EVALUATION AND UTILISATION OF EDIBLE LICHEN *Parmotrema tinctorum* (Nyl.) Hale FOR FOOD PRESERVATION

By

ANUPAMA.T .V

(2014 - 22-102)

THESIS

Submitted in partial fulfilment of the requirement for the degree of

DOCTOR OF PHILOSOPHY IN HORTICULTURE

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DEPARTMENT OF POST HARVEST TECHNOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

2018

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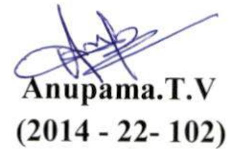
I, hereby declare that this thesis entitled **"EVALUATION AND UTILISATION OF EDIBLE LICHEN** ***Parmotrema tinctorum*** **(Nyl.) Hale FOR FOOD PRESERVATION"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.



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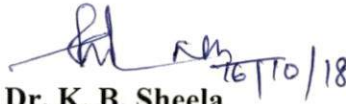

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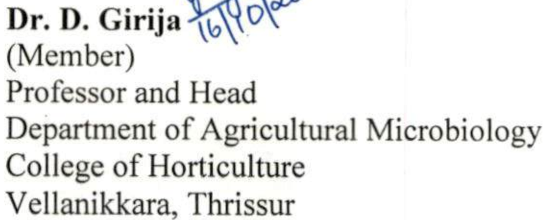

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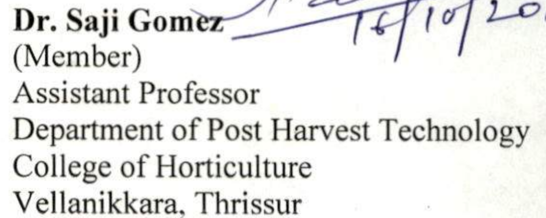

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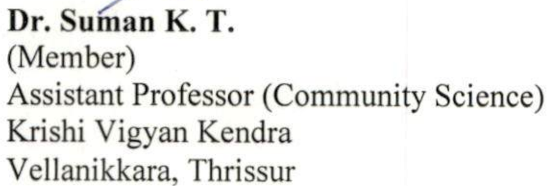

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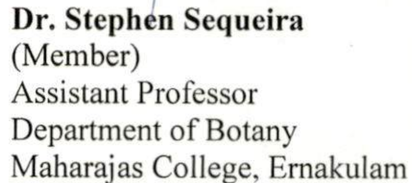

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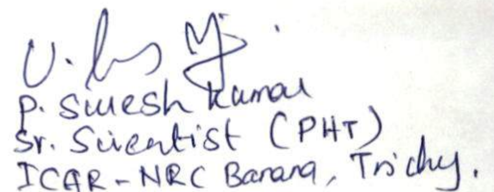

EXTERNAL EXAMINER

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LIST OF ABBREVIATIONS

ml	:	millilitre
°C	:	degree Celsius
mg	:	milligram
mM	:	milli Molar
nm	:	nanometre
l	:	litre
rf	:	retention factor
µm	:	micrometre
µl	:	microlitre
mm	:	millimetre
mcg	:	microgram
ad libitum	:	not restricted
viz.	:	namely
et al.	:	co workers
g	:	gram
min.	:	minutes
hr	:	hour
ppm	:	parts per million
%	:	percentage
CD	:	critical difference
fig.	:	figure
kg	:	kilogram
no.	:	number

INTRODUCTION

1. INTRODUCTION

Microbiological quality has become an increasingly important factor in determining the final quality of food. The use of synthetic preservatives as antimicrobial agents to prevent spoilage of food has been in practice for many years. Preservatives are chemical additives having prescribed limits of use as per law, added deliberately to the foods for prevention or delay of spoilage of food. Chemical preservatives (Class II preservatives) permitted for use as per Food Safety and Standards Authority of India (FSSAI), are sulphur dioxide and benzoic acid.

Synthetic preservatives pose health problems because of the carcinogenicity, high acute toxicity, and long degradation periods. Chemical preservatives such as sodium benzoate have raised health concerns. Benzoic acid can be converted by decarboxylation into benzene, which is a carcinogenic substance. Benzoate was reported to cause hypersensitivity in asthma sufferers (Perez-Diaz and Mcfeeters, 2010). Studies have also proved that parabens, another group of preservatives, possess genotoxic activity by acting as inhibitors of sulfotransferase enzymes.

