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Submission Address

Professor Abu-Elteen, Khaled H.
Deanship of Scientific Research and Graduate Studies
The Hashemite University
P.O. Box 330127, Zarqa, 13115, Jordan
Phone: +962-5-3903333 ext. 4399
E-Mail: jjbs@hu.edu.jo

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Preface

Now commencing its seventh year, Jordan Journal of Biological Sciences (JJBS) will continue to provide biologists with first class research articles, review articles and short communications in various disciplines and frontiers of Biological Sciences. Here, I ask active researchers from all over the world to consider JJBS as one of their first choices for submission to publish their data. JJBS is now indexed with and included in DOAJ, EBSCO, CABI, HINARI, Google Scholar, Chemical Abstract Service, Zoological Abstract, Ulrich's, Index Copernicus International, ISC, Directory of Research Journal Indexing (DRJI) and others. Moreover, the journal is under the indexing process with ISI and Scopus. As always submitted research articles will receive fair and constructive comments by peer reviewers and worthwhile articles will get published. One of the great benefits we can provide to our prospective authors, regardless of acceptance of their manuscript or not, is the mentoring nature of our review process. JJBS provides authors with high quality, helpful notes and comments to assist authors in improving their manuscripts.

Moreover, the Editorial Board of JJBS are very much interested in publishing significant review articles that outline and discuss current hot topics in the frontiers of Biological Sciences. P utting such topics in perspective and fitting pertinent data together is of utmost importance in guiding future research and helping new scholars in the field to address important and pertinent issues. I encourage experts in various fields of Biological Sciences who wish to review certain front line topics in their specialties to contact me if they wish to contribute one or more review articles. In this way, the Editorial Board hopes to include at least one major or mini review in each journal issue as of March 2014.

As in prior two years, this seventh volume of JJBS will include four issues with at least twelve articles in each issue. In the coming year, it is my vision to have JJBS publishes more outstanding articles from distinguished scholars in various areas of Biological Sciences. In addition, I will be working on the inclusion of JJBS in Scopus, ISI and other international information retrieval services, which will lead to a good impact number.

Again, I must congratulate and thank all the researchers who contributed to research and review articles published in previous issues of JJBS during the past six years.

Also, I thank my esteemed reviewers of previous articles submitted to the journal. They are assurance of high quality of published research work. To all our former contributors and potential new ones, I welcome further manuscripts for submission. Your manuscripts will receive careful consideration to maintain a high quality publication in JJBS.

I would like to thank the JJBS International Advisory board members for their continuous support. Furthermore, I would like to thank the JJBS Editorial board members for their exceptional work and continuous support to JJBS. Finally, I very much appreciate the support of The Hashemite University and Jordanian Scientific Research Support Fund for their continuous support to JJBS.

Professor Khaled H. Abu-Elteen
Editor-in-Chief , JJBS
Hashemite University
Zarqa, Jordan
jjbs@hu.edu.jo

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Jordan Journal of Biological Sciences **Review**

The Mediterranean Region: A Reservoir for CTX-M-ESBL-Producing *Enterobacteriacae*

Katia Cheaito and Ghassan M. Matar*

Department of Experimental Pathology, Immunology and Microbiology, Faculty of Medicine,

American University of Beirut, Beirut, Lebanon

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Abstract

The incidence of ESBL-producing bacteria is increasing worldwide, which represents a ch allenge for the healthcare systems. More recently, the emergence of the CTX-M group has been frequently reported as being associated with both nosocomial and community acquired infections. CTX-M-15 was identified as being the most prevalent in a l arge geographic area including Europe and the Middle East, suggesting the presence of a co mmunity reservoir of CTX-M enzymes disseminating in the Mediterranean area. Thus, this review will focus on the Mediterranean region to highlight the increasing prevalence of CTX-M-ESBL producing *Enterobacteriacae.*

Keywords: ESBL, CTX-M, Mediterranean, *Enterobacteriacae*, Multi-drug Resistance, *E. coli, Klebsiella pneumonia*, UTI.

1. Introduction

Since the initial report in Germany in 1983, extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* emerged as a m ajor pathogenic threat worldwide (Paterson and Bonomo, 2005; Livermore *et al.,* 2007; Pitout and Laupland, 2008; Qian-Hong *et al.,* 2011). The increasing prevalence of ESBLs producing pathogens and their alarming evolution can be attributed to the frequent prescription of β-lactam agents such as penicillins, cephalosporins, monobactams and carbapenems. ESBLs were identified shortly after the introduction of oxyimino-β-lactam antibiotics (Medeiros, 1997; Pitout *et al.,* 2005). Initially, the classic ESBLs occurred due to mutations in the genes encoding the common plasmid-mediated SHV-1, TEM-1, or TEM-2 beta-lactamases (Jacoby and Munoz-Price, 2005). More recently, a novel group of ESBLs, the CTX-M family, have emerged and rapidly disseminated worldwide (Canton and Coque, 2006). This CTX-M emergence led to further dissemination of ESBLs in both community and hospital settings (Woerther *et al.,* 2011).

2. Overview of ESBLs

These enzymes are termed ESBLs since they confer bacterial resistance to penicillins, all cephalosporins and aztreonam and frequently resistant to fluoroquinolones and aminoglycosides but not to cephamycin, carbapenems or β-lactamase inhibitors (Tham *et al.,* 2012; Patterson and Bonomo, 2005; Drawz and Bonomo, 2010). ESBLs are mostly encoded by genes found on large plasmids which include as well genes encoding resistance for a variety of antimicrobial agents including aminoglycosides, sulphonamides, trimetho-prim, chloramphenicol and tetracyclines (Paterson, 2000). Consequently, ESBLs are characterized by a broad antibiotic resistance extending to multiple classes (Bradford, 2001). This resistance is even extending to carbapenems which represent the treatment of choice for ESBL associated serious infections (Rahal, 2008; Doumith *et al.*, 2009). Historically, mutations in the genes encoding the common plasmid-mediated TEM-1 and SHV-1 enzymes were the initial cause of the occurrence of ESBLs (Jacoby and Munoz-Price, 2005). Currently, 175 different TEM enzymes and 127 SHV different enzymes are described (www.lahey.org/ studies/). Nevertheless, a new group of ESBLs, the CTX-M family, have emerged and spread rapidly worldwide (Canton & Coque, 2006). These enzymes are prominent among *Enterobacteriaceae* from Europe, Africa, Asia, South America and North America (Bonnet, 2004). The number of CTX-M-type ESBLs is rapidly escalating and they were identified in every inhabited continent (Patterson and Bonomo, 2005). At present, the number of CTX-M β-lactamases identified exceeds 140 allelic variants (http://www.lahey.org/ Studies/other.asp#table1) which can be classified, based

* Corresponding author. e-mail: gmatar@aub.edu.lb.

on their amino acid structure, into six distinct phylogenetic groups, namely groups 1, 2, 8, 9, 25 and 45 (Rossolini *et al.,* 2008). For a period of time, CTX-M ESBLs were mainly detected in three geographic regions: South America, the Far East, and Eastern Europe, while they appeared to be sporadic in Western Europe and North America; however, more recently, the arrival of CTX-M-type ESBLs in these regions have been reported extensively (Dechamps *et al.,* 2000). Furthermore, CTX-M-type ESBLs were largely reported in China and India, and hence CTX-M-type ESBLs can be considered as the most prevailing ESBL type (Pitout *et al.,* 2005). In addition, some of the CTX-M enzymes were identified in particular countries, like CTX-M-1 in Italy, CTX-M-9 and CTX-M-14 in Spain, and CTX-M-2 in the majority of South American countries and Japan, while CTX-M-15 was detected worldwide (Bonnet, 2004; Ben-Ami *et al.,* 2006; Canton and Coque, 2006). However, CTX-M-15 was identified as being the most prevalent in a b road geographic area which includes North America, Europe, The Middle East, and India, while a number of CTX-M-32-producing strains were recently detected in both humans and farm animals in Spain, Italy, Greece and Portugal (Eckert *et al.,* 2006). This suggested the presence of a community reservoir of CTX-M enzymes disseminating in the Mediterranean area (Cartelle *et al.,* 2004; Oteo *et al.,* 2006).

3. Predominant ESBLs Circulating in European Countries on the Mediterranean

All published studies have confirmed that the prevalence of ESBL producing isolates is higher in southern Europe than in the rest of European countries and the wide dissemination of CTX-M-15 enzyme is increasingly reported from both hospital and community settings. A nationwide Spanish study including 40 medical centers reported C TX-M-9 (27.3%), SHV-12 (23.9%), and CTX-M-14 (20.5%) as the most prevalent ESBL-producing *E. coli*, and clonal dissemination was not detected among the 170 isolates included (Hernandez *et al.,* 2005). In another Spanish study, Oteo *et al.* (2006) reported the dissemination of *bla*CTX-M-15 alleles linked to an ISEcp1-like element between the community, long-term care centers, and hospitals, and the majority of the tested isolates harbored the same three virulence genes: *iut*A and *fyu*A (siderophores), and *tra*T (serum survival factor). Moreover, Valverde *et al.* (2008) identified high rates of colonization with ESBL-producing *Enterobacteriacae* in patients with community infections and their household members with 66% of the ESBL-producing isolates being indistinguishable between patients and their healthy household contacts. These results highlight the high risk of the spread of these multi-drug resistant isolates to healthy individuals and their dissemination in both hospital and community settings (Valverde *et al.,* 2008). In 2009, Valverde *et al.* demonstrated that the dissemination of IncK plasmids is responsible for the high incidence CTX-M-14-producing *E. coli* isolates which represents a f requent cause of community-acquired urinary tract infections in Spain (Valverde *et al.*, 2009). In 2010, ESBL-producing *E. coli* was collected from 27 Spanish centers as part of European Antimicrobial Resistance Surveillance Network for susceptibility testing and typing and an increase in fosfomycin resistance was shown. The higher rate of fosfomycin resistance was 15.3% belonging to CTX-M-15-ESBL-producing isolates (Oteo *et al.,* 2010). In a recent study, Dahbi *et al.* (2013) highlighted the emergence of new variants of ST131 clonal group among extra-intestinal pathogenic CTX-M-15 ESBL producing *E. coli* with a significantly elevated virulence score and novel virulence profile. In France, before 2008 the prevalence of ESBL producing *Enterobacteriaceae* was under 1% (Galas *et al.,* 2008). However, since then, this prevalence has increasingly been reported in both community and nosocomial settings. In a study that aimed to report the evolution of ESBL production between 1999 and 2007 at a general hospital from south France, ESBL prevalence in *E. coli* augmented during this episode from 0.3 to reach 2.5% (Anastay *et al.,* 2013). Concurrently, the predominant ESBL in 1999, TEM-24 was substituted by CTX-M in 2007, with CTX-M-15 being the most prevalent (88% of CTX-M) (Anastay *et al.,* 2013). CTX-M variants were also identified predominantly in the southern coastal region of France, and the zoonotic spread was detected through gulls carrying bacteria that harbored mostly the CTX-M-1 group (Bonnedahl *et al.,* 2009). In 2006, a nationwide survey was conducted in Italy and the widespread of CTX-M-producing ESBLs was described. Although the rate of CTX-M pr oduction varied between hospitals (1.2 to 49.5% of ESBL producers), all these isolates belonged to CTX-M group 1 with CTX-M-15 and CTX-M-1 being the most prevalent variants (60% and 35%, respectively) and CTX-M-32 carried only by a minority (5%). In 2011, the dominance of CTX-M group 1 ESBL-producing *E. coli* causing urinary tract infections among outpatients was reported (Huemer *et al.,* 2011). More recently, an outbreak of colonization by ESBL producing *E. coli* sequence type 11 was identified in a n eonatal intensive care unit. This epidemiological investigation highlighted the importance of detecting the silent spread of ESBLs (Giuffre *et al.,* 2013). In addition, the emergence of *K. pneumoniae* clone carrying both VIM-1-MBL and CTX-M-15-ESBL was reported from different hospitals in Italy (Nucleo *et al.,* 2013). In Greece, a multiclonal epidemic of *K. pneumonia* producing both VIM-1 and SHV-5 was reported to be under way in the major hospitals (Psichogiou *et al.,* 2008). Sequencing analysis of ESBL-producing *Enterobacteriacae*, collected between 2007 and 2011, showed that *bla*CTX-M-3 gene is predominant, followed by the *bla*CTX-M-15 gene and *bla*SHV-5 gene (Kristo *et al.,* 2013). In 2008, Turkey was added to the list of countries concerned by community-acquired CTX-M-15-ESBL *E. coli* clone O25-ST131 among outpatients with *E. coli* urinary tract infection (Yumuk *et al.,* 2008). More recently, in a nationwide study including ESBL-producing *E. coli* isolates collected from 10 different Turkish hospitals, CTX-M-1 was the most prevalent (366/440) followed

by TEM (194/440) and CTX-M-2 (140/440) (Cicek *et al.,* 2013).

4. Prevalence and Distribution of ESBL Production in African Countries on the Mediterranean

In 2006, CTX-M production was initially detected and the high rate of ESBLs was confirmed in Egypt reaching 60% among urinary tract infections patients; three different enzymes were found CTX-M-14, CTX-M-15 and CTX-M-27 (Alagamy *et al.,* 2006). Later on, Hassan *et al.* (2013) reported the high prevalence of quinolone resistance determinants qnr, aac(6')-Ib-cr , qep A4, and their association with CTX-M positive *E. coli* isolates from Egypt (Hassan *et al.,* 2013). The extensive community acquired CTX-M-15 carriage reaching 57% was also described in a recent study (Newire *et al.,* 2013). In Tunisia, the initial identification of a CTX-M-producing strain (CTX-M-3) was recovered in 2001. Later on, CTX-M-27 originated a nosocomial outbreak in a Tunisian neonatal center. The first report of CTX-M-15 and CTX-M-16-producing *Enterobacteriaceae* in Tunisia was submitted in 2006 (Mamlouk *et al.,* 2006). Later, Dahmen *et al.* (2010) corroborated the high prevalence of CTX-M-15 with 91% of the isolates producing this enzyme. The majority of CTX-M-15-ESBL-producing *E. coli* belonged to B2 phylogenetic group and to the sequence type 131 and was associated with Qnr-like determinants (Dahmen *et al.,* 2010). It was also found that this CTX-M-15-B2-ST131 *E. coli* clone is also highly disseminating in community-acquired urinary tract infections in Tunisia (Hammami *et al.,* 2013). The molecular analysis of a collection of ESBL producers isolated between 1989 and 2009 confirmed the prominence of *bla*CTX-M-15 gene followed by *bla*CTX-M-14 gene, *bla*SHV-12 gene, *bla*SHV-2a gene and *bla*TEM-26, with the frequent dissemination of CTX-M-15 producing *E. coli* being attributed to the spread of various IncF-type plasmids (Mnif *et al.,* 2013). In Algeria, the results obtained were similar to the other Mediterranean countries, and in 2008 the prevalence of ESBLs belonging to CTX-M-1 group was noticed (Messai *et al.,* 2008). It was determined that the size of the self-transferable plasmid reached 85 kb and included in addition to the *bla*TEM and *bla*CTX-M genes, determinants for aminoglycosides and sulfonamides resistance (Messai *et al.,* 2008). Similarly, in a study that tried to determine the overall incidence of ESBL production among *Enterobacteriacae*, predominance of CTX-M-1 group was revealed, TEM and SHV were also detected. But the conjugative plasmids carrying these genes were of higher molecular weight (≥125kb) (Nedjai *et al.*, 2012). Ahmed *et al.* (2012) detected the CTX-M-15 ESBLs in the intensive care unit of an Algerian hospital and determined that this gene was genetically linked to insertion sequence lSEcp1B (Ahmed *et al.,* 2012). More recently, in a study that aimed at characterizing environmental ESBLs and quinolone resistance, it has been found that the antibiotic resistance mechanisms are similar in both the environment and the clinical setting, and wastewater treatment plant might represent a cause of dissemination of resistance genes (Alouache *et al.,* 2013). Morocco is another country where the production of CTX-M-type beta-lactamase *bla*CTX-M-15 is the most recurrently found mechanism of resistance to beta-lactams. In a nationwide study conducted over a 2-year period in the Moroccan community, *bla*CTX-M-15 was the most frequent gene detected among ESBLs causing urinary tract infections, followed by *bla*CTX-M-1, SHV-12 and PER-2. The *bla*OXA-48 and *bla*IMP-1 carbapenemases genes were also detected and qnr genes were harbored by only a small percentage of the isolates (Barquiqua *et al.,* 2013).

5. ESBL-Producing E. coli in the Middle-East

In Lebanon, an increase in the prevalence of ESBL-producing bacteria was observed from 2.3% in 2000 to reach 8% in 2005 and 16.8% in 2009 (Kanafani *et al.,* 2005; Daoud and Afif, 2011). The escalating numbers of ESBL-producing bacteria were not only noted for *E. coli*, but also for *K. pneumoniae*, *Salmonella spp*. and *Shigella spp* (Araj *et al.,* 2012). The predominance and fast emergence of the CTX-Ms has been reported in Lebanon (Moubareck *et al.,* 2005; Kanj *et al.,* 2008) and sequence analysis indicated that the *bla*-CTX-M-15 is the most prominent (Matar *et al.,* 2007; Baroud *et al.,* 2013). In a recent study, resistance to carbapenemase production was detected in ESBL *E. coli* and *K. pneumoniae* isolates (Baroud *et al.*, 2013). There is a lack of sufficient information about the prevalence of ESBLs and their molecular characterization in Syria; however, in a recently published article, the high prevalence of ESBL producing *E. coli* was revealed reaching 52% among urinary tract infections patients (Al-Assil *et al.,* 2013). In Jordan, the prevalence of ESBLs has been reported to be relatively high compared to other reported data worldwide (Youssef *et al.,* 1999; Shehabia *et al.,* 2000). More recently, clinical specimens have been collected from three major hospitals in Northern Jordan, and ESBL-producing gram negative bacteria comprised 22.9% of all isolates which included *E. coli*, *K. pneumoniae*, *K. oxytoca* and *Enterobacter cloacae* as the most prominent (Batchoun *et al.*, 2009). A study by Aqel *et al.* (2013) suggested the endemicity of ESBL producing bacteria in Jordanian hospitals, where CTX-M-1 and CTX-M-9 ESBLs were detected in two geographically distant hospitals. Moreover, the results of another recent study revealed that ESBL urinary *E. coli* isolates with high levels of *sul*2, *bla*CTX-M and *bla*TEM are circulating in the Jordanian community with associated multidrug resistance profile (Nimri and Azaizeh, 2012). Similarly, in a recently published study, ESBL producers were isolated from patients with urinary tract infections from three hospitals in the West Bank and the results showed that all isolates harbored CTX-M, TEM was also detected but none harbored the SHV gene (Adwan *et al.,* 2013). The dissemination of CTX-M sequence type 131 ESBLs was also detected in Israel among patients with community-onset bacteremia (Karfunkel *et al.,* 2013). Moreover, in an investigation conducted over 7 years, the association

between the increase of such infections and CTX-M ESBLs was suspected, and specifically it appeared to be related to the clonal extension of *bla* CTX-M-15 or *bla* CTX-M-14 carrying ST131(Karfunkel *et al.,* 2013).

6. Conclusion

The data provided in a large number of studies conducted in the Mediterranean area show that the dissemination of CTX-M genes, in both community and hospital settings, have been increasing extensively. This expansion is enhanced by environmental and zoonotic spread. Moreover, it is associated with a significantly elevated virulence score and multidrug resistance profiles, which highlights the importance of identifying the silent spread of ESBLs, and of implementing additional measures to prevent ESBL associated infections.

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Effect of Green Tea and Green Tea Rich with Catechin on Blood Glucose Levels, Serum Lipid Profile and Liver and Kidney Functions in Diabetic Rats

Usama El-Sayed Mostafa*

Ain Shams University, Faculty of Specific Education, Home Economics Department, Cairo, Egypt

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Abstract

Diabetes is a major health problem in the developed and developing world. This study aims at investigating the effects of continuous ingestion of green tea and green tea plus different doses of catechin in rats (with type 2 diabetes) that were not receiving insulin on the serum glucose, lipid profile and both liver and kidney functions. The results indicated that the sharpest decrease of serum glucose was observed in the group treated with green-tea plus 100 mg of powder catechin. Blood glucose level was significantly low in all treated group 2 h after the administration of green tea and green tea plus both doses of powder catechin suspension, whereas the level of blood glucose level remained high in diabetic rats. Treating diabetic groups fed on basal diet with different levels of green tea, green tea plus 30 mg of powder catechin and green tea plus 100 mg of powder catechin led to a significant decrease in the mean value of serum cholesterol, triglyceride, LDL and VLDL as compared to the positive control group. Green tea plus high dose of catechin tended to improve liver functions manifested by the reduction of the serum levels of (AST and ALT), as well as kidney functions manifested by the reduction in the serum levels of (uric acid, urea nitrogen and Creatinine). The tea supported with catechin, drunk for many weeks, may be beneficial for people suffering from moderate diabetes or hyperlipidemia, reducing its complications such as liver and kidney disorders.

Keywords: Green Tea, Catechin, Lipid Profile, Liver and Kidney Function, Blood Glucose.

1. Introduction

Tea is the most widely consumed beverage in the world, second only to water. The three kinds of true tea-green, black, and oolong-are all derived from the Camellia sinensis plant. At harvest, tea leaves contain high levels of catechins, a particular class of polyphenols (Cabrera *et al.,* 2006). Green tea (GT), however, is produced by heat-treating leaves soon after harvest, thereby preserving the catechins from oxidation. An average serving of 250 ml of GT contains between 50 and 100 mg of catechins. In addition, GT contains a variable amount (typically around 30 mg/serving) of caffeine (Wolfram *et al.,* 2006).

Green tea extracts are nonoxidized/nonfermented derivatives of the leaves of Camellia sinensis, which belongs to the aceae family. Polyphenols (flavonols or catechins), found in the tea, make up 30 to 40% percent of the extractable solids of dried green tea leaves. The main catechins in green tea are epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin- 3-gallate (EGCG), with EGCG being the highest in concentration. These polyphenols have been shown to exhibit some potential antioxidant, anticarcinogenic, anti-inflammatory, thermogenic, probiotic, and antimicrobial properties (Graham, 1992).

Vivo studies, using rodents, have shown that green tea extract and catechins isolated from green tea can induce a variety of health effects, including anti-obesity, hypoglycemic and hypolipidemic activities (Suzuki *et al.,* 2012).

Most research has focused on the function of the antioxidant components found in tea and the potential of these to reduce the risk of cardiovascular diseases and cancer (Wolfram *et al.,* 2006). In addition, a number of reports have been published showing that regular consumption of GT, or catechins extracted from GT (with or without added caffeine), may influence energy metabolism, body weight and body fat content (Hongqiang *et al.,* 2010).

Several studies reported that alloxan produces oxygen radicals which destroy pancreatic β-cells and cause severe hypoinsulinaemia (type I diabetes) that is responsible for the hyperglycemia seen in alloxan-treated animals. However, its action is not directed to pancreatic β-cells only, as other organs, such as the liver, kidney and bone marrow, are also affected by the

* Corresponding author. e-mail: usama127@yahoo.com.

alloxan administration as seen from the elevation of plasma markers reflecting renal cell damage (urea and creatinine levels) and the reduction of hematological parameters ((Jabber, 2013).

The present study aims at examining the effects of green tea alone or green tea supported with different levels of catechin on serum glucose, lipid profile and some liver and kidney functions in normal and diabetic rats.

2. Material and Methods

2.1. Materials

Green tea was purchased from Cairo, Egypt local market. Catechin and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Kits for biochemical analysis of serum glucose, cholesterol, triglyceride, HDL-c, AST, ALT, uric acid, urea nitrogen, and creatinine were obtained from the Gamma Trade Company for Pharmaceutical and Chemicals, Dokki, Egypt.

2.2. Preparation of Green Tea Extract (GTE)

For animal studies, 20 g of green tea leaves were added to 1000 ml of nanopure water. After being stirred for 5 min at 80 ° C, the tea leaves were removed by filtration, using filter paper (Advantec 2 filter paper, Hyundai micro Co., Seoul, Korea). The extract was dried using under-vacuum machine (temperature at 50°C, under-vacuum of 0.3 bar). A total of 3 g of dried GTE was harvested and dissolved in 10 ml of water. An equal amount of the GTE solution was mixed with different amount of powder catechin (Park *et al.*, 2009).

2.3. Animal Care

The present study was approved by the local Animal Ethics Committee. The investigation conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, Bethesda, MD 20892]. All animals were maintained on a 12 h lights and 12 h dark cycle and a temperature of 23 ± 1°C. All animals received modified basal diets (Reeves, 2004) and water ad libitum. Male Sprague-Dawley rats with average weights of about 200 g (10 wk old) were purchased from Laboratory Animal Colony, Ministry of Health and Population, Helwan, Cairo, Egypt. They were housed 5 rats/cage. All rats, except rats in group 1, were injected with alloxan in a dose of 150mg/kg of body weight as a single dose, subcutaneously to induce diabetes according to the method described by Hiroshi *et al*. (1996). Of eighty rats treated with alloxan, twenty five per cent died within the first and second weeks of alloxanization. Blood was extracted from the tail vein for glucose analysis; rats with fasting glucose ranging from 210-220 mg/dl were considered diabetic and were analyzed 48 hours after alloxan treatment. Rats were divided into the following experimental groups (n = 10 rats in each group):

- Group I: Negative control, normal healthy rats, receiving regular diet with no treatment.
- Group II: positive control, diabetic rats receiving regular diet with no treatment.
- Group III: diabetic rats receiving regular diet and receiving oral 25 ml of green tea extract.
- Group IV: diabetic rats receiving regular diet and receiving oral 25 ml of green tea extract plus 30 mg of powder catechin /kg/day.
- Group V: diabetic rats receiving regular diet and receiving oral 25 ml of green tea extract plus 100 mg of powder catechin /kg/day.

It should be noted that the green tea extract containing catechins was freshly prepared every other day. The daily dose of GTE plus powder catechin was calculated according to rat weight. This amount was divided into three portions daily; first portion was given orally in the morning after rats being fasted about 12 hours; second portion was given orally 6 hours after the first portion; third portion was orally given after 6 hours from the second portion. Oral dosing volumes should not exceed 10ml/kg.

2.4. Determination of Serum Glucose

Serum glucose concentration was determined according to the methods described by Young, (2001) using Spectrophotometer DU7400 adjusted at 500 nm.

2.5. Intraperitoneal Glucose Tolerance Test (IPGTT) and OGTT in Animals

After a 12 h fast, rats were randomly divided into 5 groups: first group (positive control) orally fed on 300 µl phosphate buffered saline (PBS), second group (negative control) (diabetic control) orally fed on 300 µl of PBS, third group orally fed on 25 ml of GTE, fourth group fed on orally 25 ml of GTE plus 30 mg of powder catechin dissolved in 300 µl PBS, and fifth group orally fed on 25 ml of GTE plus 100 mg of powder catechin dissolved in 300 µl PBS. The maximum volume that can be administered to the animal is to be calculated; oral dosing volumes should not exceed 10ml/kg. Then the distance from the oral cavity to the end of the xyphoid process (caudal point of the sternum) with the feeding needle on the outside of the restrained animal is to be measured. With the feeding needle attached to the filled syringe, the end of the feeding needle along the roof of the animal's oral cavity towards the animal's left side is to be slid. Once the feeding needle is in to the premeasured distance, the solution is to be injected, slowly as to minimize the fluid coming back up the esophagus.

Thirty minutes after the oral feeding, each rat was orally given 1 ml distilled water containing 2 g glucose/kg. For the assay of blood glucose levels, blood was drawn from the tail vein at the indicated times (Park *et al.*, 2009).

2.6. Estimation of Serum Lipid Profile

Total serum cholesterol (Cohn *et al.*, 1988), triglycerides (Foster and Dumns, 1973), HDL-c (Young, 2001), LDL-c and VLDL-c were calculated by the methods described by (FriedWald *et al.*, 1972).

2.7. Estimation of Liver and Kidney Functions

Liver functions, such as Aspartate amino transferase (AST), alanine amino transferase (ALT), and kidney functions, such as Uric acid, urea nitrogen and creatinine were determined according to methods described by Young (2001).

2.8. Statistical Analyses

The results are expressed as mean ±SEM. The SPSS (release10.0) software package (SPSS Inc., Chicago, IL) was used for the statistical analyses. For comparisons of more than two groups, significance was tested using an analysis of variance (ANOVA). Differences between groups were considered significant when $P<0.05$.

3. Results and Discussion

In the healthy control negative group, blood glucose level (BGL) slightly changed. Also, hyperglycemic rats control positive group (injected with alloxan), the BGLslightly changed, after 8 weeks. No statistical significance was observed between them. The BGL for rats injected with alloxan was much higher than the healthy group. This may be due to alloxan producing oxygen radicals that destroy pancreatic β-cells and cause severe hypoinsulinaemia that is responsible for the hyperglycemia seen in alloxan-treated animals (Jabber, 2013).

Table 1. Effect of some levels from green tea and green tea rich with different doses of catechin on serum glucose of hyperglycemic rats.

Parameter		mg/dl.	
Groups		Initial Glucose	Final Glucose
Healthy rats. Control (-)		92.51 ± 4.653 [a]	93.22 ± 4.436 [a] (NS)
Hyperglycemic rats. Control (+)		305.52 ± 7.292 [e]	301.33 ± 6.653 [e] (NS)
Hyperglycemic rats fed on basal diet and treated with	Green tea.	295.14 ± 7.221 [e]	260.66 ± 4.083 [d] (S)
	Green tea + 30 mg of powder catechin	285.55 ± 8.053 [e]	205.167 ± 5.231 [c] (S)
	Green tea + 100 mg of powder catechin	283.55 ± 7.231 [e]	160.53 ± 4.719 [b] (S)

Values are expressed as mean ± SD. Significance at $P<0.05$
[a, b, c, d], means that values which don't share the same letter in each column are significantly different at $P<0.05$.
a is the best result followed by b and c and so on (NS) none significant in the same row (S) significant in the same row.

Whereas in the green-tea and catechin treated group, BGL markedly changed after 8 weeks. The sharpest decrease was observed in diabetic rats treated with green-tea plus 100 mg of powder catechin after 8 weeks from consumption. As shown in table 1, green tea tended to lower BGL at 25 ml of GTE, and significantly lowered it at 25 ml of GTE of green tea plus 30 and 100 mg of powder catechin. These results are in agreement with Suzuki results (Suzuki *et al.*, 2012). The efforts to manage type 2 diabetes and obesity by natural green tea treatment appear to be taken cautiously because the systemically absorbed GTE blocks the cellular glucose uptake and thereby increases blood glucose (Park *et al.*, 2009).

The effect of green tea was compared among the different groups of rats, i.e., rats injected with alloxan-diabetic rats (control +), rats injected with alloxan-diabetic rats treated with oral 25 ml of GTE, rats injected with alloxan-diabetic rats treated with oral 25 ml of GTE plus 30 m g of powder catechin and rats injected with alloxan-diabetic rats treated with oral 25 ml of GTE plus100 mg of powder catechin. (Figure1). BGLs significantly went down in treated group 2 h after administration of green tea and green tea plus both doses of powder catechin suspension, whereas the level of BGL remained high in rats injected with alloxan - diabetic rats (control +).

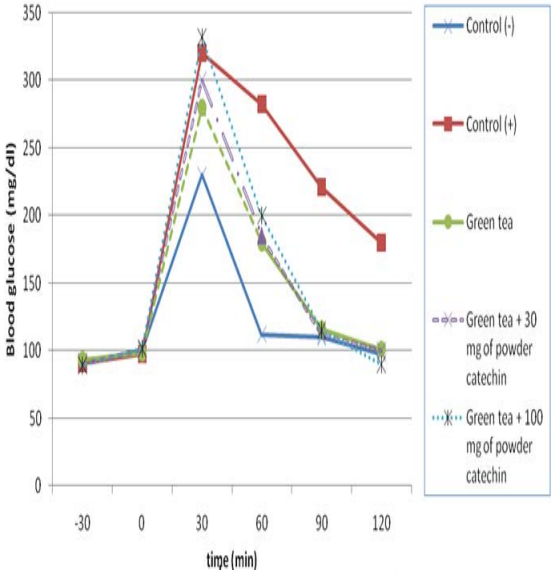

Figure 1. effect of green tea and green tea rich in catechin on glucose tolerance of hyperglycemic rats.

All tested groups showed more elevated blood glucose levels 30 min after glucose loading, compared to control negative group ($P<0.05$). This result may be due to the effect of green tea extract and catechin on glucose tolerance which may be mainly due to the two epicatechin (EC), Epigallocatechin-3-gallate (EGCG) and epicatechin-3-gallate (ECG) (Park *et al.*, 2009).

Treating diabetic groups fed on basal diet with different levels of green tea, green tea plus 30 mg of powder catechin and green tea plus 100 mg of powder catechin led to a significant decrease in the mean value of serum cholesterol and triglyceride as compared to the positive control group. Data in table 2 revealed that the mean value of total serum cholesterol and triglyceride in rats suffering from hyperglycemia decreased gradually with increasing the level of GTE enriched with catechin. Statistical analysis in this table showed that no significant change in serum cholesterol and triglycerides was observed between the groups treated with oral 25 ml of GTE and green tea plus 30 mg of catechin.

Table 2. Effect of some levels from green tea and green tea rich with different doses of catechin on cholesterol and triglycerides of hyperglycemic rats.

Parameter / Groups		mg/dl. Cholesterol	Triglycerides
Healthy rats. Control (-)		106.30 ± 4.35 [c]	60.667 ± 4.58 [c]
Hyperglycemic rats. Control (+)		162.63 ± 6.022 [a]	82.507 ± 6.04 [a]
Hyperglycemic rats fed on basal diet and treated with	Green tea.	126.83 ± 4.622 [b]	75.000 ± 4.00 [b]
	Green tea + 30 mg of powder catechin	119.66 ± 4.262 [b]	70.333 ± 3.20 [ab]
	Green tea + 100 mg of powder catechin	109.28 ± 1.966 [c]	62.000 ± 1.26 [c]

Values are expressed as mean ± SD. Significance at $P<0.05$

[a, b, c, d,] means that values which don't share the same letter in each column are significantly different at $P<0.05$.

[a] is the best result followed by [b] and [c] and so on

The best results in serum cholesterol were observed in hyperglycemic group fed on basal diet and treated with green tea plus high levels of catechin, because this treatment showed no significant differences in serum cholesterol and triglyceride as compared to the negative control group. The current results completely agreed with Ikeda (2008) who reported that green tea and catechin preparations significantly lowered serum and liver cholesterol concentrations. Moreover, another study reported that serum cholesterol concentration was 5% lower in the groups fed with GTE than in the placebo control group (Kajimoto *et al.*, 2003; Suzuki *et al.*, 2012). The reduction of serum cholesterol may be due to the inhibition of intestinal absorption of cholesterol by green tea and catechin, leading, therefore, to reducing the serum cholesterol concentrations (Ikeda, 2008). The lowering of triglyceride observed in this study was in agreement with other study which confirmed that both green tea and heat-treated tea catechins have the same triacylglycerol lowering activity (Ikeda *et al.*, 2005). The reduction of triglyceride observed in this study may be due to catechin suppress postprandial hypertriacylglycerolemia through the inhibition of pancreatic lipase, which, therefore, delayed the absorption of fat (Ikeda *et al.*, 2005).

Table 3 s howed the change in serum lipid. Significant difference in HDL-c, LDL-c and VLDL-c has been observed between control positive group and control negative group. The similar finding was reported by Kajimoto *et al.* (2005) and Suzuki *et al.* (2012).

Table 3. Effect of some levels of green tea and green tea rich with different doses of on serum lipoproteins of hyperglycemic rats.

