Exploring the Antibacterial and Antioxidant Properties and GC-MS Profile of the Indian Lichen *Parmotrema perlatum*

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Abstract :

Antimicrobial resistant necessitates development of new antimicrobial agents. Most drugs have a bacterial origin. Lichens produce pharmaceutically active metabolites from both algae and fungi as well as other metabolites that arise from the symbiotic interaction between the two. The present research investigates the pharmacological properties of lichen in terms of their ability to produce bioactive secondary metabolites. Extracts of the lichen were prepared in different solvents and a systematic phytochemical analysis of the extracts was performed. The extracts were also evaluated for their antioxidant and antimicrobial properties as well as its GC-MS profile. The investigations revealed that the lichen contains a variety of pharmacologically active agents that are active against pathogenic microbes along with high antioxidant activity and merits further investigation for therapeutic use.

***Keywords: Parmotrema perlatum*, GC-MS, antioxidant activity, antimicrobial activity.**

I. INTRODUCTION

Natural products are a significant source of new drugs, especially in the treatment of cancer, infections, hypertension, and immune and neurological disorders (Butler, 2004). Although many natural metabolites have been screened resulting in the production of many pharmaceutically important drugs, many potential sources of drug therapies are still to be investigated and one such potential source of drugs include the lichens.

A lichen is a symbiotic association between a fungus and one or more partners, the latter usually being a photosynthetic partner - a eukaryotic alga and/or cyanobacterium; or, in some cases a non-photosynthetic bacteria. Lichens are ecologically diverse and are distributed from the tropics to the Polar Regions. (Brodo. et. al., 2001).

Lichens produce a number of metabolites with potential use in medicine. The primary metabolites in lichens are produced by both the symbiotic partners. However, the secondary metabolites are produced exclusively by the fungal partner, exported outside the fungal hyphae and deposited as extracellular crystals in the thallus, often in the upper cortex or in specialized structures such as fruiting bodies (Fahselt, 1994). The metabolic interaction between the mycobiont and phytobiont is essential to the production of these secondary chemicals. This has been documented in studies where mycobionts grown without the photobiont do not produce the same metabolites as the intact lichen or produces a completely different suite of chemical products. There are reports where the photobiont, especially cyanobacteria, also produce some secondary metabolites. (Stocker-Wörgötter and Elix, 2002).

Lichens produce an impressive variety of unique secondary metabolites and have been used as ingredients in folk medicines for centuries. Over 1050 secondary metabolites have been reported for lichens and aposymbiotically cultured mycobionts (Molnar & Farkas, 2010). This metabolic diversity is due largely to the symbiotic relationship between the lichen partners (Lawrey, 1986). Lichen secondary products can comprise up to 20% of the thallus dry weight, but in most lichens the amount varies from 5–10%. Some of the compounds produced by lichens include lichenin, isolichenin, hemicellulose, pectins, disaccharides, polyalcohols, amino acids, enzymes, and pigments like algal chromophores: chlorophyll and, β-carotenes, xanthophylls, etc. (Podterob, 2008; Selbmann. et. al., 2010, Stojanovic. et. al., 2011). The medicinal properties of some lichens are mentioned in the Ayurvedic and Unani systems where they are used to treat a broad array of common ailments, including blood and heart diseases, bronchitis, scabies, leprosy, asthma, stomach disorders, etc. (Jayasri. et. al., 2009) .

Although India is home to an impressive variety of lichen species, there is limited research to examine the pharmaceutical potential of these lichens. The main goal of this research was to investigate some of the properties of a local lichen (*Parmotrema perlatum*) found in Maharashtra. *P. perlatum* is a common Indian lichen found in all regions. It is commonly known as "Dagad phool" (Marathi = stone flower) or "Kalpasi" and is a constituent of spice mixes (masalas) used in Indian kitchens (Lumbsch and Huhndorf, 2007*).*

For this study the lichen was identified by assessing the morphology, anatomy and colour and collected from amongst the lichens growing on a hard wood tree (Oak Tree) in open habitats of coastal woodlands of Chinchani, Tarapur-Boisar region of Maharashtra state in India. The systematic hierarchical classification of the lichen is as follows : Kingdom: *Fungi*, Division: *Ascomycota*, Class: *Lecanoromycetes*, Order: *Lecanorates* , Family: *Permiliaceae*, Genes: *Parmotrema* , Species: *P. perlatum*

II. MATERIALS AND METHODS:

The lichen for the study was collected after first identifying it by assessing its morphology, anatomy and colour on site from amongst the lichens found on an oak tree in the coastal woodlands of Chinchani in Maharashtra state of India.