Natural alternatives to synthetic antimicrobials are in much demand due to the increasing consumer awareness for healthier and additive free food and, because of the increasing microbial resistance to various synthetic antimicrobials or preservatives. Antimicrobial compounds derived from animal, plant and microbial sources have potential for use in food preservation processes. Spices and herbs are well known to inhibit bacteria, yeast and mould; and traditionally found use in food preservation. Natural antimicrobials are reported to increase the shelf life of foods, maintaining their quality and safety. Identification and utilization of natural preservatives is important so as to reduce the hazards associated with synthetic ones.

Lichens are the symbiotic association of fungi (the mycobiont) with a photosynthetic partner (the photobiont), usually either a green algae or cyanobacteria (Bhattarai *et al.*, 2008). They occur in varying habitats throughout the world

dominating terrestrial ecosystems and are seen as epiphytes on tree branches in rainforests. Lichens synthesise more than 1000 secondary metabolites of which 80% are unique to lichens and are not reported yet from any other natural sources. Many species of lichens are used either as food or to flavour the foods.

Lichens are eaten by different cultures across the world and it is estimated that 50 percent of all the lichen species have antibiotic properties. According to Awasthi (2007), there are about 2450 species of lichen present in India. Western Ghats, one of the bio diversity hotspot in India contain around 950 lichen species belonging to 150 genera and 54 families, and the lichen belonging to the family *Parmeliaceae* dominate the Western Ghats with 137 species and 22 genera. Upreti *et al.* (2005) reported that *Parmotrema tinctorum* is used as a spice and flavouring agent for meat and vegetable preparations by ethnic groups in India and Nepal.

Parmotrema tinctorum (Nyl.) Hale, a member of the family Parmeliaceae is being collected in huge quantities from the forests of Wayanad district by the tribes and marketed through tribal societies to North India as well as exported to the Gulf countries for using in food products. It is reported to be used as a spice and aids in preservation of foods *etc*. Antioxidant and anti diabetic properties have been reported for this lichen species indicating its potential for use as nutraceutical. Despite the large scale collection of this lichen by the tribal people from Wayanad district, the documentation on this lichen as a food source is meager.

Parmotrema tinctorum (Nyl.) Hale, an edible lichen, is a good source of biologically active compounds and found to exhibit varied biological properties such as antibacterial and antioxidant activities. The present project is proposed to explore the feasibility of utilizing *Parmotrema tinctorum* as a flavouring agent and natural antimicrobial in processed products. A detailed investigation on the biochemical constituents, antimicrobial activity and toxicological effects is a pre requisite for

recommendation for its use as an antimicrobial in foods. In this background, present investigation entitled "Evaluation and utilisation of edible lichen *Parmotrema tinctorum* (Nyl.) Hale for food preservation" was undertaken with the following objectives:

1. To evaluate the biochemical constituents and proximate composition of edible lichen *Parmotrema tinctorum* (Nyl.) Hale
2. To evaluate the antimicrobial activity and feasibility of *Parmotrema tinctorum* for food preservation
3. To study the toxicological effect of the lichen

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Lichens are composite organisms consisting of a fungus (the mycobiont) and a photosynthetic partner (the photobiont, usually green algae or cyanobacteria) growing together in a symbiotic relationship. They are cosmopolitan in nature, and occur in all of the environments on the earth and also are abundant as epiphytes on leaves and branches in rainforests (Slack, 1988).

They have been used by many different cultures across the world and it is estimated that 50 percent of the all lichen species have antibiotic properties. Lichens have been used in medicine by the ancient Chinese and Egyptians (Brightman, 1960). They have been used in Traditional systems of medicine all over the world. Since they are natural antibiotics, their metabolites exert biological actions including antimycotic, antiviral, analgesic, and antipyretic effects (Vartia, 1973). Lichens have been used in the treatment of diseases like arthritis, kidney diseases, pharyngitis, leprosy, and worm infestation. The medicinal properties of lichens are attributed to the presence of secondary metabolites like usnic acid and atranorin (Malhotra *et al.*, 2008). Joulain and Tabacchi (2009), reported that the lichens, *Evernia prunastri* ('oakmoss'), and *Pseudevernia furfuracea*, ('treemoss'), were commercially utilized for production of fragrances.