Parameter / Groups		mg/dl. HDL-C	LDL-c	VLDL-c
Healthy rats. Control (-)		63.167 [a] ± 2.978	36.950 [a] ± 5.231	12.133 [a] ± 0.918
Hyperglycemic rats. Control (+)		23.106 [e] ± 2.595	128.726 [d] ± 2.909	16.502 [b] ± 1.209
Hyperglycemic rats fed on basal diet and treated with	Green tea.	33.833 [d] ± 4.119	101.567 [c] ± 2.320	15.600 [b] ± 0.809
	Green tea + 30 mg of powder catechin	38.667 [c] ± 2.066	87.500 [b] ± 1.002	14.500 [ab] ± 0.724
	Green tea + 100 mg of powder catechin	58.833 [b] ± 1.169	35.100 [a] ± 1.079	12.400 [a] ± 0.253

Values are expressed as mean ± SD.Significance at $P<0.05$

[a, b, c, d,] means that values which don't share the same letter in each column are significantly different at $P<0.05$.

[a] is the best result followed by [b] and [c] and so on

Data showed that high-density lipoprotein of hyperglycemic rats, treated with green tea plus high levels from catechin, increased significantly at $P<0.05$, as compared to h yperglycemic groups treated with green tea plus medium level of catechin and treated with green tea alone. This result was in agreement with Kajimoto *et al.* (2005) who reported similar results.

In general, the three treated groups showed decreased serum LDL-c in rats suffering from hyperglycemia. The highest decrease in serum LDL-c recorded for hyperglycemic group, which was treated with oral 25 m l of GTE plus 100 m g of powder catechin, followed by the group treated with green tea plus low level of catechin. Decrease of LDL-c was observed in the present trial, and this result further confirmed the result of the study of Suzuki *et al.* (2012).

The data in this table showed that VLDL-c in serum diabetic rats increased significantly at $P<0.05$, as compared to non-diabetic rats (healthy group). All treated groups tended to have VLDL-c lower than hyperglycemic rats (control +). No statistical significant was observed between groups treated with green tea plus low or high level of catechin for serum VLDL-c.

Maron *et al.* (2003) reported that 120 subjects consumed green tea extract (150 mg of green tea catechins) for 12 weeks, and the green tea extracts decreased serum LDL-cholesterol by 11.3%. This study showed the decrease of LDL and VLDL-c by the consumption of green tea rich with catechin. This is in agreement with several previous observations of the experimental animals and humans (Loest *et al.,* 2002; and Maron *et al.,* 2003). This may be contributed to the excretion of cholesterol into feces (Muramatsu *et al.,* 1986), the activities of lipid enzymes (Lin *et al.,* 2002), the apoB secretion (Yee *et al.,* 2002) and uptake by cells (Bursill *et al.,* 2001).

As mentioned above, results showed that alloxan induced diabetic rats showed significant hypercholestremia as compared to control. The level of serum lipid is usually elevated in diabetes meilleitus, and such an elevation represents a r isk factor for coronary heart diseases (Ikeda *et al.,* 2005). From the data mentioned above, it could be concluded that a daily consumption of green tea rich with catechin significantly reduces risk factors for coronary artery diseases, including total cholesterol, LDL-cholesterol, and triacylglyerides, while it increased the protective HDL cholesterol. Catechins in green tea have been shown to reduce the risk of coronary heart diseases in epidemiological studies. Also, it has been reported that catechins have hypolipidemic and antioxidant effects (Gomikawa *et al.,* 2008).

Table 4. Effect of some levels of green tea and green tea rich with different doses on liver enzymes of hyperglycemic rats.

Parameter		U/L	
Groups		AST	ALT
Healthy rats. Control (-)		94.750 [a] ± 1.118	37.42 ± 1.08 [a]
Hyperglycemic rats. Control (+)		181.000 [a] ± 7.899	71.18 ± 6.52 [d]
Hyperglycemic rats fed on basal diet and	Green tea.	157.167 [d] ± 4.579	64.15 ± 3.742 [c]
	Green tea + 30 mg of powder catechin	112.833 [c] ± 4.355	42.63 ± 3.53 [b]
	Green tea + 100 mg of powder catechin	105.667 [bc] ± 3.445	35.05 ± 1.33 [a]

Values are expressed as mean ± SD.Significance at $P<0.05$

[a, b, c, d,] means that values which don't share the same letter in each column are significantly different at $P<0.05$.

[a] is the best result followed by [b] and [c] and so on

Rats treated with the three oral solutions produced a significant decrease in the levels of serum AST, ALT as compared to control positive group ($P<0.05$). (table 4). The best results in serum AST and ALT observed in hyperglycemic group fed on basal diet and treated with green tea plus high levels from catechin, because this treatment showed no significant differences in serum AST and ALT as compared to the negative control group. The reduction of the serum activities of AST and ALT by green tea and catechin indicates the ability of green tea to stabilize plasma membrane and repair hepatic tissue damages caused by oxidative stress. The protective effect of green tea is due to its antioxidant properties that scavengering free radicals formed in alloxan induced diabetic rats. It has been shown that green tea contains volatile oils antioxidant vitamins like (B, C, E and folic acid), tannins and amino acid (theanine) which is a m ajor available amino acid in green tea. Additionally, polyphenols may also function indirectly as antioxidants (Al-Khashaly, 1997).

According to the results of this study, it c ould be concluded that green tea alone or green tea plus catechin have the ability, through a m echanism related to direct and/or indirect antioxidant property, to provide protective effects against diabetic rats with induced hepatotoxicity through the reduction of hepatic oxidative stress with a subsequent improvement in liver functions manifested by reducing the serum levels of AST and ALT that improve the damage in liver tissues and makes it a good candidate to be tried clinically in this respect.

Table 5. Effect of some levels from green tea and green tea rich with different doses on kidney functions of hyperglycemic rats.

Parameter		mg/dl.		
Groups		Uric acid	Urea nitrogen	Creatinine
Healthy rats. Control (-)		1.82 ± 0.62 [a]	31.54 ± 3.08 [a]	0.71 ± 0.05 [a]
Hyperglycemic rats. Control (+)		2.95 ± 0.22 [c]	56.47 ± 3.33 [c]	1.22 ± 0.06 [c]
Hyperglycemic rats fed on basal diet and treated with	Green tea	2.36 ± 0.21 [b]	54.41 ± 2.74 [b c]	1.29 ± 0.06 [c]
	Green tea + 30 mg of powder catechin	2.03 ± 0.23 [ab]	50.17 ± 3.01 [b]	0.93 ± 0.06 [b]
	Green tea + 100 mg of powder catechin	1.91 ± 0.18 [a]	31.58 ± 2.16 [a]	0.69 ± 0.05 [a]

[a, b, c, d,] means that values which don't share the same letter in each column are significantly different at $P<0.05$.

[a] is the best result followed by [b] and [c] and so on

Table 5 illustrates the changes in serum levels of uric acid, urea nitrogen and creatinine (mg/dl) as a result of the treatments with green tea and green tea plus two doses of catechin for rats suffering from hyperglycemia. From presented data, it could be observed that alloxan injection led to abnormal changes in kidney functions. Serum uric acid, urea nitrogen and creatinine have increased significantly at $P< 0.05$ in diabetic group fed on basal diet, as compared to healthy group fed on the same diet. Serum uric acid, urea nitrogen and creatinine decreased significantly in all treated groups, as compared to control positive group. Using the high levels of catechin plus green tea showed the highest decrease in all investigated kidney functions; no statistically significant differences have been observed between these groups and control negative groups (healthy rats). These results are in agreement with a recent study reporting that the catechins present in green tea possess antioxidant potency and enable the kidney malfunctions resulting from diabetes to return to normal state (Jabber, 2013). This action may be due to its antioxidant properties. Green tea and catechin contains many antioxidant components, which have scavengering free radicals, especially superoxide anions and may thereby protect cells from oxidative stress. The use of antioxidants appears rational in the improvement of kidney diseases therapy (Graham, 1992). Antioxidant therapy has been well documented to help in the improvement of organ functions. The beneficial effect of green tea and catechin may be related to its antioxidant properties (Gomikawas *et al.,* 2008). All diabetic treated groups had much better kidney functions

than those of the non-treated groups. It may be due to the fact that the kidney of diabetic rats showed aggregation of inflammatory, neutrophil and monocytes around the blood vessels. While kidney of GT treated diabetic rats showed degeneration of the cells lining the kidney tubules, vaculation of glomerulus, and weak fatty degeneration in cells of kidney tubules (Jabber, 2013). Alloxan-induced diabetes caused increasing plasma levels of creatinine and urea. Alloxan produces oxygen radicals and oxidative stress in the body that could be helpful in this aspect (Halliwell and Gutteridge 1985).

4. Conclusion

This investigation showed that the continuous ingestion of green tea rich with catechins, especially in high amounts, reduces serum cholesterol levels, and blood glucose without the need for any lifestyle changes. Accordingly, the ingestion of a green tea extract rich with catechins might prevent or decrease the risk of cardiovascular disease and anti-diabetic. At the doses used in this study, green tea or catechin did not induce abnormal changes in hepatic and renal functions. In contrast, doses of green tea or catechin used in this study tended to improve both kidney and liver functions, especially at a high dose of catechin plus green tea.

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Genetic Variability Evaluation Among Iraqi Rice (*Oryza sativa* L) Varieties using RAPD Markers and Protein Profiling

Nawroz Abdul-razzak Tahir*

University of Sulaimanyah, Faculty of Agricultural Sciences, Bakrajo, Sulaimanyah, Iraq

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Abstract

Genetic variability and relationships among ten rice varieties were studied by using 20 decamer random primers and SDS-PAGE methods. Out of 22, 20 Random Amplified Polymorphic DNA (RAPD) primers revealed polymorphism, while the remaining 2 primers showed no reaction. The primers produced a total of 109 bands, of which 62 (56.88%) were polymorphic. The number of polymorphic fragments for each primer varied from 1 to 9 with an average of 3.1 polymorphic fragments. The primer OPC-07 produced the maximum number of polymorphic bands. The RAPD data were analyzed to determine the genetic distance coefficients which ranged from 0.30 to 0.76. Cluster analysis was performed using the Jaccard's coefficient. The dendrogram resolved the selected rice varieties into four major clusters. On the other hand, similar varieties of rice were analyzed for endosperm storage proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to estimate their genetic diversity. Seed storage profiling led to the detection of a total of 12 polypeptide bands. The molecular weights of peptides ranged from 284 to 11 kDa. Polymorphism was not evident in all seed proteins of diverse molecular weights among all rice varieties, but two polymorphic bands (63 and 120 kDa) were found in a high molecular weight region. The results of the genetic diversity, obtained during this study, will be useful for the selection of the parents for developing rice breeding varieties in the future.

Keywords: *Oryza sativa*, Seed, Genetic Variation, RAPD, Polypeptide.

1. Introduction

Rice (*Oryza sativa* L.), belonging to the family Graminae, is the staple food for one third of the world's population (Chakravarthi and Naraveni, 2006). Approximately, 90% of the world's rice is grown in the Asian continent and constitutes a staple food for 2.7 billion people worldwide (Salim *et al.,* 2003; Paranthaman *et al.,* 2009). The world's rice production has doubled during the last 25 years, largely due to the use of improved technology such as high yielding varieties and better crop management practices (Byerlee, 1996). Further scope of crop improvement depends on the conserved use of genetic variability and diversity in plant breeding programmes and use of new biotechnological tools. Molecular characterization can reveal the maximum genetic variation or genetic relatedness found in *Vigna angularis* population (Xu *et al.,* 2000). Chakravarthi and Naravaneni (2006) reported that the usefulness of preservation and conservation of genetic resources since genetic diversity provides information to monitor germplasm and prediction of potential genetic gains. DNA based molecular markers have been proven to be powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among the species of rice (Shivapriya and Hittalmani, 2006). Several molecular markers have been used for studying the genetic variation and relatedness among different populations, species and genes such as RFLP and RAPD (Tingey and Deltufo, 1993; Wu *et al*., 2006; Rahman *et al.,* 2007; Abdul-razzak, 2008; Rawashdeh, 2011; Rajani *et al.,* 2013), SSRs (Prabakaran *et al.,* 2010), AFLP and SNPs (Joshi *et al.,* 2000; Liu *et al.,* 2011).

Nowadays, the PCR-based RAPD is technically simple and cheaper compared to the others methods of molecular markers. RAPD requires small quantity of DNA, and ability to regenerate polymorphisms. In many instances, the small number of primers is necessary for polymorphism identification within species.

On the other hand, genotyping of different species is also necessary for characterization of different accession of crop germplasm, testing varietal purity and registration of newly developed cultivars (Choudhury *et al.,* 2001). Among numerous techniques available for assessing the genetic variability and relatedness among crop germplasm, seed storage protein analysis represents a valid alternative and/or improved approach to varietal identification

* Corresponding author. e-mail: nawroz.tahir@univsul.net or nawrozbiology@gmail.com.

(Mennella *et al.,* 1999; Galani *et al.,* 2011). Seed storage protein markers are polymorphic and environmental influence on their electrophoretic pattern is limited (Sadia *et al.,* 2009). It is a useful tool for studying genetic diversity via sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Abdul-razzak, 2009; Sadia *et al.,* 2009) in a short period of time (Netra and Prasad, 2007). The objective of this study is to determine the genetic relationships among several Iraqi rice varieties based on RAPD markers and protein profiling.

2. Materials and Methods

2.1. Plant Materials and Genomic DNA Isolation

The present paper was carried out at the University of Sulaimani in Iraq during 2012-2013. Ten Iraqi rice varieties (*Oryzae savtiva* L.) have been used for molecular and biochemical analysis. The genetically pure rice varieties, named [Delfino/M239 (Italian origin), Azmar56R (Iraqi origin), Bcdo/M245 (Italian origin), Loto/M269 (Italian origin), Azmar56W (Iraqi origin), Kalar-2 (Iraqi origin), Shawre (Iraqi origin), Zangiswr (Iraqi origin), Kalar-1(Iraqi origin), Pitwen (Iraqi origin)], were collected from the Agriculture Department of Ministry of Agriculture in Sulaimanyah, Iraq. Those varieties were widely cultivated at Sulaimanyah region. The analysis of these varieties leads to the collection of information about the genetic diversity at the genome level. In a 9 cm pot, the healthy seeds were covered with compost, and then the pots kept warm at 25°C. The seedlings germinate moved to a 30 cm pot. Transplants should be placed slightly deeper in the compost than they were previously growing; as this will help them produce side shoots. Plants put in full sun and kept well watered. The pots incubated in greenhouse for six weeks under natural conditions (temperature 28-30ºC). The DNA extraction was carried out from the fresh leaves following cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987) with minor modifications. Fresh 500 milligrams of young leaves were ground to a very fine powder in liquid nitrogen and dispersed in 3 mL of pre-warmed (65°C) CTAB DNA extraction buffer [2% CTAB; 1.4 M NaCl; 100 mM Tris-HCl (pH 8.0); 20 mM EDTA (pH 8.0); 0.2% 2-Mercaptoethanol (added just before use)]. Tubes containing samples were incubated at 65 °C for 60 min in a water bath. The samples were swirled every 10 min. After incubation, the mixture was cooled down to room temperature and emulsified with an equal volume of chloroform: isoamyl alcohol (24:1), and centrifuged at 8000 rpm for 10 min. Following centrifugation, the aqueous phase was collected and nucleic acid was precipitated by mixing with equal volume of chilled isopropanol. The precipitated nucleic acid was centrifuged at 12000 rpm for 10 min and the pellet was washed with 70% ethanol. The DNA pellet obtained was dried and stored in 100 µL TE buffer. In order to remove RNA, DNA was treated with 40 µg RNAse-A at 37 °C for 1 h and stored at 4 °C until use.

2.2. Determination of DNA Concentration by Spectrophotometer

The DNA samples were diluted 100 times with TE buffer. The optical density (OD) of each sample was read at wave length of (260 and 280 nm) with a spectrophotometer. The DNA concentration was calculated by this equation:

DNA concentration (µg/µl) = (50 µg/ml x ODA_{260} x dilution factor)/1000

The OD of diluted dsDNA at 260nm was measured. The dilution factor was 100 (10 µl of extracted DNA in 990 µl of high pure water or ddH_2O). The D260/280 ratio for pure DNA was 1.78-1.85 (Sambrook *et al*., 1989). The final concentration of extracted DNA was 0.5-0.6 µg/µl. A solution of 20 ng/µl was prepared by adding of 20 µl of extracted DNA in 480 µl of ddH_2O. 2.5 µl (50 ng) of this dilution was used for PCR reaction.

2.3. RAPD Amplification Procedure

Twenty two different 10-mer oligonucleotide RAPD primers (Operon Technologies Inc., USA) (Table 1) were used. Each polymerase chain reaction (PCR) was carried out in a 25 µl volume containing 50 ng template DNA, 1.5 mM $MgCl_2$, 0.32 mM dNTPs, 1X *Taq* DNA polymerase buffer, 10 pmol decanucleotide primer and 2 units of *Taq* DNA polymerase. Amplification was performed in a thermal cycler (Corbett Research, Australia) using the following conditions: denaturation at 95ºC for 3 min; 36 cycles of 1 min denaturation at 94ºC, 1 min annealing at 34, 35, 36, 37, and 38ºC and 2 min in extension at 72ºC; and a final extension at 72ºC for 5 min. The RAPD-PCR products were analyzed directly on 1.5% agarose gel in 1X TBE buffer. The DNA was stained with 0.5 mg mL^{-1} Ethidium bromide and visualized and photographed under a UV transilluminator.

Table 1. Twenty two RAPD primers, their sequences and annealing temperature.

	Name of primer	Sequence (5' to '3)	Annealing temperature
1	OPC-07	GTCCCGACGA	38
2	OPC-15	GACGGATCAG	38
3	OPD-08	GTGTGCCCCA	38
4	OPF-06	GGGAATTCGC	38
5	OPF-13	GGCTGCAGAA	38
6	OPF-14	TGCTGCAGGT	38
7	OPF-17	AACCCGGGAA	38
8	OPJ-13	CCACACTACC	38
9	OPK-11	AATGCCCCAG	38
10	OPR-2	GATTCCGCGG	36
11	OPJ-08	CATACCGTGG	35
12	OPD-05	TGAGCGGACA	34
13	OPD-06	ACCTGAACGG	34
14	OPD-07	TTGGCACGGG	34
15	OPD-02	GGACCCAACC	37
16	OPD-10	GGTCTACACC	37
17	OPD-18	GAGAGCCAAC	37
18	OPD-20	ACCCGGTCAC	37
19	OPF-03	CCTGATCACC	37
20	OPH-13	GACGCCACAC	37
21	OPG-14	GGATGAGACC	37
22	OPJ-10	AAGCCCGAGG	37

2.4. Protein Extraction

Variability of the total seed storage proteins was investigated by using SDS-PAGE.

Protein was extracted from dry seeds by grinding them into a fine powder. For total seed protein extraction from individual seed samples, 0.5 g of each variety was taken and then, 1 mL Tris urea buffer [containing 0.05 M Tris-HCl (pH 8.0); 0.4% SDS; 5 M Urea; 2.5% glycerol; 1.5% 2-Mercaptoethanol; 1 mM EDTA and 0.01% (w/v) Bromophenol blue)] was added. The sample was vortexed for 5 m in followed by incubation for 3 hours at room temperature. The crude homogenate was centrifuged at room temperature at 15000 rpm for 10 min. The extracted protein samples were collected as supernatant and pellets were discarded and then stored at (-20°C).

2.5. SDS-PAGE (Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis) Gel

The soluble seed proteins were subjected to SDS-PAGE in gel slabs of 1.5 mm thickness (3.5 cm, 4.5% stacking and 15.5 cm, 15% resolving gels) as described by Laemmli (1970). Electrophoresis was carried out at 80 V for 5 h. The gels were then fixed in solution (10% acetic acid and 40% ethanol) for 15 min with constant shaking and then stained with 0.2% (w/v) Commassie brilliant blue R250 overnight on an electrical shaker. Distaining of gels was carried out for a couple of hours followed by gel preservation, scanning and photography.

2.6. Data Analysis

The data obtained with the technique RAPD were scored in a binary form as the presence or absence (1/0) of bands per each sample. Program of SPSS (version 19) was used to calculate Jaccard coefficient and construction a hierarchical cluster analysis.

3. Results

3.1. RAPD Analysis

The genetic relationship among the ten rice varieties has been carried out using RAPD markers (Figure 1). The results of present study indicate a considerable level of genetic diversity among the studied varieties. Among the 22 primers, 20 primers showed high polymorphic bands (Table 2).

A total of 109 D NA fragments was generated by 20 primers, out of which 62 w ere polymorphic (56.88% polymorphism) (Table 2). These primers produced multiple band profiles with a number of amplified DNA fragment varying from 2 to 14.

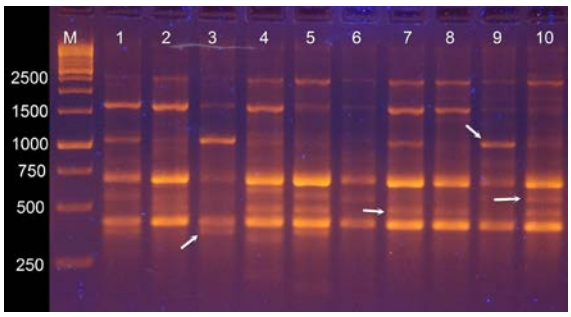

Figure 1. RAPD profiles of ten different rice varieties using primer OPC-15. Lane M: 1kb ladder, lane 1: Pitwen, lane 2: Kalar-1, lane 3: Zangiswr, lane 4: Shawre, lane 5: Kalar-2, lane 6: Azmar56W, lane 7: Loto/M269, lane 8: Bacdo/M245, lane 9: Azmar56R, lane 10: Delfino/M239. The white arrows show polymorphic bands.

Table 2. Total amplified fragments, number of polymorphic bands and percent of polymorphic bands of 20 random primers used under this study.

Primers	Total amplified fragments	Number of polymorphic bands	% of polymorphic bands
OPC-07	14	9	64.3
OPC-15	7	4	57.14
OPD-08	7	2	28.6
OPF-06	10	6	60
OPF-13	6	4	66.67
OPF-14	5	3	60
OPF-17	5	4	80
OPJ-13	5	3	60
OPK-11	9	6	66.67
OPR-2	4	4	100
OPJ-08	2	1	50
OPD-05	6	3	50
OPD-06	2	1	50
OPD-07	8	6	75
OPD-02	3	1	33.33
OPD-10	4	1	25
OPD-18	3	1	33.33
OPD-20	3	1	33.33
OPG-14	2	1	50
OPJ-10	4	1	25
Total	109	62	
Average per primer	5.45	3.1	

The primer OPJ-08 and OPJ-14 gave the minimum number of fragments (2), while the highest number of fragments (14) was obtained by primer OPC-07. Out of 20 primers, only 14 pr imers exhibited 50% or more polymorphism. The number of polymorphic fragments for each primer varied from 1 to 9 with an average of 3.1 polymorphic fragments. The primer OPR-2 yielded 4 bands that were all polymorphic (100%). The primer OPC-07 produced the maximum number of polymorphic bands (9). The genetic distance coefficients, among 10 r ice varieties based on the RAPD fragments, were used to construct a dendrogram (Figure 2).

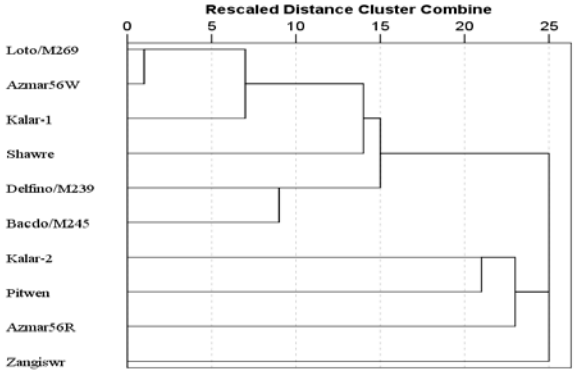

Figure 2. Adendrogram among rice varieties using polymorphic RAPD primers based on Jaccard coefficients.

The dissimilarity matrix among the 10 varieties ranged from 0.30 to 0.76 (Table 3).

Table 3. Dissimilarity matrix based on Jaccard coefficients among various rice varieties using RAPD data.

	Delfino/M239	Azmar56R	Bacdo/M245	Loto/M269	Azmar56W	Kalar-2	Shawre	Zangiswr	Kalar-1	Pitwen
Delfino/M239	0	0.692	0.421	0.561	0.55	0.61	0.524	0.683	0.591	0.619
Azmar56R		0	0.714	0.706	0.697	0.645	0.73	0.655	0.657	0.613
Bacdo/M245			0	0.486	0.375	0.658	0.526	0.737	0.486	0.632
Loto/M269				0	0.3	0.718	0.514	0.694	0.382	0.658
Azmar56W					0	0.711	0.5	0.757	0.412	0.649
Kalar-2						0	0.605	0.714	0.675	0.6
Shawre							0	0.684	0.474	0.579
Zangiswr								0	0.718	0.606
Kalar-1									0	0.579
Pitwen										0

The highest dissimilarity matrix revealed was between Azmar56W and Zangiswr (0.76), while the lowest dissimilarity matrix showed between Azmar56W and Loto/M269 (0.30). From the RAPD-based dendrogram, Zangiswr, which was the most genetically dissimilar, was separated from all other varieties. The varieties were separated into 4 groups at distance 22. Group I contains varieties including Loto/M269, Azmar56W, Kalar-1, Shawre, Delfino/M239 and Bacdo/M245. Group I could be further divided into three subgroups. Sub-group IA contained 3 varieties which include Loto/M269, Azmar56W and Kalar-1. Subgroup IB contained the Shawre variety only; subgroup 1C contained Delfino/M239 and Bacdo/M245. Kalar-2 and Pitwen comprising group II. The third group contained Azmar56R, and the last group comprised Zangiswr.

3.2. Protein Profiling

In this study, the SDS-PAGE of seed proteins of ten rice varieties was carried out to investigate the genetic diversity. Seed storage profiling showed distinct polymorphism in electrophoretic banding patterns and led to the detection of a total of 12 polypeptide bands (Figure 3).

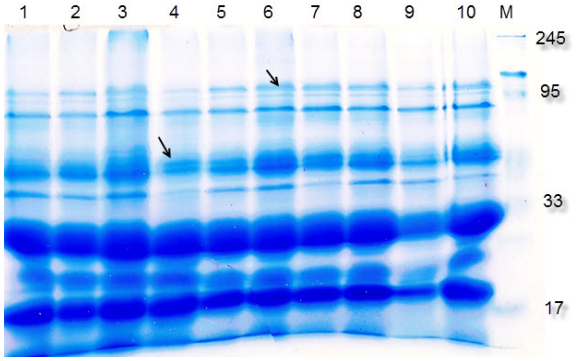

Figure 3. Proteins profiles of ten rice varieties. Lane M: Molecular weight marker size (Roti mark prestained (kDa)), lane 1: Delfino/M239, lane 2: Azmar56R, lane 3: Bacdo/M245, lane 4: Loto/M269, lane 5: Azmar56W, lane 6: Kalar-2, lane 7: Shawre, lane 8: Zangiswr, lane 9: Kalar-1, lane 10: Pitwen. The black arrows show polymorphic bands.

The number of SDS-PAGE bands per varieties varied from 11 to 12. The molecular weights of peptides ranged from 284 to 11 kDa. Polymorphism was not evident in all seed proteins of diverse molecular weights among all rice varieties but two polymorphic bands (63 and 120 kDa as shown in arrow) were found in high molecular weight region. Varieties Delfino/M239, Azmar56R and Kalar-1 possessed a band of 63 kDa. The rice varieties Kalar-2, Shawre, Zangiswr and Pitwen were showed subunit in of 120 kDa. These results suggested that by SDS-PAGE analysis, Iraqi rice varieties have a low diversity in storage protein profiles.

4. Discussion

In this study, the inter-specific genomic polymorphisms in ten rice varieties were evaluated through RAPD-PCR and SDS-PAGE methods. The molecular technique RAPD-PCR analysis is currently used to discrimination of the closely related varieties in order to determine the genetic distance and genetic diversity. In general, RAPD fingerprinting has a number of potential applications including the determination of cultivar's purity, efficient use and management of genetic resources (Ahmed, 1999). The number of amplification RAPD bands (109) was more than that of SDS-PAGE (12). The total number of all amplification bands for each primer was between (2-14). The variation in the number of bands amplified by different primers is influenced by variable factors such as primer structure and less number of annealing sites in the genome. Moreover, the total number of polymorphic bands detected by RAPD primers was much higher than SDS-PAGE. However, the results in this study suggested that the RAPD markers were superior to SDS-PAGE markers in the capacity of revealing more information about the genetic diversity. It was observed, in this study, that the level of polymorphism with primers differed among the varieties. The total number of amplified and polymorphic bands was higher compared to those obtained by Mani *et al*. (2010). The proportion of polymorphism was higher compared to some previous RAPD analysis in rice e.g., 53.85% in six different rice cultivars (Rahman *et al*., 2007), and lower compared to 67.5% (Choudhury *et al*., 2001) 85.02% (Rajani *et al*., 2013) and 72.27% (Skaria *et al*., 2011) in some Indian rice varieties. One of the reasons for this high level of polymorphism can be due to intraspecific variation among the varieties. Information on intraspecific variation from the present study might be useful in making decision for

improvement of rice cultivars. The similarity level, up to 50% in cluster analysis, is indicative of plant derived from interspecific hybridization (Marsolais *et al.,* 1993). Also, Mackill (1995) stated that the use of RAPD markers in DNA fingerprinting of U.S. rice cultivars is feasible. The dendrogram, in this study, divided the rice varieties into 4 groups or clusters. The increasing of number of groups can be due to the presence of high variation among the rice varieties. The number of clusters was higher compared to a previous study, e.g., two clusters in some Indian rice varieties (Rajani *et al.*, 2013). Ren *et al.* (2003) reported that the dendrogram, constructed using UPGMA from a genetic-similarity matrix based on the RAPD data, supported the clustering of distinct five groups with a few exceptions. The success of RAPD analysis in *O. sativa* accessions were also reported earlier (Muhammad *et al.,* 2005; Malik *et al*., 2008; Rahman *et al*., 2007). The analysis of SDS-PAGE can be used to certify the genetic makeup of germplasm (Javid *et al.,* 2004; Iqbal *et al.,* 2005). Thus, profiling of total seed storage proteins through SDS-PAGE for differentiating rice genotypes is well established (Thanh and Hirata, 2002). It also estimates the extent of genetic variation and its geographical distribution in rice germplasm (Asghar, 2004). Tehrim *et al*. (2011) was studied the diversity in protein profiling in rice varieties. The authors found low genetic distance among the studied varieties. In this study, a wide range of protein peptides (low to high molecular weights) did not show the potential for discriminating rice varieties and creates additional variability to supplement existing germplasm. Seed storage proteins polymorphism can be used as a potential molecular marker for varietal identification and economic characterization of rice germplasm as reported earlier (Netra and Prasad, 2007).

5. Conclusion

In conclusion, it can be seen from the present study that there are real variations among the varieties studied. RAPD marker was found to be powerful tool to analyze the genetic structure of different varieties of rice. Using RAPD markers in this study give an early and correct result about the variations among the varieties before starting a breeding program. This study showed that the numerical analysis of seed protein profiles were not sufficient as a typing tool for the differentiation of rice varieties.

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Macroinvertebrate Community and Pollution Tolerance Index in Edion and Omodo Rivers in Derived Savannah Wetlands in Southern Nigeria

John O. Olomukoro and Abdul-Rahman Dirisu*

Department of Animal and Environmental Biology, Faculty of Life Sciences,University of Benin, Benin City, P.M.B. 1154, Nigeria.

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Abstract

We carried out a study of the macroinvertebrates community and pollution tolerance index (PTI) in Edion and Omodo Rivers in Agbede wetlands for eight months (from March to October, 2010). Two stations, upstream and downstream, were chosen for each river. Benthic fauna was sampled fortnightly, using the modified Ekman Grab designed for shallow waters. Eight major taxonomic groups comprising 33 taxa were collected in this study. The dominant groups by percentage density occurrence were Ephemeroptera (45.4%) > D iptera (24.7%) > Decapoda (24.03%). The number of taxa was least for station 2 (14) and was highest (23) for station 4, with highest population density recorded for station 1 (211 individuals). The heterogeneity indices revealed higher values for evenness across the stations except, for station 1 (0.3574). Meanwhile, PTI values showed moderate water quality with range of values from 7 to 16.

Keywords: Macroinvertebrate, Biological Diversity, Tolerance Index, Aquatic regimes.

1. Introduction

In the last few decades, aquatic ecologists have focused more on water quality, resources management and sustainable utilization. Research in aquatic ecology becomes more challenging when coupled with climate change phenomena. Inundation, siltation, agriculture and deforestation outside industrialization and urbanization, pose greater challenges to aquatic regimes.

The use of benthic macroinvertebrates to ascertain the overall health status of aquatic environments remains the most suitable, reliable, and the most widely acclaimed method globally. In this study, we attempt to define pollution tolerance index (PTI) as a method of measuring the overall health status of aquatic bodies through the use of macrobenthic invertebrates. Macrobenthic invertebrates are useful bio-indicators in understanding the ecological health of an aquatic ecosystem, rather than using chemical and microbiological data, which at least give short-term fluctuations (Ravera, 2000; Ikomi *et al.,* 2005; George *et al.,* 2009). Odiete (1999) discussed the use of benthic macroinvertebrates in the assessment of freshwater bodies. Benthic invertebrates were used as bio-indicators for studies of impact of environmental perturbations on the aquatic ecosystems (Lenat *et al.,* 1981; Victor and Ogbeibu, 1985). They are considered important because they reflect the cumulative effects of the present and past conditions; also they have low mobility (i.e. are sedentary or sessile or nearly) and life cycles of several weeks and or years.

Biomonitoring studies and the use of macroinvertebrates to rate the quality of water bodies which include both lotic and lentic types have been widely reviewed elsewhere (Ogbeibu and Oribhabor, 2002; Imoobe and Ohiozebau, 2009; Omoigberale and Ogbeibu, 2010; and Olomukoro and Dirisu, 2012). Macroinvertebrates, which were utilized in aquatic pollution studies, included: Mayflies (Ephemeroptera), caddisflies (Trichoptera), stoneflies (Plecoptera), beetles (Coleoptera), crayfish and amphipods (Crustaceans), aquatic snails (Mollusca), biting midges (Chironomids) and leeches (Hirudinea) in Nigeria, North America and Europe.

Existing works on t he benthic fauna of Agbede wetlands are quite scanty and included: Olomukoro and Dirisu (2012) who dealt with the macroinvertebrate community of a post lindane treated stream with a

* Corresponding author. e-mail: dedonrahman10@yahoo.com.

record of 43 taxa comprising 532 individuals, and Olomukoro *et al.* (2013) on the ecological survey of macrobenthic invertebrates of selected ponds in Agbede flood plain, where they encountered 10 groups and 1,031 individuals.

The purpose of this study is, firstly, to present a general account of the benthic macroinvertebrates species composition and diversity as well as determining the water quality using the benthic fauna, and, secondly, to establish the pollution tolerance index (PTI) for the water bodies. So this study would hopefully be a reference archive for future studies of the water bodies in the subject area.

2. Materials and Methods

2.1. Study Area

Agbede wetlands are situated within a d erived savannah ecozone that lies between 06o16.3E, 06o 18.7E and 06o52.2N, 06o55.4N. The climate of Agbede and its environs is not stable. It is comparatively like that of Benin and its environs as a rhythm of rainfall occurs in conjunction with the movement of the Southern-West monsoon wind across the Atlantic Ocean and the timing of this movement varies from year to year. There are two distinct annual seasons associated with this region: the rainy season which begins in April and terminates in October, and the dry season which starts from November and terminates in March. Rainfall for 2010, ranged from 158.4 – 608.7mm with the lowest recorded in the month of May (158.4mm) and the peak recorded in the month of September (608.7mm). The mean rainfall value was (356.76mm).

Station 1: This station (06o55.4N and 06o16.4E) is directly located on the Benin and Auchi/Abuja high way by Edion River bridge. There was an occasional inundation of the surrounding banks in the months of July, August, and September. The station is surrounded by a number of ponds on its banks as well as settlements. It is subjected to all forms of human activities. The velocity of flow was determined to be 0.372m/s and the average depth 76cm. Lemna pausicostata (duckweed) was found floating here.

