaminants [13].

2.3 Preparation of Serial Dilution

Eppendorf tubes were used in order to prepare a serial dilution. These tubes were tagged from 1 to 3. The first tube was filled with 500 µl stock extract, while, the second and third Eppendorf tubes were filled with distilled water of about 250 µl using a pipette with 500 µl tips. 250 µl of stored extract from the 1st Eppendorf tube was drawn out to second, from second to third by a pipette. The stored extracts persisted in the 1st tube of about 250 µl as default or stock for the diluted extract, followed by the shaking of all the Eppendorf tubes by a Vortex mixer (DAIHAN Scientific, Germany). Finally, the diluted concentrations were formulated at 250 mg/ml, 125 mg/ml, and 62.5 mg/ml [14].

2.4 Analgesic Activity

2.4.1. Acetic acid induced activity

Swiss albino female mice (20-30 g) were brought

from the Veterinary Research Institute Peshawar, Pakistan, for the activities. The mice were kept starved for 4 hours before the initiation of the procedure, followed by the division of the animals into six sets. The first set was administered with normal saline (10 ml/kg I.P) as a negative control, while, the 2nd set was inoculated with the standard drug (Diclofenic sodium) as a positive control (50 ml/kg I.P) third, fourth and fifth sets were provided with 10, 15, and 20 mg/kg I.p. of extract, and the residual 6th set was inoculated with acetic acid. After 30 minutes of saline, diclofenic sodium, and plant extract injections, pain was fostered by introducing 1 % of acetic acid into the peritoneum of mice. The wriggle (abdominal constriction, trunk twirling and expansion of hind limbs) took place in 10 minutes and the result/effect revealed hindrance in percentage [16].

2.5 Muscle Relaxant Activity

2.5.1. Traction test

During the given test, female mice were laid on a string firmly braced from the apex. Normal mice seized the string by feet. However, by allowing them to hover freely, they would hold the cable with at least one hind foot for 4-5 seconds. The incapability of the mice to hold the cord with at least one hind foot indicated a failure in the traction [17]. During the examination, animals were classified into six sets; each administered with either saline (10 ml/kg) or the plant extracts at different doses i.e. (100, 150, and 200 mg/kg).

2.6 Acute Toxicity Activity

Swiss Albino female mice (20-30 g) were used in the said activity for the evaluation of toxic effects [18]. The caged animals accommodated under standard conditions of 12 hours of light/dark cycle, nourished with the food prepared by the Veterinary Research Institute along with water access were habituated to the laboratory environment for 14 days, preceding the experiment. All of the mice were retained famished overnight with a free approach to water followed by the random division into 10 groups, each with six mice which were constantly being scrutinized for the initial 4 hours and then the subsequent 24 hours for any possible toxic symptoms.

2.7 Antispasmodic Activity

2.7.1. Charcoal movement activity

Swiss Albino female mice (20-30 g) were kept deprived of nourishment for 5 hours before the procedure was initiated. However, they were allowed to drink water. After 60 mins, the mice were treated with standard drugs and plant extracts, followed by the oral administration of 1ml charcoal nosh (3 % deactivated charcoal in 2 % aq-tween 80) to each mouse, succeeded by the treatment of charcoal for 50 minutes. each mouse was then dissected and the interval covered by charcoal food from the pyloric region to caecum was calculated to demonstrate the inhibition induced by the extracts in percentage [18].

2.8 Statistical Analysis

The result of this activity was achieved with the use of one-way ANOVA by Dunnet's numerous juxtapositions. The acquired result was then contrasted with the vehicle observational group. $*P<0.01$ was considered to be statistically significant. The percent inhibition was calculated by the following formula;

$$\% \text{ inhibition} = [(A-B)/A] \times 100$$

Where A = Average number of writhing of the control group.

While B = Average number of the writhing of the test group

The result of the traction test was accomplished with the help of one-way ANOVA by Dunnet's various comparisons. A level of importance of $P<0.05$ was considered numerically substantial.

The result of the Antispasmodic Activity was described as mean ± S.E.M (The standard error of the mean) succeeded by execution of Statistical analysis by Student's t test. A level of significance of $P<0.05$ was considered statistically substantial.

3. RESULTS & DISCUSSION

3.1 Analgesic Activity of Ehretia acuminata on Acetic Acid Persuaded Writhing in Mice

The distilled water bark extracts of *E. acuminata* showed significant analgesic activity (**P*<0.01). The activity was dose dependent. It showed 19±1 no. of writhes at the dosage of 100 mg/kg. Similarly, both doses showed 15±1 and 10±1 no. of writhes at 200 and 300 mg/kg. The ethanol bark extracts of *E. acuminata* revealed noteworthy analgesic activity. The activity was dose dependent. It showed 21± 1 no. of writhes at 100 mg/kg. Similarly, both doses showed 17±1 and 16±1 no. of writhes at 200 and 300 mg/kg. The chloroform bark decoction of *E. acuminata* set forth notable analgesic activity. The activity was dose-dependent. It showed 22±1 no. of writhes at 100 mg/kg. Similarly, both doses showed 15±1 and 11±1 no. of writhes at 200 and 300 mg/kg Figure 1.

Numerous researchers described analgesic activities of multiple medicinal herbs that delivered significant effects on the tested animals which fully substantiate our findings e.g., Khan *et al.* evaluated the analgesic effects of methanol extracts of various parts of *Ehretia serrata* and *E. obtusifolia* and showed significant results which supports our result [19]. Al-Snafi performed analgesic activities of *Cordia myxa* and reported substantial result similar to our discovery [20]. Other researchers also executed analgesic activities of *E. microphylla* and revealed consequential findings that correspond to our results [21].

3.2 Muscle Relaxant Activity

The distilled-water bark extract of *E. acuminata* showed significant muscle relaxant activity ($P < 0.05$). The activity was dose dependent. The muscle relaxant potential was 11±1 at 100 mg/kg and 9±1 at 200 mg/kg, while at 300mg/kg; the muscle relaxant potential was 6±1 and showed significant results. The ethanol bark decoction of *E. acuminata* illustrated a noteworthy muscle relaxant experiment. The activity was dose-dependent. The muscle relaxant potential was 11± 1 at 100 mg/kg and 7±1 at 200 mg/kg while at 300mg/kg, the result was 5±1 and showed significant results. The chloroform bark extract of *E. acuminata* showed significant muscle relaxant activity. The activity was dose-dependent. The muscle relaxant potential was 12±1 at 100 mg/kg and 8±1 at 200 mg/kg while at 300mg/kg, the muscle relaxant potential was 5±1 and showed significant results Figure 2. AlBayaty explored the muscle relaxant potential of *Cordia myxa* (Boraginaceae) extract in the isolated tracheal smooth muscle of sheep. The results support our findings [22].

3.3 Acute Toxicity

The distilled water bark extract of *E. acuminata* showed significant acute toxicity. The experiment was dose dependent. The result showed no mortality at 100 mg/kg, 200 mg/kg and 300 mg/kg. The bark ethanol decoction of *E. acuminata* revealed remarkable acute toxicity. The activity was dose dependent. The result showed no mortality at the dosage of 100 mg/kg, 200 mg/kg and 300 mg/kg. The chloroform bark extract of *E. acuminata*

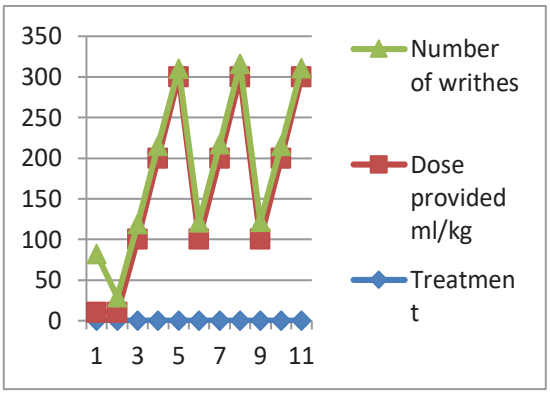

Fig. 1. Analgesic activity of *E. acuminata* bark.

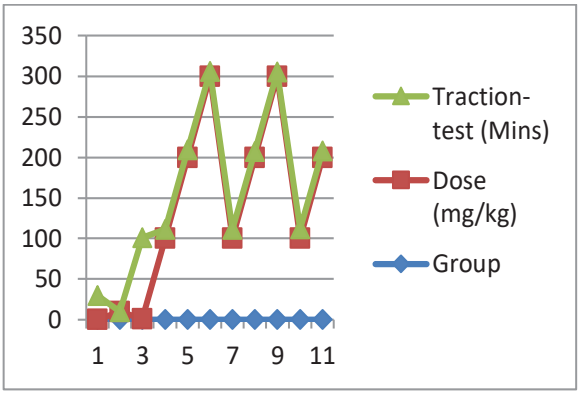

Fig. 2. Muscle relaxant activity of *E. acuminata* bark

showed significant acute toxicity. The activity was dose dependent. The result showed no mortality at 100 mg/kg, 200 mg/kg and 300 mg/kg Figure 3. Another scientist examined the acute toxic effects of *Heliotropium indicum* Linn. (Boraginaceae). Inoculation of HIEA did not cause any death in the experimented animals throughout 24 hours' duration of the acute toxicity which corresponds to our findings [23].

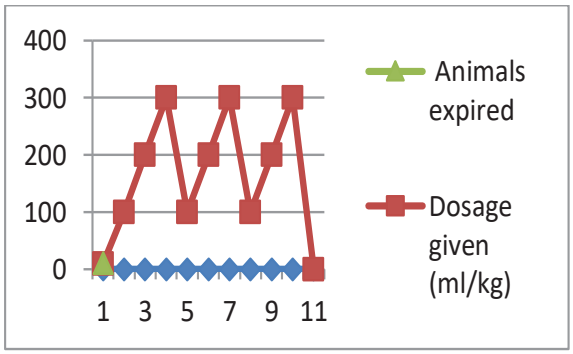

Fig. 3. Acute toxicity of *E. acuminata* bark

3.4 Antispasmodic Activity

The distilled -water bark extract of *E. acuminata* showed significant antispasmodic activity *(P*<0.05). The activity was dose dependent. The distance covered by charcoal was 19±1 and 15±1, at 100, 200 mg/kg while at 300mg/kg the 11±1 distance covered by charcoal which showed more significant. The bark ethanol decoction of *E. acuminata* revealed notable antispasmodic activity. The activity was dose dependent. The distance covered by charcoal showed 19±1 and 13±1 at 100 mg/kg and 200mg/kg while at 300 mg/kg showed 19±1 distance covered by charcoal, which showed more significant. The chloroform bark extract of *E. acuminata* showed significant antispasmodic activity. The activity was dose dependent. The distance covered by charcoal showed 20±1 and 17±1 at 100 mg/kg and 200 mg/kg while at 300mg/kg showed 11±1 distance covered by charcoal which showed more significant Figure 4. A group of scientists assessed the Methanol extracts of *Onosma griffithii* and its different parts for possible antispasmodic effects on rabbits' intestine. Rabbits of both sexes (1.0 -2.0 kg) were operated in the experiments. Studies were carried out on rabbits' jejunum (The middle part of the small intestine) preparations [24]. The results showed significant antispasmodic effects which support our findings as well.

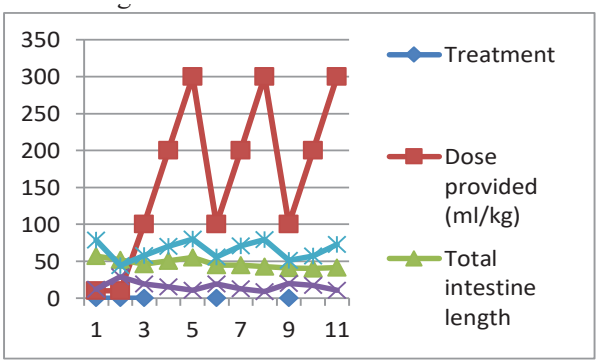

Fig. 4. Antispasmodic activity of *E. acuminata* bark

4. CONCLUSION

The present study was conducted to assess the pharmacognostic potential of bark extracts of *E. acuminata* including four activities, i.e., analgesic activity, muscle relaxant activity, antispasmodic activity, as well as acute toxicity activity, ~~in~~ all of which showed significant dose dependent effects; the higher the dose, the greater the effect. While acute toxicity of *E. acuminata* extracts did not show any effect, the plant provided significant evidence for its pharmacological use as a medicinal plant and can be used as an analgesic, muscle relaxant, and an antispasmodic drug in the future.

5. ETHICAL STATEMENT

The animal used in our research has been approved by the Ethical committee of the veterinary research institute of Peshawar.

6. DISCLOSURE STATEMENT

We wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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Pakistan Academy of Sciences

Research Article

Ecological Assessment of the Native Flora of Matta Kharari Village, Swat, Khyber Pakhtunkhwa, Pakistan

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Abstract: Plant species composition, diversity and distribution is a hierarchical expression of vegetation determined by different environmental factors. A study was conducted to document the floristic composition and biological spectrum of plant species of the Matta-Kharari region, Swat. The region has not been ecologically explored yet. We used quantitative ecological techniques for sampling plant species. Sixty-seven quadrats of 1x1 m^2 for herbs, 5x5 m^2 for shrubs and 10x10 m^2 size for trees were established. Plant species data were analyzed using multivariate statistical techniques. We evaluated 76 plant species belonging to 42 families. Asteraceae and Rosaceae were the leading families with 8 species each, followed by Ranunculaceae, Lamiaceae and Moraceae with 4 species each. Among life form classes i.e., geophytes were dominant (13 species, 17.10 %), followed by therophytes (12 species, 15.87 %), nanophanerophytes (11 species, 14.47 %), and chamaephytes (10 species, 13.58 %). Moreover, Mesophylls (36.84 %) were the dominant leaf spectra class followed by nanophyll (30.26 %), microphyll (27.63 %), and megaphyll (2.63 %). The two-way cluster analysis classified the vegetation into three clusters. The detrended correspondence analysis shows the distribution of plants in a mixed array because the locations explored are characterized by similar climatic factors and vegetation. We elucidate that the variation in climatic factors and topography brings variation in vegetation. Understanding these responses at the life form and leaf spectra level will provide a better understanding and knowledge that how plant species and their communities or associations respond to changes in climate in the future. The current study could be utilized as a baseline for large-scale studies in the future.

Keywords: Floristic Composition, Biological Spectrum, Multivariate Analysis, Detrended Correspondence Analysis

1. INTRODUCTION

Ecological assessment of a floristically rich and diverse region is crucial for determining the key elements responsible for hosting such a diverse and rich Phyto-diversity [1]. Floristic classification of a region depends on different characteristics such as habitat, species diversity, composition, and geographical features. The floristic inventory of a region is a complete set of wild, native, horticultural and agricultural plant species [2]. Precise floristic inventories can assist in a better understanding of topography, climate, soil, hydrology and many other features influencing forest diversity and composition [3]. The collective growth of the plant in a particular area characterized by a component of species or combination of species is known as vegetation. They are a key component of the ecosystem which describes different ecological patterns and facts across the landscape [4]. Classification of vegetation of a region into various associations or communities is crucial for resource management [5]. Quantitative ecological techniques are used to determine vegetation, pattern distribution and composition in a meaningful way [6]. Various qualitative and quantitative features

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of vegetation such as density, cover, frequency, abundance, richness, and phenology are focused on in the field of plant ecology [7].

Plant life forms and leaf spectral traits reflect the existing environmental conditions of a region [8]. A number of authors have disentangled the complex relationship of plant life form and leaf spectral traits with the surrounding environment i.e., Harboi rangeland Kalat [9], Mastuj valley Chitral [10], Havelian Abbottabad [11], Manoor Valley Kaghan [8], Balakot Mansehra [2] Biha valley Swat [12] and many others. It is crucial to understand the state of the floristic diversity of the unexplored areas [13]. Plant life forms and spectra are an important expression of the current climate. In some species, leaf size increases when moving from dry to most sorts of climatic regions. Multivariate statistical analyses are used to understand the complex species distribution pattern [14]. Cluster and two-way clusters are used for the classification and understanding of the distribution pattern of species [15]. On the other hand, detrended correspondence analysis (DCA) is used to identify various vegetation groups, communities and associations [16].