In India, lichens have been a household item since ancient days. *Shipal* in Atharvaveda is the first record of the use of a lichen as medicine. Lichen powder is a major ingredient of the common condiments used in food dishes, known as *Meat masala, Garam masala etc*. The Western Himalayas and Western Ghats are the main areas for lichen collection (Upreti *et al.*, 2005)

According to Awasthi (2007), there are about 2450 species of lichens present in India. Western Ghats, one of the bio diversity hotspot in India contain around 950

lichen species belonging to 150 genera and 54 families, and the lichen belonging to the family *Parmeliaceae* dominate the Western Ghats with 137 species and 22 genera (Sudarshan and Ramachandra, 2010).

Parmotrema tinctorum

Parmotrema tinctorum belongs to the family Parmeliaceae and is used for flavouring food and for extracting dyes. According to Abo-Khatwa *et al.* (1997) *Parmelia tinctorum* (syn. *Parmotrema tinctorum*) is referred to as *Al-Sheba* in Arabic and used as a spice in food. Upreti *et al.* (2005) reported that *Parmotrema tinctorum* is used as a spice and flavouring agent for meat and vegetables by ethnic groups in India and Nepal.

Parmotrema tinctorum (Nyl.) Hale, an edible lichen, is a good source of biologically active compounds atranorin, lecanoric acid, salazinic acid and usnic acid. They exhibit effects such as antibacterial, antioxidant and anti-HIV activities (Sebastian *et al.*, 2014).

2.1. Collection and characterization of lichen *Parmotrema tinctorum*

Parmotrema tinctorum is found moist or shady places in the evergreen/subtropical forests between an altitude of 750-2300 m above MSL. The localities identified in Kerala are Silent valley, Munnar, Amarambalam, Punnamala, Nelliampathy, Karimala, Parambikulam, Siruvani, Kakki damsite, and Wayanad. (Kumar *et al.*, 2009).

2.1.1. Phytochemical screening of secondary metabolites

Phytochemicals are bioactive compounds found in plants, which function as a defense mechanism to protect against biotic and abiotic stress conditions. Phytochemicals are divided into primary and secondary constituents; based on their functions. Primary constituents include carbohydrates, amino acids, proteins whereas secondary constituents consist of phenols, alkaloids, terpenoids, and flavanoids (Krishnaiah *et al*., 2009).

Saponins are glycosides of triterpenes and sterols and are used as expectorant and emulsifying agent (Belewu *et al.,* 2009). Flavonoids protect biological systems against the harmful effects of oxidation on carbohydrates, proteins, lipids and nucleic acids. They possess properties, such as anti-inflammatory, antimicrobial, oestrogenic, anti-allergic, antioxidant, and cytotoxic antitumor activity (Saxena *et al*., 2013).

Lichens synthesize a variety of secondary metabolites, which are seen as crystals deposited on the surface of hiphes. They are poorly soluble in water and are usually isolated by organic solvents (Hale, 1983; Otzurk *et al*. 1999). Most lichen substances with antibiotic activity are phenolic compounds *e.g*. usnic acid. Lichens produce over 800 secondary metabolites consisting of many compounds *viz*. amino acid derivatives, depsides, depsidones, terpenoids, aromatic and aliphatic acids, steroids, xanthones *etc*. These compounds are present as amorphous form or as crystals within the thalli (Huneck, 1999).

Lichen substances are insoluble in water and hence extracted using organic solvents (Tiwari *et al*., 2011). Ramya and Thirunalasundari (2014) reported the presence of flavanoids, alkaloids, tannins, saponins, steroids and glycosides in different lichens *viz*. *Parmotrema austrosinensis*, *Parmelia andinum* and *Parmelia sulcata*, collected from Kodaikanal, India.

Rashmi and Rajkumar (2014) had conducted phytochemical screening of nine lichen species collected from Madikeri district, Karnataka which were identified as

Flavoparmelia caperata, Roccella montagnei, Teloschistes flavicans, Physcia aipolia, Parmotrema austrosinensis, *Parmotrema. grayanum*, *Parmotrema. tinctorum*, *Parmotrema reticulatum* and *Usnea subflorida* using petroleum ether, chloroform, ethyl acetate, acetone and methanol solvents. Major phytochemicals like proteins, carbohydrates, tannins and steroids were present in most of the lichen extracts tested They also observed that methanol dissolves most of the secondary metabolites of *Parmotrema tinctorum* due to it's high polarity.