Stations 2: This station (06o55.4N and 06o16.4E) is located about 1,050m downstream of the same river Edion. There are lots of aquatic macrophytes (Lemna pausicostata) and algae (Chlorophyta sp.) here. There is vegetation of shrubs and trees like Bambusa bambusa and Anacadium occidentalis on the banks. Cattle dung is commonly associated with this station. The velocity of flow was 0.24m/s and the depth was 82cm.

Station 3: This station (06o52.2N and 06o16.8E) is the upstream of River Omodo at Odighie community, by Ewora-Idegun Road. It is surrounded by Bambusa bambusa tickets. Macrophytes are rare here. Velocity of flow was 0.27m/s and the depth was less than 35cm. In dry season, it flows in South–North direction. Human activities in this station include washing, bathing, fishing and fermentation of starch (cassava). It is the only source of domestic water to the immediate communities.

Station 4: This station (06o52.2N and 06o18.7E) is located about 840m downstream of stations 3. Here, features and human activities are similar to those in station 3 except the fermentation of starch activities. Current velocity was equally high here (0.29m/s) and the water depth did not exceeded 35cm.

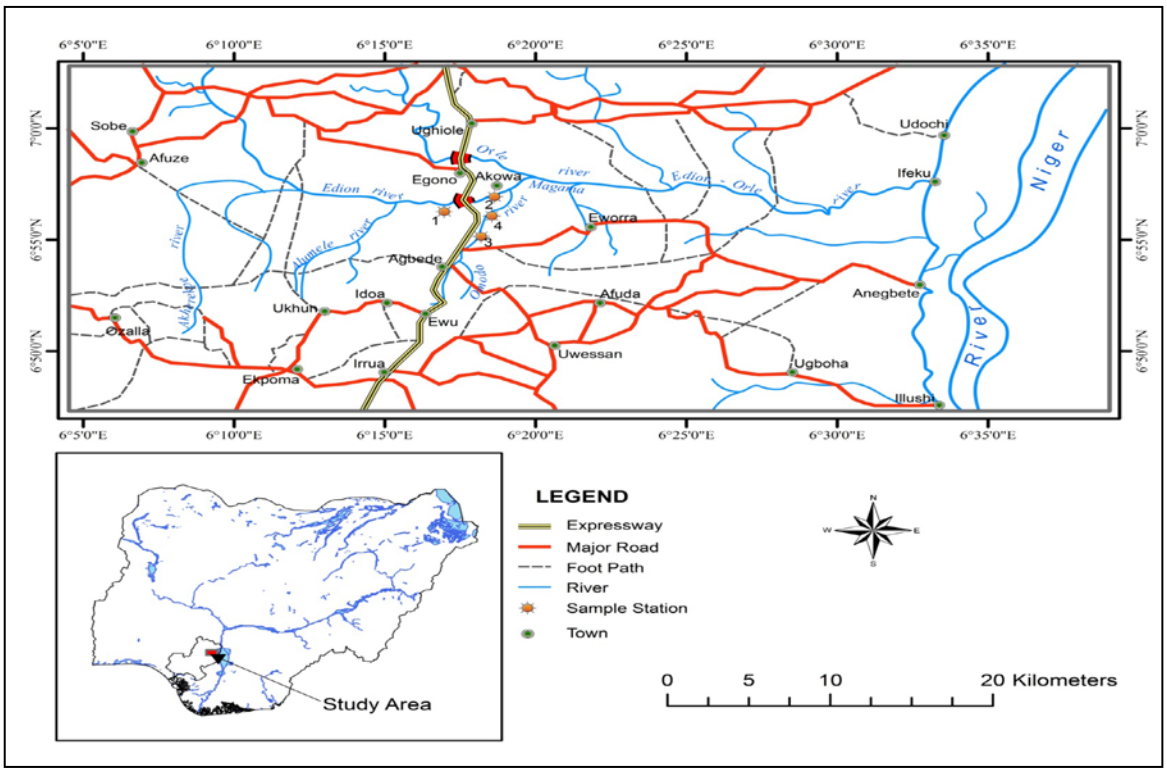

Figure 1. Map of the study area.

2.2. Benthic Fauna

Macrobenthic fauna were collected by sampling the rivers substratum using an Ekman grab (made by Hydrobios, West Germany) as recommended for sand and silt (Hynes, 1961) as well as on the Bank-roots and Macrophytes (Olomukoro and Dirisu, 2012). Contents trapped by the grab were processed as described by Olomukoro and Ezemonye (2000). For the bank-roots and macrophytes, benthos was collected using a hand net made of mesh bolting silk of 100µM. The sediment was collected in a plastic container of 15 liters volume; water was added and stirred vigorously while the floating fauna were sieved using 150, 250 and 500 micron sieve sizes and the unfloated fauna were handpicked. The benthic macroinvertebrates were identified using the literature (Olomukoro, 1996).

2.3. Determination of Pollution Tolerance Index (PTI)

Pollution tolerance Index (PTI) was determined and computed by utilizing the methods earlier used by Klemm *et al.* (1990) and Izaak Walton League of America (1994). Three groups of macroinvertebrates were chosen and assigned a multiplication factor of 3 for the pollution sensitive group (Ephemeroptera, Trichoptera and Coleoptera), 2 was assigned to the facultative or somewhat tolerant group (Anisoptera, Zygoptera and Decapoda) and 1 to the pollution tolerant group (Pond snails, Oligochaetes and Leech) as utilized in this study after which they were sum to get the PTI values for the four stations and the same was done for the monthly, and spatial variations. Values obtained were thereafter compared with established standard values. Unpolluted water had values between 23 a nd above, excellent, 17-22 good. Polluted water had 11-16 fair and < 10 poor.

The water quality of stations 2, 3 and 4 had different results. Although station 1 had a PTI value of 16; PTI values of stations 2 and 3 were 7 for each; station 4 had 10.

2.4. Statistical Analysis

Biological indices, such as taxa richness, evenness (E) and Shannon-Weiner diversity, were computed using paleontological statistics software (PAST). The graphs were plotted with MS-Word Excel.

3. Results

3.1. The Macrobenthic Fauna

A total of 33 m acroinvertebrate taxa composed of 1 species each of Oligochaeta, and Decapoda (crab), 3 species of Crustaceans (shrimps), 6 s pecies of Ephemeroptera, 3 species of Anisoptera and 3 species of Zygoptera. Others include; Coleoptera 1 species, Trichoptera 3 species, Ceratopogonidae (Diptera) 1 species, Chironomidae (Diptera) 8 species, Cullicidae (Diptera) 2 species, Mollusca 2 species. The relative percentage composition of the taxonomic groups collected from the four stations were: Oligochaeta (0.972%), Decapoda (24.027%), Ephemeroptera (45.420%), Odonata (3.611%), Coleoptera (0.139%), Trichoptera (0.833%) Diptera (24.722%) and Mollusca (0.277%). (Table1 and 2).

Table 1. Summary of macrobenthic invertebrate communities present in Edion and Omodo Rivers of Agbede Wetlands, Edo State (March to October, 2010).

	Stations				
Taxa	STN1	STN2	STN3	STN4	Total
OLIGOCHAETA					
Nais sp.				7	1
DECAPODA					
Unidentified crab larva	-	-	-	2	2
Caridina africana	14	15	40	21	90
Caridina gabonensis	14	17	8	14	53
Desmocaris trispinosa	17	8	2	1	28
EPHEMEROPTERA					
Adenophlebiodes sp.	1	2	-	-	3
Baetis sp.	45	31	32	16	124
Centroptilum sp.	10	14	2	6	32
Cloeon sp.	84	49	21	6	160
Cloeon bellum	-	-	1	2	3
Ephemerella ignita	2	2	-	1	5
ODONATA					
Anisoptera					
Aeschna sp	1		5		6
Libellula sp.	1	1	3	4	9
Aphylla sp			3	4	7
Zygoptera					
Coenagrion sp.	1				1
Enallagma sp.	1			1	2
Lestes sp.				1	1
COLEOPTERA					
Dytiscus marginalis	1				1
TRICHOPTERA					
Hydroptila sp.	1				1
Limnophilus sp.	2				1
Unidentified larva		1		2	3
DIPTERA					
Anopheles sp.		10		1	11
Ceratopogonidae					
Palpomyia sp.	2				2
Chironomidae					
Chironomus fractilobus	6	4	18	22	50
Chironomus travalensis	6	1	8	4	19
Chironomus sp.		5	17	32	54
Tanypus sp.			5		5
Tanytarsus sp.			13	12	25
Clinotanypus sp.				7	7
Pentaneura sp.			1	3	4
Insect larva				1	1
MOLLUSCA					
Hydrobia sp.	1				1
Planorbis crista	1				1
Total no. of individuals	211	160	179	170	720
Total no. of species	20	14	16	23	
Pollution Tolerance Index (PTI)	7	4	3	5	

Table 2. Relative percentage composition of taxonomic groups including; the dominant and subdominant, in the study area**.**

Groups	Taxa (%)	Number of Individuals	% Occurrence
Oligochaeta	3.03	7	0.972
Decapoda	12.12	173	24.027
Ephemeroptera	18.18	327	45.420
Odonata	18.18	26	3.611
Coleoptera	3.03	1	0.139
Tricoptera	9.09	6	0.833
Diptera	30.30	178	24.722
Mollusca	6.061	2	0.277
Total	33	720	100

A total of 720 individuals belonging to 33 species were recorded during this study. At station 1, the total numbers of taxa were 20, and the number of individuals was 211. At station 2, the numbers of taxa and individuals were 14 a nd 160, respectively. While, at stations 3 and 4, the numbers of taxa and individuals were 16 and 179, and 23 and 170, respectively. (Table 3)

3.2. Diversity Indices

Diversity indices were applied to the macroinvertebrates using the computer software package tool called PAST (Palaeontological Statistics) to determine taxa richness, evenness, Shannon diversity, dominance index and Margelef index (Table 3).

Table 3. Diversity of the macroinvertebrate community of the selected rivers, in Agbede Wetlands

Description (Indices)	Station 1	Station 2	Station 3	Station 4
Number of samples	16	16	16	16
Number of Taxa	20	14	16	23
Number of Individuals	211	160	187	170
Taxa Richness (d)	0.2236	0.1675	0.1170	0.0965
Shannon diversity (H)	1.967	2.082	2.414	2.625
Evenness (E)	0.3574	0.5729	0.6210	0.6003
Dominance Index (C)	0.7764	0.8325	0.8830	0.9035

Taxa richness was highest in station 1 (0.2236) and least in station 4 (0.0965). Station 2 and 3 had very close values. There was a gradual decrease from station 1 to 4.

Station 4 ha d the highest general diversity value (2.625), while, station 1 recorded the least value (1.967). An increasing order from station 1 t o 4 w as observed here. The evenness of these species was fairly low across the stations. The values were < 1 in each station. However, it was lowest in station 1 (0.3574). Station 4 had the highest value (0.9035) for dominance when compared with station 1 (0.7764).

3.3. Pollution Tolerance Index (PTI)

PTI was utilized to assess the overall health status of the study stretch to ascertain the extent of human impact on rivers (Table 4). Unpolluted water would have values between 23 and above, excellent, 17 – 22, good. While polluted water would have 11 –16 fair and < 10 poor.

The water quality of stations 2, 3 and 4, had different results. Although station 1 had a PTI value of 16, stations 2 and 3, PTI values of 7 each and station 4 had 10.

Table 4. Summary of the overall health status of the water quality in the wetlands

Rivers/Stations		PTI	Water Quality Status
Edion	1 (Upstream)	16	Fair
	2 (Downstream)	7	Poor
Omodo	3 (Upstream)	7	Poor
	4 (Downstream)	10	Poor

At stations 2, 3 and 4, the pollution tolerance Index values were low, indicating a poor water quality status (Table 5). This is an indication that organisms such Odonata (Zygoptera and Anisoptera), Oligochaeta, Chironomids among others were dominant. The monthly pollution tolerance indices (Figure 2) for all the stations generally recorded low water quality values. But, PTI values were generally higher in the month of September throughout the sampling period and generally lowest in the month of March. Meanwhile the PTI value (11) was highest at station 1 (Edion River), in the month of June and least in March. At station 4, PTI (9) was highest in the month of September.

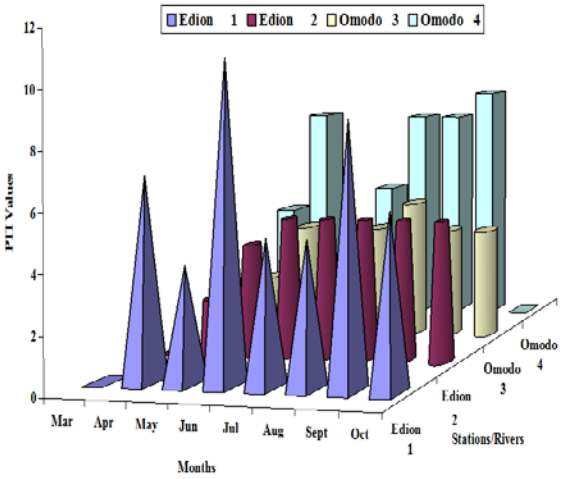

Figure 2. Monthly fluctuation of pollution tolerance index at the stations.

4. **Discussion**

All the organisms found in this study have been variously reported elsewhere in Africa and in the tropics at large (Green, 1979; Olomukoro and Ezemonye, 2000; Imoobe and Ohiozebau, 2009; and Olomukoro and Dirisu, 2012).

Oligochaeta were very poorly represented (3.030% by taxa and 0.972% by individual). Olomukoro (1996) recorded several species of Oligochaetes in Warri River including *Nais* sp. It was reported that the abundance of Oligochaeta was due to the richness of the immediate substrate in organic matter. This may be due to their feeding habits as they are deposit feeders and they are

tolerant to silting, decomposition and flow rate than other macrobenthic groups.

Among the Decapoda, a high abundance of shrimps was recorded. Three species of shrimps (*Caridina gabonensis*, *Caridina africana*, and *Desmocaris trispinosa*) were recorded. The diversity and high population density of shrimps have been widely reported in Nigeria (Ogbeibu and Victor, 1989; Olomukoro, 2002; and Omoigberale and Ogbeibu, 2010).

Ephemeroptera showed relatively high diversity. Three families (Leptophlebiidae, Baetidae and Ephemiridae) and six species which include *Ademophleboides* sp., *Baetis* sp., *Centroptillum* sp., *Cloeon* sp., *Cloeon bellum* and *Ephemerella ignita* were recorded. The abundance of these species is an indication of good water quality and may be due to habitat preference and availability of food.

Odonata are known to be facultative animals as they are mostly associated with moderately polluted waters. A total of six species of Odonata was recorded. The diversity of Odonata has been utilized in biomonitoring of fresh water bodies. Generally, the diversity was poor except in station 3 where, *Aeschna*, *Libellula* and *Aphylla* sp. (members of the suborder Anisoptera) had a little higher numbers and in station 4, respectively.

Only one species of Coleoptera (*Dytiscus marginalis*) was represented in this group. Coleoptera are known to be mostly associated with lentic water bodies such as ponds and lakes. They are sheltered by macrophytes. Only two species of Trichoptera were recorded in the month of June and high density is indicative of good water quality. The density was very low when compared with the work of Imoobe and Ohiozebau, (2009) for Okhuo River in Benin City. This may be as a result of the fact that they are mostly present in well oxygenated and fast running waters.

Diptera was the second largest group after Ephemeroptera. Three families (Ceratopogonidae (1 species), Chironomidae (8 species) and Culicidae (1 species)) were recorded throughout the study. *Chironomus* sp., *Chironomus fractilobus* and *Chironomus travalensis* were dorminant with the highest occurrence in stations 3 and 4, respectively. *Tanypus* sp., *Pentaneura* sp., *Clinotarnypus* sp. and unidentified insect larva recorded a low density and were restricted to stations 3 and 4 only. The relative abundance of these taxa has been emphasized by Wallace and Hynes (1981). They may have been so favored by the conditions of the immediate substrates, which include the alkaline pH in the study area.

Mollusca were poorly represented with two species (*Hydrobia* sp and *Planorbis crista*). The younger life forms inhabit polluted environment, hence their great importance in monitoring pollution stress of wetlands. The two species and two individuals Mollusca recorded were restricted to station 1 only. Molluscs are mostly associated with lentic ecosystems, so that the restriction may be attributed to sampled station type, with an element of backwaters during the dry season months.

The diversity of the macroinvertebrates fauna was low when compared with the number of taxa recorded in some other water bodies. Victor and Ogbeibu (1985) recorded 55 taxa in Ikpoba River, Olomukoro and Egborge (2003) recorded 138 taxa in Warri River, and Omoigberale and Ogbeibu (2010) recorded 57 taxa in Osse River. The low taxa and the total number of individuals recorded for these two Rivers in Agbede – wetlands may be very surprising. This is in contrast with the report of Victor and Victor (1992) who stated that brackish water are known to record low number of taxa. However, the low number of taxa may be due to the choice of sampling stations such that the activities impacting such habitats are colossal, and hence, do not support the ecology of benthos.

The use of pollution tolerance index was subject to problems of the inherent variations in the nature of the aquatic communities in the study area and it was observed that the list of groups of organisms had different degrees of tolerance to their environments. A high pollution tolerance index of 16 was recorded for station 1 (upstream of Edion River) in the wetlands, where organisms, such as Ephemeroptera, Coleoptera and Trichoptera, which are least tolerant to pollution, were represented in a variety of species. It obviously indicated that the waters at station 1 were of low pollution (fair), which could be described as Oligosaprobic in quality. Pollution sensitive organisms such as Trichoptera and Coleoptera had no species recorded outside station 1 and Ephemeroptera density was lower. However, Odonata and Decapoda had relatively high abundance and high species diversity; and densities in stations 2, 3 and 4. The high density of Chironomidae in these stations 2, 3 and 4 was an indication that the waters were relatively polluted or mesosaprobic in quality. The relatively high diversities of Ephemeroptera, Coleoptera and Trichoptera as utilized in this study may be due to habitat preference resulting from presence of very low pollutants/contaminants levels, as directly impacted into the immediate substrates. Trichoptera are mostly present in uplands streams or rivers which are well oxygenated when compared to low land fresh waters like in this case.

In conclusion, organisms, which are most sensitive to pollution, such as Coleoptera and Trichoptera as utilized in this study, were completely absent in stations 2, 3 and 4, an indication that the waters were relatively poor in the above stations, considering the PTI values (7, 7 and 10). Thus, the density of the families of the Ephemeroptera group in stations 2, 3, and 4 dropped when compared with that at station 1. One peculiar observation is that some of the insects like Ephemeroptera prefer slow running water environment with macrophytes, which support their ecology. We strongly advocate that organic farming should be encouraged and practiced as run-off from agricultural sites contains lots of contaminants; and washing of all kinds and channeling of industrial effluence be discouraged. Nomadic agriculture should be restricted to designated sections along the rivers catchment. The need for long-term hydrobiological investigation, with elaborate emphasis on water quality monitoring and the ecology of macrobenthic fauna is so much

recommended for the safety and conservative use of our fresh water bodies and the resources.

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Bacteriocin Typing of *Staphylococcus aureus* Isolated from Different Sources in Ibb City, Yemen

Fadhl A. Al-Gosha'ah*; Shayma M. Al-Baker and Khadijah Y. Al-Hetar

Department of Medical Microbiology – College of Science – Ibb University, Yemen

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Abstract

A total of 207 s pecimens were collected from different sources including patients, health care staff and hospital environment in Ibb city, Yemen. The study used the bacteriocin produced from active producer strains in typing of *Staphylococcus aureus*. Depending on the morphological, cultural and biochemical characteristics, 54 (26.09%) isolates of *Staphylococcus aureus* were identified. An antibiotic sensitivity test was done for the bacterial isolates, and the results showed that there were multiple resistant antibiotics. The Staphylococcin production of these isolates has been detected by using wells assay. Fifty one isolates were Staphylococcin producer. Four isolates (staph19, staph25, staph28 and staph43) were chosen as good Staphylococcin producers, and used locally as indicators in bacteriocin typing. Depending on *S. aureus* typing, the isolates fell into (9) groups. The most numerous group was characterized by susceptibility to all four staphylococcin and comprised 61.11% isolates of *S. aureus*, while the lowest numerous were found in three groups with a ratio of 1.85%; the remaining groups had little percentages ranging from 3.70% to 11.11%. We observed that about (94.44%) of the isolates were bacteriocin producers, and among them, four isolates had a strong bacteriocin production. Based on typing, most isolates had one pattern.

Keywords: Bacteriocin, Staphylococcin, Typing, *Staphylococcus aureus*, Infection,Antibiotics, Resistance.

1. Introduction

Staphylococcus aureus is one of among Staphylococci belonging to the *Micrococaceae* family, that can cause under appropriate conditions, minor skin infections (pimples, boils, cellulites, toxic shock syndrome, impetigo and abscesses) as well as life threatening diseases (pneumonia, meningitis, endocarditis and septicemia); it is also responsible for severe morbidity and mortality worldwide (Singh and Prakash, 2010) and (Noskin *et al.,* 2005). Staphylococcal infections are frequently treated with antibiotics and, consequently, acquire resistances to antibiotics that evolve (Sabra and Farag, 2012). The resistance to antimicrobial agents is an increasing global problem worldwide (Duran *et al.,* 2012). Controlling and understanding *S. aureus* in both hospital and community settings is a signii.cant public health concern that is underscored by the continuous evolution and development of antibiotic-resistant *S. aureus*.

Recent advances in biotechnology allow molecular epidemiology to play an increasing role in the control of *S. aureus* (Arbeit, 1997). Within the genus *Staphylococcus*, bacteriocins, also called Staphylococcins, have been reported from several species, and already been described and characterized in details (Hena and Sudha, 2011). Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria (Cleveland *et al.,* 2001). Bacteriocin-like inhibitory substances (BLIS) are generally described as antagonistic bacterial agents with an active protein moiety; immunity of the producer strain to its own substance is genetically determined (Saeed *et al.,* 2004). Production of bacteriocin is a highly important factor in microbial ecology. Bacteriocins have found widespread application in epidemiological studies as specific markers for bacteria. Various typing schemes have been based upon either the production of, or sensitivity to a range of different bacteriocins (Guven, 2000). Staphylococcal bacteriocins are lethal to strains belonging to the same or related species and act by binding to surface receptors followed interaction with an intracellular target. It has a broad activity spectrum against many Gram-positive (e.g. corynebacteria, listeriae, streptococci and bacilli) and Gram-negative bacteria (e.g. *Neisseria gonorrhoeae* and *Escherichia coli*) (Rogolsky and Wiley, 1977; Kader *et al.,* 1984). It is apparent from the reviews and from the recent studies about staphylococcins that the main effort is directed towards the discovery of as many bacteriocin producing strains as possible, which can be used in

* Corresponding author. e-mail: Fad974@yahoo.com.

characterization of staphylococcal strain. Bacteriocin synthesis is a valuable character of some staphylococcal strains (Skalka, 1986). On the other hand, studies on the possibility of typing *S. aureus*, on the basis of their sensitivity to bacteriocins, are rarely published. Therefore, the purpose of this study is to use the production of bacteriocin from active strains in typing of *S. aureus*.

2. Material and Methods

2.1. Bacteriological Study

A total of 207 specimens were collected from different sources including patients, health care staff and hospital environment in Ibb city, Yemen, from April to September, 2012. For the isolation and identification of *S. aureus*, each specimen was identified, depending on the morphology, cultural characteristics and biochemical reaction (Baron and Feingeld, 1990). Fifty four isolates of *S. aureus* were subjected to API Staph System tested to confirm the identification of this pathogen.

2.2. Antimicrobial Susceptibility Test

The antibiotic susceptibility pattern of all isolated *S. aureus* was tested by 9 antibiotics, determined by the modified Kirby-Bauer disc diffusion technique. In brief, *S. aureus* isolates were grown overnight on nutrient agar at 37°C, and the colonies were suspended in sterile saline water equivalent to a 0.5 McFarland standard (1.5×108 CFU/ml). The suspension (100 µl) was spread over the Mueller-Hinton agar. Then, the antibiotic disc was transferred aseptically on to the surface of the inoculated Muller Hinton plates, and the plates were incubated at 37°C for 18 hrs. (Ehinmidu, 2003). The diameter of the zone of inhibition produced by each antibiotic disc was measured and recorded, and the isolates were classified as "resistant" or "sensitive" based on the standard interpretative according to CLSI (formerly NCCLS) guidelines.

2.3. Bacteriocin typing of S.aureus

2.3.1. Investigation of the Efficient Strains Producing Staphylococcin

Five staphylococci isolates (three isolates were selected from our study which are sensitive to most antibiotics (staph4, staph11 and staph31), and two standard strains (obtained from the Central Laboratories: ATCC 12345 and ATCC 98765)) were used as basic indicator strains to determine the most producing staphylococcin isolates, by well diffusion method (Rasool *et al.,* 1996). Nutrient agar plates were inoculated with 100 µL of each basic indicator strains after growing them in a Brain-Heart infusion broth and diluting appropriately to a 0.5 McFarland standard (1.5×108 CFU/ml), then left to dry at room temperature for a period (10-15 minutes). Wells (6 mm) were cut into the plates and 100 µL of supernatant fluid after centrifuged at 5000 × g for 10 min of the isolates were placed into each well. Plates were incubated at 37 °C for 24 hrs. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells.

2.3.2. Typing of S. aureus Strains

Four staphylococcal isolates (staph19, staph25, staph28 and staph43) were chosen as good Staphylococcin producers according to their widest inhibition zone on the basic indicator isolates. Then the four isolates (producers) were tested against fifty four *S. aureus* (Indicator) by well diffusion method, as described earlier (Rasool *et al*., 1996).

3. Results

3.1. Isolation and Identification

From the 207 specimens collected from different sources, only 54 isolates were found to be *S. aureus*, with the ratio 26.09% of the total isolates (Table1). Data analysis, based on the sites of infection (Table2), shows that 16 (29.63%) samples were recovered from nose infection, 11 (20.37%) samples from wound infection, 8 (14.81%) and 5(9.26%) from urine infection and ear swabs, respectively. While samples taken from health care staff showed that 7 (12.96%) were nasal isolates and 3 (5.56%) were skin swabs. But samples taken from the hospital environment showed the lowest percentages: 1 (1.86%) were operation room swabs, and 3 (5.56%) were surgery room swabs.

Table 1. Represents the prevalence of *Staphylococcus aureus* isolated from different sources of infection

Sources of sampling	No. samples	No. isolates	(%)
Patients	125	40	32
Health care staff	40	10	25
Hospital environment	42	4	9.52
Total	207	54	26.09

Table 2. Distribution of *Staphylococcus aureus* isolated from different sources patients based on sites of sampling

Sources of sampling	Site of Sampling	Number	(%)
Patients	Wound	11	20.37
	Ear	5	9.26
	Urine	8	14.81
	Nasal	16	29.30
Health care staff	Skin	3	5.56
	Nasal	7	12.96
Hospital environment	Surgery room	3	5.56
	Operation room	1	1.86
Total		54	100

3.2. Antibiotic Susceptibility Test

All isolates (54) of *S. aureus* exposed to nine antibiotics are used in this study. The resistance phenotype obtained was as follows: all isolates were resistant to ampicillin with ratio (100%), penicillin G (94.44%), gentamycin (77.78%), erythromycin

(68.52%) and cephalexin (57.41%). Vancomycin and amikacin had moderate effects on the isolates (37.04%) and (35.19), respectively. However, most isolates were highly susceptible to ciprofloxacin (75.93%) and ofloxacin (81.48%), as shown in (Figure 1).

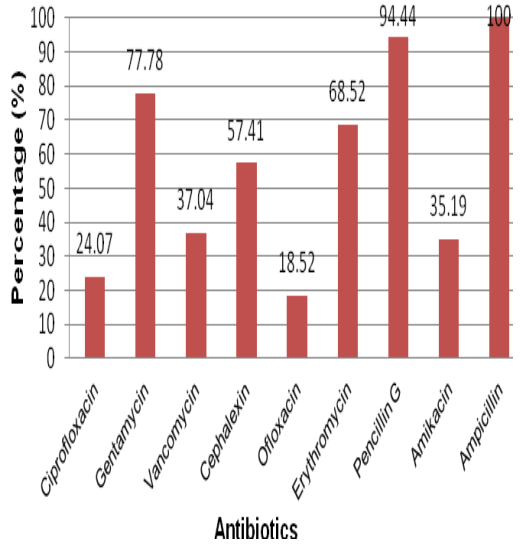

Figure 1. Percentage of Antibiotic-resistance of *S. aureus* isolates from different sites.

3.3. Bacteriocin Typing of S. Aureus

Among the 54 *S. aureus* isolates, four bacterial isolates ($staph_{19}$, $staph_{25}$, $staph_{28}$ and $staph_{43}$) produced an efficient staphylococcin, identified by wells diffusion method, depending on the widest inhibition zone and the highest sensitive number of the basic indicator isolates. These isolates were used as indicator local in bacteriocin typing, since they have characters compatible with the bacterial strains producing staphylococcin to follow the epidemic and the spread of *S. aureus* isolates in Ibb city, Yemen.

Four staphylococcal producer strains, previously selected and used as indicator in bacteriocin typing, were tested against fifty four isolates of *S. aureus.* Most of these isolates were susceptible to the staphylococcin of the producer isolates, the results showed that 51 isolates with a ratio (94.44%) were sensitive dissimilar to staphylococcin (Table 3), (Figure 2).

Stapylococcin of $staph_{19}$, $staph_{25}$, $staph_{28}$ and $staph_{43}$ were inhibited 46, 46, 41 and 45 with ratios of 85.19%, 79.63%, 75.93% and 83.33% of the tested isolates, respectively (Table 3). Depending on the sensitivity to the staphylococcin used, the isolates of *S. aureus* were classified into nine groups. The most numerous group was characterized by the susceptibility to all four staphylococcin and comprised 61.11% isolates of *S. aureus*, while the lowest numerous were found in three groups with ratio 1.85% ; the remaining groups had little percentages ranging from 3.70% to 11.11%.

Table 3. Susceptibility patterns of bacteriocin typing of *Staphylococcus aureus*

Typing	Producer isolates				Total	No. of groups
	$Staph_{19}$	$Staph_{25}$	$Staph_{28}$	$Staph_{43}$		
results	+	+	+	+	33	1
	+	+	+	-	4	2
	+	+	-	+	6	3
	+	-	-	+	1	4
	+	-	-	-	2	5
	-	+	+	+	3	6
	-	-	+	+	1	7
	-	-	-	+	1	8
	-	-	-	-	3	9
Total	46	46	41	45	54	9

+ = inhibition of growth - = without effect

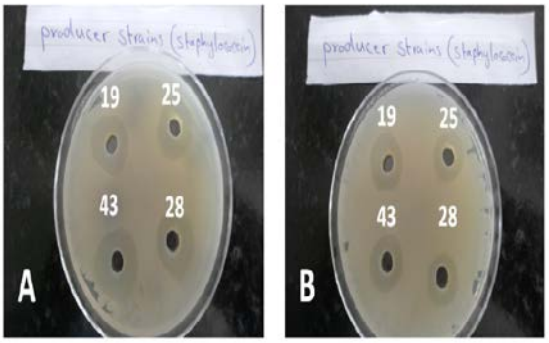

Figure 2. Effect of staphylococcin producer isolates (staph19, staph25, staph28 and staph43) on A) staph16 B) staph38

4. Discussion

The widespread use of antibiotics has been responsible for the development of numerous problems, including the emergence of multi drug resistance bacteria, an increased number of acquired infections from community and hospitals, and increased health care costs (Snyder *et al.,* 2000). In this study, all isolates of *S. aureus* were resistant to ampicillin while most of these isolates were resistant to penicillin-G, which agrees with the study by Syed *et al.* (2011). The indiscriminate use of antibiotics may be a cause of this multidrug resistance. (Aucken *et al.,* 2002). High resistance of these isolates against gentamycin, erythromycin and cephalexin approximately agrees with other previous studies (Oununga and Awhowho, 2012; Shazia and Jyothsna, 2011). This resistance against a particular antibiotic may be due to its frequent and long-term use (Sabour *et al.,* 2004). Among the nine antibiotics used in the present study, ofloxacin and ciprofloxacin are the best choices for treating *S. aureus* infection. *S. aureus* is capable of causing a variety of human infections, including fatal invasive and toxic conditions and also possesses a differential ability to spread and cause hospital associated outbreaks of infections (Aucken *et al.,* 2002).

Bacteriocin and bacteriocin-like inhibitory substances (BLIS) are natural antimicrobial agents produced by Gram positive bacteria. BLIS have potential applications against a wide range of human and animal diseases. They are ribosomally synthesized antimicrobial peptides produced by microorganism belonging to different eubacterial taxonomic branches; they are lethal to bacteria closely related to the producing bacteria, the latter being protected by an immunity phenomenon. Bacteriocins may serve as anti-competitor compounds enabling an invasion of a strain or species in an established microbial community (Ahmad *et al.,* 2003; Desriac *et al.,* 2010).

The results obtained with the typing set strains on tested isolates show that the producers isolates having a wide spectrum and a high intensity of activity against indicator strains. However, the producers isolates contributed to the achievement of greater differentiation of typed staphylococci. Furthermore, since bacteriocins, produced by bacteria, are thought to have an important role in establishing the ecosystem (Nakamura *et al.,* 1983), the bacteriocin presented here may be responsible for a part of the control mechanism of microbial ecology. Bacteriocins are found in almost every bacterial species examined to date (Riley and Wertz, 2002). Bacteriocins are part of widespread applications in epidemiological studies as specific markers for bacteria. Various typing schemes have been based on either the production of, or sensitivity to a range of different bacteriocins (Guven, 2000).

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Molecular Identification of Six *Steinernema* Isolates and Characterization of their Internal Transcribed Spacers Regions

Omar M. Darissa* and Naim M. Iraki

UNESCO Biotechnology Educational and Research Center (UNESCO BERCEN). Bethlehem University, P.O. Box 9, Bethlehem, Palestine.

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Abstract

Molecular identification and genotyping of entomopathogenic nematodes (EPNs) are prerequisites for their proper classification, biodiversity studies, and their potential use in biological control programs. In Palestine, although several isolates of EPNs have been collected, phenotypically characterized, and assessed for their tolerance to cold and heat stresses, the molecular identification and genotyping of most of these isolates have not been accomplished yet. In this study, genomic DNA was isolated from all nematode stages of six *Steinernema* isolates collected in several areas of historical Palestine. The Internal Transcribed Spacers 1 and 2 (ITS1 and ITS2) as well as the 5.8S regions of the 26S rDNA were amplified and sequenced. The obtained sequences were aligned to identify, to explore sequence variations and construct phylogenetic relationships among the isolates. Three of the isolates were identified as *S. feltiae* while the other three as *S. carpocapsae*. At the interspecies level, the 5.8S sequences were more conserved than the ITS sequences which varied in structure and length. Moreover, the ITS2 sequences contained more variable informative sites than those of ITS1. The aligned sequences of the six isolates were analyzed using the neighbor-joining method for two sets of sequences; ITS1-ITS2, and ITS1-5.8S - ITS2. In both cases, similar clustering profiles were produced with slight differences in the branch length of each cluster. These data showed that the association of the different rDNA regions of these *Steinernema* isolates with different evolutionary rates is solely at the species level but not among different isolates of the same species.

Keywords: Entomopathogenic Nematodes, Internal Transcribed Spacers, Phylogenetic Analysis, Ribosomal DNA.

1. Introduction

The use of Entomopathogenic nematodes (EPNs), belonging to the families *Steinernematidae* and *Heterorhabditae* as biocontrol agents of soil-borne pests, necessitates the availability of a collection of EPN strains with various adaptive traits that enhance their efficacy and persistence as biocontrol agents when introduced to different environmental conditions (Grewal *et al*., 2001). In historical Palestine, which is divided into five different environmental and climatic zones that support diverse sets of life forms (Salem, 2008), several isolates of EPNs have been collected, phenotypically characterized, and assessed for their tolerance to cold and heat stresses (Iraki *et al*., 2000; Salame *et al*., 2010). However, molecular identification of the most of these isolates was not conducted yet. Such identification would be a prerequisite for proper classification, biodiversity studies, and pest-management programs.