To prepare the lichen extract, the thalli of the lichen was ground finely and extracted using different solvents to give a final extract concentration of 100 mg/ml (0.1g/ml). The extracts were filtered using Whatman No.1 filter paper and stored at 4˚C until further use. (Lumbsch, 2007)

The antimicrobial effect of the lichen extracts was evaluated against various pathogenic bacteria and fungi using the standard disk diffusion method (7 mm diameter disks, 15µl of extract) on Mueller-Hinton agar for bacteria and Sabouraud agar for fungi (yeast). The antimicrobial activity was measured as of diameter of the zone of inhibition observed around the disk as compared to a suitable solvent control disk; as per CLSI standards.

Different assays were carried out to determine the antioxidant properties of the extract. The free radical scavenging activity of the extracts was measured using the DPPH (1,1-diphenyl-2-picryl-hydrazine) method. The activity was measured as percent scavenging effect as compared to ascorbic acid – a known, potent, free radical scavenger (Blios, 1958). The reducing power of extracts was determined according to the potassium ferricyanide method of Oyaizu (1986). The superoxide anion radical scavenging activity of lichen extracts was assessed using the Nitro Blue Tetrazolium (NBT) method of Nishimiki et al. (1972). Total soluble phenolic compounds in the lichen extracts were determined with Folin-Ciocalteu reagent as per the method of Slinkard (Slinkard and Singleton, 1997) using pyrocatechol as a standard phenolic compound.

The total flavonoid content was determined using the Dowd aluminium trichloride method (Meda. et. al., 2005). The total flavonoid content was determined in terms of microgram of pyrocatechol equivalent.

For GC-MS profiling the extract was prepared in HPLC grade solvents. The extract was filtered through a 0.22 µm Millipore filter. The GC–MS analyses were realised using a Hewlett-Packard 6890N gas chromatograph equipped with a fused silica capillary column DB-5MS (5% phenylmethylsiloxane, 30 m*0.25 mm, film thickness 0.25 m, Agilent Technologies, USA) and coupled to a 5975B mass selective detector from the same company. The injector and interface were operated at 250 and 380°C, respectively. The oven temperature was raised from 70–290°C at a heating rate of 5°C min^{-1} and then held isothermally for 10 min. Helium at a flow rate of 1.0 mL min^{-1} was used as the carrier gas. The samples and acetonitrile (1:100), were injected in a pulsed split mode (the flow was 1.5 mL min-1 for the first 0.5 min and then set to 1.0 mL min^{-1} throughout the remainder of the analysis; split ratio 40:1). The MS conditions were as follows: ionization voltage of 70 eV, acquisition mass range 35–500 and scan time 0.32s. The extract constituents were identified based on their linear retention indices (relative to C_{12}–C_{33} alkanes on the DB-5MS column) and by the application of the AMDIS software (auto- mated Mass Spectral Deconvolution and Identification System, Ver. 2.1, DTRA/NIST, 2002).

III. RESULTS AND DISCUSSION

1. Antimicrobial assays

The extracts of the lichen were tested against a variety of microorganisms viz. *Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi, Shigella sonnei, Micrococcus luteus, Serratia marcescens*, *Bacillus subtilis Streptococcus pyogenes* and the yeast-like fungus; *Candida albicans.* The tested lichen extracts showed strong antimicrobial activity. The zones of inhibition for the bacteria were in the range 9–24 mm for the hexane extract and 12–30 mm for the methanol extracts. The extracts were also effective against the fungal (yeast) pathogen *Candida albicans*. The strongest activity was found in the methanol extract against *S. typhi* (30 mm).

***Table 1** : Zone of inhibition for the antimicrobial testing*

Test organism	Zone of inhibition (mm)			
	Methanol extract	Hexane extract	Positive control	Negative control
P. aeruginosa	29	11	--	--
Shi. sonnei	29	12	--	--
S. pyogenes	22	15	--	--
M. luteus	15	10	--	--
S. marcescens	17	9	29	--
E. coli	20	24	15	--
B. subtilis	15	10	--	--
S. aureus	22	12	--	--
Sal. typhi	30	10	--	--
C. albicans	12	10	--	10

The intensity of the antimicrobial effect depended on the solvent used to prepare the extract and the tested microorganism. The hexane extract did not show a strong antimicrobial activity. That's probably because the active components produced by lichens methanol extract was more potent in inhibiting most organism than hexane extract. The hexane extracts of the tested lichens may also have not shown strong antimicrobial activity due to poor solubility in hexane. Methanol extract was more potent in inhibiting microorganisms as compared to the hexane extract.