The current study region Matta-Kharari lies in the floristically rich region of Swat. However, a study presenting floristic composition by using multivariate ecological techniques is missing. Therefore, the current study is the first-ever attempt to analyze life and leaf spectral data as well as plant species data through different multivariate ecological tools and it will serve as a baseline for large-scale studies in the future. The current study aimed to elaborate on floristic composition with a special focus on life form and leaf spectra traits.

2. MATERIALS AND METHODS

2.1 Study Area

The beautiful valley of Swat is geographically situated at 34^0 34' to 35^0 55' North latitude and 72^0 08' to 72^0 50' East longitude. It lies in the Norwest parts of the Khyber-Pakhtunkhwa province of Pakistan. The region has beautiful sites with an altitudinal range from 700-6000 meters [17]. Swat Valley is best known for its waterfalls, lakes, and lush green hills. Physically it forms parts of the Himalayan, Hindukush and Karakorum mountains and is considered among the most beautiful places of the subcontinent, also known as the "Switzerland of the East" [18]. Swat contains innumerable monuments of ancient civilizations, spanning 5,000 years of history. The annual rainfall in Swat ranges from 1000-1200 mm. On the other hand, the average temperature is mostly in the range of -2 ^{0}C to 37 ^{0}C [17]. Swat Valley has diverse flora distributed across its moist and temperate zones. The region is considered a hub for different fruits and vegetables. Apple, peach, plum, apricot and persimmon are among the popular fruits of the region. It has an area of 5,337 square kilometers and a population of 2,309 [19]. The region is divided into seven tehsils: (1) Barikot (2) Babuzai (3) Bahrain (4) Charbagh (5) Kabal (6) Khwazakhela (7) and Matta [19].

Matta is the leading administrative subdivision of Swat [17], [20]. Tehsil Matta has a population of about 465,996 individuals and 52,625 households [21]. It is situated at 34° 36' 59" to 35° 44' 51' N and from 72° 29' 52" to 72˚ 09' 52" E and at an altitude of 1136 meters at sea level [20]. It has a total area of 683 Km^2. Word Matta is a traditional word that means clay. Matta lies 20 Km from the main city of Mingora. Most of its population is rural [20]. Apple and peach (more than 95 %) of Swat are produced in the Matta sub-division rightly termed as the "Apple region". Matta Kharari is located near the north junction of the Swat and Haronai rivers. Matta Swat has a lot of biodiversity resources. A review of the available information shows that the livelihood and primary healthcare in traditional communities are mostly dependent upon the vegetation of the area.

2.2 Collection and Preservation

We collected various plant species from different sites in the Matta Khararai region. We used quadrat quantitative ecological techniques across the area. Quadrats of 10x10 m^2 for trees, 5x5 m^2 for shrubs and 1x1 m^2 for herbaceous plants were used. Plant samples were properly tagged and dried with their respective codes of each quadrat. We identified our samples using various updated volumes of the flora of Pakistan. All samples were cross-checked with previously collected samples by different researchers from various regions of the country. Plant samples were poisoned for preservation using mercuric chloride and ethanol solution. Preserved

samples were mounted on standard herbarium sheets. Then the collected plants were preserved and mounted on standard herbarium sheets. A complete alphabetical floristic list of species along with families and ecological descriptions was prepared. The plants with voucher numbers were submitted to the Herbarium, Department of the Botany University of Peshawar, Pakistan.

2.3 Life Form and Leaf Spectra Classes

We arranged plants into different Raunkiaer (1934) life form classes [22]. The Raunkiaer (1934) life form classes include Phanerophytes (Megaphanerophytes > 30 m, Mesophanerophytes 7.5-30 m, Microphanrerophytes 2-7.5 m, Nanophanerophytes 0.25-2 m, Chaemiphytes, Hemicryptophytes, Geophytes, Therophytes, Climber and Lianna. Moreover, we further classified these plant species into different classes based on leaf size following Raunkiaer's (1934) leaf spectra classification. The Raunkiaer (1934) leaf spectra classes are Leptophyll up to 25 mm, Nanophyll with leaf size 25-225 mm^2, Microphyll 225-2025 mm^2, Mesophyll 2025-18225 mm^2, Macrophyll 18225-164025 mm^2 and Megaphyll larger than 164025 mm^2 [23].

2.3 Data Analysis

Data analyses were carried out to know the distribution and classification patterns of vegetation. The data were put and sorted in MS Excel for hierarchical cluster and Two-Way Cluster Analysis as per the requirement of the software. Cluster and Two-Way Cluster dendrograms were constructed using PCORD software. We used presence and absence data (1,0) for Two-Way Cluster analysis to understand the distribution pattern in the study region. In Two-Way Cluster the black dots show the presence while the white empty dots show the absence of a particular species. Species are grouped in Two-Way Cluster based on similarities and dissimilarities in presence and absence frequency (Figure 3). For ordinations, we used detrended correspondence analyses. It is a type of indirect gradient analysis used to know species ordination in a particular region. The Canodraw function of CANOCO software was used for data visualization and graph creation. Vegetation ecologists mostly use detrended correspondence analyses when they are interested in understanding the relationship among species or sampling plots.

3. RESULTS AND DISCUSSION

3.1 Floristic Composition and Ecological Features

We collected 76 plant species belonging to 42 families (Table 1). Among these 42 families, only one family belongs to Gymnosperms while the remaining are Angiosperms. Angiosperms have 35 dicots and 6 monocot families. Asteraceae and Rosaceae are the top dominant families each with 8 species followed by Ranunculaceae, Lamiaceae and Moraceae each contributing 4 species. Brassicaceae and Fabaceae have 3 species each. Buxaceae, Polygonaceae, Adoxaceae, Salicaceae and Caprifoliaceae have 2 species each. Plantaginaceae, Papaveraceae, Violaceae, Diosccoreaceae, Poaceae, Juglandaceae, Berberidaceae, Hyperiacaceae, Scrophulariaceae, Cannabaceae, Ebenaceae, Rubiaceae, Onagraceae, Asparagaceae, Utricaaceae, Primulaceae, Paeoniaceae, Araliaceae, Aquifoliaceae, Geraniaceae, Saxifragaceae, Boraginaceae, Colchicaceae, Crassulaceae, Balasaminaceae, Elaegnaceae, Phyllanthaceae and Celastraceae are having 1 species each.

Shaheen *et al.* [26] documented 248 plant species in 166 genera and 38 families from the desert of Thal Punjab Pakistan. Based on their findings the most dominant families were Poaceae, Fabaceae, Amaranthaceae and Asteraceae which are in complementarity to our results. On the other hand, Nasir and Sultan [24] explored the floristic composition of the district Chakwal and revealed that the dominant families are Asteraceae and Poaceae which support our findings. The vegetation of Hayatabad Peshawar is explored by Shah and Hussain [25], they documented that Asteraceae, Brassicaceae, Poaceae and Solanaceae are the top leading families of the region. Their results strongly support our findings. Khan *et al.* [27], classified the vegetation of the dry and Coal rich Dara Adam Khel mountains into different biological spectrums. They reported 54 species belonging to 30 different families. Asteraceae was the topmost leading family followed by Lamiaceae and Solanaceae which support our findings.

3.2 Life Form

Plants are classified into different life form classes to know about the general appearance of the vegetation of a region because it reflects the impact of the environment on the existing vegetation. It assists vegetation scientists in the recognition and description of the flora of a region. Raunkiaers life form classification system is more accurate and reliable. It is based on the protection of perennating buds during adverse and unfavorable conditions [22]. Following Raunkiaer's classification, we classified all plant species into different life form classes i.e., geophytes (13 species, 17.10 %), therophytes (12 species, 15.87 %), nanophanerophytes (11 species, 14.47 %), chamaephytes (10 species, 13.58 %), Mesophnarophytes (9 species11.84 %), mega-phanerophytes (9 species, 11.84 %), hemicryptophytes (8 species, 10.52 %), and Microphanerophytes (2 species, 2.63 %) as shown in (Figure 1).

3.3 Leaf Size Spectra

In the current study, the Mesophylls (36.84 %) were the dominant leaf spectra class followed by nanophyll (30.26 %), microphyll (27.63 %), megaphyll (2.63 %) and leptophyll (1.31 %) as shown in (Figure 2). Malik *et al.* [29], explored the moist temperate vegetation of Pir Chinnasi hills in Azad Jammu Kashmir by classifying their plant species into leaf spectra classes. Their results revealed that in the spring season microphylls were the dominant class followed by nanophylls. Their results are in accordance with our findings because the Azad Jammu Kashmir has great similarity to the climatic conditions of Swat.

Moreover, the Dheri Baba hills explored by Shah and Rozina [30], documented that the microphylls and mesophylls were the top leading leaf spectra classes in the region. Leaf spectral classes reveal that the microphylls increase with a gradual increase in altitude from the lower foothills to the top peaks. According to them, Nanophylls density was higher at lower altitudes. Plant species with minute leaf sizes are the general indicators of dry and xerophytic ecosystems because small leaf size is an adaptation to dry and arid types of habitats [24]. According to Batalah and Martin [31, 33] that leaf size is significantly correlated to drought and soil conditions.

3.4 Distribution of Plant Species in the Study Area

Based on the presence and absence of the plant species in the specific quadrat using 1-0 data a two-way cluster dendrogram was constructed. Where black blocks show the presence of plant species while white blocks indicate the absence of the species in a particular quadrat. It tells us about the distribution pattern of plant species in the studied region [1,15, 32]. Species with maximum black dots show dominancy while rare species are shown by very few dots in the dendrogram (Figure 3). *Lonicera quinquelocularis, Rubus niveus, Fragaria vesca, Thalictrum hamatum, Cannabis sativa,*

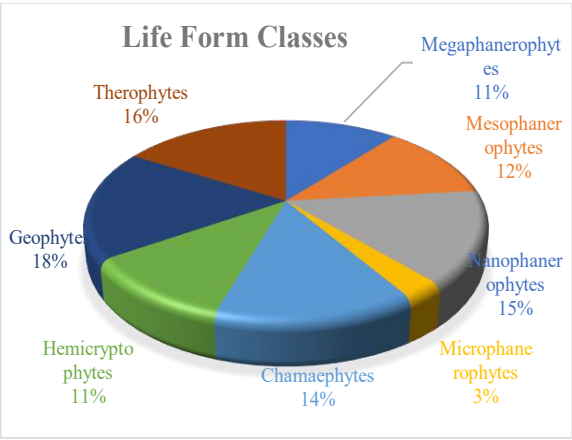

Fig. 1. Percentage of Raunkaier life form classes

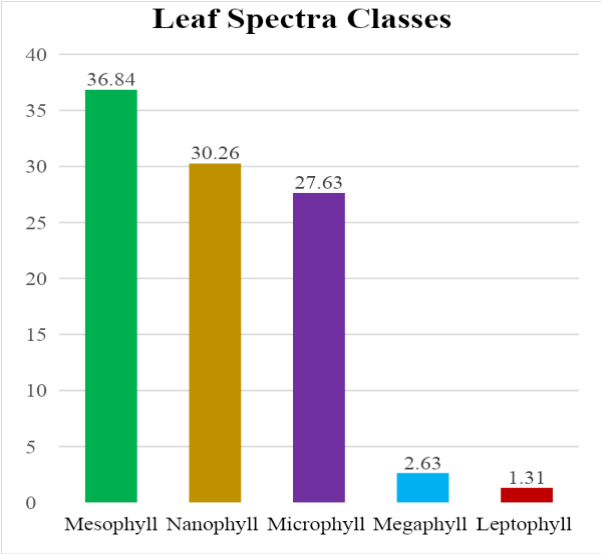

Fig. 2. Percentage of Raunkaier leaf spectra classes

Table 1. Floristic composition, life form and leaf size spectra

Family	No. of Species	Botanical name	Collection No	Local name	Life form	Leaf spectra
Pinaceae	2	*Picea smithiana* (Wall.) Boiss.	23	Mangazai	MegP	N
		Pinus wallichiana A.B. Jacks	2	Peuch	MegP	N
Asteraceae	8	*Aster flaccidus* Bunge	90	--	Th	Mic
		Onopordum acanthium L	72	Candeyary	G	Mes
		Conyza bonariensis (L.) Cronquist	66	--	Th	N
		Prenanthes brunoniana Wall. ex DC	42	--	Th	Mes
		Artemisia vulgaris L.	40	Tarkha	Ch	Mic
		Launaea secunda (C.B. Clarke) Hook. F	20	Karazay	H	L
		Leontopodium himalayanum DC.	14	Sargary	H	N
		Tussilago farfara L.	7	Nakipanra	G	Mes
Rosaceae	8	*Rubus niveus* Wall. ex G. Don	97	Boganra	MP	N
		Spirea Pall.	80	--	MP	Mic
		Prunus cornuta (Wall. ex Royle) Steud.	49	Barid	Mic	Mes
		Fragaria vesca L.	36	Zmakeen Toot	Th	Mic
		Rosa moschata Herrm	34	Qurach	MP	N
		Sorbaria tomentosa (Lindl.) Rehder	28	Jajrai	NP	Mic
		Spiraea canescens D. Don	80	Chaghge-botay	NP	Mic
		Cotoneaster himalaiensis hort. ex Zabel	73	Kharawa	MP	N
Ranunculaceae	4	*Delphinium denudatum* Wall.ex Hook. f. & Thomson	70	Leghonay	H	N
		*Thalictrum hamatu*m Maxim	21	Kekerbotay	TH	N
		Ranunculus laetus Wall. ex Royle	19	Zergolay	G	N
		Aquilegia fragrans Benth.	11	Zergolay	Ch	N
Lamiaceae	4	Origanum vulgare L.	82	Desipodina	Ch	Mic
		Isodon rugosus (Wall. ex Benth.) Codd	69	Sperkay	NP	Mes
		Mentha longifolia (L.) Huds.	58	Welanay	G	N
		Ajuga bracteosa Wall.exBenth.	13	Boti	H	Mic
Moraceae	4	*Morus nigra* - L.	81	Tor toot	MegP	Mes
		Ficus palmata Forssk.	77	Enzar	MegP	Mes
		Ficus foveolata (Wall. ex Miq.) Miq.	63	Patenzar	MegP	Mes
		Morus serrata Roxburgh	55	Toot	MegP	Mes
Brassicaceae	3	*Nasturtium officinale* R. Br	57	Tarmera	G	Mic
		Cardamine hirsuta L.	43	--	Ch	Mic
		Arabidopsis thaliana (L.) Heynh	6	--	Th	N
Plantaginaceae	1	*Plantago lancelata* L.	45	Gabai	Th	Mes
Fabaceae	3	*Robinia pseudoacacia* L.	95	Kikar	MicP	N
		Indigofera heterantha Wall.ex Brandis	60	Gowareaga	Ch	N
		Trifoliun repenes L.	46	Shawtal	Ch	N
Buxaceae	2	*Buxus wallichiana* Baill.	4	Shamshad	NP	MIc
		Sarcococca saligna (D. Don) Müll. Arg.	3	Akhtar	NP	Mic
Polygonaceae	1	*Bistorta amplexicaulis* (D.Don) Greene	51	Tarwapanra	H	Mes