Because EPNs belonging to the genus *Heterorhabditus* produce hermaphrodites in their life cycle while those of *Steinernema* do not (Muthulakshmi *et al*., 2012), molecular tools are not necessary to differentiate between these two genera. Such tools are, however, very important in order to identify and distinguish between species within each genus, where phenotypic variations become more limited. The Non-coding Internal Transcribed Spacers 1 and 2 (ITS1 and ITS2) of the Ribosomal DNA (rDNA) have provided a powerful tool to identify nematodes since these regions afford species specific polymorphism and in some cases populations specific polymorphism (Powers *et al*., 1997). These two spacers (ITS1 and ITS2) flank a conserved gene coding for the 5.8S of the rRNA. The much more conservative feature of the 5.8S sequences as compared to the two spacers indicates that they vary significantly in their evolutionary rates. Such differences

* Corresponding author. e-mail: odarissa@bethlehem.edu.

can disclose phylogenetic relationships among the different nematode species. The frequently reported intraspecific variation between rDNA sequences of nematodes disagrees with the conception that this gene family has concertedly evolved (Blok *et al*., 1998; Elbadri *et al*., 2002; Hugall *et al*., 1999; Hung *et al*., 1999; Morales-Hojas *et al*., 2001). This study aims at identifying and revealing the evolutionary relationship among six entomopathogenic nematode isolates belonging to the genus *Steinernema* by characterizing the ITS1-5.8S-ITS2 region of their rDNA.

2. Material and Methods

The EPN isolates were provided by Prof. Glazer from the Volcani Center, Bet Dagan, who recovered them from soil samples by baiting with last instar larvae of the wax moth, Galleria mellonella according to Bedding and Akhurst (1975).

2.1. DNA Extraction, Amplification and Sequencing

For each isolate, Infective Juveniles (IJs) were collected from the white trap while the other stages (adults) were gathered from the *G. mellonella* cadaver laying on the white trap. The collected nematodes were put in a 15 ml falcon tube and centrifuged at 10000 rpm for 5 min. The precipitated nematodes were transferred to an Eppendorff tube and ground using a drill. Genomic DNA was then extracted as described in the protocol of Dnasy TM System (Qiagen GmbH, Leusden, Netherlands). The ITS region from each isolate was amplified in a sterile 0.5 ml tube using the primers described by Vrain *et al*. (1992). PCR amplifications were performed according to Hominick *et al*. (1997) in a PTC-100 thermocycler (MJ research, USA). The amplified ITS regions were electrophoresed in 1% agarose (w/v) gel using 1X TBE buffer at 120 V for 1 hr. Then, they were excised from the gel and purified using QIAquick Gel Extraction Kit (Qiagen GmbH, Leusden Netherlands). Both strands of the purified PCR products were sequenced using sequence specific primers with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystem, USA) carried out on ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, USA). The identity of the sequences was confirmed by a BLAST (Basic Local Alignment Search Tool) search at NCBI (National Center for Biotechnology Information). The obtained sequences were deposited at the NCBI database with the accession numbers listed in Table 1.

Table 1. Gene bank accession numbers for ITS1-5.8S-ITS2 sequences and the result of their BLAST against the Nucleotide collection (nt) database of the NCBI website using the BLASTN 2.2.27+ software (Zhang *et al*., 2000; Morgulis *et al*., 2008).

Isolate	Accession number	Species	Query cover	E value	Max identity
SiS11	KC571262	*S. carpocapsae*	99%	0.0	99%
SiS7	KC571260	*S. carpocapsae*	99%	0.0	99%
IS34	KC571265	*S. carpocapsae*	98%	0.0	99%
SiS15	KC571263	*S. feltiae*	99%	0.0	99%
F17	KC571264	*S. feltiae*	99%	0.0	99%
SiS8	KC571261	*S. feltiae*	99%	0.0	99%

2.2. Phylogenetic Analysis

The sequence sets ITS1+ITS2 and ITS1+5.8S+ITS2 were multialigned with the default parameters of the ClustalX program (Larkin *et al*., 2007). The corresponding sequences of the EPN *Heterorabditis bacteriophora* (NCBI accession number AY321477) were used as outgroup. Phylogenetic relationships between the isolates were established using the MEGA4 program (Tamura *et al*., 2007) following the neighbor-joining (NJ) method (Saitou and Nei, 1987). The evolutionary distances were computed using the LogDet method according to Tamura and Kumar (2002) and are expressed as the units of the number of base substitutions per site. This method permits phylogenetic analysis of sequences that have different base composition such as the ITS regions. The differences in the composition bias among sequences were considered in evolutionary comparisons. Trees were constructed by clustering of associated taxa based on 1000 replicates in the bootstrap test (Felsenstein, 1985).

3. Results and Discussion

For the purpose of identifying the six isolates of EPNs at the species level, their ITS1-5.8S-ITS2 sequences were BLASTed against the nucleotide collection (nr/nt) database. Based on sequence homology, the isolates SiS11 (Sha'alabim), SiS7 (Bet Nir), and IS34 were identified as *S. carpocapsae* while the isolates SiS15 (Alonim), F17 (Karmel), SiS8 (Zichron) were identified as *S. feltiae* (Table 1). Although the length and GC content of both spacers was almost the same within species, they varied at the interspecies level (Table 2).

Such variations are indeed the basis of the frequent use of these spacers to identify nematodes at the species level (Hominick *et al*., 1997). On the other hand, the length and GC content of the 5.8S gene was constant among all isolates (Table 2).

Table 2. Lengths (in bp) and G+C contents (in %) of sequenced rDNA regions of the six *Steinernema* isolates

Isolate	ITS1		5.8S		ITS2	
	(bp)	G+C (%)	(bp)	G+C (%)	(bp)	G+C (%)
SiS11	278	35	157	41	302	39
SiS7	278	35	157	41	302	39
IS34	279	35	157	41	302	39
SiS15	264	38	157	41	306	36
F17	264	38	157	41	306	37
SiS8	264	38	157	41	306	36

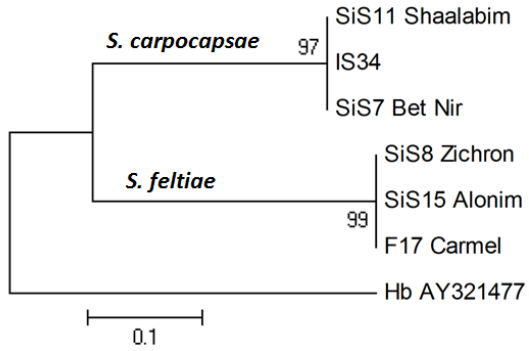
Therefore, the length variations in the rDNA sequences among different *Steinernema* species is mostly due to variations in the ITS region which is in accordance with other reports in the literature (Nguyen *et al*., 2001). Moreover, when the different regions of the rDNA sequences were multiple aligned, the number of variable and informative sites within the non-coding spacers' sequences was 175 sites, which is significantly higher than those of the coding 5.8S sequences which offered only 10 such sites along its entire length (Table 3).

Table 3. Number of variable and constant sites in the six *Steinernema* isolates: total number of sites used for coding (5.8S), noncoding (ITS1, ITS2 or both), and combined sequences (5.8S+ITS1-ITS2).

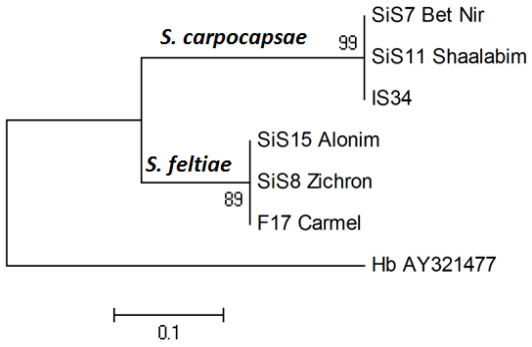
	ITS1	**ITS2**	**ITS1+ ITS2**	**5.8S**	**ITS1+ ITS2+ 5.8S**
Length (bp)	299	324	623	157	780
Constant sites	237 (79%)	209 (64%)	447 72%)	147 (94%)	592 (76%)
Variable but uninformative	0	1	1	0	1
Variable and informative	62 (21%)	114 (35%)	175 28%)	10 (6%)	187 (24%)

The results showed also that these informative sites are more available in the ITS2 (35%) than in the ITS1 (21%). Such variations in the rDNA sequence were reported to exist even at the intra-individual level in nematodes (Subbotin *et al*., 2000; Blok *et al*., 1998). The amount and nature of variable and informative sites in multiple-aligned rDNA sequences could be used for revealing the phylogenetic relationship among entomopathogenic nematodes.

Phylogenetic analyses with NJ method were performed using the non-coding ITS sequences alone or by combining the whole rDNA sequences (ITS1-5.8S-ITS2). In both cases trees with similar topologies were produced (Figure 1A, B). The six isolates were separated into two clusters: the first included the *S. carpocapsae* isolates SiS11 (Sha'alabim), SiS7 (Bet Nir), and IS34, while the second included the *S. feltiae* isolates SiS15 (Alonim), F17 (Karmel), and SiS8 (Zichron). Using *Heterorhabditis bacteriophora* as an outgroup, the clustering of the *S. feltiae* isolates was supported by stronger bootstrap value when the tree was produced based on a lignments of the non-coding sequences alone than when the whole rDNA sequences were aligned (99 compared to 89, Figure 1A, B). This might be due to the fact that the percentage of variable and informative sites in the ITS region alone is higher (~30% by dividing the number of variable and informative site over the length of the ITS region) than in the whole rDNA sequence (~25%) which would provide a more reliable phylogram.



A



B

Figure 1. Evolutionary relationships among the six *Steinernema* isolates. The evolutionary history was revealed using the Neighbor-Joining method. The percentage of replicate trees in which the associated isolates clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree is drawn to scale with branch lengths calculated using the LogDet method and expressed as the units of the number of base substitutions per site. Two different data sets have been considered for the analysis: (A), ITS1-ITS2 and (B), ITS1-5.8S-ITS2. *Heterorhabditis bacteriophora* isolate (NCBI accession no. AY321477) is included as outgroup. Phylogenetic analyses were conducted in MEGA4.

In conclusion, the data presented in this study showed that the different regions of the rDNA of the six *Steinernema* isolates encompass diverse percentage of variations as follows; ITS2 > ITS1 > 5.8S. Although, these variations were associated with different evolutionary rates at the species level, such variable

rates were not revealed within isolates of the same species.

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Diversity of Fungal Trunk Pathogens Associated with Grapevine Dieback of Grapevine in Algeria

Faiza Ammad[1, 2,*], Messaoud Benchabane[2] and Mohamed Toumi[1]

[1] *Département of Biology Ecole Normale Supérieure Kouba, BP. 92, 16050 Vieux-Kouba, Alger, Algéria.*

[2] *Faculté des sciences Agrovétérinaires et Biologiques, Université Saad Dahleb Blida,BP 270 Blida 09000 – Algérie.*

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Abstract

A significant reduction in vine production has recently been recorded in several vine regions in Algeria due to the death (total or partial) of many vines and to the pulling of many vineyards that had showed too many symptoms of decline. This study was conducted during spring 2010 - 2012 to detect the responsible agents for this disease. Samples of grapevine wood were collected in five grapevine fields in two regions (Medea and Tipaza). A cross section of infected trunks revealed many types of necrosis: sectorial brown colored, central necrosis sectorial gray and light-brown central necrosis. Several fungi were isolated from the margin between healthy and diseased tissues. *Eutypa lata* and *Fomitiporia mediterranae* were identified fungi based on the morphological characteristics of the culture and confirmed by partial sequences analysis of the nuclear ribosomal internal transcribed Spacer (ITS). The sequences submitted in GenBank under accession number revealed 94-100% homology. *F.mediterranae* was the dominant species, followed by *E. lata*. Other fungal species (*Alternaria, Fusarium, Pestolozzia, Botrytis, Rhizopus, and Penicillium*) were also isolated with high frequency. Two kinds of fruiting structures were found where one type showed the presence of perithecia (sexual form) of *Eutypa lata*.

Keywords: Grapevine, Dieback, Eutypiosis, Esca, Algeria.

1. Introduction

Grapevine growing occupied important places in the agricultural sectors of many countries. In Algeria, grapevine was cultivated principally to produce table grapes, in addition to some extent products such as fresh juice and raisins. The soil and climatic conditions in Algeria are favorable to the development and extension of this culture. Grapevines are grown in the north regions (South Mediterranean Sea) on an area of 100.200 ha according to the Ministry of Agriculture statistics done in 2012. The decline of the vine was due to fatal diseases. The most destructive diseases were esca and eutypiosis, respectively developed in vineyards (Larignon and Dubos, 1997; Mugnai *et al.,* 1999). *Eutypa* dieback, caused by the fungus *Eutypa lata*, threatened the sustainability of vineyards, especially those of eight years or older; it is becoming a serious problem in most cool climate growing regions. The fungus infects vines through pruning wounds, colonized wood tissue and caused a characteristic wedge of dead tissue. It was thought that the fungus produce toxic metabolites which were transported through vascular tissue to the foliage, causing stunting of the shoots, distortion and necrosis of leaves (Moller and Kasimatis, 1978; Molyneux *et al.,* 2002). The second disease, called esca, was a complex disease, more complicated than that of *Eutypa* dieback, including a vascular symptoms and an internal white rot in the trunk, which gradually changed the hard wood to a soft one (Mugnai *et al.,* 1999). It was attributed to a group of systematically diverse fungi that were considered to be latent pathogens. The principal pathogenic taxa associated with esca were *Eutypa lata, Phaeomoniella chlamydospora,* and various species of the genera *Botryosphaeria,Cylindrocarpon, Fomitiporia*, and *Phaeoacremonium* (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Surico *et al*., 2006).

As a result of the gravity of this phytosanitary problem of this decline in the vineyards in Algeria, this study was conducted in five vineyards located in two

* Corresponding author. e-mail: Sahraoui_a_f@yahoo.fr.

regions known by their vine vocation. This study aims at identifying and characterizing the causal agents of grapevine dieback in Algerian vineyards on morphological characteristics of culture; besides, a partial sequences analysis of the nuclear ribosomal internal transcribed Spacer (ITS) was investigated. Detection of fruiting bodies in wood samples was also investigated.

2. Materials and methods

2.1. Sampling and Isolation

A field survey was conducted on some vineyards which showed dieback symptoms on l ocal cultivars, namely Dattier de beyrouth, Muscat, Cinsault, in the north region of Algeria (Tipaza, Medea) during the spring period of 2010 and 2012 (Table 1). After carrying out the descriptive symptomatology and localization the vines with dieback symptoms, samples were collected randomly from each vineyard vines (10 among the 500 observed). Some vines showed symptoms of decline on herbaceous parts for each cultivar were cut at the base of the trunk, wood slices of 0,5 mm of large were sectioned from the margin of all necrosis categories at the frontier of necrotic tissues and apparently healthy. Slices surface were disinfected by immersion in sodium hypochlorite (NaOCl) (2%) for 4 min, after that they were rinsed and dried with sterilized filter paper. Then, they were placed on potato dextrose agar (PDA) plates and stored at 25°C. Observations of fungal development were recorded weekly. Morphological and microscopic analyses of mycelia culture of *Fomitiporia* and *Eutypa* were done according to Fischer (2002), Moller and Kasimatis (1978), respectively.

Table 1. Characteristic vine yards studied in (Médea and Tipaza) provinces during 2010 growing season.

Location	Medea		Tipaza		
site	Benchicao		Hamrelain		
Vine yards	1	2	1	2	3
Cultivar	Dattier	Muscat	Dattier	Muscat	Cinsault
Age (an)	26	45	40	10	10
Sup (Ha)	12	06	05	04	03
Mode of pruning	Guyot simple	Guyot double	Cordon double	Cordon double	Guyot
Rootstock	41B	41B	41B	41B	41B

2.2. Body Fruiting

Dead branches and wood were inspected in the vineyard for the presence or absence of the sexual forms (Perithecia and Pycnida) of the fungus implicated in the decline of vines such as the genus *Eutypa*. Infected samples, collected from vineyards, were transported to the laboratory where they were left to dry for examination. The preparation of ascosporic was conducted from fruiting in sterilized water and was placed on PDA plates. After 24 h at 25 ± 2°C, individual germinated spores were observed under a microscopic observation.

2.3. DNA Extraction and PCR Amplification

Total genomic DNA of all isolates, identified morphologically as *Eutypa* and *Fomitiporia*, were extracted from pure culture mycelia as reported by Liu *et al.* (2000). Oligonucleotide primers ITS1 (5'-TCCGTAGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGA TATGC-3') were used to amplify the ITS regions of the nuclear ribosomal DNA (including the 5.8s gene) according to (White *et al.,* 1990). The amplification reactions were performed in a 25µl volume of reaction mixture containing (1mM of each primer, 0.2 Mm of dNTP, 15 ml $MgCL_2$ and 2.5 U of Taq polymerase adjusted with purified distilled water to a final volume of 25 µl). The PCR program for ITS genes was run according to Guizhen and Mitchell (2002) included an initial denaturation at 95 C° for 2 min, followed by 35 cycles of 1 min denaturation at 94 C°, annealing for 40 s at 53 C°, and 1 min elongation at 72 C°, with final elongation step at 72 C° for 10 min. The PCR amplification products were separated by electrophoresis in 1.5% a garose gels prepared in tampon TBE 0,5 X (Tris-Borate 100 Mm ;pH 8,3; EDTA 2 mM) added 50 µg ethidium bromide (BET), and visualized under UV light (Sambrook *et al*., 1989). The PCR products were puriifed with QIAquick Wizard PCR puriifcation Kit (Promega) according to th manufacturer's instructions. The sequences were determined by cycle sequencing using the Taq Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems, HTDS, Tunisia).

2.4. Molecular Identification

The nucleotide sequences were read and edited with Chromas1.7.5(http://www.technelysium.com.au/ chromac. html) . All sequences were checked manually. They were initially analyzed by searching the National Center for Biotechnology Information (NCBI) database using the BLAST (Basic local alignment search tool) (Altschul *et al*., 1997). Reference sequences for the ITS regions for the *Eutypa* and *Fomitiporia* spp. were obtained from GenBank.

3. Results

3.1. Fungal Isolation

Based on their appearance in culture, the isolates obtained in this study were assigned to main fungal groups. The first group of isolates, occurs in the first days (2-3 days) of incubation; this later became of white color and of a cottony texture. All isolates were typical of *Fomitiporia* genus and produced high density and aerial hyphae; after 10 da ys of incubation they developed white-yellowish mycelia, which became yellow-brown over time.

The second group was characterized by having white to white-cream cottony and slow-growing an intense aerial mycelium of the developed hyphae on PDA. With age, some cultures of *Eutypa* change color, turning from white to yellow with no fruiting structures. After four weeks, the accumulation of a brown color which became darker blackish determining melanisation. The higher frequency was attributed to the genus *Eutypa* and

Fomitiporia, the fungal agents associated with *Eutypa* dieback and esca, respectively. Their genus were isolated from all vines showing disease symptoms and frequently isolated from central and sectorial necrosis of soft and hard texture (Table 2) and (Table 3). Other fungi were isolated from grapevine cankers, such as *Alternaria, Fusarium; Pestolozzia, Botrytis, Rhizopus* and *Penicillium.*

Table 2. Frequency of isolation of the fungus depending on the variety in (Médea and Tipasa) provinces during 2010.

Cultivar	*Eutypa lata spp*	Frequency of isolation	*Fomitiporia mediterrania*	Frequency of isolation
Muscat	$8^{a}/10^{ь}$	80%	$6^{a}/10^{ь}$	60%
Cinsault	4/10	40%	5/10	50%
Dattier de Beyrouth	12/30	40%	15/30	50%
Total	24/50	48%	26/50	52%

* The report is (a) number of isolated fungus, (ь) number of vines analyzed.

Table 3. Frequency of isolation of the fungus depending in (Médea and Tipasa) provinces during 2010.

Region	Location	*Eutypa lata*	Frequency of isolation	*Fomitiporia mediterania*	Frequency of isolation
Médéa	Ben chicao	$6^{a}/20^{ь}$	30%	$11^{a}/20^{ь}$	55%
Tipasa	Hamrelain	18/30	60%	15/20	75%
Total	2	24/50	48%	26/50	52%

* The report is : (^{a}) number of isolated fungus, (ь) number of vines analyzed.

3.2. Fruiting Bodies

On the surface of dead branches and debris collected in the field, many fruiting structures of various trunks diseases pathogens were found (Fig 1). Microscopic observation of these fruiting bodies showed the presence of many asci, rounded with a pedicel (Fig. 2). Each ascus contained ascospores arched liberated from an ostiole (Fig. 3). The perithecia were the infectious form of *Eutypa lata*. None of the fruiting bodies was recorded for the second genus: *Fomitiporia*. In the present study, other forms of fruiting bodies on dead wood were found (Fig. 4) and presence of asci contained ascospore (Fig.5), the line identification helped distinguish isolates genre *Hysterium* (www.lenaturaliste.net).

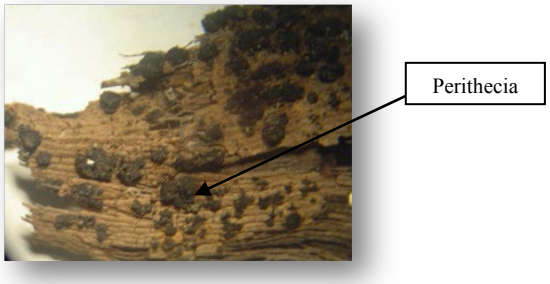

Figure 1. Body fruiting of Eutypa lata (Dattier de Beyrouth cultivar, Tipaza,2010).

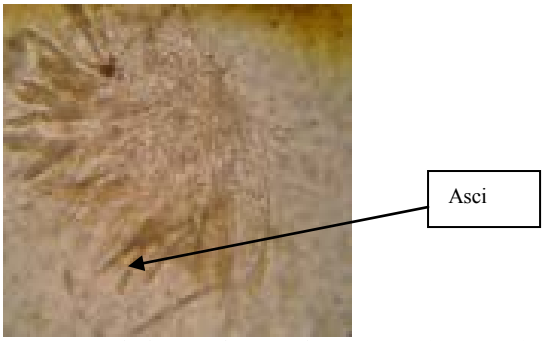

Figure 2. Asci of Eutypa lata contain ascospores (G : 40× 10). (Dattier de Beyrouth cultivar, Tipaza,2010).

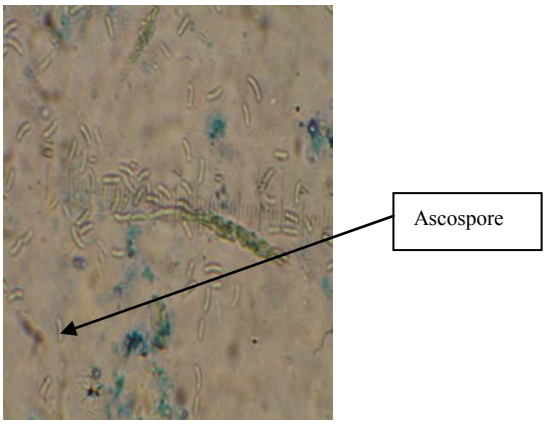

Figure 3. Liberation of ascospores of Eutypa lata (G: 40 × 10)(Dattier de Beyrouth cultivar, Tipaza,2010).

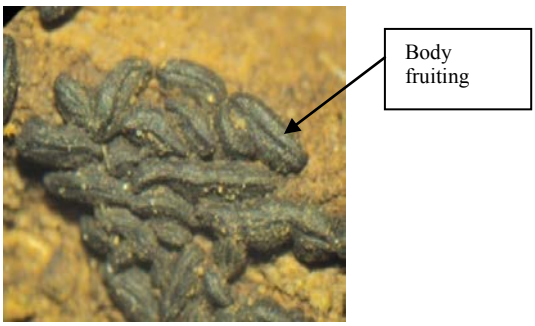

Figure 4 . Body fruiting of Hysterium sp (Cinsault cultivar, Tipaza,2010).

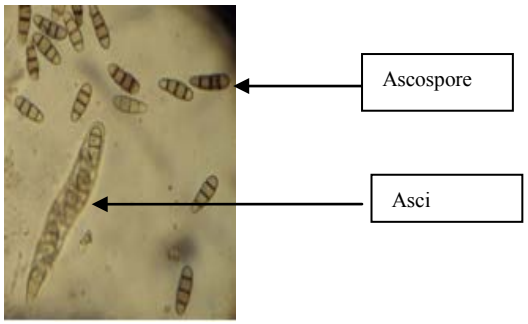

Figure 5 . Asci and ascospore of *Hysterium sp*(G : 40× 10). sp (Cinsault cultivar, Tipaza,2010).

3.3. Molecular Identification

Sequence alignments, used BLASTn in GenBank, showed that all *Fomitiporia* isolates from grapevine, which were tested with ITS sequence analysis, were identified as *F. mediterranea*. Nucleotide identity was 98-100% among sequences of *F. mediterranea* from grapevine. The second group of isolates presented 94-96% of homology with *Eutypa lata*.

4. Discussion

This study shows diversity in fungal trunk pathogens associated with wood decay symptoms on grapevine in Algeria during the period between 2010 and 2012. The result of the isolations shows the complex situation generated by many fungal trunk pathogens on grapevine in Algeria; isolates obtained were classified on clusters based on their appearance in culture and conidial morphology. Morphological and microscopic characters are compared with those reported previously by Moller and Kasimatis (1978) and Fischer (2002).

Including isolates species of *Eutypa lata*, *Fomitoporia mediterranea* and other detected species, such species could be distinguished based on DNA sequence data and unique morphological characters. Several strains of *Eutypa lata* were isolated during this study. This fungus was a major pathogen of cultivated crops such as apricot and grapevine and has been found all over the world (Carter, 1957). Those species were isolated from grapevine and known as grapevine pathogens in different regions of the world. *Eutypa lata* was the causal agent of *Eutypa* dieback, an important perennial canker disease that occurred in most countries where grapevine is cultivated (Munkvold and Marois, 1994). This species has also been reported from grapevines in Australia, Brazil, South Africa (Mostert *et al*., 2003). In many European countries this specie is considered the primary limiting factor.

The examination of the dead branch revealed the presence of Perthecia, in grapevine the occurrence of a perithecial stroma on the dead stump of a tree was first reported by Carter (1960) in Australia. According to Munkvold and Marois (1994), perithecia is more common on apricot than on grapevine. No perithecia were detected in the vineyards of Merced country. We concluded that infected trees did not develop perthecia but the presence of this sexual structure in some vineyards studied may serve as a source of inoculums (Elmomany, 2002).

In this present investigation, we report the presence of *F. mediterranea* in declining grapevine in Algeria. The importance of *F. mediterranea* as a pathogen has been widely investigated (Mugnai *et al*., 1999; Fischer, 2002; Fischer and Binder, 2004). The association between *F.mediterranea* and *Vitis vinifera* was of particular relevance, since the fungus produces an extensive white rot in the trunks of growing *Vitis* plants, was one symptom of the destructive grapevine disease complex esca, affecting vine cultivation on a global scale (Romanazzi *et al*., 2009). No fructification was detected in all vineyards for *Fomioporia*; we suggest that the conditions were not favorable for the developpement of those structures.

5. Conclusion

The eutypiosis and esca appeared as severe dieback diseases of the vine, because there were no means of control to cure the diseased vines. The majority of vine growers in Algeria have not realized the seriousness and severity of the diseases that affected the vine heritage. The present work has allowed analysis of the grapevines showing symptoms of decline showed the existence of several types of necrosis in the trunks. The analysis of the isolated from necrotic wood revealed the existence of two genus *Eutypa lata* and *Fomitiporia mediterranea* involved in the decline. It is clear that a better understanding of diseases is absolutely necessary to try to find effective solutions. It is advisable to design a diagnostic study involving vine regions to identify the likely origins of decay. Further investigations on *E. lata* and *F. mediterranea* and other wood decay agents should be conducted in order to manage the slow decline phenomenon in Algeria and other regions.

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Biochemical Response of the Cyclopoida Copepod *Apocyclops borneoensis* Exposed to Nickel

Emadeldeen Hassan Mohammed*

Department of Environment and Natural Resources, Faculty of Applied Science, Red Sea University, Port Sudan, Sudan

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Abstract

The use of biomarkers to evaluate the biological effects of pollutants in marine organisms represents a r ecent tool in biomontoring programs. The cyclopoida copepod *Apocyclops borneoensis* was exposed to different Ni treatments [0 (control), 10, 100, 1000 µg l^{-1}Ni] for 1, 2, 4, 7, 14 days. At each exposure time, acetylchlinesterase (AChE), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione (GSH) were analyzed. Additionally, lipid peroxidation (LPO) level was measured after a 14-day exposure. The results show that Ni treatment significantly stimulated copepod's antioxidants SOD, GST and GSH at environmentally relevant concentrations after a certain exposure time. On the other hand, the exposure time significantly affected SOD and GSH. In contrast, Ni exposure significantly decreased LPO level, implying that the factor involved in LPO might not significantly depend on the operations and functions in the antioxidant defense system. In addition, Ni might also be a neurotoxic agent to copepods via changing AChE activity.

Keywords: Nickel; *Apocyclops borneoensis*; Biochemical Response; Oxidative Stress; Neurotoxiciy.

1. Introduction

Nickel (Ni) is a metallic element that is ubiquitously present in the environment. Ni is released into the marine environment from the discharge of metal industries, mining, refining, power plants, waste incinerators, and direct leaching from rocks and soil (Fishbein, 1981; Denkhaus and Salnikow, 2002). Meanwhile, Ni also is present in crude oils and, in the event of an oil spill, is released into the marine environment (Sadiq, 1989). Ni concentration in estuaries and streams generally ranges from 1 to 75 µg L^{-1} (Eisler, 1998) and could reach as high as 500 to 2000 µg L^{-1} in natural waters near industrial sites (Chau and Kulikovsky-Cordeiro, 1995).

The metabolism of a great variety of pollutants, including metals, can enhance reactive oxygen species (ROS) production (Regoli *et al*., 2002; Limón-Pacheco and Gonsebatt, 2009). At high concentrations, ROS can be important mediators of damage to cell structures, including lipids, membranes, proteins and nucleic acids and often leading to oxidative damage in organisms (Poli *et al*., 2004; Valko *et al*., 2006). The biological defense mechanisms against ROS include enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), and glutathione peroxidases (GPx), and non-enzymatic antioxidants, such as glutathione (GSH), ascorbic acid (vitamin C), α-tocopherol (vitamin E), β-carotene, and urate (de Zwart *et al*., 1999; Valko *et al*., 2006). Higher Ni concentrations can generate ROS, subsequently it can induce oxidative stress in organisms and alter the cellular antioxidant defense system (Denkhaus and Salnikow, 2002; Kasprzak *et al*., 2003). Measurement of antioxidants response in aquatic organisms has been used as a biomarker of heavy metals effect (Paris-Palacios *et al*., 2000; Elumalai *et al*., 2002; Brown *et al*., 2004; Cunha *et al*., 2007; Elumalai *et al*., 2007).

Acetylchlinesterase (AChE) is a k ey enzyme in the nervous and sensory systems in most species. AChE terminates the transmission of neural impulses by the rapid hydrolysis of acetylchline (ACh) into the inactive products of choline and acetic acid (Barnard, 1974). Previous studies demonstrated that heavy metals may be neurotoxic agents to aquatic organisms, via affecting their AChE activity (Forget *et al*., 1999; Tsangaris *et al*., 2007; Pretto *et al*., 2009).

Copepods are widely distributed and are key secondary producers in the ocean (Zhong *et al*., 1989). They play an important ecological role in aquatic ecosystems, because of their position in the trophic chain (i.e., essential link between the phytoplankton and the higher trophic levels), their rapid turnover (Runge, 1988), and their role in ocean biogeochemical cycles (Wang and Fisher, 1998). As they are the essential link between the primary producer, phytoplankton, and other organisms of higher trophic

* Corresponding author. e-mail: emadeldeenelfahal@yahoo.com.

levels, they also play significant roles in transportation of aquatic pollutants across the food chain (Raisuddin *et al*., 2007). Although several studies have highlighted the effect of heavy metals on survival, growth, development, reproductive performance and the community structure of copepods (Hook and Fisher, 2001; Lee and Correa, 2005; Kwok *et al*., 2008; Lee *et al*., 2008; Mohammed *et al*., 2010), few studies have been carried out on the biochemical response of copepods to heavy metals exposure (Barka *et al*., 2001; Wang and Wang, 2009, 2010). Using the cyclopoida copepod *Apocyclops borneoensis*, the present study primarily aims at assessing the biochemical response to sublethal Ni treatments in a long exposure time, via measurement of AChE, SOD, GST; GSH, and LPO as biomarkers.

2. Materials and Methods

2.1. Copepod Collection and Maintenance

In most cases, the species belong to the genus *Apocyclops* are dominant in coastal brackish waters (Støttrup, 2006). *Apocyclops borneoensis* was collected using 64 µm mesh size plankton net from Xiamen bay, PR. China. Since collection, copepods had been maintained at 28 to 31° C and 18 to 22 ppt salinity in continuous stock cultures in our laboratory under static-renewal conditions and under 12D: 12L photoperiod cycle. The culture water was 0.45 µm Millipore filtered seawater (cellulose filter paper), with 7 to 7.9 mg L^{-1} dissolve oxygen and a pH ranging from 7.90 to 8.25. Copepods were fed a mixed algal diet of *Isochrysis galbana* and *Platymonas subcordiformis*. The algae were cultured in filtered seawater contain f/2 enriched media at 20°C.

2.2. Test Solutions

Nickel was provided as a chloride salt ($NiCl_2 .6H_2O$) from Guang Fu Chemical Institute, China (≥ 98.0% pure). Stock solutions were prepared in double distilled water. The stock solutions were subsequently diluted in different volumes of 0.45 µm filtered seawater to create various Ni nominal test concentrations (0, 10, 100, 1000 µg L^{-1}).

2.3. Exposure

Adult copepods were exposed to Ni test concentrations for 1, 2, 4, 7, 14 days. The experimental salinity and temperature were 20 ppt and 30°C, respectively. The experiments were carried out as semi-static (renewal) tests, with daily renewal of half of the exposure solution, each treatment with 2-L exposure solution. After the daily renewal of the test solutions, copepods were fed with *Isochrysis galbana* at a density of 5×10^5 cell mL^{-1} during 14 days exposure. At the end of each exposure time, about 1000 adult copepods of mixed gender were collected from each treatment and immediately stored at -80°C.

2.4. Biochemical Analysis

In order to determine biochemical parameters, samples were homogenized by digital sonifier cell disrupter (model 450, Branson, USA) for 2 min with 20 mmol/L Tris-buffer (pH 7.6, containing 1 mmol/L EDTA, 0.25 mol/L sucrose, 0.15 mmol/L NaCl and 1 mmol/L dithiothreitol) at 4°C. The homogenate was centrifuged at 15,000g for 20 min at 4°C, and the supernatant was used for the biochemical analysis. Protein determination was performed using the method of Bradford (1976) with bovine serum albumim as a standard.

The activity of GST, SOD, and AChE, and the level of GSH and LPO (as MDA) were measured spectrophotometrically with the test kits supplied by Jian-Cheng Bio-engineering Institute of China. The test kits were made of reagents from Sigma-Aldrich Co.

The content of GSH was determined based on spectrophotometric method of Rahman *et al*. (2006). DTNB (5,5-dithiobis-2-nitrobenzoic acid) was developed for the detection of thiol compounds. DTNB and GSH react to generate 2-nitro-5-thiobenzoic acid (TNB) and glutathione disulfide (GSSG). The rate of formation of TNB is proportional to the concentration of GSH in the sample. The content of GSH was expressed as micrograms per milligram of protein.

The GST activity was determined according to Habig *et al*. (1974), by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH. The rate of GSH content decrease is directly proportional to the GST activity in the sample. One unit of GST is defined as the amount of enzyme that will reduce 1µmol/L GSH in the reaction system at 37° C per minute in 1 mg protein.

The SOD activity was determined based on its ability to inhibit the reduction of cytochrome-*c* by the O_2^- generated in the xanthine oxidase/hypoxanthine system (McCord and Fridovich, 1969). One unit of SOD will inhibit the rate of oxidation of hydroxylamine by 50% in a coupled system, using xanthine and xanthine oxidase at 37°C in 1.0 mg/ml protein concentration of tissue homogenate.