2.1.2. Proximate composition of lichen Parmotrema tinctorum

2.1.2.1 Moisture content

Lichens lack stomata, cuticle and any water storage system, leading to loss of water vapour readily from the whole surface of lichens. So they are able to survive long periods of dry conditions in a dormant stage (Lumbsch, 2008). The moisture content of *P. tinctorum* is reported to be 9.12% (Kambar *et al*., 2014) and 9.13% (Raj *et al*., 2014).

2.1.2.2 Crude fibre

Crude fibre is the residue remaining after defatting followed by digestion with dilute acid and alkali (Ranganna, 1997). Cellulose and lignin form major part (97%) of crude fibre. Insoluble fibre passes through the intestines undigested, absorbing water and organic toxins (Chavan and Patil, 2015). Kambar et al. (2014) observed a fibre content of 16.36 % in *Parmotrema tinctorum*.

2.1.2.3. Crude fat

Kambar *et al*. (2014) reported a fat content of 1.3% in *Parmotrema tinctorum* and according to Raj *et al*. (2014), it contained a fat content of 1.8%.

2.1.2.4. Total Protein

Lal and Rao (1956) reported high protein content in *Parmotrema tinctorum* (13.8%). According to Behadur *et al*. (2015), certain lichen species recorded high crude protein content and thus have good food value *viz*. *Dermatocarpon moulnsii* (20%), *Lobaria isidiosa* (20%), *Roccella montagnei* (14%) and *Parmotrema tinctorum* (14%). Kambar *et al*. (2014) also reported a higher protein content of 11.3 % in *Parmotrema tinctorum*.

2.1.2.5. Total ash

The ash content represents the mineral content of organic samples. A high ash value suggests the high percentage of mineral matter in the sample (Ooi *et al*., 2012).

Lal and Rao (1956) also observed a high ash value (12.6%) for *Parmotrema tinctorum*. Another edible lichen *Ramalina conduplicans* also recorded a significant ash content of 10.0% (Vinayaka *et al*., 2009). Raj *et al*. (2014) reported an ash content of 11.65 % for *Parmotrema tinctorum*.

2.1.2.6. Total carbohydrates

Lal and Rao (1956) observed the carbohydrate content of *Parmotrema tinctorum* to be 25.0 g/100 g. It is the major component of lichen species *viz*. *Parmotrema tinctorum* (72.13%) *Ramalina conduplicans* (79.80%), *Ramalina hossei* (59.9%) and *Parmotrema pseudotinctorum* (53.2%) as reported by Kambar *et al*.

(2014). Raj *et al.* (2014) also reported a similar value for carbohydrate (32.35%) in *Parmotrema tinctorum*.

2.1.2.7. Total phenols

Phenols are the largest group of phytochemicals, widespread in plant kingdom; more than 4000 phenolic compounds had been recognised which vary from <1 mg/kg to 3000 mg/kg in plants (King and Young, 1999). Most lichen substances with antibiotic activity are phenolic compounds (Manojlovic *et al.*, 2010). It has been reported that the antioxidant activity is correlated to the phenol content in the lichens (Stanly *et al.*, 2011; Kosanic and Rankovic, 2011).

According to Proestos *et al.* (2006), plant poly phenols are considered to be antimicrobial agents, and hence proposed as potential food natural preservatives. Lichens synthesise a large number of bio active compounds called lichen substances (mostly phenols). They constitute more than 30% of dry mass of the thallus (Prashith *et al.*, 2013).

2.1.2.8. Total free amino acids

The taste of edible mushrooms was primarily due to the presence of several small, water-soluble substances including free 5'-nucleotides, free amino acids, and soluble carbohydrates. Fresh mushrooms contained high amounts of alanine, aspartic acid, glutamic acid, and phenylalanine. Aspartic and glutamic acids are monosodium glutamate-like components, which give the most typical mushroom taste (Tseng and Mau, 1999).

Mau *et al*. (2001) evaluated the commercially available mushrooms in Taiwan *viz. Dictyophora indusiata, Grifola frondosa, Hericium erinaceus* and *Tricholoma giganteum* for their non volatile taste components. Total free amino acid contents ranged from 7.41 to 12. 3 mg/g.