The antibacterial effect was found to be stronger compared to the antifungal activity. These results are in line with the expectations. Numerous tests have proved that bacteria are more sensitive to antibiotics compared to fungi. The reason of different sensitivity between the fungi and bacteria can be found in difference in the cell wall. The cell wall of Gram-positive bacteria consists of peptidoglycans (mureins) and teichoic acids, the cell wall of the Gram-negative cells consists of lipopolysaccharides, and lipoproteins whereas the cell wall of fungi consists of polysaccharides such as chitin and glycans (Yang, 1999).

2. **DPPH radical scavenging**

Free radical scavenging action is considered to be one among the various mechanisms for antioxidation (Sini and Devi, 2004). This assay is used as a preliminary test to provide information on the reactivity of a test compound with a stable free radical. DPPH gives strong absorption band at 517 nm (purple colour) and when it is quenched by the extract, there is a decrease in absorbance and discoloration from purple to yellow. This method is rapid and does not require expensive reagents or sophisticated instruments (Anandjiwala. et. al., 2008). The scavenging DPPH radicals of the studied lichen extracts in hexane and methanol showed good scavenging activity against DPPH. The scavenging effects of lichen extracts were 32-58%. The percentage inhibition on DPPH radical of hexane and methanol extracts of lichen were 32.33% and 58.03% respectively.

3. **Reducing Power Activity**

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing properties are generally associated with the presence of reductones. Gordon (1990) reported that the antioxidant action of reductones is based on the breaking of the free radical chain by donating a hydrogen atom. The reduction of ferrous ion (Fe^{3+}) to ferric ion (Fe^{2+}) is measured by the intensity of the resultant blue-green solution which absorbs at 700 nm. The result of reducing power activity of the lichen extracts indicate a marked ferric reducing power activity due to presence of polyphenols which may act in a similar fashion as reductones by donating the electrons and reacting with free radical to convert them into more stable products and terminate free radical chain reactions (Sasikumar. et. al. 2010).

4. **Super oxide anion radical scavenging activity**

Numerous biological reactions generate superoxide radical which is a highly toxic species. Although they cannot directly initiate lipid oxidation, superoxide anion radical are potential precursors of damaging oxygen species and thus the study of the scavenging of this radical is important (Jayasri. et. al. 2009). In the PMS/NADH-NBT system, superoxide anion is generated using a non-enzymatic reaction of phenazine methosulphate in the presence of NADH and molecular oxygen (Robak and Gryglewski, 1998). The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture (Gulcin, 2004). The superoxide radical scavenging activities of extracts were evaluated based on their ability to quench the superoxide radical generated from the PMS/NADH reaction. The lichen extracts revealed a good superoxide anion scavenging activity, although the activity was lower than ascorbic acid which was 30.43% and that for methanol and hexane was 20.58% and 21.73%respectively.

5. **Total Phenolic and Flavonoid Content**

Phenolic components are potential antioxidants, free radical terminators (Shahidi and Wanasundara, 1992; Kaushik. et. al. 2010). These compounds are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups (Sawa. et. al. 1999). The amount of total phenolic compounds was determined as the pyrocatechol equivalent using an equation obtained from a standard pyrocatechol graph. Results of the study showed that the phenolic compound of the tested lichen extracts varied from 228.58 µg of pyrocatechol equivalents for methanol extract and 366.67 µg of pyrocatechol equivalents for hexane extract. The value of phenolic content for the standard was 276.10 µg of pyrocatechol equivalents.

Table 2 *: Results of antioxidant properties and phenol-flavonoid content*

Absorbance O.D for	DPPH assay at 517nm	Reducing power assay at 700nm	Superoxide anion radical scavenging assay at 560nm	Total phenolic content at 760nm	Total flavonoid content at 415nm
Standard	0.11	0.10	0.16	0.58	0.26
Methanol extract	0.47	0.12	0.27	0.48	0.21
Hexane extract	0.90	0.15	0.54	0.77	0.85
Control methanol	1.12	0.68	0.34	0.3	0.2
Control hexane	1.33	0.09	0.69	0.10	0.5
Blank	0.83	0.00	0.23	0.00	0.00

Flavonoids are the most important natural phenolics and they possess a broad spectrum of chemical and biological activities including radical scavenging properties (Mohammed. et. al. 2010). The total flavonoid content of the standard

was found to be 123.81 µg of pyrocatechol equivalents. The amount of total flavonoid compounds was also determined as the pyrocatechol equivalents. The flavonoid content was found to be 100 µg of pyrocatechol equivalents in methanol extract and 404.77 µg of pyrocatechol equivalents in hexane extract.