The lipid peroxidation (LPO) level was assessed by the thiobarbituric acid (TBA) assay. The measurement of thiobarbituric reactive species (TBARS) quantified as MDA equivalents was based on the method previously described by Ohkawa *et al*. (1979). MDA is formed as an end product of lipid peroxidation which reacts with TBA to generate a colored product that measured spectrophotometrically. The amount of MDA formed was expressed as nmol MDA per mg of protein.

The AChE activity was spectrophotometrically determined according to Ellman method (Ellman *et al*., 1961). Acetycholine is hydrolyzed by acetylchlinesterase producing acetic acid and thiocholine. Thiocholine reacts with the Ellman reagent DNTB (5,5-dithiobis-2-nitrobenzoic acid) to produce the anion of 5-thio-2-nitrobenzoic acid (TNB) and the increase of its absorption indicates AChE enzyme activity. One unit of AChE catalytic activity is defined as the amount of enzyme that will cause decompose of 1 µmol acetycholine per 6 minutes at 37° C in 1.0 mg protein of tissue homogenate.

2.5. Statistical Analysis

Two statistical programs (Microsoft excel 2003 package; SPSS 17.0, Chicago, IL, USA) were used to analyze the data. All biochemical measurements were replicated at least three times. The data were expressed as mean values ± standard deviation (S.D). Prior to any statistical analysis, data were log-transformed to meet ANOVA assumption of normality and variance homoscedasticity. The statistical analysis was carried out using one-way ANOVA and Fisher's least significant

difference (LSD) test to evaluate whether the means were significantly different among Ni treatments at particular exposure time. Significant differences were indicated at $p< 0.05$.

A two-way factorial ANOVA was used to evaluate whether Ni treatments and exposure time used had significant effects on the biochemical parameters tested (significant at $p< 0.05$).

3. Results

3.1. Effect Of Ni Exposure on Various Biochemical Parameters in The Copepod A. Borneoensis

The GSH level was significantly (one-way ANOVA, $p< 0.05$) stimulated by day 1 under the highest Ni treatment (1000 µg L^{-1}), and this stimulation reached its peak at day 2, and then was significantly decreased with the increase of exposure time (figure 1A, $p< 0.05$). For 10 and 100 µg L^{-1} Ni treatments, the level of GSH was significantly stimulated by day 7 with a significantly higher level under100 µg L^{-1} than 10 µg L^{-1} and then was significantly decreased at day 14 for both treatments (fig. 1A, $p< 0.05$).

For all Ni treatments, the GST activity was not significantly different from control at 1, 2, 4 da ys; however, it increased by day 7 with the increase of Ni concentration, except the highest concentration, and was significantly stimulated at 100 µg L^{-1} Ni (fig. 1B, one-way ANOVA, $p< 0.05$). The highest Ni treatment (1000 µg L^{-1}) had a noticeably inhibition effect on GST activity at day 14, but not other treatments ($p< 0.05$).

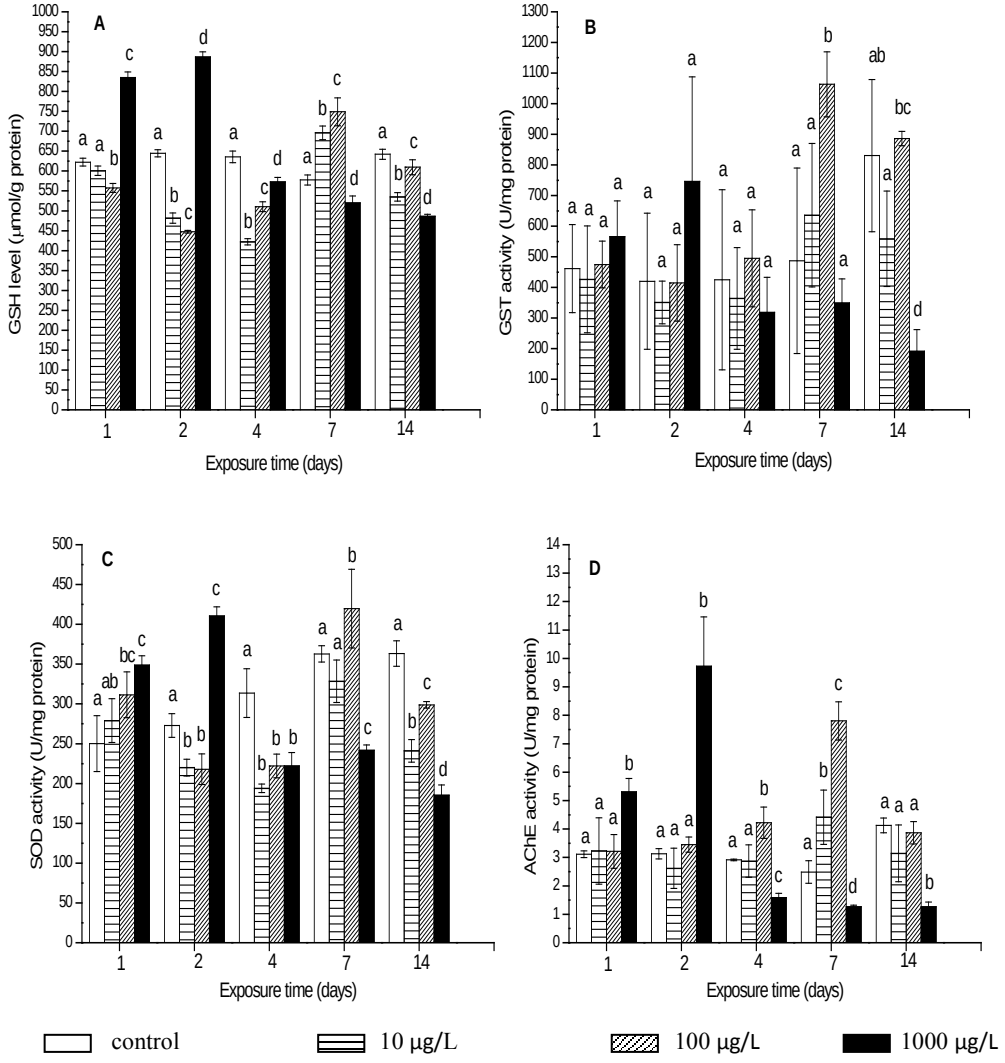

Figure 1. Effect of Nickel on various biochemical parameters (**A**) GSH level (**B**) GST activity (**C**) SOD activity (**D**) AChE activity in *Apocyclops borneoensis*. Data are described as mean ± standard deviation. Different letters indicate a significant difference among different Ni treatments at $p < 0.05$.

Figure 1C shows the response of copepod's SOD activity to different Ni treatments. By day 1, both 100 µg L^{-1} and 1000 µg L^{-1} Ni treatments were significantly stimulated copepod's SOD activity, but not the lowest Ni treatment which showed a little effect (Figure 1C, $p< 0.05$). The highest Ni treatment 1000 µg L^{-1} reached its peak stimulation of SOD activity by day 2, and then was significantly decreased at 4, 7, 14 days ($p< 0.05$). Both 10 µg L^{-1} and 100 µg L^{-1} Ni treatments significantly decreased the SOD activity at days 2 and 4; however, by day 7, 100 µg L^{-1} Ni treatment significantly stimulated its activity but not 10 µg L^{-1} ($p< 0.05$). The inhibition effect of all Ni treatments on copepod's SOD activity was shown by day 14.

In the case of copepod's AChE activity, the highest Ni treatment (1000 µg L^{-1}) displayed a significant stimulated effect at day 1 and attained its peak by day 2, and then was significantly decreased with the increase of exposure time (Figure 1D, $p< 0.05$). The other Ni treatments had insignificant effect on AChE activity at 1, 2, 4 days; however, by day 7 they significantly stimulated its activity (Figure 1D, $p< 0.05$). The highest Ni treatment (1000 µg L^{-1}) had a noticeably inhibition effect on copepod's AChE activity at days 4, 7 and 14, but not other treatments ($p< 0.05$).

The influence of Ni on copepod's LPO was measured as MDA level at day 14. Figure 2 shows that the copepod's MDA level significantly decreased with the increase of Ni concentration ($p< 0.05$). There was no significant difference in MDA level between 10 µg l^{-1} and 100 µg l^{-1} Ni treatments.

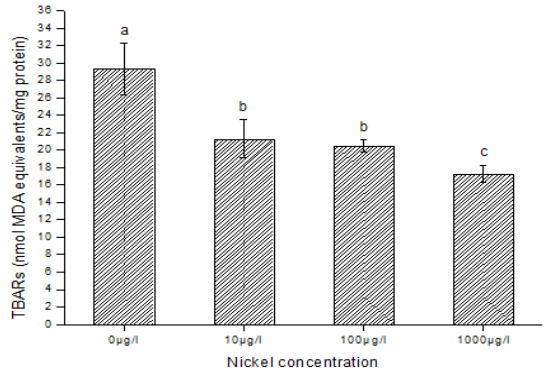

Figure 2. Effect of Nickel on LPO measured as MDA level in *Apocyclops borneoensis* exposed for 14 days. Data are described as mean ± standard deviation. Different letters indicate a significant difference among different Ni treatments at $p < 0.05$.

3.2. Effect of Ni Exposure Time and Treatment on Biochemical Responses in The Copepod A. Borneoensis

The two-way ANOVA statistical analysis of the effect of Ni treatment and exposure time (table 1) confirmed the significant influence of Ni treatment on the biochemical parameters GSH ($p<0.001$), SOD ($p<0.001$), AChE ($P<0.001$) and GST ($p< 0.05$). Also, Ni exposure time significantly ($p<0.001$) influenced GSH, SOD, and AChE; however, its influence on GST was insignificant ($p>0.05$). Moreover, the two-way ANOVA showed a significant interaction between the effect of Ni treatment and exposure time on the copepod biochemical parameters GSH ($p<0.001$), SOD ($p<0.001$), AChE ($p<0.001$) and GST ($p< 0.05$).

Table 1. Effect of Ni treatment and exposure time on biochemical parameters in *Apocyclops borneoensis*

	***P*-value**			
	GSH	GST	SOD	AchE
Effect of Ni treatment	<0.001*	0.024*	<0.001*	<0.001*
Effect of exposure time	<0.001*	0.142	<0.001*	<0.001*
Treatment × time	<0.001*	0.005*	<0.001*	<0.001*

Note: * indicates a significant effect, two-way ANOVA ($P< 0.05$)

4. Discussion

Exposure to some xenobiotics, especially toxic chemical pollutants, plays an important role in the mechanistic aspects of oxidative damage. Such a diverse array of pollutants stimulates a variety of toxicity mechanism, such as oxidative damage to membrane lipids, proteins, and DNA and changes to antioxidant enzymes activity and non-enzymatic antioxidant content (Poli *et al*., 2004; Valko *et al*., 2006; Valko *et al*., 2007). Living organisms have the ability to synthesize and control specific enzymatic systems, which can be used for repairing and removing of the damaged proteins, lipids, and DNA. Also, since oxidative stress levels may vary from time to time, organisms are able to adapt to such fluctuating stresses by inducing additional synthesis of enzymatic and non-enzymatic antioxidants to regulate oxidative damage (Valavanidis *et al*., 2006). Thus, the balance between prooxidant endogenous and exogenous factors (pollutants) and the antioxidant defenses (enzymatic and non-enzymatic) in the biological systems can be used to assess toxic effects of chemical pollutants on living organisms. With respect to other aquatic organisms, few studies have been carried out on the biochemical response of copepods to heavy metals. The major findings of the present study were that Ni treatment significantly stimulated some of the measured biochemical parameters in the cyclopoida *A. borneoensis* after certain exposure time.

GSH is the main intracellular thiol antioxidant and has a key role in the detoxification process of pollutants, not only as a substrate of antioxidant enzymes, but also as a direct reducing agent and a nucleophile able to block the toxicity of heavy metals and organic chemicals with thiol affinity by covalent binding (Vasseur and Leguille, 2004). It has an important role in scavenging of cellular ROS (such as H_2O_2, $O_2^{\bullet -}$ and $^{\bullet}OH$). A variety of environmental pollutants are known to change the GSH level in aquatic organisms, including heavy metals (Canesi *et al*, 1999; Wang *et al*., 2008). Our results show that the copepod's GSH level was significantly ($p< 0.05$), increasing by day 1 under the highest Ni treatment used (1000 µg L^{-1}), and by day 7 under the lowest Ni treatments (10 µg L^{-1} and 100 µg L^{-1}), and then significantly decreased by day 14 for all Ni treatments (figure 1A, $p< 0.05$). The induction of GSH in the tissue of the copepod *A. borneoensis* exposed to Ni was probably due to a primary defense system in which the GSH is involved to protect copepod from oxidative stress. Similarly, an increase in GSH level has been reported in different tissues of aquatic animals exposed to heavy metals (Schlenk and Rice, 1998; Zaroogian and Norwood, 2002; Atli and Canli,

2008). However, Wang and Wang (2009; 2010) demonstrated that heavy metals (Cd and Ni) cause a decrease in the GSH level of the benthic copepod *Tigriopus japonicus* in early exposure time (1 day) and an increase in its level in late exposure time. The decrease of GSH level in early exposure time is due to the GSH's ability to become the first defense line to heavy metal attack. Our results show that Ni significantly inhibited GSH content at the end of exposure time for all Ni treatments. This inhibition might be explained by Ni accumulation and the reduction of the cellular ability to eliminate ROS, so that it raises the general oxidative stress potential in the cells.

Among phase II enzymes, the expression of GST is a family of multifunctional enzymes related to cellular antioxidant defenses. GST conjugates different electrophilic toxic chemicals with GSH during phase II of biotransformation reactions, enhancing the polarity of these chemicals by neutralizing their active electrophilic sites and subsequently making the toxic chemical compound more water soluble in order to enable their excretion (Sáenz *et al*., 2010). The present study show that Ni treatments increased GST activity at day 7, and was significantly different from control under 100 µg L^{-1} Ni treatment (figure 1B, $p< 0.05$). However, the highest Ni treatment (1000 µg L^{-1}) had a noticeable inhibition effect on GST activity at day 14 ($p< 0.05$). In agreement with our results, Wang and Wang (2009; 2010) found that heavy metals (Cd and Ni) significantly increased GST activity of the benthic copepod *Tigriopus japonicus* after 7 days exposure. Additionally, previous studies showed a significant change of GST activity in other marine invertebrates exposed to heavy metals (Canesi *et al*., 1999; Cunha *et al*., 2007; Attig *et al*., 2010). Canesi *et al*. (1999) reported that Cu and Hg significantly stimulated GST activity in gill and digestive gland of mussel *Mytilus galloprovincialis*. Also Attig *et al*. (2010) observed an increase of the digestive gland GST activity of mussel *M. galloprovincialis* after being exposed to Ni. However, Cunha *et al*. (2007) found that Cu caused a significant reduction of marine gastropod *Nucella lapillus* GST activity but had no effect on *Monodonta lineate* GST, while Cd had no significant effects on the GST activity of either species. Therefore, GST biomarker response might be dependent on species, type of metal, metal concentrations and experimental time. The mechanism of impact of Ni on this enzyme is still unknown and needs more investigation.

The cellular defense system against metal toxicity includes induction of SOD, which catalyses scavenging of superoxide anion radicals ($O_2^{\bullet -}$) to hydrogen peroxide H_2O_2 (Limón-Pacheco and Gonsebatt, 2009). Our study reveals that Ni increased copepod's SOD activity by day 1; this induction was significantly different from control under 100 µg L^{-1} and 1000 µg L^{-1} Ni treatments (fig. 1C, $p< 0.05$). The inhibition effect of all Ni treatments on the copepod's SOD activity was shown by day 14. The induction of SOD by day 1 might be to eliminate ROS caused by Ni, and so counteract oxidative stress. Thus, SOD might act as the first defense line against ROS attraction in the copepod *A. borneoensis* exposed to Ni, dismutating superoxide to H_2O_2. H_2O_2 is then subsequently detoxified by several enzymes such as, CAT and GPx (Limón-Pacheco and Gonsebatt, 2009). Similarly, Wang and Wang (2009; 2010) reported an increase of SOD activity in the benthic copepod *T. japonicus* after being exposed to Cd and Ni. Inhibition of the copepod's SOD activity at the end of exposure time (14 days) might be associated with Ni accumulation, accumulated H_2O_2 and the oxidative damage potential in the copepod cells. Significant inhibition of SOD activity was reported in aquatic animals exposed to heavy metals (Chandran *et al*., 2005; Vutukuru *et al*., 2006).

Heavy metals may alter the structure of cell membranes by stimulating the lipid peroxidation process, a complex sequence of biochemical reactions, broadly defined as oxidative deterioration of unsaturated fatty acids. Lipid peroxidation results in the production of lipid radicals and in the formation of a complex mixture of lipid degradation or secondary products (malondialdehyde and other aldehydes such as formaldehyde, acetaldehyde, etc.), which are extremely toxic to cells due to their high affinity to thiol and amino groups of peptides, enzymes, and nucleic acids (Roméo and Gnassia-Barelli, 1997; Benedet and Shibamoto, 2008). Malondialdehyde (MDA), which is a major cytotoxic product of lipid peroxidation, acts as oxidative stress biomarker of heavy metal exposure in aquatic animals (Roméo and Gnassia-Barelli, 1997; Jena *et al*., 2009; Wang and Wang, 2009; Attig *et al*., 2010). Unexpectedly, *A. borneoensis* LPO measured as MDA level significantly decreased with the increase of Ni concentration at the end of exposure time (14 days) (fig. 2, $p< 0.05$). Taking into account that Ni treatment significantly inhibited GSH level, GST and SOD activity at day 14, it is quite probable that the copepod cells suffered from oxidative stress or even oxidative damage, which might lead to mutagenic and carcinogenic effects to the copepod, since MDA is a reactive electrophile reacts with protein and DNA (Marnett, 1999; Nair *et al*., 2007). Similarly, Wang and Wang (2010) found that Ni treatment strictly prohibited LOP level in the copepod *T. japonicus* after 12 days of exposure.

Previous studies demonstrated that heavy metals may be neurotoxic agents to copepods, via affecting AChE activity (Forget *et al*., 1999; Wang and Wang, 2009; 2010). *A. borneoensis* AChE activity was not significantly affected after being exposed to lowest Ni treatments (10 and 100 µg L^{-1}) for 1, 2, 4 days except for 100 µg L^{-1} Ni treatment of day 4; however, by day 7, they significantly stimulated its activity (fig. 1D, $p< 0.05$). The highest Ni treatment (1000 µg L^{-1}) significantly stimulated the copepod's AChE activity at day 1 and reach its peak by day 2, and then had a noticeable inhibition effect with the increase of exposure time. Thus, Ni might cause neurotoxicity to the copepod by stimulating AChE activity during certain exposure time. In agreement with our results, Cd and Ni significantly increased AChE activity in the copepod *T. japonicus* after a certain exposure time (Wang and Wang, 2009; 2010). However, no significant change is found on AChE activity in the copepod *T. brevicornis* after a 96 h exposure to LC_{50} concentration of Cd, but similar exposure to As or Cu exerts an inhibitory effect (Forget *et al*., 1999). Taken together, exposure of copepods to heavy metals causes different responses of AChE activity. Thus, AChE biomarker response might

vary with copepod species, type of metal and metal concentration.

5. Conclusion

Biological markers or biomarkers have been used to diagnose environmental contamination and to assess its effects on living organisms. Particularly, copepods are considered sensitive indicators of metal toxicity, being employed ecotoxicological studies both in the laboratory and in the field (Raisuddin *et al*., 2007). The present study investigated the biochemical response of the cyclopoida copepod *A. borneoensis* following exposure to sublethal Ni treatments. In conclusion, our results showed that Ni treatment had a significant effect on the copepod's AChE, SOD and GST activity and GSH level at environmentally relevant concentrations after a certain exposure time. Thus, changes in SOD, GST and GSH might be detoxificative defense of copepods against Ni addition. In addition, Ni might also be a neurotoxic agent to copepods via changing AChE activity. In contrast Ni exposure significantly decreased the LPO level of copepods compared to control, implying that the factor involved in LPO might not significantly depend on the operations and functions in the antioxidant defense system. Thus, we suggest that AChE, SOD and GST activities and GSH level be used as suitable biomarkers for Ni pollution.

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Newbouldia laevis (Seem) as an Entomocide Against *Sitophilus oryzae* and *Sitophilus zeamais* Infesting Maize Grain

Olaniyi C. Ogungbite* and Emmanuel A. Oyeniyi

Department of Biology, Federal University of Technology, P.M.B 704, Akure, Nigeria.

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Abstract

This study was conducted under laboratory conditions at temperature of 28±2°C and relative humidity 75±5% to investigate the entomocidal flurry of *Newbouldia laevis* against *Sitophilus oryzae* and *Sitophilus zeamais* infesting maize grain in storage. The powders of stem and root of *N. laevis* were prepared at 0.1, 0.2, 0.3, 0.4 and 0.5g dosages, while their extracts were prepared at 1, 2, 3, 4 and 5% concentrations. The control experiments were also set up. None of the powders of stem and root of this plant was able to achieve complete mortality of the two insects within 96h of application, except for the root powder which was able to achieve 100% mortality of *S. oryzae* at 0.5g dosage only. Only the root extract at 4% was able to achieve complete weevils mortality within 96h and its effect was significantly different (p<0.05) from the extracts of the stem except at 4 and 5% concentration of stem extract. The fiducial limits showed that a lower concentration of root extract was needed to cause 50% mortality in *S. zeamais* (0.00-1.64 %) when compared to the concentration needed for *S. oryzae* (0.10-1.72 %). Both the stem and root extracts of the plant were more effective than their powders. The powders and the extracts of this plant either reduced or prevented the emergence of adult weevils and inhibit the developmental period of the insects. The powders and the extracts of *N. laevis* also reduced or prevented the weight loss of the treated maize grains. Therefore, with the result obtained in this research, both the powder and extract of *N. laevis* root and stem could go a long way in the quest of providing alternative wherewithal to the use of chemical insecticides for protecting maize grain in storage. Root extract of *N. laevis* could, however, offer more protection against *S. oryzae* and *S. zeamais* infesting maize grain than its stem extracts.

Keywords: Entomocide, Plant Extract, *Newbouldia laevis*, *Sitophilus oryzae, Sitophilus zeamais*, Adult Emergence, Weight Loss.

1. Introduction

Crop protection plays a vital and integral role in ensuring food security in a particular country. In many developing countries, security of food has been threatened due to infestation of their farm produce by many stored product insect pests starting from the field to storage where it is more pronounced. Loss of stored grains which amounts to 5-10% in the temperate countries and 20-30% in the tropical zones has been associated with the infestation by many stored product insect pests (Dubey *et al.,* 2008; Rajashekar and Shivanandappa, 2010). Maize, one of the major staple foods of the world, has been attacked by a wide range of insect pests including beetles, weevils and moths.

In recent decades, controlling many of these destructive insect pests has profoundly relied upon the use of synthetic chemical insecticides (Akinkurolere, 2007; Akinneye and Ogungbite, 2013), which has been reported to have many cons that impede their widespread use nowadays. These include high cost of procurement, pest resurgence and resistance, poisonous residue accumulation in foods, risks of user's contamination, effect on both human and environmental health (Ashamo and Akinnawonu, 2012; Akinneye and Ogungbite, 2013). Public awareness of the adverse effects of the synthetic chemical insecticides has called for the urgent need to look for safer alternatives that could comparably contend with chemical insecticides in action both preferably and adequately.

In order to obviate the use of these synthetic chemical insecticides, research studies have been focused on the plant kingdom as a new tool of controlling insect pests of stored products. Prior to the discovery and commercial success of the synthetic chemical insecticides in the late 1930s and early 1940s, botanical base insecticides have remained important weapons in the farmers armory in managing the insect pests of their farm produce (Forim *et al.,* 2012). Hitherto, in spite of the effectiveness of many botanical insecticides, their insecticidal activity is yet to be comparable to many synthetic chemical insecticides; and the once that are believed to be comparable with chemical insecticides have not commanded more than 1% of the global insecticide market (Isman, 2000;

* Corresponding author. e-mail: olaniyi2oguns@gmail.com.

Begum *et al.*, 2013) because they are believed to live for a short period of time and to lose their potency over time (Oruonye and Okrikata, 2010). Therefore, there is still a need to search for other plant species which could have high efficacy against stored product insect pests.

Sitophilus oryzae and *Sitophilus zeamais* are important stored product insect pests of maize, whose infestations have brought about diminution of the quality and the marketability of this grain. *Newbouldia laevis* is a medicinal plant whose insecticidal effect has been proved against *Callosobruchus maculatus* infesting cowpea seeds (Ashamo *et al.*, 2013). This study, therefore, investigates *N. laevis* (Seem) as an entomocide against *S. oryzae* and *S. zeamais* infesting maize grain.

2. Materials and Methods

2.1. Insect Culture

The culture of *S. oryzae* and *S. zeamais* was obtained from an infested maize grain at Food Storage Research Laboratory, Federal University of Technology, Akure, Nigeria. This was reared on non-infested clean maize grains obtained from Agricultural Development Project (ADP) Akure, Nigeria. The experiment was setup in the laboratory at temperature of 28±2°C and relative humidity of 75±5%.

2.2. Collection of Plant Materials and Maize Seeds

Different parts of *N. laevis* were collected from Oke-Odo Aratunsin area of Akure, Ondo State, Nigeria. Collected plants were taken to Natural History Museum Obafemi Awolowo University, Ile-Ife for identification. The yellow variety of maize grains, used for the experiment, was obtained from the Agricultural Development Project (ADP), Akure, Nigeria. The seeds were cleaned of foreign matter and disinfested by being kept in a freezer at -5°C for 7 days. They were then air-dried to avoid mouldiness of the grains.

2.3. Preparation of Plant Powder and Extracts

The plant parts (stem bark and root bark) used were collected fresh and sun dried. The plant parts were ground into fine powder using electric blender and the powders were further sieved to pass through 1mm^2 perforations before being stored in separate plastic containers with tight lids for subsequent use. 0.1, 0.2, 0.3, 0.4 and 0.5g of the powders were weighed using Metler beam weighing balance. To prepare the extract, twenty grams of each pulverized plant materials (stem bark and root bark) was put in a muslin cloth and transferred into the thimble and extracted with methanol in a soxhlet apparatus. The extraction was carried out for 3-4 hr depending on the plant material. The extraction was terminated when the solvent in the thimble became clear. Then, the thimble was removed from the unit and the solvent recovered by distilling in the soxhlet extractor. The resulting extracts contained both the solvent and the oil. The solvent was separated from the oil using rotary evaporator, after which the oil was exposed to air so that traces of the volatile solvents evaporate, leaving the oil extract. This is important so as to avoid making false concentrations.

From this main stock solution, different concentrations of 1, 2, 3, 4 and 5% oil concentrations were made. A concentration of 1% was made by diluting 0.1ml of plant extract in 9.9ml of methanol (solvent). 2% concentration was made by diluting 0.2ml of plant extract in 9.8ml methanol. Also, 3%, 4% and 5% concentration was made by diluting 0.3ml, 0.4ml and 0.5ml of the plant extract with 9.7ml, 9.6ml and 9.5ml of the solvent respectively. The various concentrations were made using small glass bottles and graduated syringes. After each dilution, the syringe was rinsed with the solvent while different syringes were used for different plant part extracts.

2.4. Effect of N. Laevis on Mortality, Adult Emergence and Weight Loss

2.4.1. Plant Powder

Twenty grammes of the maize grains were weighed into 250ml plastic containers. Plant powders weighing 0.0g (control), 0.1g, 0.2g, 0.3g, 0.4g and 0.5g was weighed and thoroughly mixed with the maize seeds inside the plastic containers using glass rod. The experiment was set up in a complete randomized design and each treatment was replicated five times. Ten pairs of *S. zeamais* and *S. oryzae* were separately introduced into those treated maize grains immediately and weevil mortality was assessed at 72 and 96 h post treatment. Both dead and live insects were removed on the fourth day and experiments were left for 42days to allow for emergence of F1 generation and the number of adult emerged was counted. Inhibition rate (%IR) in adult emergence was calculated using the method described by Tapondju *et al.* (2002). The weight loss of the stored

$$\%IR = \frac{C_n - T_n}{C_n} x \frac{100}{1}$$

grains was calculated using the formula below:

Where C_n is the number of insects that emerged in the control treatment and T_n is the number of adult

$$\%\text{weight loss} = \frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} x \frac{100}{1}$$

insects that emerged in the treated grains

2.4.2. Oil Extracts

20g of maize gains was weighed into 250ml plastic containers and 1ml of plant extracts concentration of 1, 2, 3, 4 and 5% was separately mixed with the maize and was left for 1hr to ensure the escape of the methanol solvent. Two control experiments were set up, one with solvent alone and one with neither solvent nor extract (untreated control). Ten pairs of 0-24 adult insects were introduced into containers with treated grains and were arranged in a complete randomized design and each treatment was replicated five times. Adult mortality was observed after 72 and 96h of application. Both dead and live insects were removed on the fourth day and experiments were left for 42 days to allow for emergence of F_1 generation and the number of adult

emerged was counted. %IR and the weight loss of the stored grains were calculated using the formula described above

2.5. Statistical Analysis

All the data obtained were subjected to one-way analysis of variance at 5% significant level and means were separated with New Duncan's Multiple Range Tests using SPSS version 17. Also data, obtained from weevil's mortality, were subjected to regression analysis to calculate the LD_{50} of the powders and LC_{50} of the extracts using probit analysis (Finney, 1971).

3. Results

3.1. Effect of stem and root powders of N. laevis on mortality of S. oryzae and S. zeamais

Table 1 presented the percentage mortality observed in *S. oryzae* and *S. zeamais* exposed to stem and root powder of *N. laevis*. Irrespective of the plant powder, weevil mortality increased with the increase in the dosage of the plant powder and the exposure time. However, significant differences (p<0.05) existed among the mortality values of *S. oryzae* and *S. zeamais*. Likewise, there was a significant difference (p<0.05) in the adult mortality of both weevils treated with both plant powder when compared to control. Generally, mortality values, observed in *S. zeamais*, were observed to be lower than those observed in *S. oryzae* and 100% mortality was only achieved in *S. oryzae* exposed to root powder of *N. laevis* at 0.5g/20g of maize grains after 96 h.

Table 1. Percentage mortality of *S. oryzae* and *S. zeamais* on maize seeds treated with plant powders of *N. laevis*

Plant material	Dosages (g)	Mean % mortality ± SE (hours)			
		S. oryzae		*S. zeamais*	
		72	96	72	96
Stem	0.1	30.00± 0.58^{b}	50.00 ± 0.58^{b}	26.20± 0.33^{b}	33.30± 0.88^{b}
	0.2	53.20± 0.88^{c}	69.00 ± 0.73^{c}	46.70±0.28^{c}	53.33± 1.45^{c}
	0.3	63.30 ± 0.67^{c}	80.00±0.58^{c}	53.28± 0.88^{c}	70.00 ± 0.00^{d}
	0.4	74.20± 0.33^{cd}	86.70±1.33^{cd}	66.40± 0.88^{cd}	76.70 ± 0.33^{de}
	0.5	78.67± 0.33^{cd}	93.30± 0.33^{cd}	76.70± 0.24^{d}	88.00 ±0.28^{e}
Root	0.1	46.00± 0.66^{b}	53.00± 0.68^{b}	35.00± 0.55^{b}	41.00± 0.58^{b}
	0.2	52.00 ± 0.93^{b}	62.00 ± 0.75^{b}	47.00 ± 0.60^{b}	56.00± 0.66^{c}
	0.3	69.00 ± 0.73^{c}	77.00 ± 1.36^{c}	56.00± 0.66^{b}	78.00± 1.22^{d}
	0.4	88.00 ± 0.75^{d}	99.00 ± 0.20^{c}	76.00 ± 1.07^{bc}	88.20± 0.63^{de}
	0.5	93.00 ± 0.40^{d}	100.00 ± 0.00^{c}	81.00 ± 0.80^{c}	94.00 ± 1.29^{e}
Control	0.0	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan Multiple Range Test.

3.2. Effect of stem and root extracts of N. laevis on mortality of S. oryzae and S. zeamais

The mortality of weevils treated with extracts of stem and root of *N. laevis* is presented in Table 2. The percentage of weevil mortality, observed in both *S. oryzae* and *S. zeamais*, was significantly different (p<0.05) from the controls with or without methanol solvent. Significant differences also existed among percentage mortality values at different concentrations of stem and root extracts in both weevils. Generally, weevil mortality increased with the increase in exposure time and concentration of stem or root extract. However, regardless of the exposure time, root extract achieved higher percentage mortality (53-100%) in both weevils at each of the concentrations when compared to stem extract (40.30-100%). This shows that the root extract was more toxic to both weevils when compared to stem extract.

Table 2. Percentage mortality of *S. oryzae* and *S. zeamais* on maize seed treated with extracts of *N. laevis*

Plant material	Concentrations (%)	Mean % mortality ± SE (hours)			
		S. oryzae		*S. zeamais*	
		72	96	72	96
Stem	1	43.30±0.33^{b}	53.30±1.45^{b}	40.30±0.33^{b}	56.30± 0.24^{b}
	2	63.30±0.33^{c}	68.00±0.58^{c}	54.70±0.24^{bc}	66.30± 1.00^{bc}
	3	66.70±0.88^{c}	83.30±0.67^{d}	59.30±0.88^{c}	74.00 ± 0.80^{cd}
	4	76.70±0.33^{cd}	90.40±0.22^{de}	72.00±0.24^{cd}	89.78 ± 2.03^{e}
	5	86.70±1.33^{d}	100.00±0.00^{e}	83.20±0.67^{de}	100.00 ± 0.00^{e}
Root	1	53.30±1.45^{b}	53.00± 0.68^{b}	60.00± 0.53^{c}	67.24± 0.88^{bc}
	2	66.24±0.28^{c}	62.00 ± 0.75^{bc}	68.30 ± 0.44^{cd}	79.00± 0.33^{cd}
	3	83.30±0.67^{d}	77.00 ± 1.36^{cd}	86.00± 0.67^{e}	88.00± 0.24^{de}
	4	96.70±0.30^{e}	100.00 ± 0.00^{e}	98.26 ± 0.22^{e}	100.00 ± 0.00^{e}
	5	100.00 ± 0.00^{e}	100.00 ± 0.00^{e}	100.00 ± 0.00^{e}	100.00 ± 0.00^{e}
Treated control		0.00±0.00^{a}	2.00±0.33^{a}	2.24±0.88^{a}	2.24±0.88^{a}
Untreated control		0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan Multiple Range Test.

3.3. Lethal dose (LD_{50}) and lethal concentrations (LC_{50}) of N. laevis powder and extract in S. oryzae and S. zeamais after 72 h

Table 3 shows that lower amounts of root powder and extract of *N. laevis* were needed to achieve 50% mortality in both weevils when compared to the amount required using stem powder and extract. This shows that the root of this plant material was more toxic than the stem. Also, higher amount of stem powder, root powder and stem extract of *N. laevis* were needed to kill 50% of *S. zeamais* when compared to the amount needed for *S. oryzae*. However, fiducial limits revealed that a lower amount of root extract was needed to cause 50% mortality in *S. zeamais* (0.00-1.64) when compared to the amount needed for *S. oryzae* (0.10-1.72). This further revealed that *S. oryzae* were more susceptible to stem and root powder as well as stem extract, while *S. zeamais* insects were, however, more susceptible to root extract.

Table 3. Lethal dose (LD_{50}) and lethal concentration (LC_{50}) of *N. laevis* powders and extracts required to achieve 50% mortality in *S. oryzae* and *S. zeamais* after 72 hours post treatment

Insect	Plant powder		Plant extract	
	LD_{50}(gm)		LC_{50}(%)	
	Stem	Root	Stem	Root
S. oryzae	0.19	0.14	1.32	1.09
	(0.16-0.22)	(0.01-0.22)	(0.95-1.62)	(0.10-1.72)
S. zeamais	0.23	0.19	1.61	0.96
	(0.20-0.27)	(0.10-0.27)	(1.22-1.95)	(0.00-1.64)

Values in parenthesis represent 95% fiducial limits.