2.1.2.9. Ascorbic acid

Ascorbic acid is the water-soluble vitamin which is found in many biological systems and is the most abundant antioxidant in plants (Lee and Kader, 2000). Ascorbic acid is an effective antioxidant which can react both directly, by reaction with aqueous peroxyl radicals, and indirectly, by restoring the antioxidant properties of fat-soluble vitamin E (Bendich *et al*., 1986).

2.1.2.10. Mineral composition of lichen

Minerals are inorganic substances, present in all body fluids and tissues. Although they do not yield energy, their presence is required for the maintenance of essential life processes (Malhotra, 1998). These elements are classified generally into major and minor elements based on their daily requirement. The major elements are required in amounts greater than 100 mg/day and the minor elements are required in amounts less than 100 mg/day (Soetan *et al*., 2010).

Bones are made up mainly of phosphorus, calcium, and magnesium, and iron is a component of blood. Minerals like zinc, molybdenum, copper, manganese, and magnesium are either structural part or part of enzyme systems. Sodium and potassium are vital for water and acid base balance of cellular fluids (Gopalan *et al*.,

2002). Copper is involved in the iron absorption, neurotransmission and lipid metabolism and Zinc is a co-factor in many enzymes (Amaradivakara *et al*., 2015).

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) technique provides simultaneous estimation of a number of elements, hence, it is the most commonly used for elemental analysis and studies have been conducted to validate this technique for element analysis of large variety of samples. (Lachman *et al*., 2007; Naozuka *et al*., 2011; Kumaravel and Alagasundaram, 2014). The use of nitric acid for digestion of organic matrix is the most common approach used for trace element analysis of samples (Bressy *et al*., 2013).

Lal and Rao (1956) reported higher value for calcium (1780 mg/100g) and phosphorus (118mg/100g) content in *Parmotrema. tinctorum*. Other species of *Parmelia* also recorded significant calcium content; 3.1% was detected in *Parmelia sulcata* (Larry *et al*., 1988). Vivek *et al*. (2014) reported significant calcium content both in two lichens of *Parmotrema* genus *viz*. *P. grayanum* (3546.33 ppm) and *P. praesorediosum* (2965.35 ppm)

In the case of potassium, *Parmotrema grayanum* (2568 ppm) and *Parmotrema praesorediosum* (3805 ppm) were also good sources (Vivek *et al*., 2014). Kambar *et al*. (2014) observed high content of iron in *Parmotrema tinctorum* (8250.52 ppm) collected from Shivamogga district, Karnataka.

2.1.3. Evaluation of antioxidant activity

Antioxidants have considerable importance in human health since they are effective in protecting the body against damage by reactive oxygen species (ROS). In normal values, Reactive Oxygen Species (ROS) are required for cell function, but in

high concentrations they lead to oxidative stress and to the development of aging and a large number of diseases such as arthritis, carcinogenesis *etc*. As a part of normal cellular function, free radicals are constantly generated in all living cells. Free radicals originating from endogenous or exogenous sources cause structural damage to lipids, proteins and nucleic acid leading to degenerative diseases such as diabetes mellitus, cancer, atherosclerosis, ageing *etc*. (Chatterjee *et al*., 2007; Sangameswaran *et al*., 2009).

Antioxidants have relevant role in food quality by preventing oxidative deterioration mainly of lipid components and thus extending shelf life. Widely used synthetic antioxidants such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tertbutylhydroquinone (TBHQ) and propyl gallate (PG) are reported to have toxicity and carcinogenicity (Zhang *et al*., 2009). There is an increasing interest in natural antioxidants due to the safety concerns of synthetic antioxidants. In the search for novel antioxidants, natural products are widely explored (Lu *et al*., 2010).

Free radical scavenging action is one of the major mechanisms for antioxidation (Sini and Devi, 2004). Radical scavenging activity of the extracts was studied by evaluating their possibility to decolourise the stable DPPH radical. This method is based on the formation of non radical form DPPH-H in the presence of alcoholic DPPH solution and hydrogen donating antioxidant (AH) by the reaction DPPH + AH → DPPH-H + A (Anandjiwala *et al*., 2008).

The electron-donating ability of the different extracts and pure compounds were measured by the degree of bleaching of a purple coloured methanolic diphenyl picryl hydrazyl (DPPH). DPPH scavenging activity is denoted by IC_{50} value, which is

the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution (Cao *et al*., 2009).