Family	No. of Species	Botanical name	Collection No	Local name	Life form	Leaf spectra
Liliaceae	2	Gagea elegansWall. ex D. Don.	9	Spinsakay	G	N
		Tulipa clusiana Redouté	41	Gantol	G	Mes
Adoxaceae	2	*Viburnum grandiflorum* Wall. ex DC	1	Gultan	MP	Mes
		Viburnum cotinifolium D. Don	54	Inzargul	MP	Mes
Salicaceae	2	*Salix tetrasperma* Roxb.,Pl. Corom	55	Wela	Mp	Mes
		Populus nigra L.	96	Toorsperdar	Mp	Mes
Papaveraceae	1	*Corydalis diphylla* Wall.	17	Shamdana	Mp	Mg
Violaceae	1	*Viola canescens* Wall	16	Banafsha	Th	Mic
Diosccoreaceae	1	*Dioscorea deltoidea*Wall.ex Griseb.	62	Kanrhiz	G	Mes
Poaceae	1	Dactylis glomerata L.	61	Wakha	G	N
Juglandaceae	1	*Juglans regia* L.	65	Guz	MesP	Mic
Berberidaceae	1	*Berberis pseudoumbellata* subsp. gilgitica Jafri	59	Kwaray	NP	N
Hypericaceae	1	*Hypericum perforatum* L.	84	Desi shin chay	Ch	N
Scrophulariaceae	1	*Verbascum thapsus* L.	86	Warmagu	Th	Mg
Cannabaceae	1	*Cannabis sativa* Linn	91	Bang	Th	Mic
Ebenaceae	1	*Diospyros kaki* L.	98	Soramlook	MegP	Mes
Rubiaceae	1	*Galium aparine* L.	94	--	Th	N
Onagraceae	1	*Oenothera speciosa* Nutt	39	--	H	Mic
Asparagaceae	1	*Polygonatum verticillatum* (L.)	50	Noryalam	G	Mic
Caprifoliaceae	1	*Lonicera quinquelocularis* Hardwicke in Hook	48	--	NP	N
Urticaceae	1	*Urtica dioica* Linn.	38	Galbang	Th	Mic
Primulaceae	1	*Androsace rotundifolia* Hardwicke	37	Kanrkan	H	Mes
Paeoniaceae	1	*Paeonia emodi* Wallich ex Royle	35	Mameakh	Ch	Mes
Caprifoliaceae	1	*Lonicera myrtillus* Hook. f. & Thoms.	33	Aday	Np	Mes
Araliaceae	1	*Hedera nepalensis* K. Koch, Hort. Dendrol.	30	Palul	NP	Mes
Aquifoliaceae	1	*Ilex dipyrena* Wall.	29	Banj	MegP	Mes
Geraniaceae	1	*Geranium nepalense* Sweet.	27	--	Ch	Mic
Saxifragaceae	1	*Bergenia ciliata* Haw.	26	Gut panra	G	Mes
Boraginaceae	1	*Myosotis alpestris* F. W. Schmidt, Fl. Boem	22	--	H	Mic
Cochicaceae	1	*Colchicum luteum* Linn.	5	Zargulay	G	N
Crassulaceae	1	*Sedum ewersii* Ledeb.	88	Warkharay	G	Mes
Balsaminaceae	1	*Impatiens bicolor* Royle.	67	Pratai	Th	Mes
Elaeagnaceae	1	*Elaeagnus umbellata*	76	Ganamranga	NP	Mic
Phyllanthaceae	1	*Andrachne cordifolia* (Wall. ex Decne.) Muell.	83	Shrub	Th	Mes
Celastraceae	1	*Maytenus wallichiana* Spreng.	53	Bampora	NP	Mic
Polygonaceae	1	*Rumex dentatus* Linnaeus	24	Shalkhy	Ch	Mes

and Verbascum thapsus are the species clumped together and shown by many presence values than the other species. They mostly prefer similar sort of climatic conditions and grow in associations.

3.5 Detrended Correspondence Analysis (DCA)

Plant species are classified in various quadrants via

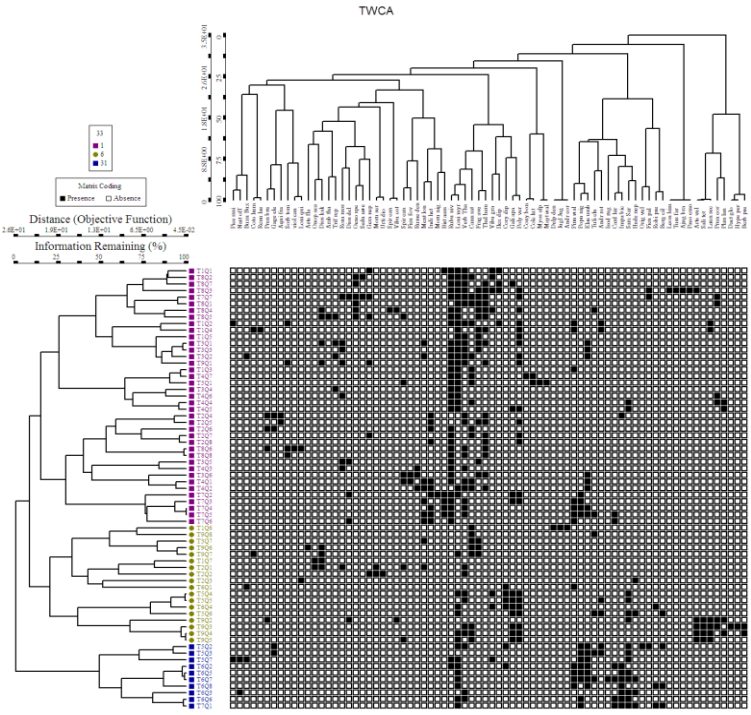

Fig. 3. Two-way cluster dendrogram presenting species distribution

Detrended correspondence analysis (DCA) using CANOCO software. Detrended correspondence analysis was carried out for all plant species in the studied region (Figure 4). The DCA diagram shows the distribution of species in the studied region. In DCA ordination the maximum length recorded for axes 1 was 5.203 with an eigenvalue of 0.913, the length of the gradient for axis 2 was 4.2 with an eigenvalue of 0.734, the length of the gradient for axis 3 was 5.572 with an eigenvalue 0.512, length of the gradient for axis 4 was 3.282 with eigenvalue 0.399. The total inertia recorded for this DCA was

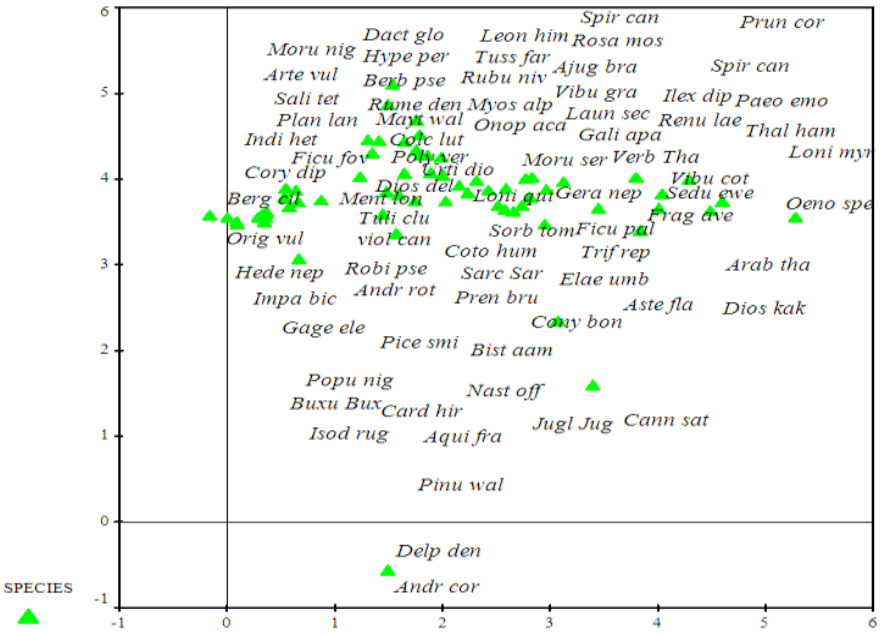

Fig. 4. Detrended correspondence analysis showing plant species distribution and ordination

Table 2 Summary of Detrended Correspondence Analysis

Axes	1	2	3	4	Total inertia
Eigenvalues	0.913	0.734	0.512	0.399	11.308
Lengths of gradient	5.203	4.2	5.572	3.282	
Cumulative percentage variance of species data	8.1	14.6	19.1	22.6	
Sum of all eigenvalues					11.308

11.308. The cumulative percentage variance of species data was 8.1 for axis 1, 14.6 for axis 2, 19.1 for axis 3 and 22.6 for axis 4 (Table 2).

4. CONCLUSION

We conclude that multivariate ecological techniques such as Two-Way Cluster analysis and detrended correspondence analysis are the most important methods for vegetation classification and ordination. Leaf spectral classes reveal that the microphylls increase with a gradual increase in altitude from the lower foothills to the top peaks. Moreover, nanophyll's density was higher at lower altitudes. Plant species with minute leaf sizes are the general indicators of dry and xerophytic ecosystems because small leaf size is an adaptation to dry types of habitats, that leaf size is significantly correlated to drought and soil conditions. Understanding these responses at the life form and leaf spectra level will provide a better understanding and knowledge that how plant species and their communities or associations respond to changes in climate in the future, which is already predicted with a decrease in water and snow and an increase in temperature. The current study could be utilized as a baseline for large-scale studies in the future.

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6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research Article

Distribution and Damage Potentials of Tomato Leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae) Sindh, Pakistan

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Abstract: Tomato leafminer (TLM), *Tuta absoluta* (Lepidoptera: Gelechiidae), is a serious oligophagous pest of tomatoes. Although originated in South America, it has now been introduced and spread in almost all the tomato-growing areas of the globe. Therefore, studies were undertaken to determine the infestation of *T. absoluta* on tomato parts at various locations of six main tomato growing areas of Sindh i.e., Badin, Thatta, Mirpur Khas, Hyderabad, Sanghar and Shaheed Benazirabad. Five tomato fields (locations) were randomly selected from each district to determine the infestation of TLM based on its characteristic mines on tomato leaves and fruits. *Tuta absoluta* infestation was recorded on tomato leaves and fruits observed at all the sampling locations of six districts with significant differences among plant parts, locations, and districts. The highest infestation on leaves (19.36±0.92 %) and fruits (1.36±0.28 %) was observed from location one of Mirpur Khas district. District-wise, overall, the highest infestation of *T. absoluta* on tomato leaves (17.04±0.38 %) and stems (1.10±0.11 %) was recorded at Mirpur Khas, whereas Shaheed Benazirabad and Hyderabad districts suffered the lowest infestation on leaves (4.18±0.22 %) and fruits (0.26±0.04 %), respectively. The highest infestation of *T. absoluta* at Badin (14.10±0.28 %), Thatta (11.10±0.27 %), Sanghar (8.22±0.34 %), Hyderabad (5.32±0.18 %) and Shaheed Benazirabad (4.18±0.22 %) was also recorded on leaves. Therefore, early monitoring and management should be taken by growers to restrict the losses of *T. absoluta* on tomatoes, whereas government should adopt strict quarantine measures on the movement of tomato nurseries to restrict its spread.

Keywords: Infestation, leafminer, Solanaceae, survey, tomato

1. INTRODUCTION

Globally among vegetables, tomato (*Solanum lycopersicum* L.) (Solanaceae) is cultivated and consumed on a large scale, mainly because of its nutrient contents (Vitamins A, C, K, and minerals) that are essential for human health [1-2]. In Pakistan, tomatoes are cultivated in all four provinces as total area and production during 2018-2019, were 54.5 thousand hectares and 560.6 thousand tons, respectively [3]. In Pakistan, Sindh province contributed a major share in the area and production of tomatoes. The area and total production during 2018-19 in the province stood at 21.0 thousand hectares and 153.3 thousand tons, respectively [3]. However, in Pakistan, yield potential of tomatoes is not fully exploited because of many constraints with insect pests being one of the key factors for significant yield reduction [4].

Tomato leafminer (TLM), *Tuta absoluta* (Lepidoptera: Gelechiidae), originated from South America, is a highly invasive pest of tomato and other solanaceous plants including potato [5, 6], along with other cultivated crops such as eggplant [7, 8]. Its larvae cause severe damage to tomatoes, after emergence from egg, it penetrates leaves, apicals buds, flowers, fruits and stems [9]. It forms extensive galleries in the feeding areas, which hinders the normal growth and development of the plants as the plants may bear a necrotic appearance [10]. The yield losses due to the attack TLM may

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be enormous as in severe cases it may cause 100 % loss to the produce in both green house and open field tomatoes [1, 5, 11].

Tuta absoluta was first reported outside South America in 2006 and then spread rapidly in almost all tomato-growing areas in Europe, Africa and Asia including China [1, 5, 12-15]. Thus, within ten years of its first invasion, it has attacked up to 60 % of the tomato-growing regions of the world including China, Spain, Italy, Iran, Egypt, Turkey and India, seven of the top ten tomato producers [16, 17]. Therefore, it is surprising that these countries including the European Union and USA did not take serious quarantine measures to restrict its invasion despite knowing its status as a key pest of tomatoes in its native region [5, 11]. Among the main reasons for its invasion includes the commercialization and importation of tomato seedlings and fruits from the infested areas [5, 18, 19] as well as its ability to survive on plants other than the Solanaceae family [20].

Tuta absoluta was first reported from Pakistan in tomato-growing areas of Southern Punjab during 2020 [21], whereas [4] also confirmed its presence in the Charsadda district of Khyber Pakhtunkhwa province. Although, no confirmed reports of TLM infestation have been reported from Sindh province, it has been mentioned that having information about the prediction, relative abundance, and geographical distribution potential of the invasive species is key for their restriction and proper management [22, 23]. Therefore, this study was undertaken from the main tomato-growing areas of Sindh to determine the presence and infestation level of TLM.

2. MATERIALS AND METHODS

Six districts i.e., Badin, Thatta, Mirpur Khas, Hyderabad, Sanghar, and Shaheed Benazirabad were selected for the survey because they are the main tomato-growing areas of Sindh. Survey was conducted at the time of fruit set to confirm the damage percentage of *T. absoluta* as its attacks on all the above-ground growing parts of the tomato. Five fields were randomly selected from each district (Table 1). Fifty plants were randomly selected from each field to determine the infestation percentage by observing the characteristic mines (Figure 1) in leaves and fruits [10], and also by counting the number of healthy and infested leaves, stems and

Table 1. Names and GPS co-ordinates of locations where samples were collected

District	Location	Address	Latitude	Longitude
Badin	One	Village Ibraheem Junejo	24.60222	68.78219
	Two	Village Mevo Jaskani, UC Kazia Wah, Taluka Badin	24.60622	8.78184
	Three	Village Mevo Jaskani, UC Kazia Wah, Taluka Badin	24.60367	68.7754
	Four	Village Mosa Junejo, UC Kazia Wah, Taluka Badin	24.58904	68.79211
	Five	Village Mosa Junejo, UC Kazia Wah, Taluka Badin	24.58974	68.79142
Mirpur Khas	One	Village Muhammad Yousif Rajar Taluka Shujabaad	25.42368	69.00844
	Two	Goth Haji Rajar Taluka Shujabaad	25.42508	69.04349
	Three	Goth Ismail Rajar Taluka Shujabaad	25.43213	69.0401
	Four	Village Allah Bachayo Rajar Taluka Shujabaad	25.417267	69.02167
	Five	Village Hamid Rajar Taluka Shujabaad.	25.420116	69.02388
Sanghar	One	Village Muhammad Saeed Araen, UC Kurkli, Taluka Sinjhoro	25.93791	68.81557
	Two	Village Khan Muhammad Brohi, UC Kurkli, Taluka Sinjhoro	25.93769	68.81793
	Three	Village Kurkli, Taluka Sinjhoro	25.93955	68.81306
	Four	Village Ali Gul Qambrani, Taluka Sinjhoro	25.93182	68.8228
	Five	Village BK Daas, Taluka Sinjhoro	25.96131	68.7912
Shaheed Benazirabad	One	Village M. Safar Dahari Deh 78 Nusrat Taluka Daur	26.43979	68.34136
	Two	Village M. Safar dahari Deh 78 Nusrat Taluka Daur	26.44101	68.3429
	Three	Village Haji Meeran Mari 60-Miles, Taluka Daur	26.41035	68.44041
	Four	Village Haji Meeran Mari 60-Miles, Taluka Daur	26.41373	68.4308
	Five	Village Syed Mithal shah deh 16 Dad, Taluka Nawab Shah	26.52263	68.33262

District	Location	Address	Latitude	Longitude
Thatta	One	Village Karam Khan Magsi, UC Faqueer Goth, Taluka Thatta	24.74726	67.95761
	Two	Village Karam Khan Magsi, UC Faqueer Goth, Taluka Thatta	24.74706	67.95755
	Three	Village Faqueer jo goth, UC Faqueer Goth, Taluka Thatta	24.74989	67.95202
	Four	Village Faqueer jo goth, UC Faqueer Goth, Taluka Thatta	24.75075	67.95215
	Five	Village Karam khan Magsi, UC Faqueer Goth, Taluka Thatta	24.74726	67.95761
Hyderabad	One	Village Haji Allahdad, near Channel Mori, Tando Jam Hyderabad	25.38768	68.40959
	Two	Village Haji Allahdad, near Channel Mori, Tando Jam Hyderabad	25.38691	68.412
	Three	Village Naseer Morio, near Channel Mori, Tando Jam Hyderabad	25.40691	68.40977
	Four	Village Naseer Morio, near Channel Mori, Tando Jam Hyderabad	25.41033	68.40599
	Five	Village CH Nizam Din, near Channel Mori, Tando Jam Hyderabad	25.41777	68.4599

fruits. The percentage infestation was calculated using the following formula:

The collected data were subjected to analysis of variance using Statistix 8.1 computer software and their means were compared for significant differences using Least Square Difference (LSD) at a 5 % probability level.