6. **GC-MS Chemical analysis**

The GC-MS profile of the acetonitrile and methanol extract of the lichen ***Parmotrema perlatum*** was obtained. The acetonitrile profile was dominated by cinnamic acid (44.4%) in the methyl ester form. Significantly lower amounts of a structurally related compound, Disperse blue 26, was found at 10.7%. This is a type of anthraquinone dye which the lichen pigments may have accumulated. It is not a naturally occurring dye and it is synthetically produced for various commercial purposes. Its presence in lichen extracts may be due to its absorption in the lichen thallic cells as lichen survives in effluent water bodies and serves as a bioindicator species (Glew, 2010*)*. 5-bromo2,4bis(methulsulfanyl)-pyrimidine, a pyrimidine derivative found in aromatic compounds was detected at 5.03%. This is used in a wide variety of old drug formulations, either in combination with other compounds or on its own. It finds various pharmaceutical applications including general anaesthetics, anti-epilepsy medications, anti-malarial medications, drugs for treating high blood pressure, as HIV medications etc. and shows different levels of toxicity. The compound may be contributing to the antimicrobial activity of lichen *Parmotrema perlatum species.* Methaqualone (2.44%), detected in the profile is an ethaqualone - a sedative or a tranquilizers that increases the activity of the GABA receptors in the brain and nervous system. It has wide applications in drug formulations but its overdose can cause delirium, convulsions, hypertonia, hyperreflexia, vomiting, kidney failure, coma, and can be lethal. (Van Zyl, 2001).

The methanol profile was also dominated with Cinnamic acid (56.3%) along with cinnamic acid, lignin, flavonoids, isoflavonoids, coumarins, aurones, stilbenes, catechin, and phenylpropanoids. A volatile ethyl ester of cinnamic acid, ethyl cinnamate, is the principal flavour component of the lichen which produces a distinct aroma when lichen is used in cooking as a spice. Cinnamic acid has various properties including antituberculous, antidiabetic, antimicrobial, as a fragrance material, hepatoprotective, CNS depressant, anticholesterolemic, anti-fungal and fungitoxic, antihyperglycemic, antimalarial, antiviral, anxiolytic, cytotoxic, anti-inflammatory and UV absorbent. (Sharma, 2011). Cinnamic acid also has different levels of toxicity, especially in its Trans-Cinnamic acid state (Cinnamic acid trans-3-phenylacrylic acid) causing serious eye irritation, skin irritation, is harmful when absorbed through skin and may cause respiratory irritation. (MSDS, 2012).

IV. CONCLUSION

The antimicrobial and antioxidant properties and GC-MS profile of *Parmotrema perlatum* was studied. The extract of lichen sample was prepared in different solvents and used in the study. The antimicrobial activity had a potential to inhibit a variety of microbes including Gram positive and Gram negative bacteria as well as yeast. Methanol extract was more potent in inhibiting the organism as compared to the hexane extract. The strongest activity was found in the methanol extract against *S. typhi* and in hexane extract against *E. coli*. Thus it can be concluded that the lichen species of *Parmotrema perlatum* has a good potential for use as an antimicrobial and needs further exploration for therapeutic use in humans. The study of antioxidant activity of lichen extracts was also found to be strong. The lichen extract showed high antioxidant properties due to the presence of phenolic and flavonoid contents. The strong relationships between total phenolic and flavonoid contents and the antioxidative activities of tested extracts suggest that these compounds play important role in antioxidant activity. The present study shows that tested lichen species demonstrated a strong antioxidant activity and can be considered as good sources of natural antioxidants. The GC-MS profile of the acetonitrile, methanol and hexane extract of the lichen *Parmotrema perlatum* was dominated by cinnamic acid (44.4%) and 5-bromo-2,4bis(methulsulfanyl)pyrimidine (5.03%) – substances used in old drug formulations with a variety of pharmacological applications. These have different levels of toxicity. Thus it can be concluded that lichen secondary metabolites merit further investigation for therapeutic use.

V. AUTHOR CONTRIBUTIONS

Irin Tandel carried out the laboratory work, Vikas Gupta co-supervised the laboratory work, Mohammed Mubeen Shaikh contributed to the writing, Ganesh Lad conceived and coordinated the study and wrote the manuscript.

All authors gave final approval for publication.

VI. COMPETING INTERESTS

The authors do not have any competing interests.