3.4. Adult emergence, Inhibition rate (%IR) in adult emergence of S. oryzae and S. zeamais and weight loss in maize grains treated with N. laevis powders

Table 4 reveals more *S. oryzae* (33.24%) and S. *zeamais* (37.21%) emerged from the control maize grain which was significantly higher (p<0.05) than those observed in the treated maize grains. Weevil's emergence in the treated maize grains also decreased with increasing dosage of both plant powders. However, significantly fewer (p<0.05*) S. oryzae* and *S. zeamais* emerged from the maize grains treated with root powder when compared to those treated with stem powder. Percentage reduction in F1 progeny also increased with the increase in the dosage of stem and root powder of *N. laevis*; this was significantly different (p<0.05) from that of control. Regardless of the weevil used to infest the maize grains, high percentage reduction in F1 progeny, which ranges from 89.85-100%, was observed in maize grains treated with root powder of *N. laevis* when compared to their counterpart treated with stem powder of this plant material which ranges from 81.60-89.29%. 100% reduction in F1 progeny was, however, observed only at 0.4g and 0.5g of root powder/20g of maize grains infested with *S. oryzae*. But for maize grains infested with *S. zeamais*, 100% reduction was only observed at 0.5g of root powder/20g of maize grains. Weight loss in control maize grains was significantly higher (p<0.05) than in the treated maize grains. Significant differences also existed among maize grains treated with both plant parts powders and infested with both weevils. Generally, weight loss in maize grains reduced with increasing stem and root powder of *N. laevis*. No weight loss was, however, observed at 0.4 and 0.5g of root powder/20g of maize grains infested with *S. oryzae* and 0.5g of root powder/20g of maize grains infested with *S. zeamais*.

Table 4. Number of adult emergence, inhibition rate (%IR) in adult emergence of *S. oryzae* and *S. zeamais* and weight loss in maize grains treated with *N. laevis* powders.

Plant materials	Dosage (g)	*S. oryzae*			*S. zeamais*		
		Mean number of adult emergence±S.E	%IR	(%)Weight loss	Mean number of adult emergence	%IR	(%)Weight loss
Stem	0.1	7.93±0.44^{d}	81.60±0.08^{b}	9.00±0.58^{c}	8.10±0.08^{ef}	73.62±0.01^{b}	9.78±0.39^{d}
	0.2	5.74±1.33^{c}	83.79±1.43^{b}	8.60±0.40^{c}	7.50±1.55^{e}	77.08±0.22^{b}	9.60±1.33^{d}
	0.3	4.44±2.03^{c}	86.00±0.33^{b}	7.67±0.33^{bc}	4.87±3.02^{d}	82.30±0.02^{c}	7.96±0.24^{c}
	0.4	4.02±0.34^{c}	86.95±1.77^{b}	7.33±0.67^{bc}	4.56±0.53^{d}	85.82±1.02^{c}	7.58±4.01^{c}
	0.5	3.97±2.88^{c}	89.29±0.33^{bc}	6.11±1.06^{b}	4.29±1.48^{d}	88.37±2.36^{cd}	6.11±1.06^{bc}
Root	0.1	2.70±0.34^{b}	89.85±1.17^{bc}	9.43±1.32^{c}	2.89±0.23^{c}	89.40±0.01^{cd}	9.54±0.03^{d}
	0.2	2.10±1.28^{b}	92.00±0.32^{cd}	6.28±0.33^{b}	2.47±0.13^{c}	91.49±0.22^{d}	7.13±1.04^{c}
	0.3	0.13±0.01^{a}	99.98±0.01^{d}	4.67±2.88^{b}	1.44±2.04^{b}	95.04±1.67^{d}	5.78±0.23^{bc}
	0.4	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	1.00±0.33^{ab}	97.86±2.24^{e}	3.86±0.33^{b}
	0.5	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	0.00±0.00^{a}	100.00±0.00^{e}	0.00±0.00^{a}
Control	0.0	33.26±2.06^{e}	0.00±0.00^{a}	73.30±0.01^{d}	37.21±2.45^{g}	0.00±0.00^{a}	81.32±0.28^{e}

Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan Multiple Range Test.

3.5. Adult emergence, Inhibition rate (%IR) in adult emergence of S. oryzae and S. zeamais and weight loss in maize grains treated with N. laevis extracts

Table 5 shows there was a significantly higher (p<0.05) adult emergence of both weevils in maize grains of treated and untreated control when compared to grains treated with stem and root extracts of *N. laevis.* Adult emergence decreased with the increase in concentration of plant part extract irrespective of the plant part extract used. However, grains treated with root extract and infested with both weevils showed a lower adult emergence than those treated with stem extract of *N. laevis.* No weevil emerged at 4 and 5% of stem extract/20g of maize grains infested with *S. oryzae* and 5% of stem extract/20g of maize grains infested with *S. zeamais.* Likewise, no weevil emerged at 3, 4 and 5% of root extract/20g of maize grains infested with *S. oryzae* and *S. zeamais*. Percentage reduction in F1 progeny of both weevils increased with increase in concentration of both extracts and 100% reduction in F1 progeny was observed at 4 and 5% of stem extract/20g of maize grains infested with *S. oryzae* and 5% of stem extract/20g maize grains infested with *S. zeamais*. At 3, 4 and 5% of root extract/20g of maize grains infested with both weevils; 100% reduction was also observed in progeny development. Weight loss in maize grains of treated and untreated control was significantly higher (p<0.05) than in the maize grains treated with extract of *N. laevis*. Weight loss at 4 and 5% of stem extract/20g of maize grains infested with both weevils was significantly lower (p<0.05) when compared to weight loss at 1, 2 and 3% respectively. Likewise, weight loss at 3, 4 and 5% of root extract/20g of maize grains infested with *S. oryzae* was also significantly lower than at 1 and 2%. But, no significant different (p>0.05) existed among the weight loss observed in maize grains treated with root extract and infested with *S. zeamais*.

Table 5. Number of adult emergence, inhibition rate (%IR) in adult emergence of *S. oryzae* and *S. zeamais* and weight loss in maize grains treated with *N. laevis* extracts.

Plant materials	Conc. (%)	*S. oryza*			*S. zeamais*		
		Mean number of adult emergence	%IR	(%)Weight Loss	Mean number of adult emergence	%IR	(%)Weight loss
Stem	1	5.25±2.57^{e}	86.42±2.08^{b}	6.82±1.44^{d}	8.10±0.08^{ef}	73.62±0.01^{b}	7.25±0.01^{d}
	2	3.74±1.28^{d}	88.36±1.22^{b}	5.96±4.20^{cd}	7.50±1.55^{e}	77.08±0.22^{b}	6.25±2.01^{d}
	3	1.22±4.01^{c}	92.24±3.34^{c}	4.67±0.01^{c}	4.87±3.02^{d}	82.30±0.02^{b}	4.98±0.67^{c}
	4	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	0.58±0.24^{ab}	97.44±4.01^{c}	2.42±2.67^{ab}
	5	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	0.00±0.00^{a}	100.00±0.00^{c}	0.00±0.00^{a}
Root	1	1.70±1.33^{c}	95.67±1.33^{cd}	2.74±2.88^{b}	1.98±1.33^{c}	93.74±2.44^{c}	2.73±0.01^{ab}
	2	0.52±1.28^{ab}	97.82±0.02^{d}	2.14±0.01^{b}	0.58±4.13^{ab}	97.44±0.28^{c}	0.60±3.01^{a}
	3	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	0.00±0.00^{a}	100.00±0.00^{c}	0.00±0.00^{a}
	4	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	0.00±0.00^{a}	100.00±0.00^{c}	0.00±0.00^{a}
	5	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	0.00±0.00^{a}	100.00±0.00^{c}	0.00±0.00^{a}
Untreated control		33.26±2.06^{f}	0.00±0.00^{a}	73.30±0.01^{e}	37.21±2.45^{g}	0.00±0.00^{a}	81.32±0.28^{d}
Treated control		34.78±1.33^{f}	0.00±0.00^{a}	73.89±0.10^{e}	35.82±1.55^{g}	0.00±0.00^{a}	78.67±2.67^{d}

Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan Multiple Range Test.

4. Discussion

The use of plant materials as a protectant against stored product pests is a common practice mostly in many developing countries of the world. This practice has been suggested as one of great hope for controlling stored product pests (Singh, 2011) due to several limitations associated with the use of synthetic insecticides and fumigants. As a result, several powders and extracts of different plants have been shown to possess insecticidal activity against stored product pests (Ileke and Olotuah, 2012; Akinneye and Ogungbite, 2013; Ashamo *et al*., 2013).

In this study, both the powders and extracts of *N. laevis* were observed to cause adult mortality and reduced adult emergence and progeny development of both weevils. They also reduced weight loss of maize grains treated with this plant material. However, the efficacy depended on the plant part used (stem or root), dosage or concentration of *N. laevis* and exposure time. Stem and root extracts were also observed to be more effective than the powders and this may be linked to the presence of more bioactive compounds in the methanolic extracts of this plant when compared to the powder. Powder and extract of *N. laevis* root also proved to be more effective in controlling both weevils than the stem as revealed by their $LD_{50}$and LC_{50} values. This can be corroborated by the findings of Ashamo and Akinnawonu (2012) in which the efficacy of different plant oils were found to be effective than their powders. It was also noted that the root extracts were more effective than the stem extracts. This result agreed with the work of Ashamo *et al*. (2013) in which the root extract of *N. laevis* was found to be more effective than the extracts of its leaf and stem in controlling *Callosobruchus maculatus* on cowpea. The high mortality effect of these *N. laevis* oil extracts and powders could be due to the inability of the insects to feed on the maize grain that have been coated with these oils and powders thereby leading to their starvation. The oils and powders of this plant may have also disrupted the normal respiratory activities of these insects leading to the asphyxiation and subsequent death (Ashamo *et al.,* 2013).

The higher efficacy of root powder and extract of *N. laevis* may be ascribed to some toxic phytochemical compounds in roots that may not be present in stem of this plant. *N. laevis* root bark had been reported to contain alkaloids, tannins, flavonoids, saponins, cyanogenetic glycosides, cardiac glycosides and phenylpropanoids (Germann *et al.,* 2006; Akerele *et al.,* 2011) while the stem barks contained tannins, flavonoids, and alkaloids (Anaduaka *et al.,* 2013). Most of these compounds had been reported for their considerable toxicity and antifeedant effect towards insects (Yang *et al*., 2006). However, the actual compound responsible for the higher insecticidal activity of root of *N. laevis* on weevils when compared to that of the stem remains to be explored.

The results of this study reveal that *S. zeamais* tolerates stem and root powder as well as stem extract more than *S. oryzae* while *S. oryzae* tolerates the root extract more than *S. zeamais*. The high tolerance of *S. zeamais* insects to the plant material might be due to the thicker exoskeleton and larger size when compared to that of *S. oryzae* (Buhler, 2013). Thicker exoskeleton in insects reduces the penetration of plant materials (Delorme *et al.,* 1988) while larger size affords them the ability to withstand the effect of a poison (Buhler, 2013).

Powders and extracts of *N. laevis* also significantly reduced or prevented the adult emergence of both weevils when compared to the control. This could be linked to the inability of the weevil eggs to develop adult due to the death of their larvae, which cannot cast off their old exoskeleton which typically remain linked to the posterior part of the abdomen (Oigiangbe *et al.,* 2010). This further suggests that *N. laevis* may have an obvious effect on the post embryonic survival of both weevils, which, in turn, prevents and significantly reduces adult emergence from treated maize grains when compared to control (Ashamo *et al.,* 2013). Also, different chemical compositions of these plants as mentioned earlier could be accountable for the inability of the adult insects to emerge as they are found to disrupt growth and reduced larva survival as well as disruption of life cycle of insects (Yang *et al.,* 2006). This result showed that both the powders and extracts of *N. laevis* had high rate of inhibition on the emergence of the adult weevils and this agreed with the work of Akinneye and Ogungbite (2013) in which the powder and extract of *Zanthoxylum. zanthoxyliodes* was found to inhibit the development of *S. zeamais.*

Powders and extracts of *N. laevis* significantly reduced or prevented the weight loss of treated maize grains. At the highest experimental dosage and concentration, the plant material completely prevented weight loss in infested maize grains with the exception of stem powder. The plant root powder and extract however reduced weight loss in infested maize grains than the stem powder and extract. This reduction in weight loss may be due to the inability of the larvae of both weevils to feed on the treated maize grains. Similar observation has been reported by Jayakumar *et al.* (2003) and Asawalam *et al.* (2007) on cowpea seeds treated with plant materials.

N. laevis is a plant with so many medicinal properties as it is used in the treatment of malaria fever, constipation coughs, tooth ache, sexually transmitted diseases and breast cancer (Iwu, 2000). The plant is readily available and it is native to tropical Africa and grows from Guinea Savannahs to dense forests, or moist and well-drained soils (Burkhill, 1984). Therefore, with the result obtained in this research, both the powder and extract of *N. laevis* root and stem could go a long way in the quest of providing alternative wherewithal to the use of chemical insecticides for protecting maize grain in storage. Root extract of *N. laevis* could however offer more protection against *S. oryzae* and *S. zeamais* infesting maize grain than its stem extracts. Further investigations is required to identify the main active compounds responsible for the higher toxicity of both powders and extracts of *N. laevis* stem and root on weevils.

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Entomocidal Activity of Powders and Extracts of Four Medicinal Plants Against *Sitophilus oryzae* (L), *Oryzaephilus mercator* (Faur) and *Ryzopertha dominica* (Fabr.)

Kayode D. Ileke[1,*] and Olaniyi C. Ogungbite[2]

[1]*Department of Environmental Biology and Fisheries, Faculty of Science, Adekunle Ajasin University P.M.B 001, Akungba-Akoko,Ondo State, Nigeria* [2]*Department of Biology, School of Science, Federal University of Technology P.M.B 704, Akure, Ondo State, Nigeria.*

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Abstract

Powders and extracts of *Azadirachta indica, Zanthoxylum zanthoxyloides, Anacardium occidentale* and *Moringa oleifera* were assessed in the laboratory at ambient temperature of 28±2°C and relative humidity of 75±5% for their insecticidal activity against *Sitophilus oryzae, Oryzaephilus mercator* and *Ryzopertha dominica* infesting paddy rice. The powders of these plants were tested against these insects at 0.4g, 0.6g, 0.8g and 1g/50g of paddy while the extracts were tested at 2%, 4%, 6% and 8% concentrations. Both the powders and extracts of these botanicals evoked a high mortality effect on the insects. Moreover, both the extracts and powders of *A. indica* and *Z. zanthoxyloides* showed greater insecticidal bustle than the powders and extracts of *A. occidentale* and *M. oleifera* as they both achieved 100% insect mortality within a short period after treatment. The extracts of these plants were also able to prevent the emergence of adult insects. *S. oryzae* and *R. dominica* were more affected with all the powders and extracts of these botanicals than *O. mercator* which was more vulnerable to the extracts and powders of *Z. zanthoxyloides* than other botanicals. Moreover, all the plants used showed a greater insecticidal effect and could be integrated into pest management system.

Keywords: *Azadirachta indica, Zanthoxylum zanthoxyloides, Anacardium occidentale, Moringa oleifera, Sitophilus oryzae, Oryzaephilus mercator, Rhyzopertha dominica*

1. Introduction

Loss of cereal grains via insect infestation during storage is a serious problem, particularly in the developing countries, where damage to stored grains and their products by insects may amount to 5 - 10% in the temperate countries and 20 - 30% in the tropical zones (Dubey *et al*., 2008; Rajashekar and Shivanandappa, 2010; Ileke and Oni, 2011; Akinneye and Ogungbite, 2013). Rice is the seed of the monocot plant *Oryza sativa* (Ashamo and Akinnawonu, 2012). It is the grain with the second highest worldwide production, after maize (FAO, 2010). Rice helped Africa to conquer its famine of 1203 (NRC, 1996; Ashamo and Akinnawonu, 2012). Rice, being one of the staple foods mostly consumed by many parts of the world, has been infested by many important insect pests such as *Sitotroga cerealella, S. oryzae, S. granarius, R. dominica, O.mercator* as well as *Scirpophaga incertulas* and *Scirpophaga innotata* among others (Sarwar, 2012; Ashamo and Akinnawonu, 2012). Therefore, crop protection plays a vital and integral role in modern agricultural production; and the ever-lasting demands on yield as well as intensification of farming practices have increased the problem of pest damage and hence control (Martins *et al.,* 2012).

Infestation control of stored grains insect pests is primarily achieved by the use of synthetic chemical insecticides, such as methyl bromide and phosphine. In several countries, due to environmental concerns and human health hazards, several chemical insecticides have either been banned or restricted (Tapandjou *et al.,* 2002). The adverse effects of the most novel chemical insecticides have led researchers to try to find new avenue of insect control, which has led to the discovery of plant products as an alternative way of controlling insects (Sutherland *et al*., 2002; Zibaee, 2011). Moreover, tropical regions are believed to be endowed with many plant species with insecticidal properties and some of them are with medicinal properties (Ileke and Oni, 2011). Therefore, this research investigates the entomocidal of powders and oil extracts of four medicinal plants against *S. oryzae, R. dominica*, and *O. mercator* which are important insect pests of paddy rice in storage.

* Corresponding author. e-mail: kayodeileke@yahoo.com.

2. Materials and Methods

2.1. Insect Culture

The adults *S. oryzae, R. dominica* and *O. mercator* used were obtained from the existing cultures in the Department of Environmental Biology and Fishery Research Laboratory, Adekunle Ajasin University, Akungba Akoko, Ondo State, Nigeria. Clean un-infested paddy rice variety FARO-36, collected from the International Institute for Tropical Agriculture, Ibadan, Nigeria, was used to rear the insects inside 2 litres jars covered with muslin cloth. The jars were kept at 28±2°C and 75±5% relative humidity in insect cages and the culture was maintained by replacing the devoured grains with fresh uninfested one.

2.2. Preparation of Plant Materials

The used plant materials, *A. indica* seeds, *Z. zanthoxyloides* roots, *A. occidentale* nuts and *M. oleifera* seeds were sourced fresh from a farm at Ipinsa, Akure, Ondo State, Nigeria. The plants were sun dried, separately milled into fine powders, sieved to pass through 1mm^2 mesh and kept inside different plastic containers with tight lids at 4^{o}C in a refrigerator at prior to use.

2.3. Preparation of the Extracts

Methanolic extracts of *A. indica, Z. zanthoxyloides, A. occidentale* and *M. oleifera* were carried out using cold extraction method. About 150g of the powders were soaked separately in an extraction bottle containing 450ml of absolute methanol. The mixture was stirred occasionally with a g lass rod and extraction was terminated after 72 h. The resulting mixture was filtered using a double layer of Whatman No. 1 filter paper and the solvent was evaporated using a rotary evaporator at 30 to 40^{o}C with rotary speed of 3 to 6 rpm for 8 h (Udo, 2011). The resulting materials were air dried in order to remove traces of solvents. From this stock solution, different concentrations of 2%, 4%, 6% and 8% were prepared (Ashamo and Akinnawonu, 2012; Ileke and Bulus, 2012; Ileke *et al*., 2013).

2.4. Toxicity of Plant Powders to Adult Insects

Fifty grams of paddy rice (FARO-36) was weighed into 250ml plastic containers and the plants powders weighing 0.4g, 0.6g, 0.8g and 1.0g were added to each weigh. The powders and the paddy were thoroughly mixed together to ensure uniform spread of the powders. Untreated paddy rice was set as control. Ten pairs of adult insects were introduced into each container.

Four replicates of each treatments and untreated controls were laid out in Complete Randomized Design. Adult mortality was counted and recorded after 48 and 72 hours of application. Both dead and alive insects were removed away on the fourth day and experiments were left for 35days to allow for emergence of F1 generation and the number of adults emerged was counted. Inhibition rate (%IR) in adult emergence was calculated using the method described by Tapandjuo *et al.* (2002).

$$\%IR = \frac{C_n - T_n}{C_n} x \frac{100}{1}$$

where Cn is the number of emerged insects in the control and Tn is the number of emerged insects in the treated container.

2.5. Effect of Oil Extracts on Mortality of Adult Insects

Fifty grams of paddy rice (FARO-36) were weighed into 250ml plastic containers and 1ml of plant extracts concentrations of 2, 4, 6 and 8% were separately mixed with the paddy and were left for 1hr to ensure the evaporation of the volatile solvent. Two control treatments were set up, one with solvent alone and one without solvent or extract (untreated control). Ten pairs of adult insects were introduced into each container. Four replicates of the treatments and untreated controls were laid out in Complete Randomized Design. Adult mortality was observed after 48 a nd 72hours of application. Both dead and alive insects were removed on the fourth day and experiments were left for 35days to allow for adult emergence of F1 generation and the number of emerged adults were counted. Inhibition rate (%IR) in adult emergence was calculated as described above.

2.6. Analysis of Data

All the data obtained were subjected to one-way analysis of variance at 5% significance level and means were separated using New Duncan's Multiple Range Tests.

3. Results

3.1. Effect of Plant Powders on Mortality of S. oryzae, O. mercator and R. dominica

Table 1 pr esents the effect of plant powders on mortality of *S. oryzae, O. mercator* and *R. dominica*. All the plant powders had a significantly high mortality effect on the three tested stored product insects at (p<0.05). Within 48h of application, only the powder of *A. indica* at 1.0g concentration achieved 100% mortality of *S. oryzae* and was significantly different from other powders. Also, the powder of *Z. zanthoxyloides* at 1g affected significantly with 100% mortality of *O. mercator* within 48h of exposure at (p<0.05). However, none of the tested powders was able to achieve complete mortality of *R. dominica* within 48h of application. After 72h of exposure, the powder of *A. indica* and *Z. zanthoxyloides* at 1.0 g caused 100% mortality for the three tested insects. In addition , both previous powders at 0.8 g achieved 97.63 - 100% mortality for the three tested insects. However, *R. dominica* was the most sensitive one to the three powders, *A. indica* ,*Z. zanthoxyloides* and *A. occidentale* at 0.8 and 1.0 g (100% mortality) On the other hand, powder of *M. oleifera* was less potent on the three tested insects, mortality ranged between 30.00 to 72.24% at all the used concentrations.

Table 1. Percentage mortality of *S. oryzae, O. mecator* and *R. dominica* on paddy rice treated with plant powders

Plant materials	Dosage (g)	% Mortality (Mean ± SE)					
		S. oryzae		*O. mecator*		*R. dominica*	
		48 hrs.	72 hrs.	48 hrs.	72 hrs.	48 hrs.	72 hrs.
A. indica	0.4	54.00±0.24^{d}	76.00±0.84^{e}	49.40±2.40^{cd}	54.24±2.58^{c}	46.70±0.33^{c}	70.58±0.58^{d}
	0.6	76.00±0.88^{ef}	98.00±1.22^{gh}	56.00±1.28^{d}	80.58±0.58^{ef}	72.50±2.50^{e}	92.70±1.33^{f}
	0.8	85.24±0.67^{f}	100.00±0.00^{h}	59.27±0.88^{d}	95.30±0.33^{f}	80.30±0.67^{f}	100.00±0.00^{g}
	1.0	100.00±0.00^{g}	100.00±0.00^{h}	67.00±1.30^{e}	100.00±0.00^{fg}	91.00±0.66^{g}	100.00±0.00^{g}
Z. zanthoxyloides	0.4	50.00±0.58^{cd}	68.00±0.67^{de}	58.00±1.67^{d}	76.00±0.84^{e}	45.00±0.24^{c}	65.00±0.24^{cd}
	0.6	70.00±1.80^{e}	92.00±0.24^{g}	86.00±0.88^{f}	100.00±0.00^{g}	72.00±0.88^{e}	89.68±0.88^{ef}
	0.8	80.00±1.00^{f}	97.63±1.33^{gh}	92.67±2.44^{f}	100.00±0.00^{g}	79.86±2.67^{ef}	100.00±0.00^{g}
	1.0	86.67±0.33^{f}	100.00±0.00^{h}	100.00±0.00^{fg}	100.00±0.00^{g}	89.06±0.22^{fg}	100.00±0.00^{g}
A. occidentale	0.4	46.70±0.33^{c}	57.00±2.24^{cd}	40.25±0.67^{c}	55.00±0.24^{c}	46.00±0.88^{c}	68.00±0.28^{cd}
	0.6	56.70±0.88^{d}	66.00±2.24^{d}	53.00±2.43^{d}	65.25±0.88^{d}	73.23±1.67^{e}	93.00±0.24^{f}
	0.8	66.00±0.58d^{e}	88.24±1.00^{fg}	57.24±2.88^{d}	89.00±1.67^{f}	81.00±2.67^{f}	100.00±0.00^{g}
	1.0	72.70±0.88^{e}	90.00±0.88^{g}	67.12±0.88^{e}	97.86±2.24^{fg}	90.00±0.24^{g}	100.00±0.00^{g}
M. oleifera	0.4	30.20±2.44^{b}	44.00±0.28^{b}	30.00±4.08^{b}	43.08±0.28^{b}	31.00± 0.58^{b}	42.00±2.33^{b}
	0.6	42.24±0.88^{c}	48.59±2.45^{b}	37.50±2.50^{b}	46.00±0.58^{b}	49.30± 1.00^{c}	52.47±0.67^{b}
	0.8	57.24±2.88^{d}	63.00±1.87^{d}	49.00±2.89^{cd}	58.00±1.33^{c}	63.00± 1.00^{d}	66.00±0.24^{cd}
	1.0	59.12±0.67^{d}	69.10±2.67^{de}	56.50±1.33^{d}	69.82±2.67^{d}	68.67±0.33^{de}	72.24±0.88^{d}
Control	0.0	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Each value is a mean ± standard error of four replicates. Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan's Multiple Range Test.

3.2. Effect of Plant Extracts on Mortality of S. oryzae, O. mercator and R. dominica

The effect of different plant extracts on mortality of *S. oryzae, O. mercator* and *R. dominica* was presented in Table 2. All the used plant extracts showed a higher mortality effect on the three insects than the powders and their effect increased as the period of exposure increased. The extracts of *A. indica* and *Z. zanthoxyloides* at all concentrations were able to achieve complete mortality of *S. oryzae* within 48h of exposure and their effect was significantly (p<0.05) different from other extracts, in addition, the extract of *A. occidentale* at 6% and 8% concentrations achieved 95% and 100% insect mortality of *S. oryzae* at the same time of examination, respectively. Moreover, all the extracts, at all tested concentrations, achieve 100% mortality of *S. oryzae* within 72h of exposure except extract of *M. oleifera* at 2 and 4 % which achieved 72% and 87.59% mortality, respectively. The extract of *Z. zanthoxyloides* at all tested concentrations was more active and able to achieve complete mortality of *O. mercator* within 48h and 72h post-treatment and its effect was significantly (p<0.05) different from other extracts. Also, the extract of *A. indica A. occidentale* at all concentrations significantly at $p\leq$ 0.05 achieved 89.45 - 100% mortality of *O. mecator* within 72h of application. *R. dominica* again proved to be sensitive to the first three extracts and achieved 100% mortality within 72h post-treatment at lower concentration (2%) except the extract of *M. oleifera* which only achieved complete beetle mortality at 8% concentration. However, effect of all the plant extracts on the three insects was significantly (p<0.05) different from the controls.

Table 2. Percentage mortality of *S. oryzae, O. mecator* and *R. dominica* on paddy rice treated with plant oil extracts

Plant materials	Dosage (%)	% Mortality (Mean ± SE) in hours					
		S. oryzae		*O. mecator*		*R. dominica*	
		48 hr	72 hr	48 hr	72 hr	48 hr	72 hr
A. indica	2	100.00±0.00^{f}	100.00±0.00^{d}	79.27±0.88^{e}	100.00±0.00^{e}	91.00±0.66^{fg}	100.00±0.00^{e}
	4	100.00±0.00^{f}	100.00±0.00^{d}	87.00±1.30^{fg}	100.00±0.00^{e}	100.00±0.00^{h}	100.00±0.00^{e}
	6	100.00±0.00^{f}	100.00±0.00^{d}	100.00±0.00^{h}	100.00±0.00^{e}	100.00±0.00^{h}	100.00±0.00^{e}
	8	100.00±0.00^{f}	100.00±0.00^{d}	100.00±0.00^{h}	100.00±0.00^{e}	100.00±0.00^{h}	100.00±0.00^{e}
Z. zanthoxyloides	2	100.00±0.00^{f}	100.00±0.00^{d}	100.00±0.00^{h}	100.00±0.00^{e}	89.06±0.22^{ef}	100.00±0.00^{e}
	4	100.00±0.00^{f}	100.00±0.00^{d}	100.00±0.00^{h}	100.00±0.00^{e}	98.00±1.30^{gh}	100.00±0.00^{e}
	6	100.00±0.00^{f}	100.00±0.00^{d}	100.00±0.00^{h}	100.00±0.00^{e}	100.00±0.00^{h}	100.00±0.00^{e}
	8	100.00±0.00^{f}	100.00±0.00^{d}	100.00±0.00^{h}	100.00±0.00^{e}	100.00±0.00^{h}	100.00±0.00^{e}
A. occidentale	2	78.82±0.28^{cd}	100.00±0.00^{d}	69.45±0.24^{d}	89.45±0.67^{d}	75.56±3.40^{d}	100.00±0.00^{e}
	4	87.70±0.88^{de}	100.00±0.00^{d}	89.00±2.67^{fg}	100.00±0.00^{e}	83.23±1.88^{e}	100.00±0.00^{e}
	6	95.00±0.00^{ef}	100.00±0.00^{d}	92.28±0.24^{f}	100.00±0.00^{e}	89.00±0.33^{ef}	100.00±0.00^{e}
	8	100.00±0.00^{f}	100.00±0.00^{d}	99.20±0.02^{fg}	100.00±0.00^{e}	100.00±0.00^{h}	100.00±0.00^{e}
M. oleifera	2	68.88±0.24^{bc}	72.00±0.28^{b}	48.00±2.36^{b}	62.00±0.28^{b}	56.82± 0.88^{b}	66.00±0.67^{b}
	4	70.24±0.88^{c}	87.59±2.45^{c}	57.50±2.50^{c}	75.00±2.58^{c}	69.00± 1.67^{c}	72.47±0.43^{c}
	6	87.24±0.28^{de}	100.00±0.00^{d}	65.00±2.89^{d}	88.00±1.63^{d}	76.00± 2.20^{d}	96.80±0.88d^{e}
	8	93.12±0.28^{e}	100.00±0.00^{d}	82.50±1.33^{e}	100.00±0.00^{e}	88.63± 2.46^{ef}	100.00±0.00^{e}
Solvent control		0.00±0.00^{a}	3.30± 0.33^{a}	0.00±0.00^{a}	0.24±0.23^{a}	0.00±0.00^{a}	1.35±0.67^{a}
Untreated Control		0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Each value is a mean ± standard error of four replicates. Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan's Multiple Range Test.

3.3. Effect of plant powders on number of adult emergence and their inhibitory effect on progeny development of adult S. oryzae, O. mercator and R. dominica

All the plant powders significantly reduced the number of adult emergence of the three tested insects (Table 3). Powders of *A. indica, Z. zanthoxyloides* and *A. occidentale* at 0.8 and 1g w ere able to prevent the emergence of adult of the three tested insects being 97.86 - 100 % and 95.04 – 100 % adult reduction, respectively. *A. indica, Z. zanthoxyloides* and *A. occidentale* powders at 0.8 g were more potent on *R. dominica* (100% adult reduction). In contrast, the powders of *M. oleifera*, at the same concentration, were less potent on this beetle. Moreover, the effect of these plant powders was significantly (p<0.05) different from the controls.

Table 3. Effect of plant powders on the adult emergence of three stored product insect pests infesting rice

Plant materials	Dosage (g)	% Mortality (Mean ± SE)					
		S. oryzae		*O. mecator*		*R. dominica*	
		Mean number of adult emergence	%IR	Mean number of adult emergence	%IR	Mean number of adult emergence	%IR
A. indica	0.4	1.83±1.34^{b}	96.87±0.01^{f}	2.30±1.10^{c}	91.24±0.22^{d}	1.70±0.01^{b}	96.79±0.06^{c}
	0.6	1.20±0.01^{b}	98.20±0.02^{fg}	2.01±0.33^{bc}	95.58±0.03^{d}	1.20±0.01^{b}	97.70±0.02^{c}
	0.8	0.00±0.00^{a}	100.00±0.00^{g}	1.35±1.18^{b}	97.30±1.02^{e}	0.00±0.00^{a}	100.00±0.00^{e}
	1.0	0.00±0.00^{a}	100.00±0.00^{g}	0.89±0.01^{a}	99.75±0.01^{f}	0.00±0.00^{a}	100.00±0.00^{e}
Z. zanthoxyloides	0.4	2.35±0.55^{c}	92.00±0.20^{e}	1.73±0.32^{b}	96.22±0.01^{d}	1.89±0.02b^{c}	94.00±0.21^{c}
	0.6	1.92±1.30^{b}	96.00±0.14^{f}	0.89±0.02^{a}	99.84±0.01^{f}	1.02±0.08^{b}	98.68±0.02d^{e}
	0.8	0.88±0.01^{a}	99.83±0.13^{g}	0.00±0.00^{a}	100.00±0.00^{f}	0.00±0.00^{a}	100.00±0.00^{e}
	1.0	0.00±0.00^{a}	100.00±0.00^{g}	0.00±0.00^{a}	100.00±0.00^{f}	0.00±0.00^{a}	100.00±0.00^{e}
A. occidentale	0.4	2.70±0.34^{c}	89.85±1.17d^{e}	2.89±0.23^{c}	89.40±0.01^{cd}	2.03±0.88^{c}	94.08±0.03^{c}
	0.6	2.10±1.28^{c}	92.00±0.32^{e}	2.47±0.13^{c}	91.49±0.22^{d}	1.78±1.67^{b}	96.20±0.15^{c}
	0.8	0.13±0.01^{a}	99.98±0.01^{g}	1.44±2.04^{b}	95.04±1.67^{d}	0.00±0.00^{a}	100.00±0.00^{e}
	1.0	0.00±0.00^{a}	100.00±0.00^{g}	1.00±0.33^{ab}	97.86±2.24^{e}	0.00±0.00^{a}	100.00±0.00^{e}
M. oleifera	0.4	7.93±0.44^{f}	81.60±0.08^{b}	8.10±0.08^{ef}	73.62±0.01^{b}	6.95±0.23^{e}	79.78±1.12^{b}
	0.6	5.74±1.33^{e}	83.79±1.43^{b}	7.50±1.55^{e}	77.08±0.22^{b}	6.84±1.33^{e}	80.47±0.79^{b}
	0.8	4.44±2.03^{d}	86.00±0.33^{c}	4.87±3.02^{d}	82.30±0.02^{c}	5.47±2.03^{d}	83.89±0.01^{b}
	1.0	4.02±0.34^{d}	86.95±1.77^{c}	4.56±0.53^{d}	85.82±1.02^{c}	5.23±0.34^{d}	84.97±2.67^{b}
Control	0.0	35.26±2.06^{g}	0.00±0.00^{a}	38.26±3.02^{g}	0.00±0.00^{a}	37.21±2.45^{f}	0.00±0.00^{a}

Each value is a mean ± standard error of four replicates. Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan's Multiple Range Test.

3.4. Effect of plant extracts on number of adult emergence and their inhibitory effect on progeny development of adult S. oryzae, O. mercator and R. dominica

Table 4 shows the effect of the plant extracts on the emergence of adult *S. oryzae, O. mercator* and *R. dominica*. All extracts of tested plants show a greater effect on the emergence of the adult insects. The extracts of *A. indica* and *Z. zanthoxyloides* at all concentrations were able to inhabit completely the emergence of adult *S. oryzae* and *O. mercator* and 100% adult reduction. Meanwhile, at 4, 6 and 8% concentration, extracts of *A. occidentale* also inhabit almost emergence of the three tested adults species. In contrast, *M. oleifera* was less potent on the emergence of the three tested adults species (83.46- 94.97% adult reductions). All the tested extracts were significantly (p<0.05) different from the controls in their effects.