3. RESULTS

The survey results confirmed the presence of *T. absoluta* from all the sampling locations of six districts of Sindh, Pakistan. The infestation of *T. absoluta* was recorded from both leaves and fruits of tomatoes. A three-way ANOVA results (Table 2) confirmed a highly significant difference ($F = 6.97$, $P < 0.0001$, $n = 20$) in the infestation level of *T. absoluta* on leaves and fruits of tomato fields of five randomly selected locations of six districts of Sindh. Among the sampling districts, significantly the highest infestation of *T. absoluta* was observed at Mirpur Khas, whereas its attack was more on leaves than fruits. Accordingly, location one of Mirpur Khas district suffered the highest infestation of *T. absoluta* on leaves (19.36±0.92 %), followed by location five (17.36±0.95 %) of the same district, also observed on leaves. Among other districts i.e., Badin, Thatta, Sanghar, Hyderabad, and Shaheed Benazirabad, the highest infestation percentage of *T. absoluta* recorded was 14.80±0.64 % (location two), 13.84±0.83 % (location one), 13.68±0.63 % (location five), 6.36±0.50 % (location two), and 6.04±0.53 % (location two), respectively, all recorded on tomato leaves (Table 3).

Although the infestation of *T. absoluta* on tomato fruits was very low as compared to leaves, the highest infestation on fruits (1.36±0.28 %) was recorded at location one of Mirpur Khas district, followed by 1.32±0.29 % infestation observed at location two of the same district. Among sampling locations of other districts i.e., Badin, Thatta, Sanghar, Hyderabad, Shaheed Benazirabad, the maximum infestation of *T. absoluta* on tomato fruits were recorded as 1.24±0.29 % (location one), 0.72±0.21 % (location two), 0.40±0.14 % (location two), 0.36±0.13 % (location two), and 0.40±0.16 % (location two), respectively (Table 3).

Fig. 1. Damage symptoms of *Tuta absoluta* on tomato leaf and fruit observed during the study

Table 2. Analysis of Variance for mean percentage infestation of *Tuta absoluta* on tomato leaves and fruits at various sampling locations of six districts of Sindh, Pakistan.

Source	df	Sum of Squares	Mean sum of squares	F-value	P-value
Districts	5	9050.3	1810.1	422.05	0.0000
Plant Parts	1	33210.2	33210.2	7743.62	0.0000
Farmers	4	396.5	99.1	23.11	0.0000
District * Plant part	5	6858.5	1371.7	319.84	0.0000
District * Farmers	20	659.2	33.0	7.68	0.0000
Plant part * farmers	4	344.1	86.0	20.06	0.0000
District * Plant part * farmer	20	597.9	29.9	6.97	0.0000
Error	1440	6175.8	4.3		
Total	1499	57292.4			

Results regarding the overall mean infestation percentage of *T. absoluta* observed on leaves and fruits of tomatoes at various sampling districts illustrated a highly significant difference ($F = 319.84$, $P < 0.001$) (Figure 2). At all the sampling districts, as compared to fruits, tomato leaves suffered a significantly highest infestation of *T. absoluta* as the highest overall infestation (17.04±0.38 %) was recorded on tomato leaves from various sampling locations of Mirpur Khas district, whereas the lowest infestation on tomato leaves was recorded at Shaheed Benazirabad (4.18±0.22 %). The overall infestation on leaves recorded at Badin, Thatta, Sanghar, and Hyderabad districts was 14.10±0.28, 11.10±0.27, 8.22±0.34, and 5.32±0.18 %, respectively, which were all significantly different from each other. As compared to tomato leaves, very little infestation of

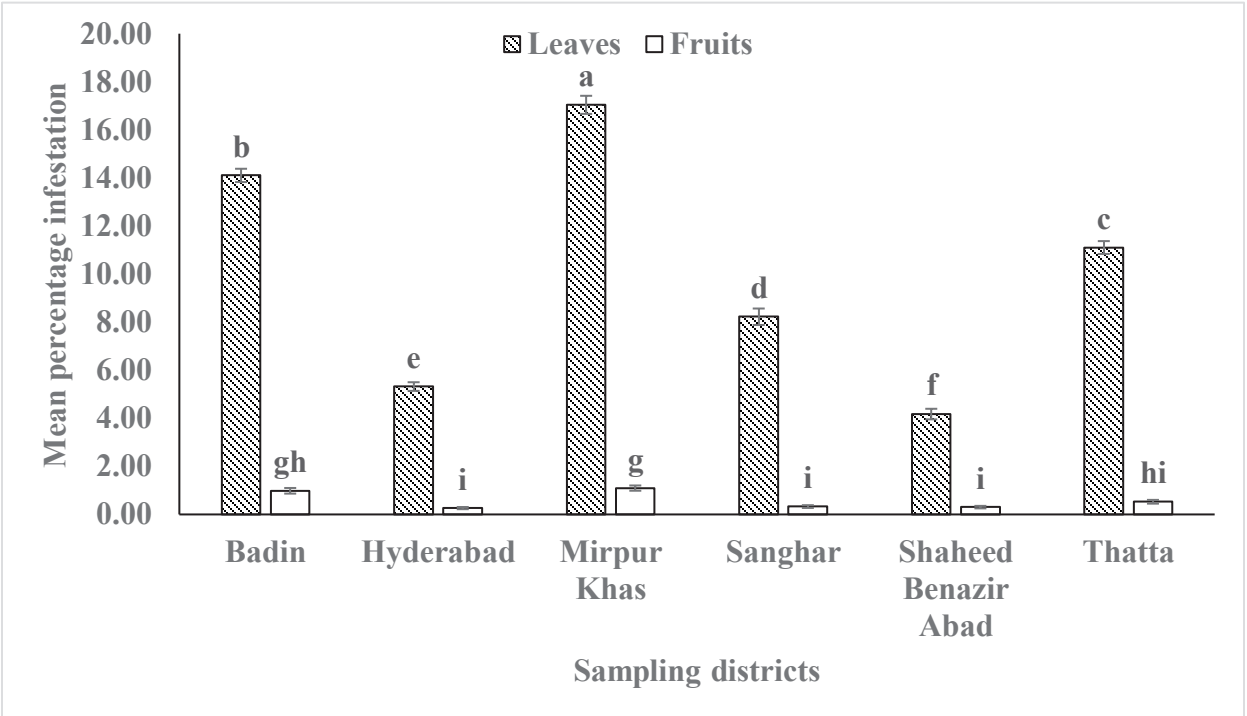

Fig. 2. Overall mean infestation of Tuta absoluta on tomatoes (leaves and fruits) at various sampling districts of Sindh, Pakistan (*Mean followed by the same letters are not significantly different from each other (LSD = 0.5139, $P < 0.05$)

Table 3. Mean percentage infestation of *Tuta absoluta* on tomato leaves and fruits at various sampling locations of six districts of Sindh, Pakistan.

District	Locations	Plant Part	Percentage Infestation
Badin	One	Leaves	14.56±0.77cde
		Fruits	1.24±0.29pqr
	Two	Leaves	14.80±0.64cd
		Fruits	1.16±0.29pqr
	Three	Leaves	13.52±0.62e
		Fruits	0.68±0.21pqr
	Four	Leaves	14.00±0.57cde
		Fruits	1.16±0.29pqr
	Five	Leaves	13.64±0.47e
		Fruits	0.68±0.21pqr
Hyderabad	One	Leaves	5.60±0.46jkl
		Fruits	0.28±0.09
	Two	Leaves	6.36±0.50ij
		Fruits	0.36±0.13pqr
	Three	Leaves	4.48±0.33lm
		Fruits	0.24±0.10pqr
	Four	Leaves	4.88±0.32klm
		Fruits	0.16±0.07r
	Five	Leaves	5.28±0.28jkl
		Fruits	0.28±0.11pqr
Mirpur Khas	One	Leaves	19.36±0.92a
		Fruits	1.36±0.28p
	Two	Leaves	17.28±0.73b
		Fruits	1.32±0.29pq
	Three	Leaves	14.84±0.54c
		Fruits	0.84±0.20pqr
	Four	Leaves	16.36±0.79b
		Fruits	1.16±0.22pqr
	Five	Leaves	17.36±0.95b
		Fruits	0.80±0.21pqr
Sanghar	One	Leaves	7.40±0.47i
		Fruits	0.32±0.11pqr
	Two	Leaves	8.72±0.62h
		Fruits	0.40±0.14pqr
	Three	Leaves	5.80±0.32jk
		Fruits	0.36±0.15pqr
	Four	Leaves	5.52±0.33jkl
		Fruits	0.20±0.10qr
	Five	Leaves	13.68±0.63de
		Fruits	0.36±0.14pqr

District	Locations	Plant Part	Percentage Infestation
Shaheed Benazirabad	One	Leaves	5.40±0.53jkl
		Fruits	0.24±0.09pqr
	Two	Leaves	6.04±0.53j
		Fruits	0.40±0.16pqr
	Three	Leaves	3.04±0.16no
		Fruits	0.28±0.11pqr
	Four	Leaves	2.60±0.33o
		Fruits	0.28±0.11pqr
	Five	Leaves	3.80±0.37mn
		Fruits	0.32±0.11pqr
Thatta	One	Leaves	13.84±0.83cde
		Fruits	0.52±0.17pqr
	Two	Leaves	9.92±0.40g
		Fruits	0.72±0.21pqr
	Three	Leaves	11.28±0.40f
		Fruits	0.56±0.19pqr
	Four	Leaves	9.28±0.41gh
		Fruits	0.48±0.17pqr
	Five	Leaves	11.20±0.37f
		Fruits	0.36±0.11pqr

*Means followed by the same letters are not significantly different from each other (LSD = 1.1490, P < 0.05)

T. absoluta was recorded on fruits with the highest overall percentage infestation observed at Mirpur Khas district (1.10±0.11 %), not significantly different from overall infestation recorded at Badin (0.98±0.12 %). The lowest overall infestation of *T. absoluta* fruits was recorded Hyderabad district (0.26±0.04 %) that was not significantly different from the infestation recorded at Shaheed Benazirabad (0.30±0.05 %), Sanghar (0.33±0.06 %) and Thatta (0.53±0.08 %) districts (Figure 2).

4. DISCUSSION

The comprehensive survey of *T. absoluta* presence and infestation in the main tomato-growing areas of Sindh, Pakistan confirmed its presence in all the sampling locations, however, there was a significant difference among various locations and districts regarding its infestation. Moreover, characteristic symptoms of *T. absoluta* mines were only observed on tomato leaves and fruits. The infestation of *T. absoluta* in Pakistan was first time reported in the tomato-growing areas of the Multan district of Punjab [21]. However, a recent comprehensive survey study confirmed its presence from Charsadda district of Khyber Pakhtunkhwa, besides Multan and Rawalpindi districts of Punjab with significantly more attacked samples (15.54 %) collected from Charsadda, followed by Rawalpindi (1.45 %) and Multan (0.52 %), whereas no infestation of *T. absoluta* was observed from Muzaffargarh, Lodhran and Rahim Yar Khan districts [4]. However, in our study, the infestation of *T. absoluta* was recorded from all the sampling locations of six major tomato-growing districts of Sindh showing significant differences among locations and districts. Such a rapid spread of *T. absoluta* in new locations including recent infestation observed in this study may be attributed to the free movement of the tomatoes or their nurseries from the infested areas to the new locations. Generally, no strict quarantine measures are observed in different areas of Pakistan when there is a growing demand for tomatoes, hence it facilitates the transportation of infested tomatoes from areas of infestation to the new locations [4].

It has been mentioned by McNitt *et al.* [24] that in the absence of proper quarantine measures and large-scale self-mediated, unmitigated spreading

potential of the pest among cities, *T. absoluta* can invade almost all the key vegetable-growing areas of Southeast Asia within a short period of 5 to 7 years. Similar kind of findings was reported by Tonnang *et al*. [25] and Kinyanjui *et al*. [26] who observed the trade of infested tomato fruits among the neighbouring cities of Kenya as its main source of spread in the country. Many other studies also identified that the main reason for the rapid spread of *T. absoluta* in new localities and regions was the commercialization and importation of tomato fruits and seedlings [5, 18-19, 27-28].

Moreover, Pakistan has a long border with China, which is the largest producer and exporter of tomatoes [29], where the presence of *T. absoluta* has been confirmed by Zhang *et al.* [6]. It has been also reported that *T. absoluta* has the potential to spread around 800 kilometers in a year [5, 30]. Therefore, having such an invasive characteristic along with a long border with China can easily facilitate the movement of *T. absoluta* into Pakistan, as the climatic conditions and long tomato growing seasons of tomatoes in Pakistan can facilitate its spread all over the country [4].

A significant difference in the level of *T. absoluta* infestation of various plant parts (leaves and fruits) at various locations surveyed of six districts may be attributed to various tomato cultivars grown or the cultural practices adopted by the respective growers, besides the geographical location and weather conditions of the survey areas. The recent study of Sadique *et al.* [4] also significant effect of rising temperatures on the mean capture of *T. absoluta* moths in pheromone traps in Pakistan. Another study also mentioned that *T. absoluta* showed a preference for some cultivars, whereas some showed relative resistance against it [31]. Kinyanjui *et al.* [26] also found similar results while evaluating the infestation of *T. absoluta* at various locations in Kenya as the number of mines and larvae differ significantly at the locations. Moreover, a relatively lower level of infestation on fruits has been reported by Kinyanjui *et al.* [26] as the same support the findings of our study. However, 80-100 % damage to open-field tomatoes has been reported by Mohamed *et al.* [32] in Sudan which may be due to the susceptibility of the cultivars grown or the free movement of the pest.

5. CONCLUSION

Tuta absoluta infestation was recorded from tomato leaves and fruits observed at all the surveyed locations of six districts i.e., Badin, Thatta, Mirpur Khas, Hyderabad, Sanghar and Shaheed Benazirabad. However, the level of infestation varies among various locations, districts, and plant parts. The highest infestation of tomato leaves and fruits was recorded in the Mirpur Khas district, whereas Shaheed Benazir Abad and Hyderabad tomato fields showed the lowest infestation of leaves and fruits. Tomato leaves were more attacked by *T. absoluta* than fruits. Therefore, appropriate monitoring and management measures should be taken by growers against *T. absoluta* to restrict its damage. Moreover, the government should strictly quarantine measures regarding the movement of transplanting tomato seedlings to restrict the spread of *T. absoluta* in new areas.