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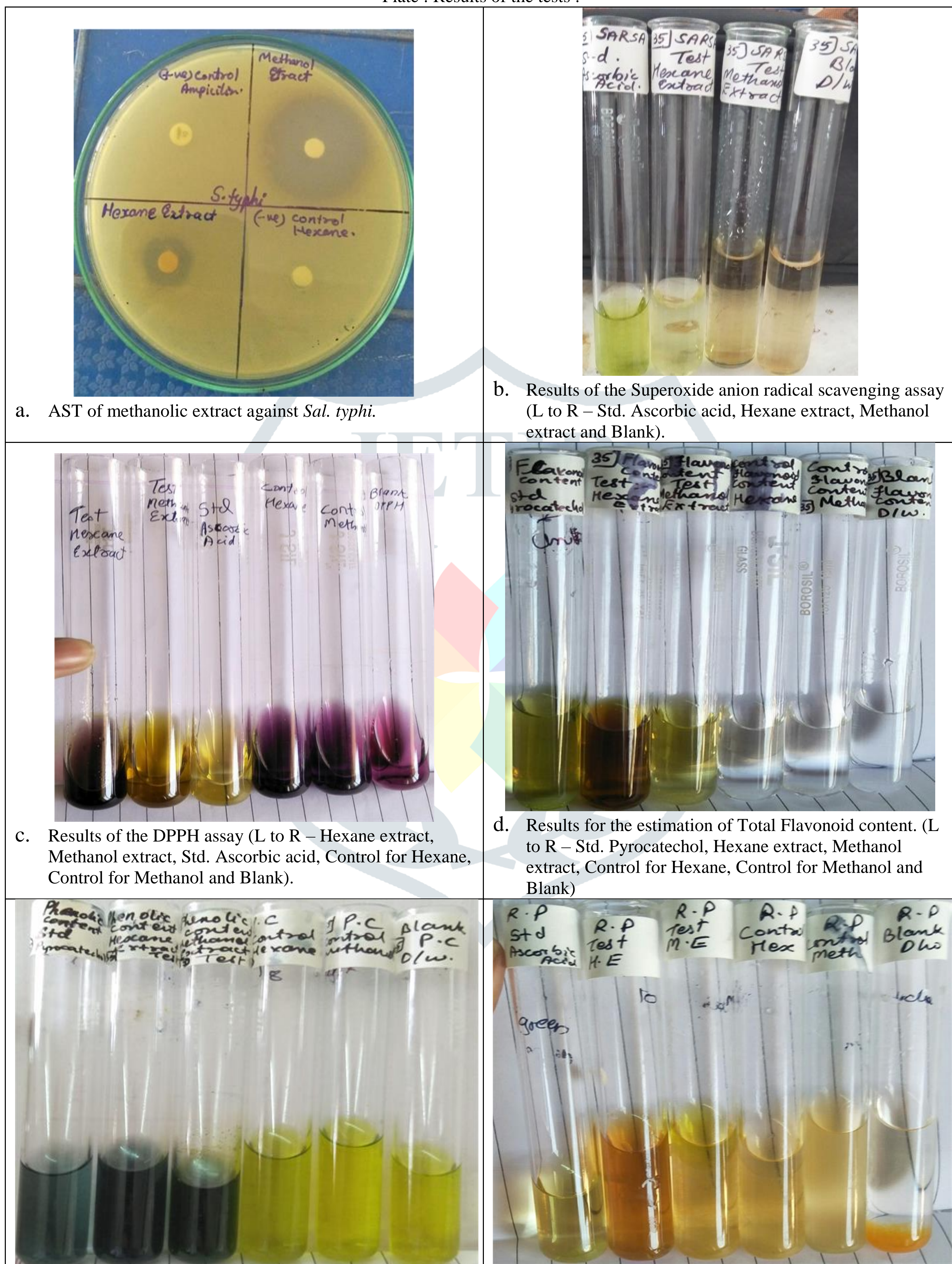
Plate : Results of the tests :



a. AST of methanolic extract against *Sal. typhi.*

b. Results of the Superoxide anion radical scavenging assay (L to R – Std. Ascorbic acid, Hexane extract, Methanol extract and Blank).

c. Results of the DPPH assay (L to R – Hexane extract, Methanol extract, Std. Ascorbic acid, Control for Hexane, Control for Methanol and Blank).

d. Results for the estimation of Total Flavonoid content. (L to R – Std. Pyrocatechol, Hexane extract, Methanol extract, Control for Hexane, Control for Methanol and Blank)

e. Results for estimation of Phenolic content. (L to R – Std. Pyrocatechol, Hexane extract, Methanol extract, Control for Hexane, Control for Methanol and Blank)	f. Results of Ferric Reducing Power Activity. (L to R – Std. Ascorbic acid, Hexane extract, Methanol extract, Control for Hexane, Control for Methanol and Blank)