Table 4. Effect of plant oil extracts on the adult emergence of three stored product insect pests infesting rice

Plant materials	Dosage (%)	% Mortality (Mean ± SE)					
		S. oryzae		*O. mecator*		*R. dominica*	
		Mean number of adult emergence	%IR	Mean number of adult emergence	%IR	Mean number of adult emergence	%IR
A. indica	2	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}
	4	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}
	6	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}
	8	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}
Z. zanthoxyloides	2	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}
	4	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}
	6	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}
	8	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}
A. occidentale	2	1.45±0.33^{b}	97.35±0.08^{cd}	1.24±0.24^{b}	96.40±1.01^{c}	0.00±0.00^{a}	100.00±0.00^{d}
	4	0.00±0.00^{a}	100.00±0.00^{d}	0.87±2.33^{a}	99.49±0.31^{d}	0.00±0.00^{a}	100.00±0.00^{d}
	6	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}
	8	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}	0.00±0.00^{a}	100.00±0.00^{d}
M. oleifera	2	4.42±4.08^{e}	87.72±0.13^{b}	4.87±3.02^{d}	83.46±1.32^{b}	4.64±3.21^{d}	86.59±1.47^{b}
	4	3.14±1.33^{d}	89.85±4.34^{b}	4.56±0.53^{d}	85.82±0.33^{b}	3.44±1.08^{c}	88.21±0.24^{b}
	6	3.03±1.33^{d}	92.60±2.03^{b}	3.96±2.44^{c}	87.23±0.46^{b}	3.23±1.33^{c}	91.89±0.33^{bc}
	8	2.00±3.05^{c}	94.97±033^{bc}	3.06±0.53^{c}	90.41±1.33^{b}	2.46±2.24^{b}	93.68±2.67^{c}
Solvent control		35.87±3.21^{f}	0.00±0.00^{a}	37.68±0.24^{e}	0.00±0.00^{a}	37.54±4.06^{e}	0.00±0.00^{a}
Untreated Control		35.26±2.06^{f}	0.00±0.00^{a}	38.26±3.02^{e}	0.00±0.00^{a}	37.21±2.45^{e}	0.00±0.00^{a}

Each value is a mean ± standard error of four replicates. Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan's Multiple Range Test.

4. Discussion

Botanical source insecticides may serve as alternatives to popularly used synthetic chemical insecticides as many of them have often been used against a number of species of stored product insect pests, including Coleoptera and Lepidoptera (Nathan *et al.,* 2007). They are believed to be easily biodegradable and not toxic to none target organisms. Moreover, prior to the discovery of the organochlorine and organophosphate insecticides in the late 1930s and early 1940s, botanical insecticides have remained an important weapon in the farmers armory in managing insect pests of their farm produce (Forim *et al.,* 2012). Many Nigerian plant species are medicinal and they are proved to be effective against different a wide range of insect pests (Akinkurolere *et al.,* 2006; Ileke and Olotuah, 2012; Akinneye and Ogungbite, 2013).

In this study, the oil extracts of *A. indica* and *Z. zanthoxyloides* show a higher effectiveness than the oil of *A. occidentale* and *M. oleifera* as they presented 100% mortality of *S. oryzae* within 48h of application at all concentrations. Moreover, the extract of *Z. zanthoxyloides* shows the greatest mortality of *O. mercator* as it was the only one that achieved 100% mortality within 48h at all concentrations. The powders of these four botanicals also show greater a mortality effect on the three insects tested, but their effect was less than their oil extracts. The high mortality effect of these botanical oil extracts and powders could be due to the inability of the insects to feed on the paddy rice that has been coated with these botanicals, thereby leading to their starvation. The oils and powders of these botanicals may have also disrupted the normal respiratory activities of these insects leading to the asphyxiation and death. The oils and the powders may have also blocked the spiracles of these insects which therefore led to suffocation. This results agree with the previous studies in which powders and oils of *A. indica, Z. zanthoxyloides, A. occidentale* and *M. oleifera* have been used as protectant against different storage insects (Onu and Baba, 2003; Ileke and Oni, 2011; Ileke and Olotuah, 2012; Akinneye and Ogungbite, 2013).

The toxicity of *A. indica* to these three insects could be attributed to the presence of many chemical ingredients such as triterpenoids, which includes azadirachtin, salanin, meliantriol, etc. (Ileke and Oni, 2011). The toxic effect of *Z. zanthoxyloides* could be related to the presence of secondary phenolic compound known as zanthoxylol and this had been reported to have mortality and ovicidal effect on stored product insect pests (Udo, 2011; Akinneye and Ogungbite, 2013). Also, the high mortality, effect evoked by the oils and powders of *A. occidentale*, could be linked to the occurrence of anacardic acid, cardinal, quercetin and kaempferol glycosides as suggested by Oparaeke and Bunmi (2006).

All the concentrations of the extract of *A. indica* and *Z. zanthoxyloides* prevented the emergence of the adults of these insects and also showed 100% inhibition rate while only the extract of *A. occidentale* at higher concentrations was able to prevent the emergence of these insects and achieved complete inhibition of the insects. However, both the extract and powder of *M. oleifera* could not completely inhabit (100%) the emergence of the three insects. However, the oil and powder of *M. oleifera* greatly reduced the emergence of these insects and achieved greater inhibition rate when compared to the controls.

The inability of these insects to emerge may be due to the death of the insect larvae which may occur due to inability of the larvae to fully cast off their exoskeleton which remained linked to the posterior part of their abdomen. This is in agreement with the observation made

by Oigiangbe *et al.* (2010) who worked on insecticidal properties of an alkaloid from *Alstonia boonei*. Also, different chemical compositions of these plants as mentioned earlier could be responsible for the in ability of the adult insects to emerge as they are found to disrupt growth and reduced larval survival as well as disruption of life cycle of insects (Mordue-Luntz and Nisbet, 2000; Yang *et al.,* 2006).

This result agrees with the work of various researchers in which the extracts and powders of *A. indica, Z. zanthoxyliodes, A. occidentale* and *M. oleifera* were used to prevent the emergence of adult insects as well as the inhibition of their development (Udo, 2005; Ileke and Oni, 2011; Udo, 2011; Akinneye and Ogungbite, 2013). The powders and oil extracts of the four used botanicals are medicinal and risk-free to mammals. Therefore, they could be integrated with other insect pest management system.

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Effects of Smoking on Lipid Profile and Homocysteine in Coronary Heart Disease

Fawzi M. Al-Sheyab[1], Faisal I. Mohammed[2], Iman K. Shehadeh[3], Nabil A. Bashir[4] and Rehan T. BaniHani[1,*]

[1]*Department of Biological Sciences, Faculty of Science and Arts, Jordan University of Science and Technology,* [2]*Department of Physiology and Biochemistry, College of Medicine, Jordan University, Amman,* [3]*Directorate of Mother and Child, Ministry of Health, Amman,* [4]*Department of Biochemistry and Molecular Biology, College of Medicine, Jordan University of Science and Technology, Irbid, P.O. Box 3030 Irbid 22110, Jordan*

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Abstract

Smoking produces adverse effects on lipid profile and homocysteine, thus increasing the cardiovascular disease risk in coronary heart disease patients**.** The present study was undertaken to evaluate plasma lipid profile, lipoprotein (a) and homocysteine in coronary heart disease of smoker male and female patients in two age groups; below and above 50 years old, comparing them with nonsmokers healthy matched control. Triglyceride plasma level **(**291.33 ±101 mg/dl , mean ± SD, *P* value=0.01) was elevated significantly in smoking coronary heart disease males less than 50 years old as compared to controls, while cholesterol (233.54 ±44.9 mg/dl, *P* value= 0.02), LDL- cholesterol (174 ±58.3 mg/dl, *P* value=0.09) , lipoprotein (a) - Lp(a) (65.06 ±25.2 mg/dl, *P* value=0.05), and triglycerides (188.18 ±62.0 mg/dl, *P* value=0.01) are significantly increased in males more than 50 years old. No lipid profile parameter was significantly elevated in smoking coronary heart disease female less than 50 years old, while only homocysteine (28.83 ±5.7 mg/dl, *P* value= 0.02) was elevated significantly in females more than 50 years . In conclusion, smoking affects lipid profile and homocysteine and increases the cardiovascular disease risk among smokers.

Keywords: Cholesterol ,Low Density Lipoprotein Cholesterol, High Density Lipoprotein Cholesterol, Triglycerides, Homocysteine.

1. Introduction

As reported by different research groups, smoking increases the concentration of serum total cholesterol, triglycerides, LDL-cholesterol, VLDL-cholesterol and decreases the levels of antiatherogenic HDL cholesterol (Adam *et al*., 2011; Austin, 1991; John and Rajat, 2004; Kavita *et al*., 2013; Muscat *et al.,* 1991). Smoking, in different forms, is a major risk factor for atherosclerosis and coronary heart disease (Fagerström, 2002; Vlassis, 2009). Passive smoking could affect blood lipid metabolism in women, which might contribute to coronary heart diseases (He *et al.,* 2007). A positive association between elevated plasma total homocysteine levels and a n umber of cardiovascular risk factors, smoking, particularly, was shown, in a study conducted in Norway (Wilcken, 2002), to be well associated. In the comprehensive analysis, now reported from the large European Concerted Action Project case control study, Callaghan and colleagues provide convincing evidence for an amplifying effect of cigarette-smoking on homocysteine associated cardiovascular risk (Callaghan *et al.*, 2002). Smokers (male and female) had significantly lower high-density lipoprotein levels and significantly higher very low-density lipoprotein total cholesterol and plasma triglyceride levels than nonsmokers (Meenkshisundaraum *et al*., 2010). The aim of the study is to find out the effect of smoking on serum lipid profile and homocysteine in coronary heart diseases in male and female patients of more and less than 50 years old. It also aims at assessing the association between smoking and the alteration in plasma concentration of lipid profile and lipid peroxides

2. Materials and Methods

A total number of 180 subjects were studied. Ninety were adult patients (of both sexes) with coronary heart diseases; they were admitted at coronary care unit in

* Corresponding author. e-mail: rtbanihani8@just.edu.jo.

Princess Basma Teaching Hospital in Irbid City, the other 90 were age, and gender matched controls that referred to the lab of Princess Basma Teaching Hospital in Irbid City. All subjects did not suffer from obesity or blood pressure. Venous blood samples were collected after 12 hours of an overnight fast into plain and EDTA tubes. Serum or plasma was obtained by low speed centrifugation at 1000g for 15 minute, and samples were immediately separated into aliquot and stored at –20o C until analysis. The subjects were interviewed and a paper questionnaire and consent were completed, which included gender, age, smoking, DM, hypertension, type of CHD, previous history of CHD, and family history of CHD. Lp(a) was quantitatively estimated in serum by enzymatic immunosorbent assay (ELISA). IMMUN-OZYM Lp(a) kit manufactured by Progen, GMBH, Germany. Homocysteine (Hcy) in blood was quantitatively estimated in plasma by enzymatic immunosorbant assay (ELISA). Homocysteine Micro-plate Enzyme immunoassay kit manufactured by BIO-RAD, USA. Cholesterol, Triglyceride, and High-density lipoprotein/Low density lipoprotein were quantitatively estimated in the serum by enzymatic colorimetric test-CHOD-PAP by commercially available kits made by ARCOMEX, Jordan. Data are expressed as means and standard deviation (SD); means of the two groups were compared by student's t test. One-way analysis of variance test was used for comparison between more than two groups. Spearman's correlation was used to determine whether there was a relationship between Lp(a) and other variables. These statistical tests were performed using the Statistical Package for the Social Science (SPSS). The level of significance was $P<0.05$.

3. Results

Cholesterol (230.93±48.3 mg/dl, *P* value= 0.011), LDL-cholesterol (126.96±65.2 mg/dl, mean ± SD, *P* value=0.003), triglycerides (183.85±70.4 mg/dl, *P* value=0.00), and homocysteine(24.35 ±11.6 mg/dl, *P* value=0.009) plasma levels significantly increased in coronary heart disease smokers (males and females less and more than 50 years old), while HDL- cholesterol (57.48 ±24.4 mg/dl, *P* value=0.027) significantly decreased as compared to control (Table 1). As shown in Table 2 only triglycerides (205.28 ±108.14 mg/dl, *P* value=0.025) significantly increased in smoking males and females coronary heart disease patients less than 50 years old. Plasma level of cholesterol (232.5 ±46.7 mg/dl, *P* value=0.02), LDL- cholesterol (184.20 ±67.9 mg/dl) (*P* value= 0,003), Lipoprotein (a) (60.22 ±24.6 mg/dl, *P* value=0.03), triglycerides (179.17 ±60.8 mg/dl, *P* value=0.00), and homocysteine (29 ±9.9 mg/dl, *P* value=0.00) significantly increased in smoking males and females coronary heart disease patients more than 50 years old, while HDL-cholesterol (55.83 ±24.1 mg/dl, *P* value=0.03) significantly decreased as compared to matched controls (Table 3). Cholesterol (233.3 ±43.3 mg/dl, *P* value=0.01), LDL-cholesterol (172.3 ±58.7 mg/dl, *P* value=0.00), triglycerides (198.86 ±72 mg/dl, *P* value=0.00) significantly increased and HDL- cholesterol (56.84 ±24.7 mg/dl, *P* value=0.00) significantly decreased in coronary heart disease male smokers (less and more than 50 years old) as compared to the matched control, while in females only homocysteine (26.4 ±5.8 mg/dl, *P* value=0.05) increased significantly (Table 4). As shown in table 5, only triglycerides (291.33 ±101 mg/dl, *P* value= 0.01) significantly increased in smoker coronary heart disease males less than 50 years old. Cholesterol (233.54 ±44.9 mg/dl, *P* value=0.02), Lipoprotein (a) (65.06 ±25.2 mg/dl), triglycerides (188.18 ±62.0 mg/dl, *P* value=0.01), LDL- cholesterol (174 ±58.3 mg/dl, *P* value=0.09) significantly increased in smoking coronary heart disease males more than 50 years old, while homocysteine (28.83 ±5.7 mg/dl, *P* value=0.02) significantly increased in females as compared to control (Table 6).

Table 1. Mean values and standard deviation of dyslipidemia indicators and homocysteine in smoker CHD and control mixed males and females more and less 50 years old

Parameter ±SD	Non CHD	CHD	*P*
CHL(mg/dl)	200.2 ±40.5	230.93 ±48.3	0.011
LDL-CHL(mg/dl)	129.8 ±68.2	126.96 ±65.2	0.003
Lp(a) (mg/dl)	39.5 ±18.4	53.9 ±24.4	0.102
TG(mg/dl)	114.2 ±51.1	183.85 ±70.4	0.00
HDL-CHL(mg/dl)	83.65 ±51.5	57.48 ±24.4	0.027
Hmcy(mg/dl)	14.84 ±6.9	24.35 ±11.6	0.009

CHD: coronary heart disease, LDL-CHL: low density lipoprotein; Lp(a): lipoprotein(a); TG: triglyceride; HDL-CHL: high density lipoprotein; Hmcy: homocysteine

Table 2. Mean values and standard deviation of dyslipidemia indicators and homocysteine in smoker mixed males and females CHD and controls <50 years old.

Parameter ±SD	Non CHD	CHD	*P*
CHL(mg/dl)	205.2 ±43.3	223.57 ±58.4	0.446
LDL-CHL(mg/dl)	122.1 ±52.2	154.43 ±48.0	0.199
Lp(a) (mg/dl)	24.8 ±6.5	32.5 ±8.7	0.18
TG(mg/dl)	111.8 ±60.06	205.28 ±108.14	0.025
HDL-CHL(mg/dl)	82.19 ±39.0	65.03 ±26.05	0.317
Hmcy(mg/dl)	7.62 ±4.1	15.06 ±9.7	0.20

CHD: coronary heart disease, LDL-CHL: low density lipoprotein;LP (a): lipoprotein (a); TG: triglyceride; HDL-CHL: high density lipoprotein; Hmcy: homocysteine.

Table 3. Mean values and standard deviation of dyslipidemia indicators and homocysteine in smoker mixed males and females CHD and controls >50 years old.

Parameter ±SD	Non CHD	CHD	*P*
CHL(mg/dl)	195.6 ±38.5	232.5 ±46.7	0.02
LDL-CHL(mg/dl)	131.2 ±82.3	184.20 ±67.9	0.03
Lp(a) (mg/dl)	43.09 ±21.2	60.22 ±24.6	0.03
TG(mg/dl)	116.9 ±43.4	179.17 ±60.8	0.00
HDL-CHL(mg/dl)	83.85 ±62.4	55.83 ±24.1	0.03
Hmcy(mg/dl)	17.25 ±5.9	29 ±9.9	0.00

CHD: coronary heart disease, LDL-CHL: low density lipoprotein; Lp(a): lipoprotein(a); TG: triglyceride; HDL-CHL: high density lipoprotein; Hmcy: homocysteine.

Table 4. Mean values and standard deviation of dyslipidemia indicators and homocysteine in smoker CHD and control males and females of mixed ages more and less 50 years old .

	Males			Females		
Parameter ±SD	Non CHD	CHD	P	Non CHD	CHD	*P*
CHL (mg/dl)	193.6 ± 40.5	233.3 ± 43.3	0.01	212 ± 40.1	224.0 ± 62.6	0.63
LDL-CHL (mg/dl)	115.03 ±51.4	172.3 ± 58.7	0.00	147.9 ± 90.7	197.84 ± 82.2	0.22
Lp(a) (mg/dl)	39.61 ± 17.9	61.3 ± 20.5	0.57	39.38 ± 24	47.15 ± 44.6	0.45
TG (mg/dl)	114.78 ±54.8	198.86 ±72	0.00	113.19 ± 74.8	140.3 ± 24.4	0.21
HDL-CHL (mg/dl)	84.02 ± 35.3	56.84 ± 24.7	0.00	81.3 ± 6.8	59.33 ± 8.2	0.39
Hmcy (mg/dl)	12.50 ± 6.3	22.5 ± 14.3	0.07	17.85 ± 11.9	26.4 ± 5.8	0.05

CHD: coronary heart disease, LDL-CHL: low density lipoprotein; Lp(a): lipoprotein(a); TG: triglyceride; HDL-CHL: high density lipoprotein; Hmcy: homocysteine.

4. Discussion

The data show that only plasma level of triglycerides increased significantly in CHD smoking mixed population of males and females who were less than 50 years old, while all other lipid parameters increased, and HDL decreased in patients who were above 50 years old.

Table 5. Mean values and standard deviation of dyslipidemia indicators and homocysteine in smoker males and females CHD and controls <50 years old

	Male			Female		
Parameter ±SD	Non CHD	CHD	*P*	Non CHD	CHD	*P*
CHL (mg/dl)	197.7 ± 44.9	231.34 ±33.6	0.26	228 ± 35.6	217.7 ±77.2	0.84
LDL-CHL (mg/dl)	103.36 ±40.6	157.67 ±72.1	0.12	178.3 ±47.7	152 ± 32.3	0.42
Lp(a) (mg/dl)	24.9 ± 8.0	31.5 ±9.4	0.36	25.6 ± 8.0	37 ± 25.4	0.10
TG (mg/dl)	108.82 ±63	291.33 ±101	0.01	118.3 ±62.1	140.7 ±59.1	0.65
HDL-CHL(mg/dl)	93.54 ±36.6	66.4 ±24.2	0.28	48.4 ±26.8	64 ±25.5	0.46
Hmcy (mg/dl)	6.16 ± 3.6	15.87 ± 11	0.21	12	11	0.99

CHD: coronary heart disease, LDL-CHL: low density lipoprotein; Lp(a): lipoprotein(a); TG: triglyceride; HDL-CHL: high density lipoprotein; Hmcy: homocysteine.

Table 6. Mean values and standard deviation of dyslipidemia indicators and homocysteine in smoker males and females CHD and controls >50 years old.

	Male			Female		
Parameter ±SD	Non CHD	CHD	P	Non CHD	CHD	*P*
CHL(mg/dl)	188.47 ±36.8	233.54 ±44.9	0.02	204.05 ±42.8	228.2 ±58	0.43
LDL-CHL(mg/dl)	130.03 ±63.4	174 ±58.3	0.09	132.69 ±106	228.4 ±93	0.13
Lp(a) (mg/dl)	41.8 ±17.2	65.06 ±25.2	0.05	47.08 ±26.3	63.58 ±13.1	0.43
TG(mg/dl)	122.45 ±45.7	188.18 ±62.0	0.01	110.64 ±44.2	140.1 ±37.9	0.24
HDL-CHL(mg/dl)	71.77 ±32.0	55.74 ±77.2	0.16	97.5 ±87.2	56.21 ±25.6	0.28
Hmcy(mg/dl)	15.66 ±4.8	29.25 ±15.4	0.87	18.83 ±6.9	28.83 ±5.7	0.02

CHD: coronary heart disease, LDL-CHL: low density lipoprotein; Lp(a): lipoprotein(a); TG: triglyceride; HDL-CHL: high density lipoprotein; Hmcy: homocysteine

This finding may explain why all parameters increased ($p<0.05$) in mixed ages in those patients. Lipoprotein (a) increased but not remarkably, while homocysteine got significantly elevated. It could be concluded that the effect of smoking on the level of lipoprotein (a) is moderate while its effect on homocysteine is strong.

Smoking increased only the plasma level of triglycerides in CHD males whose age was less than 50 years, while it increased significantly the levels of cholesterol, lipoprotein (a), and triglcerides in CHD male patients above 50 years old. On the other hand, smoking had no effect on any lipid parameters in CHD female patients less than 50 years old, and only had a significant effect on increasing the homocysteine level in female patients more than 50 years old. It was observed that smoking affects lipid parameter in males more than 50 years old, while it affected homocysteine levels in females who were above 50 years old, and had no effect on lipoprotein (a) level in CHD males and females of different age groups. This sex role is due to the differences in the type and the dose of hormones between sexes. Estrogen has beneficial effects on cardiovascular health. A recent study suggested that estrogen may exert these effects by regulating the activity of liver enzymes involved in cholesterol metabolism (Szafran and Smielak-Korombel, 1998).

In general, smoking increased many lipid parameters and decreased the HDL plasma levels in CHD patients when compared to the control group. At the same time the level of homocystein also increased in those patients. It seems that an increase in both the homocystein and lipid profiles amplifies the risk factors toward cardiovascular diseases; this is in agreement with the findings reported by O'Callaghan and colleagues (Callaghan *et al*., 2002). A positive association is observed between elevated plasma total homocysteine and a number of cardiovascular risk factors including, smoking as reported by Nygard *et al.* (1995). This is in agreement with our results. Callaghan *et al.* showed that cardiovascular risk in smokers was markedly increased when plasma homocysteine also increased. Smokers with plasma homocysteine levels more 12 µmol./l had, a 12-fold increase of cardiovascular risk when compared with the risk in non-smokers with plasma homocysteine less than 12 µ mol./l. A recent evidence for an association between elevated homocysteine and enhanced oxidative stress was shown by Yamamoto *et al.* (2000). Since it is now well established that cigarette smoking is also associated with an increase in markers of oxidative stress .Oxidative effects via free radical generation in smokers have been widely investigated. Oxidative stress causes lipid peroxidation, oxidation of proteins and damage to mainly tissues (Ozguner *et al*., 2005). Amplification by smoking of this homocysteine-related effect may be a mechanism contributing to the findings by O'Callaghan and colleagues of synergism between the two factors .Our results showed an increase in lipoprotein (a) plasma level in CHD patients who were more than 50 years old and specifically in males as a direct role of sex hormones (Szafran and Smielak-Korombel, 1998). This result is in agreement with what Barbagallo *et al*. reported in 1992. The increase in apolipoprotein (a) level could be due to oxidation or/and inhibition processes caused by smoking.

In conclusion, smoking produces adverse effects on lipid profile and homocysteine, thus increasing the cardiovascular disease risk. Further studies are needed to establish that smoking-related alterations have influences on the atherosclerotic lesions of smokers.

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Two New Records of *Astragalus* species of the Section *Chronopus* Bge. and *Harpilobus* Bge. in Saudi Arabia

Sherif M. Sharawy*

Botany Department, Faculty of Science, Ain Shams University, Abbaseya 11566, Cairo, Egypt

Present Address: Biology Department, Faculty of Science, Hail University, Hail, Saudi Arabia

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Abstract

Astragalus trigonus DC. and *Astragalus trimestris* L. are new records in Saudi Arabia. They belong to sections *Chronopus* Bge. and *Harpilobus* Bge.

Keywords*: Astragalus*, Section *Chronopus*, Section *Harpilobus*, Flora, Saudi Arabia.

1. Introduction

The genus *Astragalus* L. of the family Fabaceae (Leguminosae) is one of the largest genera of vascular plants (Polhill, 1981; Podlech, 1986, 1991, 1999; Maassoumi, 1998, 2005; Taeb *et al.,* 2007; Lozano *et al.*, 2010) with an estimated number of 2500-3000 species. Many species are narrowly endemic, while relatively few are wide-spread, distributed in the Northern hemisphere, especially in Central Asia and Western North America (Podlich, 1986; Lock and Simpson, 1991; Maassoumi, 1998). In Egypt, the genus is represented by 32-35 species (Taeckholm, 1974; Boulos 1999). Zohary (1972) recorded 50 *Astragalus* species from Palestine; Post and Dinsmore (1932) recorded about 133 species of *Astragalus* in Syria. In Qatar, the genus *Astragalus* is represented by eight species (Norton *et al*., 2009). In Saudi Arabia, the genus is represented by 25-26 species (Migahid, 1996; Collenette, 1999; Mandaville, 1999; Chaudhary, 2000), distributed in different phytogeographical regions and placed in several sections. In Hail province, North Central Saudi Arabia, Collenette and Tsagarakis (2001) recorded 6 species of *Astragalus* in the Aja Mountains, Turki and Al-Olayan (2003) recorded 9 species in Hail, while Alshammari and Sharawy (2010) recorded 6 species in the Hema Faid region and recently Llewellyn *et al*. (2011) recorded 7 species in the Aja Mountains.

In this paper, two new records of *Astragalus* species are presented from Hail province; these are recorded in Saudi Arabia for the first time. One of them is from section *Chronopus* Bge. (*A. trigonus* DC.), and the other from section *Harpilobus* Bge. (*A. trimestris* L.).

During extensive field studies in Hail province of North Central Saudi Arabia, *Astragalus trigonus* and *A. trimestris* were collected fresh from different localities (Figure 1). Herbarium specimens of the two species are deposited at the Herbarium of Biology Department, Faculty of Science, Hail University, Hail, Saudi Arabia.

* Corresponding author. e-mail: sherifsharaawy@yahoo.com.

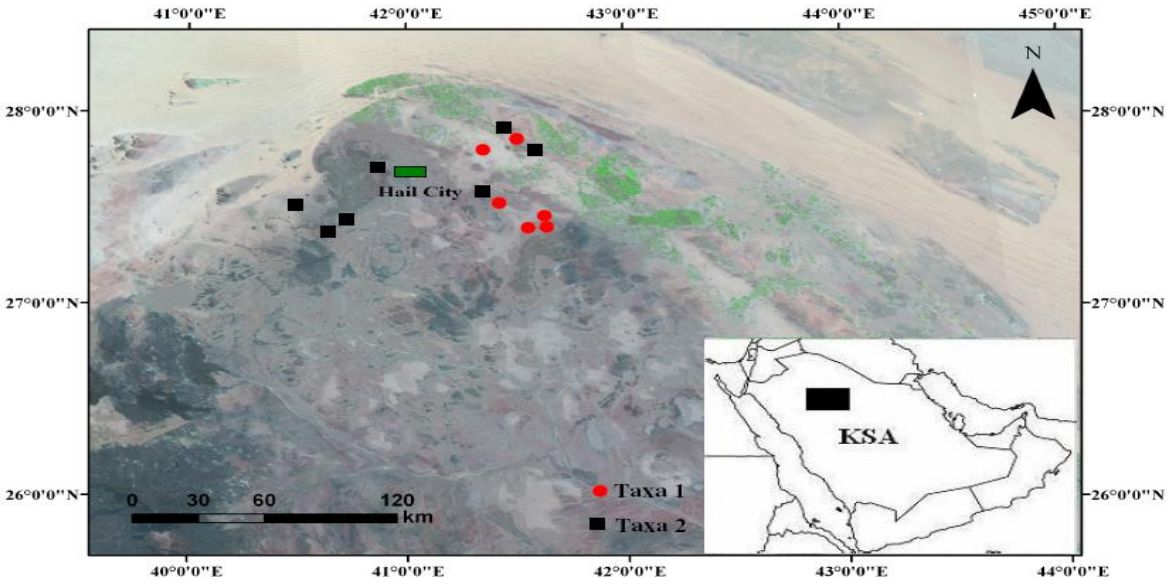

Figure 1. Distribution map of *Astragalus trigonus* (Taxa 1) and *A. trimestris* (Taxa 2) in Hail Province, Saudi Arabia

***Astragalus trigonus* DC. (Sect. *Chronopus* Bge.) – (Figure 2)**

Astragalogia 186 (1802).
= *Astragalus leucanthus* Boiss., Diagan. P1 Orient. 9:93 (1849).
= *Astragalus pseudotrigonus* Batt. & Trab., Bull. Soc. Bot. Fr. 58: 670 (1912).

Description: Subshrub 20-30 cm branched from the base and also above; stem and branches densely furnished with persistent spinous, 2-4 cm, generally rather stout leaf rachides; young shoots densely rather persistently white-tomentose. Leaves of two kinds: (1) those of the young shoots large, producing the persistent spines, oblong in outline with 5-7 pairs of ovate to oblong or suborbicular, minutely mucronate leaflets, 2-4 × 2-3 mm., flat leaflets which are sparsely appressed hairy on t he lower surface, almost glabrous on the upper; (2) those on the old wood reduced to fascicles of 2-5 cuneate-obovate, 3-15 × 1.5-5 mm leaflets; stipules triangular-deltoid, long acuminate 3-5 mm, membranous, white-ciliate. Inflorescence of 2-4 subsessile flowers; bracts deltoid, acute, membranous, 1-2 mm. Calyx 1-1.5 cm, shortly cylindrical, furnished with appressed black and white hairs, teeth about 3 mm. Corolla yellow or creamy white; standard 7-9 mm, the lamina oblong, blunt or notched; keel 5-6 mm, the lamina oblong, subequalling the claw. Style 1.5-2 mm, pilose basally or glabrous; ovary rather thinly to more densely appressed-pilose. Pod 2-2.5 × 0.3-0.6 cm including the sharp beak, straight or slightly curved, woody reticulate-veined, almost glabrous; seeds 2-5 mm, quadrangular, compressed brown or yellow and smooth

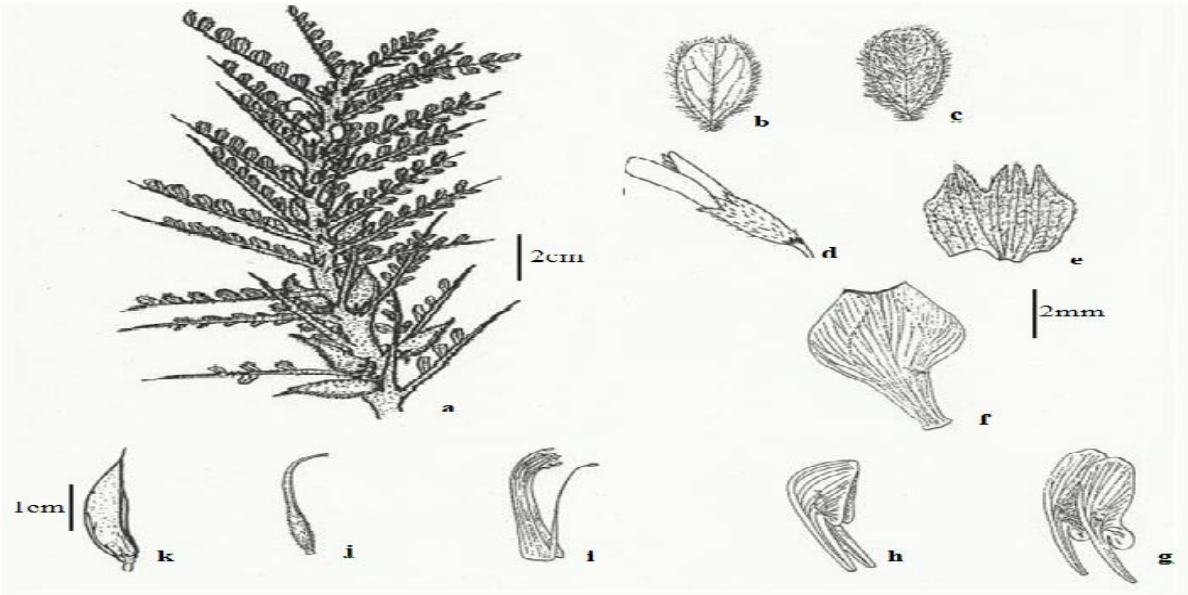

Figure 2. *Astragalus trigonus* DC. a, habit; b, leaflet, upper surface; c, leaflet, lower surface; d, flower; e, calyxopened out, outer surface; f, standard; g, wings; h, keel; i, androecium; j, gynoecium; k, pod.

***Astragalus trimestris* L. (Sect. *Harpilobus* Bge.) – (Figure 3)**

Sp. Pl., ed. 1,761 (1753).

Description: Annual, 8-22 cm branched from the base and frequently also a little above; stems decumbent or ascending, sulcate, rather weak, glabrous or sparsely pilose above with white and sometimes a few black hairs. Leaves 4-8 cm long, shortly petiolate, lamina oblong in outline; leaflets in 8-12 pairs, oblong-elliptic, 5-1 × 3-5 mm, obtuse or retuse at apex, white-strigose on the lower surface, glabrous on t he upper; stipules lanceolate-triangular, long acuminate, 2-3 mm, membranous, white ciliate. Inflorescences of 2-6 subsessile flowers in short but rather lax racemes on l ong peduncles mostly about equaling the leaves and clad with appressed black and white hairs; bracts deltoid, acute, membranous, 1-1.5 mm. Calyx shortly cylindrical, 4-5 mm, furnished with appressed white hairs, teeth about half as long as the tube. Corolla white or creamy white. Standard 10-12 mm, the lamina oblong-obovate, obtuse or retuse, about twice as long as the claw; wings 6-7 mm, the lamina oblong, blunt or notched; keel 4-5 mm, the lamina bluntly oblong, subequalling the claw. Style glabrous, 1 mm; ovary linear, 25-35 ovulate. Pods sessile, glabrous, erect, arcuate- to strongly fish-hook shaped, linear, with a short beak. Seeds quadrate-wedge shaped, 2-mm, brown and smooth.