6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research Article

Effect of Different Organic Amendments on Growth, Yield and Quality of Broccoli (*Brassica oleracea* var. italica)

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Abstract: To study the effect of different organic amendments on growth, yield and quality of broccoli a field experiment was conducted during 2021–2022 in the Agriculture Research Farm, Lovely Professional University, Punjab A Factorial Randomized Block Design (FRBD) with two factors was used. Factors 1 and 2 are varieties (V1: Palam Vichitra and V2: Palam Kanchan) and biofertilizers (T1: Phosphate Solubilizing Bacteria @ 2 % + Azotobacter @ 2 %, T2: Phosphate solubilizing @ 2 %, T3: Azotobacter @ 2 %, and T0: Control). A total of eight treatments were used, i.e., T1V1 (Palam Vichitra X Phosphate Solubilizing Bacteria @ 2 % + Azotobacter @ 2 %), T2V1 (Palam Vichitra X P.S.B @ 2 %), T3V1 (Palam Vichitra X Azotobacter @ 2 %), T0V1 (Palam Vichitra X Control), T1V1 (Palam Vichitra X Phosphate Solubilizing Bacteria @ 2% + Azotobacter @ 2 %), T0V1 (Palam Kanchan X Phosphate solubilizing bacteria @ 2 %), T3V2 (Palam Kanchan X Azotobacter @ 2 %), T0V2 (Palam Kanchan X Control). The experiment reveals that among all treatments like growth, quality and yield parameters T1 showed the best result for V1 and in V2, T3 showed the best result for almost all the parameters. Floral bud initiation was early when treated with T1 in both the varieties, i.e., V1 (63 DAT) and V2 (63.67 DAT). Days to harvest were the same for all the treatments in V1 (90 DAT) and in V2, T1 (90 DAT) took the least amount of time for harvesting. It can be concluded that, combination of Azotobacter @ 2 % with other organic amendments and treatment T1 give higher yield and better quality of broccoli.

Keywords: Broccoli, Azotobacter, Phosphate Solubilizing Bacteria, Yield, Growth and Quality

1. INTRODUCTION

Broccoli is a very well-known cole crop for its high nutritive content. It is a winter-season vegetable crop and requires an optimum temperature of 20-25 °C for proper growth. People mainly consume broccoli, as it has antioxidant properties and prevents certain cancer types. It is consumed either cooked or as a salad form but given the widespread use of chemical pesticides and insecticides, when eaten as a salad, the nutrient content is depleted, and it is also dangerous to human health. The crop contains a chemical compound called Sulforaphane that reduces the risk of cancer [1]. It is rich in ascorbic acid, protein, iron, fibre, and potassium content, respectively.

Most cruciferous vegetables, including broccoli, contain glucosinolates, which prevent chronic diseases. Fresh broccoli leaves are high in vitamins, an effective natural antioxidant and immune modulator that aids in the fight against flu, causing viruses. Broccoli leaves contain a sufficient amount of other antioxidant vitamins, vitamin-A, which helps in maintaining the integrity of skin and mucus membranes which is also required for vision [2]. Broccoli is similar to cauliflower but the difference is its relatively small flower heads which are green or purple in colour. There is commonly two type of broccoli – heading and purple or green broccoli. Green broccoli has a bunch of green, immature buds and a thick fleshy flower stalk that forms a head, whereas heading broccoli produces curd-like cauliflower. The purple type of broccoli is usually grown in Europe and North America. The crop is originated from Asia Minor and Eastern Mediterranean region. The ancestor is *Brassica oleracea* var. sylvestris.

It is observed that the use of different

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chemical fertilizers decreases the beneficial nutritional contents of the crop, while organic manures reduce the health hazards and the level of chemical residues. In recent years, bio-fertilizers application is gaining popularity among farmers, and environmentalists. They help N_2-fixing or P mobilization but also produce several vitamins and plant growth hormones needed for plant growth and hence, can be used as a bio-control agent by inhibiting many harmful pathogens and microbes. Bio-fertilizers like Azotobacter fix the nitrogen while PSB as phosphorous solubilizer. Broccoli is introduced newly to India, but it is quickly gaining popularity due to its low-fat, low-calorie but high vitamin C content, and also it is a good source of calcium, vitamin A and vitamin B2 [3]. In view of the negative effects of inorganic fertilizer on broccoli's nutritional quality, this study was conducted to analyze the effect of different organic amendments on growth, yield, and quality.

2. MATERIALS AND METHODS

The Department of Vegetable Science, School of Agriculture, Lovely Professional University, Phagwara, is where the trial was carried out. The experiment was carried out in Factorial Randomized Block Design with two factors, factor 1 consists of varieties (V1: Palam Vichitra and V2: Palam Kanchan) and factor 2 consists of biofertilizers (T1: Phosphate Solubilising Bacteria @ 2 % + Azotobacter @ 2 %, T2: Phosphate solubilizing bacteria @ 2 %, T3: Azotobacter @ 2 % and T0: Control). 3 replications with a total of 8 treatment combinations were used. Spacing of 45 x 45cm was adopted and transplanting was done on the 11th of November, 2021. The total area was 184.26 m^2. Seeds were treated with Phosphate Solubilizing Bacteria @ 2 % + Azotobacter @ 2 %, Phosphate Solubilizing Bacteria @ 2 % and Azotobacter @ 2 %. After 30 DAT for insect management, a 2 L mixture of neem oil was applied on the leaves and after sometime go for nutrition management, 3 L of Panchagavya were applied at 45 days after transplanting (DAT). Observations on the number of leaves per plant DAT, plant spread (cm^2), plant height(cm), days to flower bud initiation DAT, days to harvesting, the weight of the floral bud (g), floral bud diameter (mm), chlorophyll index (SPAD value), dry matter content (%), vitamin C (mg/100 g) and TSS (°Bx) were recorded.

3. RESULTS AND DISCUSSION

3.1 Growth Parameters

The growth parameter of broccoli was considered with regard to plant height (60 days after transplanting), Number of leaves per plant (60 days after transplanting), plant spread (N-S and E-W) (cm^2), floral bud initiation (DAT) and days to harvesting (DAT). As per Table 1, it is observed that T1 i.e. (Phosphate Solubilizing Bacteria @ 2 % + Azotobacter @2 %) showed the maximum plant height for both V1 (166.6 cm) and V2 (172.5) Number of leaves was found to be similar for all the treatments in V_1 i.e. (45 DAT) and also in V2 (46 DAT) Except for control, T0V1 (44.7 DAT) and T0V2 (46.7 DAT) [4]. It has been reported that an effective and healthy Azotobacter strain in the rhizosphere resulted in increased atmospheric nitrogen fixation to be used by the plant, resulting in strong plant development [5].

Plant spread (cm^2) was observed with the help of scale and statistical significance was found (Table 2). In plant spread (N-S) it is seen that V1 treated with T1 (60.95 cm^2) showed the highest plant spread and V2 treated with T3 (64.87 cm^2) showed the maximum plant spread. For plant spread (E-W) it is observed that the combination for T1V1 (57.4 cm^2) showed the maximum plant spread for V1 and the V2 combination for T3V2 (63.3 cm^2) showed the maximum plant height.

The level of significance is determined as shown in Table 3. It is observed that the application of T1 (63 days) and T3 (63 days) gave the shortest number of days for the initiation of floral bud in V1 and for V2 combination of T1V2 (63.67 days) gave the shortest number of days for the initiation of floral bud. Control treatment took the longest days for floral bud initiation in both the varieties T0V1 (63.33 days), and T0V2 (74.57 days). Days to harvesting were the same for all the treatments in V1 (90 days) and in V2, T1 (90 days) showed the least days to harvest followed by T2 (96.67 days). Early maturity from sowing might be due to the reason that appropriate balance in the levels of nitrogen and phosphorus maintained through the application of Azotobacter, PSB and FYM helps early maturity [6].

Table 1. Effect of different organic amendments on plant height and Number of leaves per plant in broccoli.

Varieties/ Treatments	Plant height (cm) at 60 DAT			Number of leaves 60 DAT		
	V_1	V_2	MEAN (Treatment)	V_1	V_2	MEAN (Treatment)
T1	166.6	172.5	169.55	45	46	45.5
T2	166.4	171.5	168.95	45	46	45.5
T3	166.2	172.1	169.15	45	46	45.5
T0	166	171.1	168.55	44.7	45.7	45.2
MEAN (Variety)	166.3	171.8		44.9	45.9	
	C.D.	**SE(d)**	**SE(m)**	**C.D.**	**SE(d)**	**SE(m)**
Treatment	1.247	0.576	0.407	0.306	0.141	0.100
Variety	1.764	0.814	0.576	0.432	0.200	0.141

Note: T_1: Phosphate Solubilizing Bacteria @2 % + Azotobacter @2 %. T_2: Phosphate Solubilizing Bacteria @2 %. T_3: Azotobacter 2 %. T_0: Control. V_1: Palam Vichitra V_2: Palam Kanchan.

Table 2. Effect of different organic amendments on plant spread (cm²) in broccoli (*Brassica oleracea* var. italica).

Varieties/ Treatments	Plant spread (N-S) (cm^2)			Plant spread (E-W) (cm^2)		
	V1	V2	MEAN (Treatment)	V1	V2	MEAN (Treatment)
T1	60.95	64.43	62.69	57.4	63.2	60.3
T2	60.85	64.29	62.57	56.8	62.2	59.5
T3	60.83	64.87	62.85	56.5	63.3	59.9
T0	59.83	63.58	61.84	56.2	60.8	58.5
MEAN (Variety)	60.6	64.3		56.7	62.4	
	C.D.	**SE(d)**	**SE(m)**	**C.D**	**SE(d)**	**SE(m)**
Treatment	0.809	0.374	0.264	1.311	0.605	0.428
Variety	1.144	0.528	0.374	1.854	0.856	0.605

Note: T_1: Phosphate Solubilizing Bacteria @2 % + Azotobacter @2 %. T_2: Phosphate Solubilizing Bacteria @2 %. T_3: Azotobacter 2 %. T_0: Control. V_1: Palam Vichitra V_2: Palam Kanchan.

3.2 Quality Parameters

Chlorophyll index was determined with the help SPAD meter by putting the SPAD meter in the leaves. As per Table 4, T_1 showed the best results in both V_1 (56.8) and V_2 (67.2) compared to the other treatments. In terms of interactions between treatments and varieties, $T1V_2$ had the highest Chlorophyll index (67.2), which was statistically significant as compared to the other interactions. These findings are very similar to those of Patidar *et al.* [7]. Variations were noted in the dry matter content samples, In V1, T1V1 (70.87 %) had the highest dry matter content amongst different interactions and in V2, T3V2 (72 %) recorded the highest dry matter content.

Table 3. Effect of different organic amendments on floral bud initiation (DAT) and days to harvesting (DAT) in broccoli (*Brassica oleracea* var. italica).

Varieties/ Treatments	Floral bud initiation (DAT)			Days to harvesting (DAT)		
	V1	V2	MEAN (Treatment)	V1	V2	MEAN (Treatment)
T1	63	63.67	63.34	90	90	90
T2	63.11	70.90	67.01	90	96.67	93.33
T3	63	74.44	68.72	90	100	95
T0	63.33	74.57	68.95	90	104.45	97.22
MEAN (Variety)	63.11	70.90		90	97.78	
	C.D.	**SE(d)**	**SE(m)**	**C.D**	**SE(d)**	**SE(m)**
Treatment	1.546	0.714	0.505	2.562	1.183	0.837
Variety	2.186	1.009	0.714	3.623	1.673	1.183

Note: T_1: Phosphate Solubilizing Bacteria @2 % + Azotobacter @2 %. T_2: Phosphate Solubilizing Bacteria @2 %. T_3: Azotobacter 2 %. T_0: Control. V_1: Palam Vichitra V_2: Palam Kanchan.

Floral buds were analyzed and recorded for vitamin C. According to Table 5, T1 showed the best results in both V1 (85.76 mg/100 g) and V2 (70.83 g/100 g) compared to the other treatments. After harvest, each sample's total soluble solids (TSS) were analyzed, and variations across treatments, and varieties, including their combinations were discovered. In V1, treating with T1 (39.7 °Bx) resulted in maximum TSS and in V2, (42 °Bx) combination of both T1V2 and T3V2 resulted in the highest TSS. The combined inoculation with Azotobacter + PSB was more helpful in improving all the above metrics due to increased solubility of phosphorus and higher nitrogen fixation, leading to increased availability of nitrogen and phosphorus [8].

Table 4. Effect of different organic amendments on chlorophyll index (SPAD value) and dry matter content (%) in broccoli.

Varieties/ Treatments	Chlorophyll index (SPAD value)			dry matter content (%)		
	V1	V2	MEAN (Treatment)	V1	V2	MEAN (Treatment)
T1	56.8	67.2	62	70.87	71.767	71.317
T2	56.6	66.6	61.9	70.70	71.433	71.067
T3	56.1	67	61.55	70.57	72.000	71.283
T0	55.87	64.1	59.98	70.47	70.033	70.25
MEAN (Variety)	56.34	66.3		70.65	71.308	
	C.D.	**SE(d)**	**SE(m)**	**C.D**	**SE(d)**	**SE(m)**
Treatment	2.047	0.945	0.668	0.412	0.190	0.135
Variety	2.895	1.337	0.945	0.583	0.269	0.190

Note: T1: Phosphate Solubilizing Bacteria @2 % + Azotobacter @2 %. T2: Phosphate Solubilizing Bacteria @2 %. T3: Azotobacter 2 %. T0: Control. V1: Palam Vichitra V2: Palam Kanchan.

Table 5. Effect of different organic amendments on Vitamin-C (mg/100 g) content and TSS (°Bx) in broccoli.

Varieties/ Treatments	Ascorbic acid (mg/100 g)			TSS (°Bx)		
	V1	V2	MEAN (Treatment)	V1	V2	MEAN (Treatment)
T1	85.76	70.83	78.3	39.7	42	40.85
T2	85.42	70.68	78.05	39	41	40.15
T3	85.46	70.71	78.1	39	42	40.4
T0	84.8	70.52	77.66	37	41	39
MEAN (Variety)	85.35	70.69		38.8	41.5	
	C.D.	**SE(d)**	**SE(m)**	**C.D**	**SE(d)**	**SE(m)**
Treatment	2.551	1.178	0.833	0.865	0.400	0.283
Variety	3.607	1.666	1.178	1.224	0.565	0.400

Note: T_1: Phosphate Solubilizing Bacteria @2 % + Azotobacter @2 %. T_2: Phosphate Solubilizing Bacteria @2 %. T_3: Azotobacter 2 %. T0: Control. V_1: Palam Vichitra V_2: Palam Kanchan.

3.3 Yield Parameters

According to Table 6 Fresh weight of the floral bud for V1 was shown to be better when treated with T1 (663.0 g) and we can observe that in V2 treated with T3 (974.2 g) showing the maximum fresh weight of floral bud T0 showed the lowest Fresh weight of floral bud in both varieties V1T0 (646.2 g) and V2T0 (819.7 g). Floral bud diameter was observed at the time of harvesting V_1, T1(49.06 mm) recorded the highest floral bud diameter and T_0 (47.98 mm) recorded the lowest floral bud diameter and in V_2, T_3 (57.83 mm) showed the highest floral bud diameter whereas, T_0 (57.15 mm) showed lowest floral bub diameter. Different combinations of Nitrogen doses applied directly and Azotobacter inoculated in the seedlings yielded meaningful outcomes for nearly all growth and yield attributes [9].

Table 6. Effect of different organic amendments on Fresh weight of floral bud (g) and floral bud diameter (mm) in broccoli (*Brassica oleracea* var. italica).

Varieties/ Treatments	Fresh weight of floral bud			Floral bud diameter		
	V1	V2	MEAN (Treatment)	V1	V2	MEAN (Treatment)
T1	663.0	954.6	808.8	49.06	57.78	53.42
T2	655.7	895.6	775.65	48.3	57.32	52.81
T3	656.53	974.2	815.36	48.07	57.83	52.95
T0	646.2	819.7	732.95	47.98	57.15	52.565
MEAN (Variety)	655.4	911.0		48.35	57.52	
	C.D.	**SE(d)**	**SE(m)**	**C.D**	**SE(d)**	**SE(m)**
Treatment	61.845	28.558	20.194	1.747	0.807	0.570
Variety	87.461	40.387	28.558	2.470	1.141	0.807

Note: T_1: Phosphate Solubilising Bacteria @2 % + Azotobacter @2 %. T_2: Phosphate Solubilising Bacteria @2 %. T_3: Azotobacter 2 %. T_0: Control. V_1: Palam Vichitra V_2: Palam Kanchan.

4. CONCLUSION

It can be concluded from this experiment that, in the province of Punjab use of Azotobacter @ 2 % in combination with some other organic amendments such as FYM, vermicomposting, neem oil, and Panchagavya give higher yield and better quality and treatment T1 (P.S.B @ 2 % + azotobacter @ 2 %) generated better outcomes in Palam Vichitra. For getting continuous yield Palam Kanchan can be grown, which can compensate for the high perishability problem in broccoli broccoli (Brassica oleracea var. italica).

5. CONFLICT OF INTEREST

The authors declared no conflict of interest

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Research Article

Encounter of Asiatic Black Bear (*Ursus thibetanus*) from the Low Elevated Area of District Battagram, Pakistan

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Abstract: Asiatic black bear (*Ursus thibetanus*) is widely distributed in various countries of Asia including different areas of Pakistan such as District Mansehra, Swat, Kohistan, Battagram, Gilgit Baltistan, and Azad Jammu and Kashmir. The present research was conducted in District Battagram, Khyber Pakhtunkhwa, Pakistan where new and abundant evidence of the Asiatic Black bear was reported. Data were collected during field reports, questionnaire-based surveys and oral interviews, and discussions with the local community from March 2022 to December 2022. The black bear was encountered by local peoples at a low elevated area (1259 m) of Jesol village (N 34° 41' 53" E 73° 03' 05") and was killed collectively (May 12, 2022). During such an encounter, a young man was also injured in conflict. Within one day, local people carried every part of the bear's body and left the remaining skeleton. The wildlife department of Khyber Pakhtunkhwa took immediate action against the involved people. Similarly, the second black bear was also observed in the same village after one week but this time local people did not take any action against the black bear due to strict enforcement of wildlife law implemented by the wildlife department. On the other hand, questionnaire-based surveys and oral interviews were conducted in different areas of Battagram such as Baliga, Chail, and Shumlai about the black bear population. According to the perception of the local community, a quite good population of bears n=14 (6 males, 4 females, and 4 cubs) are present in the entire district. During the field survey, bear signs were also observed in Balija Mountain such as pug marks, track routing in snow, scat samples, and setting place. Bone samples collected from a killed black bear will be genetically identified for further genotypic study. The black bear population could be conserved by the strict implementation of rules and regulations by the government and conservation agencies. Awareness among the local community also plays an important in black bear conservation.

Keywords: Killed Black Bear, New Evidence, Conflict, Population, Battagram, *Ursus thibetanus*

1. INTRODUCTION

Eight bear species have been reported worldwide as of late, according to historical perspective and fossil records (Polar bear, *Ursus maritimus*, Asiatic black bear, *Ursus thibetanus*, Sloth bear, *Ursus ursinus*, American black bear, *Ursus americanus*, Brown bear, *Ursus arctos*, Sun bear, *Helarctos malayanus*, and Giant Panda). In addition to these three species, there were two in Europe, one in South America, and six in Asia. The northern regions of Pakistan and Azad Jammu Kashmir are home to two species of bears: black bears and brown bears [23].

The population of the Asiatic black bear (*Ursus thibetanus*) is widely distributed in various countries of Asia including Afghanistan, Pakistan, India, Iran, Japan, Korea, Indo-China, Laos, Taiwan, and Vietnam [1]. Black bears live in a variety of environments that include broad-leaved and coniferous woods and range in height from sea level to 4300 metres (14,108 feet) [1]. Several studies were conducted on the population density of black bears in different countries throughout the world [2].

According to some studies in Asia, the Population of Asiatic black bears in China ranges from 15000 to 46,000 [3], however later an official government survey was conducted in 2003, indicating that there

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is about 28,000 Asiatic black bear present in China [4]. Japan formerly hosted quite a big population of Asiatic black bears, it is estimated that about 8,000–14,000 bears are present on Honshu Island. Russian biologists have presented abundant density estimated at 5,000–6,000, but the reliability of these is unreal [5]. Similarly, rough density was also estimated in some portions of India and Pakistan as 7000-9000 in India [4], and 1000 in Pakistan [5]. Black bears may be found in several regions of Pakistan in numbers of about 1000. According to earlier estimations, the population density ranges from one to ten bears per square kilometre, with the average density being around two bears per square kilometre [6].

Feeding habits and geographical diversity indicated that the black bear is one of the adjustable and adoptable animals. The black bear is a nocturnal species that mostly comes out at dawn and Dusk, goes to hibernate in the winter season and remains highly active in summer searching for better food to gain more fat [7, 21]. Seasonally, individual bears migrate to various habitats and altitudes in their hunt for food [8]. Springtime foods contain succulent plants (shoots, forbs, and leaves), summertime foods include a range of fruits from trees and shrubs, and autumnal foods include nuts [8]. In certain areas, a substantial component of the diet consists of flesh from mammalian ungulates that are either killed or scavenged [9].

Recently in Pakistan, various studies have been conducted on human-black bear conflict and their causes including crop damage, local hunting, and attack on livestock. One of the recent human-black bear conflicts was reported from Siran and Kaghan valleys which highlighted the severe rate of incidence regarding the human-Asiatic black bear conflict in 2022 [22]. In addition to other difficulties, illicit trade and hunting are seen to be the greatest obstacles to conservation, particularly in developing nations [10]. Due to overhunting and illicit exports, some intriguing species, such as rhinoceroses (*Rhinoceros* sp.), snow leopards (*Panthera uncia*), and tigers (*Panthera tigris*), are becoming endangered [10, 11]. The population of wild tigers and rhinoceroses has dropped dramatically (by 90 %) during the previous fifty years, signalling a worrisome condition of their conservation [12]. According to some assessments, the greatest danger to wildlife in the developing world is the illegal wildlife trade [13]. The region with the highest demand for illegal trafficking in wildlife and animal parts is thought to be Asia, particularly South and Southeast Asia [13]. The increased national, regional, and worldwide initiatives for effective wildlife legislation in accordance with the CITES accords are thought to be the cause of this rise in demand for illegal wildlife trading. The demand for wildlife species' parts exchanged for nutrition and medicine is largest in Asian nations such as China, India, Bhutan, South Korea, Nepal, and Japan [14]. For instance, the number of brown bears in Mongolia is fast decreasing as a result of illicit shootings and trade with the neighbouring East Asian nations, where body parts are sought after for use in traditional medicine [15].

The current study indicated the new and frightening evidence of a dead Asiatic black bear during the conflict with a young man (injured) in a low elevated area of Battagram, Khyber Pakhtunkhwa, Pakistan, similarly, another black bear individual was also observed in the same after the incidence and local community facing a severe threat from bear attacks.

2. MATERIALS AND METHODS

2.1 Study Area

The present study was conducted in District Battagram in Khyber Pakhtunkhwa, Pakistan, from 2020 to 2022. The district's coordinates are 34° 33' to 34° 47' (N) and 72° 54' to 73° 15' (S) (E). Geographically, District Battagram is situated in Khyber Pakhtunkhwa's extremely significant ecological zone, which is also a section of the Himalayan mountain ranges. District Mansehra, Shangla, Kohistan, Tor Ghar, and a portion of Gilgit Baltistan's northern regions encircle District Battagram. According to the 2018 census, Battagram has a population of 476,612 people living in a 1,301 km^2 area [24-28] (Figure 1).

2.2 Methods

2.2.1. Field Sighting and dead body observation of black bear

A dead body of an Asiatic black bear was observed

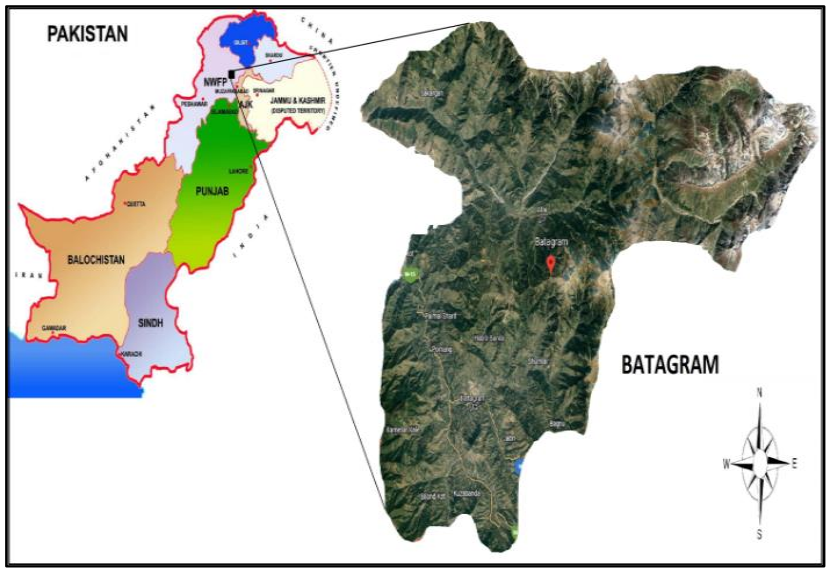

Fig. 1. Map of the study area (District Battagram)

on May 12, 2022, in the low elevated area of district Battagram. Black bear usually inhabits higher elevation areas of the mountain of the district but this time bears came down to the local village Jesol about 1259 m (N 34°41′53 E 73°03′05) [16-18]. The second black bear was also reported by the local community from the same villages and pointed out the route of bear migration from the mountains (Figure 2).

Evidence of the bear population was also estimated by following a questionnaire-based survey (Appendix-I), oral interviews, and a sign survey [17, 19]. Data were collected from different potential sites in the study area such as Balija Maidan, Shumlai (Donga and Tapka), and Chail Mountain. During a field, survey researchers visited the field for search bear signs, questionnaire filling, and informal interviews with the local community of the area. A total of 50 structured questionnaires were filled out from the study area. Among the totals, 18 questionnaires were filled from Donga and 12 from Topka villages (parts of the Shumlai mountain) followed by Chail (18) and Balija (7). Each questionnaire is composed of multiple questions related to the black bear population and

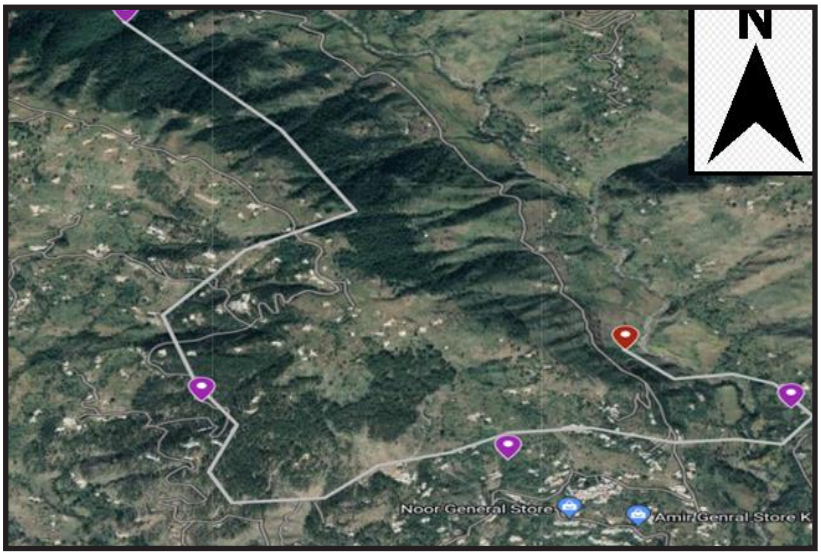

Fig. 2. Route of irregular movement and destination where a black bear was killed in Jesol

human-bear conflict. Questions were asked in the local language "Pashto" and then translated into English during questionnaire filling.

3. RESULTS

3.1 Field Report

Firstly the black bear was observed by local people in populated villages such as Arghashori, Bazargay, and Jesol and then move back toward the mountainous region. During such movement of bears in the local villages, the local people get scared and panic is created among the community. Among the local community, a young man who took a gun trying to protect other people from a bear attack was encountered in the side area, due to this severe conflict, the person was afraid and then jumped from a steppe rock and was seriously injured (Figures 3). After such an incident, people of the nearby village fired on the black bear and were killed moment (Figure 3).

The dead body of the black bear was seized by the local community and cut every part and transported for multiple purposes and left the bear skeleton (Figure 3). One of the witnesses points out that peoples in the area are not aware of the bear's importance subjectively but they try to use its parts like skin, fat, paws, and many internal organs for different medicinal purposes to treat some incurable diseases. The wildlife department of District Battagram and Khyber Pakhtunkhwa took immediate action against those people who killed the innocent black bear and warn them to avoid such incidents and conflicts in the future time.

After one week on May 19, 2022, another individual black bear was also encountered in the same villages during the daytime, but this time people did not take any action against the black bear and moved back to its natural habitat. The key factors of bear conservation are local community awareness and the implementation of rules and regulations against illegal hunters and traders to protect these animals when they are displaced. The population of black bears is not been recorded from the district Battagram previously, this incident indicated that quite a good population of black bears is present in the district. But here in the district bears face multiple threats and challenges such as illegal killing, parts trading, shortage of food sources, habitat degradation, and increasing the rate of grazers in the mountain.

3.2 Activity Timing of Asiatic Black Bear

Most of the black Bears attacked livestock, crops, and human at night time in different localities of the study area. In Balija, 16% Local community told

Fig. 3. Evidence of killed black bear in Jesol, Battagram (**A:** claw of black bear; **B:** head region; **C:** the full body of bear; **D:** 2nd day bear remains; **E:** 4th day of bear remains; **F:** injured victim due to conflict)

that black bears attack their livestock at night time and 35% of people in the Balija community told that black bears attacked livestock during the day time. The local community of Chail Mountain told that black bears mostly attack in the daytime, 6% of people observed that black bears attack during the daytime and 13% of local people of the area told that black bear attacks at night time. Similarly, 40% local community of Donga and Tapka tell that bear attack at night time and 25% of people told that black bears attacked their livestock during the day time and other people told that they have no idea about black bear activities timing (figure 4).

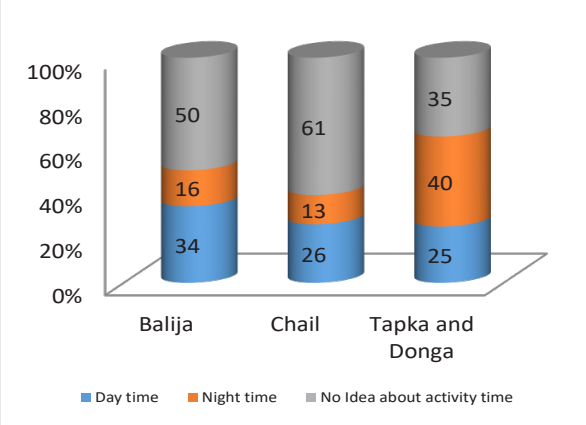

Fig. 4. Activity timing of Asiatic Black bear in Balija, Chail, and Tapka & Donga

3.3 Black Bear Population

According to the questionnaire-based survey, the local community responded that few families of black bears were seen in the different areas of the forest. In Balija Mountain there are 4 black bear individuals were observed by local people of the area. According to the perception of the locals, three bears (with a single cub) were observed on Chail Mountain. Similarly, in Tapka and Donga very high population of bears was recorded, a total of seven black bears (with two cubs) were seen in the forest multiple times. A total of (n=14) black bears were seen in the study area. Among all these, a total of 6 males, 4 female individuals, and 4 cubs were recorded from three potential sites in the area (figure 5).