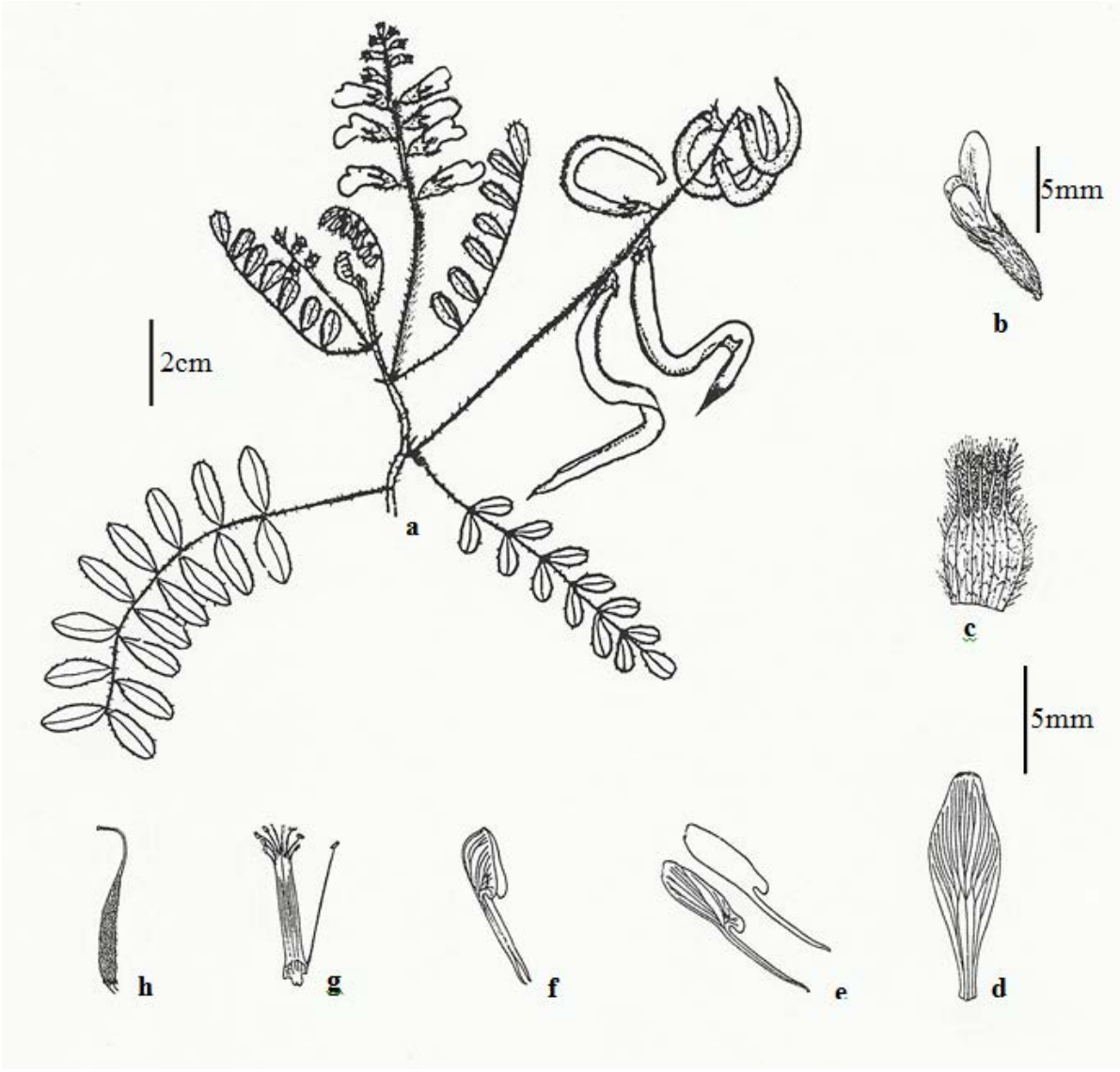

Figure 3. *Astragalus trimestris* L. a, habit; b, flower; c, calyx opened out, outer surface; d, standard; e, wings; f, keel; g, androecium; h, gynoecium.

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Secondary chromosomal association in kidney bean (*Phaseolus vulgaris* L.)

Girjesh Kumar and Nitu Chaudhary*

Plant Genetics Laboratory, Department of Botany, University of Allahabad -211002, U.P., India.

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Abstract

The present study documents the mutagenic efficacy of gamma ray and sodium azide on the chromosomal association pattern of bivalents and meiotic behavior in *Phaseolus vulgaris* L. The seeds were irradiated with different doses of gamma rays viz.10 krad, 20 krad, and 30 krad from a ^{60}Co source and thereafter, the seeds were treated with 0.3% of freshly prepared sodium azide solution for three hours, respectively. The results clearly show the formation of various types of secondary chromosomal association among bivalents. Secondary association is defined as the tendency of bivalents to lie in pairs having diffused connections. The phenomenon of secondary pairing manifested from metaphase I stage and persisted upto metaphase II stage. The bivalents lie side by side and end to end to form secondary pairing. A secondary association between bivalents is considered to be of great significance as it is being taken as an indicator of ploidy in plants. Apart from secondary chromosomal association, other meiotic abnormalities were also noticed. These include precocious movement of chromosomes at metaphase I/II (2.83%), stickiness at metaphase I/II (3.45%), stickiness at anaphase I/II (2.20%), bridge (1.25%), unorientation (0.94%), micronuclei (1.88%) etc. This phenomenon, along with other meiotic aberrations affects the pollen fertility considerably.

Keywords*: Phaseolus vulgaris* L., Secondary chromosomal association, Chiasma frequency, Meiotic aberrations, Pollen sterility.

1. Introduction

Paradoxically meiosis is an event of high evolutionary significance which aims to precisely half the chromosome complement and ensures the viability of gametes. All organisms, irrespective of their evolved complexity, meiotically reduce the chromosome number at the start of sexual reproduction, compensating for fertilization and maintaining the diploid chromosome set from generation to generation (Golubovskaya, 1979). All steps in the process of meiosis are so well orchestrated that even a miniscule change can lead to severe alterations in the phenotypic expression. So, the study of the meiotic process is essential for efficient planning of breeding programmes.

Secondary associations or secondary pairing is defined as the close proximity of bivalents or chromosomes in pairs having diffused connections during meiosis (Darlington 1965). Kuwada (1910) was credited for the discovery of this phenomenon in *Oryza sativa*. Later on, Ishikawa (1911) reported this phenomenon in *Dahlia variabilis* followed by Marchal (1912) in *Amblystegium*, Darlington (1928) in *Prunus* and Lawrence (1931) in the species of *Dahlia*. Since then, the phenomenon had been observed in many plant species. It is of great significance as it presents a clue to the analysis of polyploidy where numerical considerations are not available or fail to elucidate it (Matsura, 1935., Agarwal, 1983). Regarding the origin of secondary associations of bivalents, different views have been put forth by different authors in different plants (Darlington, 1928; Lawrence, 1931; Heilborn, 1936; Jacob, 1957; Gupta and Roy, 1973).

The kidney bean (*Phaseolus vulgaris* L.) is the world's most important grain legume for direct human consumption (Goncalves *et al*., 2008). It is a rich source of protein (21.25%), fat (1.7%) and carbohydrate (70%). Besides this, it also contains 0.16 mg iron, 1.76 mg calcium and 3.43 mg zinc per 100gm of edible part (Kaur and Mehta, 1994). Brazil is the world's greatest common bean producer, producing more than 2.2 million tons, which represents 17.3% of the world's production (Goncalves *et al*., 2008). The phenomenon of secondary association has earlier been reported in many plants such as *Cicer arietinum*, *Prunus*, *Taraxacum*, and *Ocimum*, etc. The present study is an illustration of the behavior of secondary association of bivalents in *Phaseolus vulgaris* L.

* Corresponding author. Tel.:e-mail: nituchaudhary23@gmail.com.

2. Materials and Methods

2.1. Procurement of Seeds

Seeds of inbred lines of *Phaseolus vulgaris* L. variety PDR-14 (Uday) were obtained from Indian Institute of Pulses Research (IIPR), Kanpur, India.

2.2. Treatment Procedure

Dry and healthy seeds of *Phaseolus vulgaris* L. variety PDR-14 (Uday) were selected for gamma irradiation. The seeds were irradiated with three doses of gamma rays viz. 10 krad, 20 krad, and 30 krad, respectively from a ^{60}Co source at IARI, New Delhi. Thereafter the gamma irradiated seeds were treated with 0.3% of freshly prepared sodium azide (NaN_3) solution for about 3 hours with intermittent shaking, respectively. The seeds were then thoroughly washed with running tap water to remove the residual traces of sodium azide. Excess moisture was blotted off with the help of filter paper. One set of untreated seeds was kept in distilled water to act as control. The treated seeds were then sown in experimental pots in replicates along with the control to raise the M1 population.

2.3. Cytological Preparation

At the time of flowering, randomly selected floral buds of appropriate size were fixed in Carnoy's fixative (1:3, glacial acetic acid: absolute alcohol) for 24 hours at room temperature (25 ±2ºC). The fixed buds were later transferred to 70% alcohol (ethanol) and stored at 4 ºC for preservation. The slides were prepared using acetocarmine squash technique. Photomicrographs were taken from temporary slides. The cytological data were scored from the permanent slides which were made by passing the temporary waxed slide over a mixture of glacial acetic acid: butyl alcohol for a few minutes, followed by mounting on Canada balsam. Pollen sterility was calculated on the basis of stainability of pollen grains with 2 % acetocarmine (Shinde and More 2010).

3. Results

The course of meiosis was normal in control plants with 11 bivalents at diakinesis and metaphase I (Figure-1A) and normal segregation of 11:11 at anaphase I (Figure-1B).

The phenomenon of secondary association manifested from diplotene stages, whereas no glimpse of this phenomenon has been reported during pachytene stage. At diakinesis stage 1 or 2 tetravalent were found (Figure-1C). Secondary pairing by the bivalents has been observed in two patterns - end to end (Figure 1D-F) and side by side (Figure 1G and 1H). Multivalents were also observed (Figure-1I). At metaphase I/II stage, the bivalents and univalents showed different configurations and were found associated in the groups of II, III, IV, V, VI, VII and VIII. The type and frequency of pollen mother cells with secondary association has been listed in table-1. It is observed that the spectrum of pollen mother cells showing secondary association formation increased with an increase in the dose of mutagen.

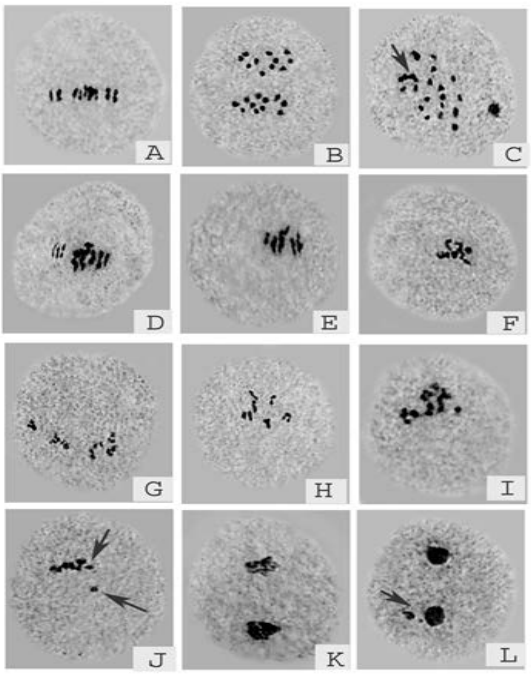

Figure 1. A. Normal metaphase (n=11); **B**. Normal anaphase (11:11); **C.** Tetravalent association of bivalents at diakinesis; **D-F-** Bivalents showing end-to-end secondary associations at metaphase; **G-H-** Bivalents showing side by side secondary associations at metaphase; **I**.A multivalent; **J**. Precocious movement of chromosomes at metaphase I; **K**. Stickiness at both pole during anaphase-I; **L**.Micronuclei at telophase I.

Table1. Secondary chromosomal associations among bivalents at metaphase-I/II in *Phaseolus vulgaris* L

Treatment doses	Total no. of PMCs observed	% of PMCs showing secondary association	% of PMCs showing secondary association among bivalents at M –I/II in different groups (I-VIII)								Other abnormalities	Pollen sterility (%)
			I	II	III	IV	V	VI	VII	VIII		
Control	365	-	-	-	-	-	-	-	-	-	-	2.87±0.13*
10kr+0.3%NaN_3	307	28.99	4.23	15.96	2.28	5.21	-	1.30	-	-	10.42	18.22±0.34*
20kr+0.3%NaN_3	339	40.70	4.12	19.17	3.83	6.78	2.35	2.06	0.88	1.47	13.27	30.07±0.22*
30kr+0.3%NaN_3	318	49.05	3.45	21.06	5.03	8.80	4.08	3.45	-	2.83	17.92	40.23±0.67*

Abbreviations : PMCs= Pollen mother cells, M-I/II=Metaphase I /II, NaN_3= Sodium Azide.
*Mean±SE

During the cytological observation, it was found that the tetravalent configuration was the common form of chromosomal association after bivalents which form the major share and were reported in 21.06 % PMCs. The percentage of PMCs showing tetravalent association varies from 5.21% at 10 krad+0.3% NaN_3 to 8.80% at 30 krad+0.3% NaN_3. Association of chromosomes in the configuration of VII was observed only at 20 krad+0.3% NaN_3 and was found to be 0.88%. Lowest number of univalents (3.45%) was found at 30 krad+0.3% NaN_3.Chromosomal associations in the group of III and VI were observed at all the doses. The treated sets also exhibited a wide array of other meiotic abnormalities apart from secondary chromosomal association. These include precocious movement of chromosomes at metaphase-2.83% at 30 krad+0.3% NaN_3 (Figure-1J), stickiness at anaphase-2.20% at 30 krad+0.3% NaN_3 (Figure-1K), micronuclei at telophase-1.88% at 30 krad+0.3% NaN_3 (Figure-1L), etc. A dose dependent increase in meiotic irregularities was observed with the mutagenic treated sets which ranged from 10.42% at10 krad+0.3% NaN_3 to 17.92% at 30 krad+ 0.3% NaN_3. The pollen sterility for control plants was found to be 2.87% which increases upto 40.23% at highest dose of treatment, i.e., 30 krad+0.3% NaN_3. The pollen sterility displayed an increasing trend along with the increasing doses of treatment.

4. Discussion

Although different views have been given regarding the formation of secondary associations, a satisfactory explanation has not yet been achieved. According to Thomas and Revell (1946), the fusion between heterochromatic regions of the involved bivalents is directly responsible for the formation of secondary associations. However, Lawrence (1931) and Malgwi *et al*. (1997) are of the opinion that homology existed between the paired bivalents, which resulted in a side by side association of bivalents in groups. There is a clear demarcation between multivalent formation and secondary pairing. Primary pairing at zygotene and chiasma formation among two or more homologous chromosomes resulted in multivalents whereas secondary association results from the loose association of bivalents without the existence of chiasmata (Katayama, 1965).

The occurrence of the phenomenon of secondary association has been considered to be a result of an artifact by many workers which may either be induced due to squash technique as obtained in *Luzula* by Brown (1950) and in *Carex* by Heilborn (1936) respectively or due to fixation as reported by Propach (1937). There has been a serious debate on the involvement of homologous chromosomes in the process of secondary pairing. Hirayoshi (1957) disagreed with the hypothesis which considers the involvement of homologous chromosome in secondary association and after examining careful results in *Oryzeae* and *Zizanieae* gave the conclusion that the secondary association may be a phenomenon operating under bio- and physico-chemical reactions and has no relation to the true homology of chromosomes.

Secondary pairing between bivalents has been considered as an indicator of polyploid nature of a species as reported in *Ocimum* (Mukherjee and Datta, 2005), and *Uraria picta* (Bhattacharya and Datta, 2010). According to Stebbins (1950), secondary association can be considered a phenomenon which depicts the polyploid nature of a species or genus, but elaborate phylogenetic predictions cannot be drawn from this as the secondary pairing between bivalents is considerably modified by other chromosomal changes. But amidst these explanations, Bhattacharya and Datta (2010) concluded that no inferences should be drawn regarding the polyploidy origin of species solely on the basis of secondary pairing. As an alternative, the cytological data must be co-related with locus specific molecular markers using FISH (Fluorescent in situ hybridization) to get a precise decision (Litcher, 1997). The phenomenon of secondary associations, along with other meiotic abnormalities, has some impact on pollen fertility as it was found to be significantly decreasing along with increasing concentrations of gamma ray +sodium azide.

Conclusively, it can be drawn from the above facts that the estimation of the strength of forces involved in the secondary association makes a foundation for assessing the impact of environmental factors on chromosomal association. The environmental factors modify the chiasma frequency by either altering chiasma formation or chromosome pairing. Since secondary pairing between bivalents is independent of chiasma formation, it provides accurate details about the effect of environmental factors on chromosome pairing.

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Study on the Production of Bacterial Cellulose from *Acetobacter xylinum* using Agro-Waste

Puji Lestari[1,*], Nitariani Elfrida[2], Ani Suryani[2] and Yadi Suryadi[1]

[1]*Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD-IAARD), Jl. Tentara Pelajar No. 3A Bogor 16111, Indonesia*

[2]*Bogor Agricultural University, Dramaga, Bogor, Indonesia*

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Abstract

Bacterial cellulose (BC), produced by *Acetobacter xylinum*, has been given a great attention due to its high potency for many industrial applications. An optimized substrate is important for higher BC production; thus, an alternative natural product, as a carbon source, should be determined. This study aims at investigating the BC of *A. xylinum* cultured in coconut water and pineapple juice-based media and to predict its fermentation kinetics. The BC was produced on two stages of fermentation system, the shaking culture for propagation and the static culture fermentation for BC production. *A. xylinum* exhibited exponential phase at 48 h w hich showed BC production associated with its cell growth on both substrates. Fermentation kinetics of *A. xylinum* using coconut water and pineapple juice revealed Rp 0.117 and 0.051 g/l/h, Rx 0.309 and 0.133 g/l/h, Rs 0.079 and 0.215 g/l/h, Yx/s 1.408 and 0.240 g biomass/g glucose, Yp/s 3.612 and 0.599 g cellulose/g glucose, Yp/x 2.235 and 2.452 g cellulose/g biomass, μmax 0.0132 and 0.0082/h, σ 0.028 and 0.0173/h, respectively. Overall parameters of fermentation kinetics revealed a high rate of BC formation and efficient conversions of glucose to biomass and BC by *A. xylinum* on coconut water substrate. Thus, coconut water proved to be a more suitable substrate to produce BC in comparison with pineapple juice.

Keywords: *Acetobacter xylinum*, Bacterial cellulose, Coconut water, Fermentation kinetic, Agro-waste.

1. Introduction

Cellulose is a l inear glucose polymer of ß-1,4-glycosidic bond with various polymerizations. Cellulose is the main component of plant cell walls (Hardjo *et al.,* 1989), which is generally used as a r aw material for paper, board, or rayon fibers in industrial manufactures. At present, wood becomes the major source of cellulose because of its quite high content. However, cellulose from wood pulp is still contaminated with polysaccharides, such as hemicellulose and lignin. Thus, it is necessary to find other alternative sources of high purity cellulose.

Acetobacter xylinum is a t ype of bacteria that produces cellulose with favorable physical properties (Suwannapinunt *et al*., 2007). *A. xylinum* is identified as a gram negative bacterium with short rod, which is capable of oxidizing glucose to gluconic acid and organic acids simultaneously. This bacterial cellulose (BC) has been known as secondary metabolite from glucose with the release of acetic acid into the environments (Tomita and Kondo, 2009). Unlike the cellulose from wood pulp, the BC has a high purity, unique strength, an ultra-fine structure and is biodegradable. Isolation and purification of this BC are also simple. These properties allow BC to be used as a substitute for wood raw material in the high-quality paper industry, low-calorie foods membrane ingredients (ultra filtration) and other materials (Iguchi *et al*., 2000).

Several studies have dealt with BC biosynthesis using various strains and cultivations systems (Ishikawa *et al*., 1995; Chao *et al*., 2000; Kongruang, 2008). The BC has been produced using different carbon sources (Ramana *et al.,* 2000; Chawla *et al.,* 2009), various nitrogen sources (Budiono *et al*., 1999; Melliawati *et al*., 2006) and other environmental factors (Chao *et al.,* 2001; Ishida *et al*., 2002). The cultivation method and the growth media influence the ability of *A. xylinum* to produce BC (Coban and Biyik, 2011). The high nutritious media induce *A. xylinum* cells growth with higher BC being formed. However, a standard media solely for producing BC is expensive even though a kind of sorbitol, glucose, lactose and mannitol are good

* Corresponding author. e-mail: plestari129@yahoo.com.

as a carbon source. Thus, more investigations using natural resources could be useful for the efficiency of BC production.

A number of fruit extracts, including orange, apple, pear, pineapple and coconut, is available every season. Because of the vitamin content and the proteolytic enzymes present in the pineapple, this fruit is good for food uses (Hebbar *et al*., 2008). The increasing production of pineapple processed items, results in a massive waste which is unsuitable for human consumption. Coconut juice is discarded from many applications of agro-industries in Southeast Asian countries. Because the residues still contain carbon and nitrogen sources, they could be utilized as a substrate for producing a good quality BC (Kongruang, 2008; Kurosumi *et al*., 2009). In Indonesia, abundant coconuts and pineapples are produced and need to be used efficiently. Before we determine them as fermentation media for producing BC, their function as the best substrate should be defined. Therefore, the present study aims at determining an efficient substrate from agro-residues for the production of BC. The fermentation kinetics of *A. xylinum* and related parameters were investigated.

2. Materials and Methods

2.1. Bacteria and Culture Media

A. xylinum strain, used in this study, was obtained from Bioprocess Lab., at the Faculty of Industrial Technology, Bogor Agricultural University. We prepared media of starter and fermentation for *A. xylinum.* A starter medium of Hassid-Barker was made per 1 liter of distilled water containing 10% sucrose, 0.25% yeast extract, 0.5% K_2HPO_4, 0.6% $(NH_4)_2SO_4$, and 0.2% $MgSO_4$. Fermentation medium for the BC production is the Hassid-Barker enriched with 0.25% ammonium sulfate and 0.25% calcium sulfate using two types of solvents as treatment, coconut water and pineapple juice. Pineapple solvent was made by filtering blended pineapple and mixing it with coconut water at ratio of 1:3. The media pH was adjusted using glacial acetic acid to 4.0 and sterilized. The composition of coconut water and pineapple juice-based media are presented in Table 1.

Table 1. Composition of fermentation media used in this study.

Coconut water-based medium		Pineapple-based medium	
Sucrose	100 g/l	Sucrose	100 g/l
Yeast extract	2.5 g/l	Yeast extract	2.5 g/l
K_2HPO_4	5 g/l	K_2HPO_4	5 g/l
$(NH_4)_2SO_4$	6 g/l	$(NH_4)_2SO_4$	6 g/l
$MgSO_4$	2 g/l	$MgSO_4$	2 g/l
Ammonium sulfate	2.5 g/l	Ammonium sulfate	2.5 g/l
Calcium sulfate	2.5 g/l	Calcium sulfate	2.5 g/l
Coconut water	1 l	Pineapple juice	333,33 ml
		Coconut water	666,66 ml

2.2. Bacterial Growth Pattern

A single colony of *A. xylinum* from stock agar was inoculated on nutrient agar and incubated for 4 days at 28ºC for regeneration. The four-day old bacterial cells (Pae *et al*., 2011) were inoculated into 50 ml of starter medium of Hassid- Barker, then incubated with shaking at 125 rpm for 48 hours at 28ºC. At the end of the incubation, the 50 ml of inoculums were added to the 500 ml of fermentation medium of Hassid-Barker in flask, homogenized and shaked for the next 5 days. To investigate the growth pattern of *A. xylinum*, culture was sampled periodically in a certain period and determined its Optical Density (OD_{620}) using spectrophotometer.

2.3. Fermentation Process

BC production was carried out with two cultivation steps, starting with shaking culture for microbial propagation and fermentation without shaking (static condition). Propagation step was prepared in 50 ml of starter liquid medium of Hassid-Barker and incubated according to pre-determined time of *A. xylinum* growth. The 50 ml propagated culture was transferred into 500 ml fermentation medium depending on the treatment of substrates and incubated in a static condition for 12 days. The lids of the flasks containing *A. xylinum* culture were kept loose to ensure the transfer of oxygen. Several parameters, such as pH, biomass which was represented by cell dry weight, and reducing sugars, were performed. Reducing sugar in the form of glucose was determined using a 3.5-Dinitrosalicylic Acid (DNS) and BC yield with gravimetric method (AOAC, 1984). Based on the value of reducing sugar, the parameters below were further determined:

- Used sugar (%) = (IS-FS) / IS x 100
- Sugar converted into BC (%) = Y / (IS-FS) x100, where: IS = initial sugar concentration (g / l), FS = final sugar concentration (g / l), Y = BC yield (g / l).

2.4. Fermentation Kinetics

To identify the efficiency of using substrate, the fermentation kinetics during BC production was measured. Parameters, such as X as biomass (g/l) is obtained from the cell dry weight, S is the substrate by

reducing sugar content in the form of glucose (g/l) and P is the weight of wet BC product per volume of fluid (g/l). Fermentation kinetics parameters were then measured as follows:

Rx = the growth rate or biomass formation (g / l / h) = dx / dt

Rs = the utilization rate of glucose substrate (g / l / h) = ds / dt

Rp = the formation rate of BC (g / l / h) = dp / dt

Yx / s = the biomass yield toward glucose substrate (g / g) = (X-Xo) / (So-S)

Yp / s = the BC yield toward glucose substrate (g / g) = (P-Po) / (So-S)

Yp / x = the BC yield toward biomass (g / g) = (P-Po) / (Xo-X)

μmax = the maximum specific growth rate (per h) = slope of ln X = f (t)

σ = the specific rate of BC formation (per h) = slope of ln P = f (t)

3. Results and Discussion

3.1. Determination of the Optimum Growth Phase of A. xylinum for Inoculum

Bacterial growth can be represented by biomass or cell number which is estimated with optical density (OD_{620nm}). In this study, the growth of *A. xylinum* was observed to determine its maximum cell number at log phase. *A. xylinum* showed a rapid exponential phase from beginning (OD: 0.201) to 36 h incubation as expected. The growth increased slower before reaching the highest OD (1.182) after 48 h, suggesting that its logarithmic phase could support for optimum *A. xylinum* growth from propagated culture to static cultivation system. The growth pattern *A. xylinum*, which was preceded by a short adaptation (lag) phase, indicates enough growth-induced substrate (Figure 1). Since *A. xylinum* cells consume nutritional products in parallel with metabolites secretion at specific period before stationary phase, thus, Hassid-Barker medium which was combined with carbon source is assumed to be enough for *A. xilynum* growth.

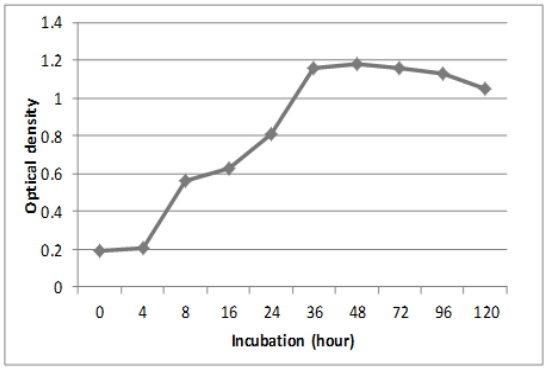

Figure 1. The growth curve of *A. xylinum* in the stirring cultivation to determine the exponential phase.

Based on this result, the carbon source concentration in fermentation medium seems to affect the *A. xylinum* growth cells. Sucrose 10% and coconut water contribute to cells regeneration, which is consistent with a previous study showing that coconut water in HS medium promotes *A. xylinum* growth rate (Kamarudin *et al*., 2013). Additionally, the adaptation phase of bacteria, including *A. xylinum*, may be influenced by the number of inoculated cells, age and physiological state (Rolfe *et al*., 2012). This initial investigation of *A. xylinum* growth is important in order to obtain the optimum BC production, thus, 48 h was determined as the best timing to transfer *A. xylinum* culture into fermentation process in this study.

3.2. pH Changes

The pH value of the fermentation media decreased on both coconut water and pineapple juice substrates, reaching approximately pH 3.5 as demonstrated in this study (Figure. 2). The pH value decreased faster in the early period of incubation up to 48 h which was proportional with the lag phase. Decrease in the pH value in this study is relatively faster than that in other formulated media with coconut water which was the only one carbon source and the other inorganic carbon sources-based media (Kamarudin *et al*., 2013). It is believed that it is more likely due to the released gluconic acid, acetic acid and acidic-by product (Ndoye *et al*., 2007; Kongruang, 2008) which lead to the inhibition of BC production because acidic medium can be toxic to *A. xylinum* cells. Furthermore, organic acids from coconut and pineapple juices may contribute to decline pH due to spontaneous fermentation.

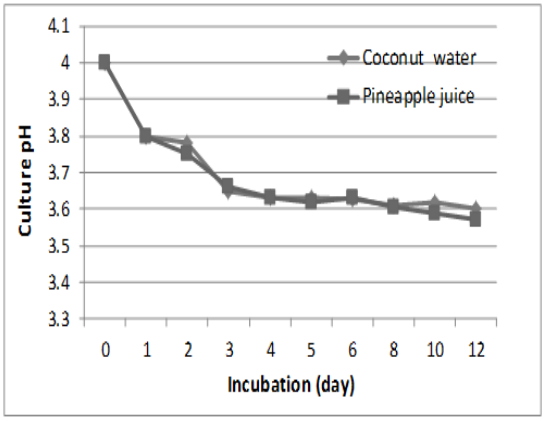

Figure 2. Pattern of pH change of *A. xylinum* culture on static culture fermentation.

These results agree with a previous study using two different strains of *A. xylinum* which revealed higher fold of acetic acid levels in Hestrin-Schramn medium with coconut juice as substrate than that with pineapple juice (Kongruang, 2008). The acidity of liquid fermentation of *A. xylinum*, however, can be adjusted with a buffer solution for optimum BC production (Pae *et al*., 2011), though it is still difficult to be controlled (Ishikawa *et al*., 1995). Overall, a decrease in the pH of culture medium is very influential on cell growth and productivity of BC from *A. xylinum* (Mathew *et al*., 2005; Kongruang, 2008).

3.3. Pattern of BC Formation

A. xylinum grown in coconut water and pineapple substrates showed a similar pattern of cell biomass, BC yield and reducing sugar (Figure 3). In both substrates, BC yield increased in parallel with cell biomass, but in contrast to declining of reducing sugars during the fermentation process. Cell biomass representing bacterial growth increased to be the greatest after 6 days, and showed higher on coconut water (19.34 g/l) than on pineapple (10.04 g/l). Even though *A. xylinum* grew relatively slow in pineapple substrate, but this fruit waste is also promising as carbon source for producing BC (Kurosumi *et al*., 2009; Upadhyay *et al*., 2010). Especially in this study, both substrates had a similar decrease in pH which is in the range of optimum production of BC, 3.5 to 6 (Pae *et al*., 2011). Moreover, the time course of maximum growth of *A. xylinum*, in our study, is relatively comparable with other *A. xylinum* strain in static culture fermentations (Coban and Biyik, 2011).

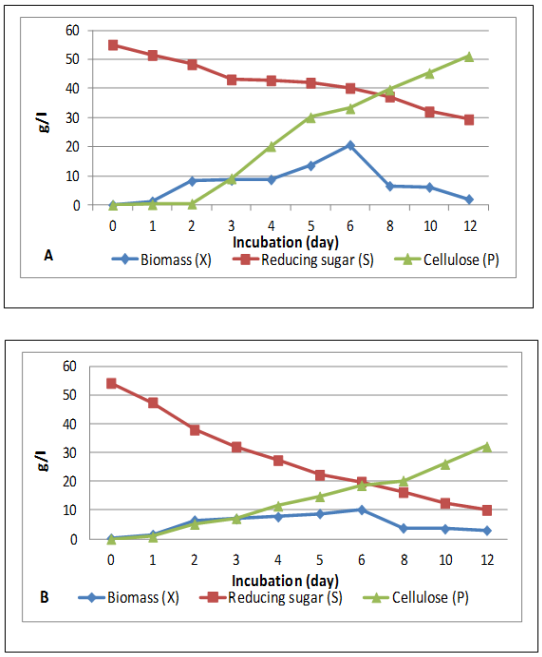

Figure 3. The relationship between the cell dry weights, BC products of *A. xylinum* and reducing sugar in cultivation system two stages. A) coconut water, B) pineapple juice

This study illustrates the *A. xylinum* growth with decreasing substrate concentration as represented by reducing sugar. Reducing sugars were found to have decreased in media containing pineapple juice more than that in media containing coconut water. *A. xylinum* used sugar in coconut water was lower in comparison with that in pineapple substrate, showing 50.8% and 81.1%, respectively. It seems that sugar converted by *A. xylinum* on coconut water was much higher (184.9%) than in pineapple juice (72%). Thus, it reveals that *A. xylinum* had higher efficiency of 2.6-fold of metabolization for monosaccharide and disaccharide in coconut water than in pineapples subtrate, which supports previous findings in the same subtrate (Kongruang, 2008; Kamarudin *et al*., 2013). *A. xylinum* presumably uses glucose from pineapple substrate more for cell metabolism than for the formation of BC.

Formation of BC can be predicted based on the pattern of substrate utilization. This study demonstrated that the BC yield achieved in maximum on day 12 when utilization of sugar on substrate was to be stationary as expected. In coconut water, *A. xylinum* produced 1.6-fold of BC yield and 0.6-fold of cell biomass with more efficient consumption of substrate of 2.9-fold than those in pineapple substrate during fermentation (Figure 3). Thus, we found out that coconut water in Hassid-Baker medium is clearly more efficient that pineapple-based medium to produce BC, convincing the flexibility of coconut water as substrate of *A. xylinum* (Kamarudin *et al*., 2013).

Our results could also explain that in the BC formation, glucose and fructose from decomposition of sucrose and other carbon sources were possibly polymerized to form BC which usually occurs via oxidative major pathway of *A. xylinum* (Ross *et al*., 1991). There is a phenomenon in which *A. xylinum* uses a specific substrate like coconut water which leads the cells to get adapted to consume the substrate. Formation of BC, which was also positively correlated with bacterial cells, indicating that the BC begins to form early cell metabolism is in line with prior study (Kongruang, 2008). Ishikawa and coworkers (1995) also reported that *A. xylinum* subp. *sucrofermentans* in the static culture fermentation formed BC pellicle and then bacterial cells were absorbed in the pellicle. Instead of various chemical nitrogen (such as casein hydrolisate, ammonium sulfate, yeast extract) and carbon sources (monosaccharides, oligosaccharides, alcohols, sugar alcohols and organic alcohols) (Jung *et al*., 2010; Coban and Biyik, 2011), coconut water, therefore, could replace carbon source to maximize the BC production with low cost.

3.4. Fermentation Kinetics

Microbial growth can be viewed as a series of biochemical reactions that convert a substrate for the synthesis of cell material and extracellular products. Analysis of the kinetics of *A. xylinum* growth is needed to determine the growth rate, the rate of substrate utilization and the product formation rate. *A. xylinum* fermentation kinetic parameters, indicating the difference between the substrates of coconut and pineapple juices, are shown in Table 2.

Table 2. Parameters of fermentation kinetics of *A. xylinum.*

Parameters	Substrate	
	Coconut water	Pineapple juice
R_p	0.117 g/l/h	0.051 g/l/h
R_x	0.309 g/l/h	0.133 g/l/h
R_s	0.079 g/l/h	0.215 g/l/h
$Y_{x/s}$	1.408 g biomass/g glucose	0.240 g biomass/g glucose
$Y_{p/s}$	3.612 g BC/g glucose	0.599 g BC/g glucose
$Y_{p/x}$	2.235 g BC/g biomass	2.452 g BC/g biomass
μ_{max}	0.0132/h	0.0082/h
σ	0.0258/h	0.0173/h

The rates of *A. xylinum* growth and of BC formation were 2.32- and 2.29-fold higher on coconut water than those in pineapple juice, respectively. Surprisingly, the greatest growth and BC productivity of *A. xylinum* on coconut water substrate were followed by much lower rate of glucose utilization (Rs:0.079 g/l/h) than that in pineapple juice (Rs:0.215 g/l/h). Based on yield values, the conversion of substrate to biomass and BC were 5.87- and 6.03-fold greater, respectively, in coconut water (Yx/s:1.408 g biomass/g glucose and Yp/s:3.612 g BC/g glucose) than in pineapple juice (Yx/s:0.24 g biomass/g glucose and Yp/s:0.599 g BC/g glucose). The more convincing results were the maximum specific growth rate (μ_{max}) in the exponential phase which was 1.61-fold higher in coconut water compared to pineapple juice. The specific rate of BC formation of *A. xylinum* was also higher in coconut water (σ:0.0258/h) than that in pineapple (σ:0.0173/h). Compared with the study of Kongruang (2008) which used the bigger scale up of cultivation, the growth kinetics parameters in our study were somewhat lower; however, our result with small laboratory scale was able to determine the efficiency of agro-residues as substrate for *A. xylinum*. Thus, it could be noted that coconut water may be preferred for a better fermentation of *A. xylinum* than other agro-wastes. However, taken together, abundant pineapple fruit waste in addition to coconut water should be further investigated for their best formulation in conjunction with cultivation methods for production of BC.

4. Conclusion

Maximum growth of *A. xylinum* reached in exponential phase at 48 h and was determined to be the best time to transfer the propagation culture to static fermentation system. BC produced by *A. xylinum* was positively correlated with the cell growth and negatively related to the reducing sugar. Overall parameters of fermentation kinetics suggest that coconut water is more efficient to be used as a substrate for producing BC by *A. xylinum* compared with pineapple juice.

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