3.4 Oral Interview

Data about black bears were also collected from

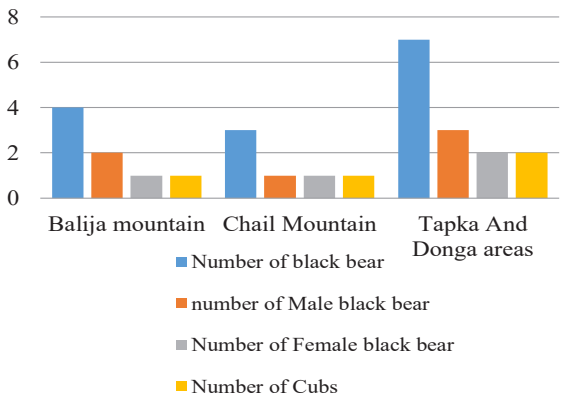

Fig. 5. Population of Asiatic black bear in three potential sites.

local people through oral interviewees and informal discussions. The local community of the district claimed that the black bear population is present in different areas of the district and mostly the population has been confirmed by local villagers, farmers, hunters, and wildlife staff in the district. Similarly, black bear families were also observed during the winter season in Shumlai (Donga, Tapka, Ghabrai), Balija, and Chail Mountains.

3.5 Sign Survey of Black Bear

During the field survey, a pug mark, scat sample, following track, and a setting place of a black bear was observed in Balija Mountains. On the other hand, a mature black bear was sighted on 14 April 2021 at Balija Maidan during the late winter (Figure 6).

Fig. 6. A: Pug Mark; **B:** Following Track; **C:** Scat Samples; **D:** Setting Place

4. DISCUSSION

Hunting poaching evidence of Asiatic black bears was reported from the Kaghan and Siran valleys of district Mansehra, Khyber Pakhtunkhwa Province. We discover that residents in both valleys actively monitor hunting and poaching in the study region. The structured interviews from the local community of both valleys claimed that peoples of District Battgarm and Kohistan target black bear mothers in both valleys, and carry their cubs into different megacities of Pakistan illegally. Every winter, parties of 10 to 25 hunters and dealers were spotted with their poaching equipment, which included firearms, pistols, and well-trained domestic dogs. Rout of black bear poaching and parts trading were also identified such as Kunda Gali, Door Gali, and Raam Gali into neighbouring districts. During each season, on average bases, approximately two to five mothers are killed and carry 12 to 20 cubs [18]. Field report and questionnaire-based survey of the present study indicated that quite a good population of black bears is present in district Battagarm. Recently a mature black bear was killed in a low elevated area (Jesol) of Battagram district where the human population is high. Parts of black bears have very high market demand which is why people in the local area seized different parts of the dead body illegally. A young man has also injured during the conflict in the populated area the entire village was dreaded by a bear attack. Similarly, the second black bear was also observed in the same village after one week. Black bear observation in low elevated and the populated area is not usually black bear was displaced from their natural habitat and come down to the village and frightening conflicts were created.

The least well-known species of animal is the Asiatic black bear, although the study is being done to learn more about its condition and dietary habits, especially in Pakistan's northwest. In the Pakistani western Himalayan area of the Kaghan Valley, we looked at the dietary preferences and altitude distribution of the Asiatic black bear [8]. The result of the questionnaire-based survey from this study indicated that there (n=14) bears present in three sites in the area. In Topeka and Donga, a very high population of bears was recorded, a total of seven black bears (with two cubs) were seen in the forest multiple times followed by Balija (4) and Chail three mature with a single cub. Among all these, a total of 6 males, 4 female individuals, and 4 cubs were recorded from three potential sites in the area. A mature black bear and bear signs such as pug mark, walking track and a scat sample were also observed in Balija Mountain which indicated that a quite good population of black bears is present in the district Battagram that is previously reported.

5. CONCLUSION

The Asiatic black bear is distributed to different areas of Pakistan including the District Battagram, where the present study was conducted and new frightening evidence of the Asiatic Black bear was reported. A black bear was encountered by local peoples of a low elevated area (1259 m) of Jesol village (N 34°41'53" E 73°03'05") and dread was spread among the community, and finally, the black bear was killed collectively on May 12, 2022. During such an encounter, a young man was also injured in conflict. After the immediate conflict, local people carried every part of the bear's body illegally and left the remaining skeleton. The wildlife department of Khyber Pakhtunkhwa took immediate action against the involved people. Similarly, the second black bear was also observed in the same village after one week but this time local people did not take any action against the black bear due to rules implemented by the wildlife department. On the other hand, questionnaire-based surveys and oral interviews with the local community revealed that a quite good population of bears n=14 (6 males, 4 females, and 4 cubs) are present in different areas of the district. Such evidence was also confirmed by a sign survey where multiple signs of black bears were observed. The black bear population faces a severe threat from the local community and immediate conservation efforts are needed with the help of the wildlife department and other conservation agencies.

6. CONFLICT OF INTERESTS

All the authors declare that there is no competing interest in this article.

7. ETHICS APPROVAL CONSENT TO PARTICIPATE

Data were collected from dead black bears with proper permission from the wildlife department of District

Battagram. However, there are no living organisms involved in this study. On the other hand, the young man was injured during the conflict with the black bear, proper permission was taken from the relatives and families of the victim during data collection.

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Appendix-I

Recent encounter of Asiatic black bear in Battagram

Structured Interview Questionnaire for collecting information about black bear (*Ursus thibetanus*) population and conflicts

Form No:

Date	Time	Interview	GPS location
/ /	:		

1) Personal Information:

Name _____ Age_____
Address_____ district _____
Occupation_____
Education: i) Illiterate ii) Primary iii) Secondary iv) Higher Secondary v) University

1. Do you have idea about black bear appearance in your local area? (Yes No)
2. If yes, specific their number and proper location ----------
3. Do you think that human- black bear conflict increased in the area (Yes No)
4. How many times you suffered from livestock attack by black bear? (----------)
5. Which type of loss----------and about ----------PKR.

Type of Livestock	Sheep	Goat	Dog	Poultry	Others	Total
Killed						
Injured						

6. Black bear population in the study area has increased? (Yes No)
7. What the causes of black bear population declining in the area ----------?
8. **Attack on human:** Where the attack took place?

In village:____in field:_____ in forest:_____Edge of forest:_____
In summer pastures_____ during grazing livestock_____ if any other_____

Animals present nearby at the time of attack
i) Dog ii) Cattle iv) Sheep v) Goat vi) others

Explain time and date of attack
Time____:_____ Day____ Night_____Date_____/_____/_____ (dd/mm/yy)

9) Consequences of attack
i) Victim injured ii) victim killed

10) What action you have taken against the black bear?
i) Chased ii) Fire gun iii) loud noise iv) Stand still v) Did nothing

11) Predator was killed?
i) Yes ii) No if yes, then

First seen in your area	Number of black bear seen,	Male	Female	Cubs
When was last seen (Approximate Date/ brief details)				
Do you have any idea about black bear killed in your area?	Bear Poaching	Observation of black bear population since ------?		
Have you seen any black bear family in the forest? Yes: No:	if yes, where:	and when:		
Activity timing (attack time)	Day or night			
Specific Remarks about black bear:				

Much thanks for your precious time

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Research Article

Effects of Graded Doses of Vitamin E on Blood and Serum Biochemistry of Sheep

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Abstract: Vitamin E (Vit. E) is primarily responsible for the increased antioxidant capacity observed in animal studies. The present research aimed to investigate vitamin E's effect on haematological and serum biochemical parameters in Kail sheep. Eighteen (18) Kail breed ewes older than two years but not yet pregnant were chosen for this experiment. The animals were randomly divided into three groups (Control, T1, and T2). During the entire experiment, the control group had access to pure water. Vitamin E was administered orally to both groups of ewes daily for 30 days, with Group T1 receiving 150mg/kg body weight and Group T2 receiving 200mg/kg body weight. Blood samples were collected on days 0, 15, and 30. The results revealed a significant increase in blood biochemistry parameters such as RBC, HGB, RDW%, WBC, LYM concentration, and LYM (%) in sheep fed Vitamin E. The serum concentration of albumin, globulin, total protein, and AST was significantly increased ($P< 0.05$). We conclude that the haematological and serum biochemical parameters in Kail sheep were enhanced after an oral dose of vitamin E.

Keywords: Kail Sheep, Vitamin E, Haematological parameters

1. INTRODUCTION

Vitamin E is a biologically active substance that primarily functions as a lipid antioxidant, preventing the proliferation of free radicals when fat is oxidized [1, 2]. It is required to develop and maintain animal health because it is an essential component of the antioxidant defence system [3]. Although only trace amounts are needed in animal diets, it plays a crucial function in farming animals [4].

Vitamin E is abundant in immune cells compared to other cells in the body, so its deficiency can impair the immune system's ability to function normally in humans and animals. By reducing free radicals, it protects humans and animals from the majority of diseases and disorders, including respiratory infections, allergic diseases (asthma), and the majority of chronic diseases [5, 6]. Vitamin E protects cells from reactive oxygen species (ROS) by reducing free radicals [7, 8]. Excessive ROS production defeats the antioxidant defence mechanism, resulting in oxidative destruction of living molecules and disruption of metabolism [9, 10]. Vitamin E is also absorbed by the small intestine (20 to 40 %) and is conveyed into the blood, generally by lipoproteins [11, 12]. Vitamin

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E reduces the risk of cardiovascular diseases in humans. A recent study found that patients with Myocardial Infarction (MI) have low levels of Vitamin E in their plasma and that supplementation with Vitamin E has a positive effect [12]. Vitamin E plays an important role in connective tissue growth, which is essential for the worth of meat. According to a study, vitamin E supplementation in broilers' diets improved the oxidative stability of breast meat and had no negative effects [7]. Lambs supplemented with Vitamin E, 15 days before slaughter improves meat and colour stability [13]. In Holstein dairy cows injection of Vitamin E and selenium increase serum albumin level, blood glutathione peroxidase activity and lactation performance [14]. When supplemented with 3000mg of Vitamin E 10 days before gestation, it reduces birth stress and protects the liver in cows [15]. Amar *et al.* performed a study on lambs by adding Vitamin E and garlic oil to their diet and found that body weight and blood parameters significantly improved [16]. A study on lambs supplemented with Vitamin E found that it dramatically affects subcutaneous fat and upregulates the genes involved in the metabolic pathway related to Vitamin E metabolism, which may be involved in meat quality [17].

Vitamin E also reverses the effect of heat stress in sheep. Sheep fed a diet high in selenium, and vitamin E have lower respiration rates, and rectal temperatures than sheep fed a diet low in selenium and vitamin E [18]. Vitamin E could boost antibody production and cellular immune responses. A study reported that Vitamin E was found in lower concentration in a group of sheep with mastitis caused by *Staphylococcus aureus* compared to the control group [19]. Vit. E deficiency completely disturbs immune functions [20]. Because of the numerous applications and roles of vitamin E, a study was designed to determine the effect of graded doses of vitamin E on blood and serum biochemical parameters in Kail sheep.

2. MATERIALS AND METHODS

2.1 Experimental Animals and Site

The study was conducted at the Experimental Livestock Farm Khaigala, Department of Veterinary Clinical Sciences, University of Poonch Rawalakot (UPR), Azad Kashmir. This experiment included 18 non-pregnant ewes of the Kail breed ranging in age from 2-3 years. The animals were divided into three groups of six each: control, T1, and T2. During the experiment, the Control group was given clean water. Animals in Group T1 received an oral dose of 150 mg/kg body weight of Vitamin E daily for 30 days, whereas animals in Group T2 received an oral dose of 200 mg/kg body weight of Vitamin E daily for 30 days. All of the animals were fed concentrates, wheat bran, wheat straws, and maize in addition to open grazing.

2.2 Blood Sampling

Blood samples were collected from experimental animals on days 0, 15, and 30. Blood samples were collected in two tubes, one with an anticoagulant, K3-ethylenediaminetetraacetic acid (K3-EDTA), for whole blood and the other without for serum isolation. The samples were kept in an icebox with ice packs before being transferred to the laboratory for further processing. Serum was stored at -20 °C after extraction [21].

2.3 Blood and Serum Parameters

Blood samples in EDTA tubes were checked for different parameters i.e., WBC count, RBC count, numbers of lymphocytes and percentage, granulocytes, haemoglobin concentration, mean cell volume, hematocrit, mean corpuscular h aemoglobin value, mean corpuscular haemoglobin concentration, MID cell, GRA percentage, and mean platelets volume [22]. A haematology analyzer (Cell-Dyn 3700; Abbott, Abbott Park, IL) processed whole blood according to the company's protocols. Suitable commercial test kits determined the serum parameters. Serum concentrations of albumin, total protein, and AST were analyzed using an ELISA reader by commercially available kits (Calbiotech, USA) [23]. The difference between albumin and total serum protein was used to calculate the concentration of globulin [24].

2.4 Statistical Analysis

Data was analyzed statistically through repeated measure ANOVA using a statistical package for social sciences (SPSS Inc, version 16, Chicago, USA). The graphing software used was Graph Pad Prism. (Graph Pad Software Inc., San Diego, CA,

USA). Data are expressed as the means ± SEM. P<0.05 was considered significant.

3. RESULTS

3.1 Blood Parameter

3.1.1. Effect on the concentration of RBCs

On day 0, the RBCs concentration (Table 1) showed that all three groups (Control, T1, and T2) had RBCs ±SE values of 3.3±1.1, 3.2 ±0.2, and 3.6±0.8, respectively. On day 15, the control and T1 groups had similar RBC values (3.7±0.8 and 3.4±0.5), whereas the T2 group (4.2±0.9) had a significant increase (P<0.05) in RBC count compared to the control group. On day 30, the RBC values in the T1 and T2 groups were 5.1±1.3 and 4.9±0.8, respectively, and showed a significant increase when compared to the control group; however, there was no significant difference between the RBC values in the T1 and T2 groups. HCT values in the T2 group followed a similar pattern (Table 1), with values increasing on day 30. Throughout the experiment, there was no significant difference in MCV and MPV concentrations (Table 1) among the three groups (Control, T1, and T2).

3.1.2. Effect on values of WBC and HGB

On day 0, the values of WBCs ±SE (Table 2) were 12.0±1.6, 13.5±2.1, and 11.9±1.7 for the control, T1, and T2 groups, respectively. On day 15, however, values of Group T1 and T2 (14.1±1.7 and 13.95±1.8, respectively) showed a significant increase (P<0.05) in WBC count as compared to the control group (12.2±1.9), and on day 30, T1 had significantly (P<0.05) higher values (12.8±1.9) as compared to the control group (11.2±1.6). Similarly, the value of the T2 group (13.5±1.2) was also increased as compared to the control group.

The HGB concentrations (Table 2) in the control and treated groups (T1 and T2) differ significantly throughout the experiment. On day 15, the T1 and T2 groups had significantly higher HGB levels (8.1±0.6 and 8.1±0.7) than the Control group (7.4±0.3). On day 30, the value of HGB in the T1 group (7.7±0.2) showed an upward trend when compared to the control group (7.6±0.5), whereas the T2 group showed significantly higher values of HGB (8.0±0.8) when compared to T1(7.7±0.2). Throughout the experiment, there were no significant differences in the MCH concentration values between and within the three experimental groups (Table 2).

3.1.3. Effect of Vit. E on other blood parameters

Results of MCHC, GRAN, LYM (Mx/Dl), and LYM (%) are arranged in Table 3. In comparison to the control group (79.5 ±4.6), the percentage value

Table 1. Effect of Vit. E supplementation on the RBC, MCV, HCT% and MPV of sheep.

Parameters	Days	Groups		
		Control n=6	T1(5g) n=6	T2(7g) n=6
RBC	0 day	3.3 ± 1.1^{Aa}	3.2 ± 0.2^{Ba}	3.6 ± 0.8^{ABa}
	15 day	3.7 ± 0.8^{Aa}	3.4 ± 0.5^{Aa}	4.2 ± 0.9^{Aab}
	30 day	4.6 ± 1.1^{Aa}	5.1 ± 1.3^{Ab}	4.9 ± 0.8^{Ab}
MCV (fL)	0 day	36.6 ± 1.2^{Aa}	36.4 ± 0.4^{Aa}	36.8 ± 2.3^{Aa}
	15 day	37.4 ± 0.8^{Aa}	36.6 ± 0.7^{Aa}	37.2 ± 2.4^{Aa}
	30 day	35.9 ± 0.6^{Aa}	36.7 ± 0.7^{Aa}	37.4 ± 1.1^{Aa}
HCT%	0 day	15.7 ± 3.8^{Aa}	11.8 ± 2.0^{Aa}	14.6 ± 5.3^{Aa}
	15 day	15.6 ± 3.3^{Aa}	12.5 ± 1.96^{Aa}	14.7 ± 5.1^{Aa}
	30 day	15.1 ± 6.4^{Aa}	15.8 ± 3.8^{Aa}	18.2 ± 4.3^{Aa}
MPV	0 day	19.8 ± 4.6^{Aa}	21.1 ± 0.9^{Aa}	18.3 ± 3.7^{Aa}
	15 day	21.9 ± 0.9^{Aa}	21.4 ± 0.95^{Aa}	18.6 ± 3.8^{Aa}
	30 day	18.98 ± 5.4^{Aa}	22.2 ± 0.9^{Aa}	18.5 ± 5.95^{Aa}

^{AB} superscript denotes the significant difference between the groups (P<0.05). ^{ab} superscript denotes the significant difference (P<0.05) within the groups. T1= Treatment 1, T2= Treatment 2

Table 2. Effect of Vit. E supplementation on the WBC, HGB and MCH parameters of sheep

Parameters	Days	Groups		
		Control n=6	**T1(5g) n=6**	**T2(7g) n=6**
WBC	0 day	12.0± 1.6^{Aa}	13.5± 2.1^{Aa}	11.9 ±1.7^{Aa}
	15 day	12.2± 1.9^{ABa}	14.1± 1.7^{Aa}	13.95±1.8^{Ba}
	30 day	11.2± 1.6^{Aa}	12.8 ±1.9^{Ba}	13.5± 1.2^{ABa}
HGB (g/dL)	0 day	7.9± 0.4^{Aa}	7.1± 0.7^{Ba}	7.2 ±0.8^{Ba}
	15 day	7.4± 0.3^{Aa}	8.1 ±0.6^{Aa}	8.1 ±0.7^{Aa}
	30 day	7.6± 0.5^{Aa}	7.7 ±0.2^{Aa}	8.0±0.8^{Aa}
MCH(pg)	0 day	19.5± 4.5^{Aa}	21.3±0.9^{Aa}	19.8± 5.1^{Aa}
	15 day	20.1 ± 3.6^{Aa}	22.0±1.8^{Aa}	19.9 ± 4.8^{Aa}
	30 day	19.6 ± 8.1^{Aa}	17.5± 3.9^{Aa}	22.3± 4.9^{Aa}

^{AB} superscript denotes the significant difference between the groups (P<0.05). ^{ab} superscript denote the significant difference (P<0.05) within the groups.

of LYM at day 15 was significantly higher (P<0.05) in Group T1 and Group T2 (82.1±4.8 and 85.8±6.8, respectively). At day 30, the concentration of LYM (Mx/D1) in Group T1 (11.4±1.1) was higher than that of the control group. Throughout the experiment, the values of all other parameters in Table 3 did not change significantly (P>0.05) between and within groups.

In Table 4 we can access the RDW%, GRA%, MID%, and MID concentration data. No statistically significant differences existed between any of the groups on day 0. (Control, T1, and T2). Compared to the T1 and control groups, the MID% figure for the T2 group increased dramatically by day 15. On day 30, the RDW% in the T1 group increased (P<0.05) compared to the control group. Furthermore, there

Table 3. Effect of Vit. E supplem entation on the MCHC, LYM conc., GRAN and LYM% of sheep.

Parameter	Days	Groups		
		Control n=6	**T1(5g) n=6**	**T2(7g) n=6**
MCHC(g/dL)	0 day	53.1± 12.2^{Aa}	57.6 ± 5.5^{Aa}	53.9± 13.6^{Aa}
	15 day	53.7± 11.0^{Aa}	60.1± 6.1^{Aa}	54.1± 13.8^{Aa}
	30 day	51.3 ±24.2^{Aa}	50.4±11.3^{Aa}	58.9±12.2^{Aa}
LYM(Mx/Dl)	0 day	9.6±1.3^{Aa}	10.3±1.3^{Aa}	9.7± 0.9^{Aa}
	15 day	9.7±1.6^{Aa}	10.8±1.4^{Aa}	9.9 ±0.9^{Aa}
	30 day	9.7± 1.4^{Aa}	11.4 ± 1.1^{Ba}	10.4±1.4^{ABa}
GRAN	0 day	0.5±0.2^{Aa}	0.6± 0.5^{Aa}	0.3±0.1^{Aa}
	15 day	0.5±0.3^{Aa}	0.4±0.3^{Aa}	0.4±0.1^{Aa}
	30 day	0.4±0.3^{Aa}	0.4±0.1^{Aa}	0.4±0.1^{Aa}
LYM%	0 day	79.9± 4.1^{Aa}	79.5± 4.9^{Aa}	72.2± 31.6^{Aa}
	15 day	79.5± 4.6^{Aa}	82.1±4.8^{Aa}	85.8±6.8^{Ab}
	30 day	86.3±1.4^{Aa}	86.9±6.2^{Aa}	89.4±2.7^{Ab}

^{AB} superscript denotes the significant difference between the groups (P<0.05). ab superscript denote the significant difference (P<0.05) within the groups.

Table 4. Effect of Vit. E supplementation on the RDW%, GRA%, MID, and MID% of sheep.

Parameter	Days	Groups		
		Control n=6	T1(5g) n=6	T2(7g) n=6
RDW%	0 day	7.2±1.2^{Aa}	5.6±1.4^{Aa}	7.7±4.7^{Aa}
	15 day	7.4±1.4^{Aa}	5.9±1.4^{Aa}	9.4±5.0^{Aa}
	30 day	9.1±4.6^{Aa}	10.7±4.6^{Ab}	10.1±4.9^{Aa}
GRA%	0 day	4.4±1.5^{Aa}	2.9±0.8^{Aa}	3.2±1.6^{Aa}
	15 day	4.7± 2.2^{Aa}	3.6±1.3^{Aa}	3.3±1.6^{Aa}
	30 day	3.8±2.1^{Aa}	3.1±0.3^{Aa}	3.9±0.9^{Aa}
MID Conc.	0 day	1.9±0.5^{Aa}	4.3±5.8^{Aa}	1.5±0.7^{Aa}
	15 day	1.9±0.5^{Aa}	4.5±5.7^{Aa}	1.5±0.7^{Aa}
	30 day	1.4±0.4^{Aa}	1.9±0.5^{Aa}	1.5±0.6^{Aa}
MID%	0 day	15.6±2.9^{Aab}	14.5±2.9^{Aa}	11.6±5.0^{Aa}
	15 day	12.8±2.5^{Aa}	12.7±3.2^{Aa}	15.3±5.4^{Aa}
	30 day	11.5±2.9^{Ab}	12.9±4.2^{Aa}	13.8±2.4^{Aa}

^{AB} superscript denotes the significant difference between the groups (P<0.05). ^{ab} superscript denotes the significant difference (P<0.05) within the groups.

was no significant difference between groups on days 0-15 or 30-day MID concentration or GRA % (P>0.05) (Table 4).

3.2 SERUM PARAMETERS

3.2.1. Effect on albumin, globulin, AST, and total protein concentration

Figure 1 demonstrates the effect of vitamin E supplementation on the levels of several enzymes in the blood. Both albumin and total protein concentrations on day 0 were similar (P>0.05) for the Control, T1, and T2 groups. On day 15, however, the T1 and T2 groups' albumin concentration and total protein were higher than the control group's by a significant margin (P<0.05). Additionally, T2 exhibited a higher albumin concentration than T1 and control on day 30, with a significance level of P<0.05. On day 30, there was an increasing tendency in the T1 group's total protein concentration (P>0.05), whereas the T2 group's total protein concentration improved significantly compared to the control group. (Fig 1A, B).

On day 15, globulin values were found to be significantly higher in the T1 and T2 groups

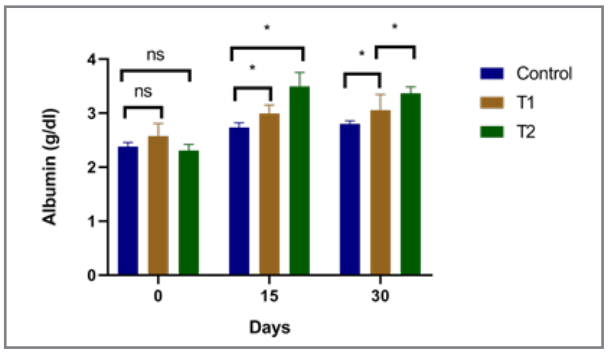

Fig 1A: Effect of graded doses of Vit. E on serum Albumin

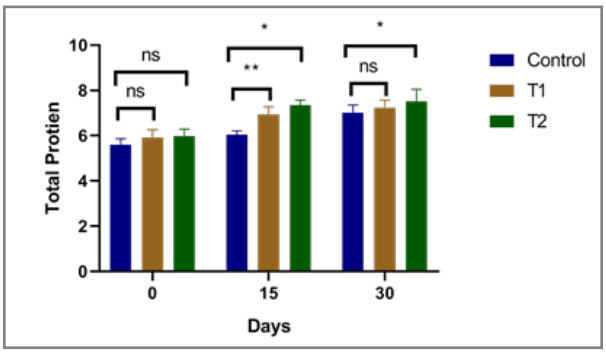

Fig. 1B. Effect of graded doses of Vit. E on total protein

compared to the control group. On day 30, globulin values were trending upward in the T1 group and increased significantly in the T2 group compared to the control group (P>0.05). (Fig 1C).

The AST enzyme value on days 15 and 30 increased in the T1 and T2 groups (P< 0.05) compared to the control group. There was no difference in AST enzyme levels between the control, T1, and T2 groups on day 0. (Fig 1D).

4. DISCUSSION

Vitamin E is crucial to the antioxidant defence system and vital to animal and human development and health [25–27]. As an antioxidant, it helps protect the cell membranes of immune system cells and has been linked to disease prevention. The effects of vitamin E supplementation on sheep blood and serum biochemistry were investigated in this study.

Vitamin E supplementation significantly affected the HCT/PCV and HGB values in this experiment. It has been demonstrated that supplementation with vitamin E significantly improved (P<0.05) PCV in sheep [28]. Mohri *et al.* found that calves fed with Se and Vitamin E in the age of third and fourth weeks had higher HCT and HGB values/levels. This study reported that HGB and PCV values increase significantly in treated groups as compared to control groups (p<0.05) [29]. Our findings are similar to these.

Previous data indicated no differences in PCV between treated and control lambs when supplemented with vitamin E, which contradicts the current findings [30]. Intake of Vitamin E did not affect rats' HCT value or HGB concentration [31]. Mohri *et al.* found that Se and Vitamin E supplementation had a negligible effect on HCT and HGB [29]. Because of the various doses utilized, these results do not correspond with the data we obtained.

The current studies found that vitamin E supplementation significantly improved (P<0.05) WBCs and RBCs in the treated group. A previous study reported that white blood cells had higher values in the test group in calves due to the antioxidant effect of vitamin E, thus increasing RBC and WBC lifespan [32]. Similarly, the administration of vitamin E to rats increased the number of white blood cells in the treated group compared to the untreated group [31]. Broiler breeders' white blood cell and lymphocyte counts improve when vitamin E is added to their diet [33]. In a study, Vit. E supplementation in poultry reduced the toxic effect of chromium by boosting haematological parameters (RBC, WBC, Hb, PCV, and MCHC) at a significant level [34]. Vitamin E and selenium supplementation dramatically increased red blood cell, white blood cell, and neutrophil counts in Markhoz offspring, as reported by Shokrollahi *et al.* [35]. Finding agreed with research by H_Asadi *et al.*, which found that vitamin AD3E injections in Arabi rams increased red blood cell (RBC) concentration [36].

Our data showed that the lymphocyte counts of the experimental animals improved dramatically (P< 0.05) after receiving vitamin E supplements. Our findings are consistent with those of Hassan and Mustafa, who found that giving ram lambs vitamin E improved their lymphocyte counts [28]. Vitamin E intake may be related to the protection of organelles and cell membranes through the vitamin's antioxidant effects on lymphocyte cell numbers [37]. However, Shokrollahi *et al.* discovered the opposite in Markhoz's offspring, finding that varying doses of vitamin E and selenium treatment had a negligible impact on lymphocyte counts [35].

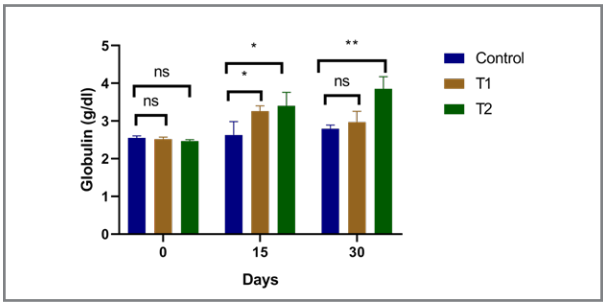

Fig. 1C. Effect of graded doses of Vit. E on serum Globulin

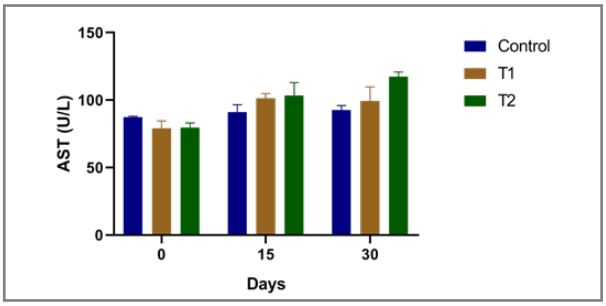

Fig. 1D. Effect of graded doses of Vit. E on serum AST

A study that was done on rats showed that there was no statistically significant difference (P>0.05) in mean cardiac output (MCV), mean cardiac hemodynamic output (MCHC), or mean cardiac output (MCH) between groups that were given or not given vitamin E [31].

The albumin, globulin, AST, and total protein levels increased by a statistically significant amount (P<0.05) in our experiment when Vitamin E was provided as a supplement. Previous research indicated that vitamin E and selenium significantly affected albumin and total protein concentrations in sheep serum [38]. Serum total protein concentration in Markhoz's progeny was reported to increase rapidly after supplementation with vitamins E [35]. El-Shahat and Abdel Monem found that taking vitamin E and selenium supplements increased globulin and tissue-specific protein levels by a statistically significant amount (P<0.05) [39]. Vitamin E plays a vital role in maintaining protein and albumin synthesis and a substantial part in cellular protein synthesis [40]. Vitamin E's capacity to boost immunoglobulins may be responsible for the rise in albumin and globulin concentration. These results accord with those found by Abdelatif *et al*. in their research on Nubian goats [32]. We hypothesized that this caused the overall increase in protein concentration that we saw in our experiment.

5. CONCLUSION

The results of the current study suggest that vitamin E has beneficial effects on several essential blood and serum parameters in sheep. RBC, WBC, HGB, LYM, RDW%, albumin, globulin, total protein, and AST were all improved significantly by vitamin E supplementation. It was concluded that vitamin E enhanced important blood parameters; hence, it is necessary for a variety of body functions as well as the sustenance of good animal health.

6. FINANCIAL SUPPORT

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7. CONFLICT OF INTEREST

The author declared no conflict of interest.

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