Re *et al*. (1999) described the ABTS assay, applicable to both hydrophilic and lipophilic antioxidants, for screening of antioxidant activity, including carotenoids and flavonoids. The pre-formed radical monocation of 2,2 9-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•1) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants. The ABTS assay can determine antioxidant activity at different pH and can measure both lipophilic and hydrophilic antioxidants (Johnston *et al.,* 2006).

2.1.3.1. Evaluation of antioxidant activity of lichens

Phenolic compounds from the lichen *Parmotrema stuppeum* (Nyl.) Hale (Parmeliaceae) *viz*. atranorin, lecanoric acid, orsellinic acid, and methyl orsellinate, showed moderate antioxidant activity (Jayapraksha and Rao, 2000). Gulcin *et al*. (2002) evaluated the antioxidant activity of aqueous extract (50, 100, 250, and 500 µg) of lichen *Cetraria islandica* which revealed higher antioxidant activity than 500 µg of α-tocopherol.

In vitro antioxidant activities of methanol and water extracts of three lichen species, *Usnea longissima* , *Usnea florida,* and *Lobaria pulmonaria* were determined by Odabasoglu (2004). The methanol extracts of *Lobaria pulmonaria* and *Usnea longissima* showed potent antioxidant activities. *L. pulmonaria* also had the highest total phenolic content (87.9 mg/g lyophylisate).

Bhattarai *et al*. (2008) reported that there exists the need for developing antioxidants of natural origin, due to high carcinogenicity of synthetic antioxidants.

They observed that extracts of lichen species *Lecanora sp, Stereocaulon alpinum, Ramalina terebrata, and Caloplaca regalis* showed a high number of (50%) of antioxidant constituents. Microalgae and cyanobacteria forming the base of the food chain in aquatic ecosystems are promising natural sources of antioxidants (Guedes *et al.*, 2013).

Manojlovic *et al.* (2012) analysed the antioxidant capacity of three Parmeliaceae lichen *viz. Parmelia caperata, Parmelia saxatilis and Parmelia sulcata* by DPPH method and found that salazinic acid from all these lichens showed significant radical scavenging activity (IC_{50}-91.57 μg/ml). Kosanic *et al.*, (2012) reported that the acetone extract of *Parmelia saxatilis* showed the highest DPPH radical-scavenging activity (55.3%). The scavenging activity of *Parmelia caperata* was also high (46.37%), while *Parmelia sulcata* showed a slightly lower activity (38.79%).

According to Vinayaka *et al.* (2009) over 50 percent scavenging activity in all the concentrations of the methanolic extract of edible lichen *Ramalina conduplicans* had been reported using DPPH assay. Methanol and ethanol extract of *Parmotrema reticulatum* have exhibited DPPH radical scavenging activity (Sharma and Kalikoty, 2012). Raj *et al.* (2014) reported significant antioxidant activity of ethyl acetate extract of *Parmotrema tinctorum* indicated by the IC_{50} values against DPPH (396.83±2.98 μg/ml) and ABTS (151.34±1.79 μg/ml).

2.1.4. Thin Layer Chromatography

Thin layer chromatography is a chromatography technique used to identify the organic compounds present in a given substance, determine the purity of a substance and to monitor the progress of a reaction. The behavior of an individual compound in

TLC is characterized by a quantity known as Retention factor (Rf) and is expressed as a decimal fraction (Bele and Khale, 2010). The techniques such as TLC, HPLC, GC-MS are widely used for analyzing the chemical components from lichen species (Liu *et al.*, 2014).

Cuberson (1972) had described chromatographic data and *Rf* classes for 149 lichen products in three standard solvent systems. Jayaprakasha *et al.* (1998) suggested three solvent systems *viz.* hexane-ethyl acetate 80:20, chloroform-methanol 98:02 and benzene-ethyl acetate 95:05 for TLC of *Parmotrema tinctorum* acetone extract. The major compounds identified were atranorin, methyl orsellinate, orsellinic acid and lecanoric acid. The structures of these compounds were identified by Nuclear Magnetic Resonance spectrascopy. Thin layer chromatography profiling revealed the presence of atranorin and lecanoric acid in *Parmotrema pseudotinctorum* collected from the forests of Bhadra wildlife sanctuary, Karnataka (Vinayaka *et al.*, 2009).

Din *et al.* (2010) evaluated the chemical profile of ten lichen species from Peninsular Malaysia and found that *Parmotrema tinctorum* showed a retention factor of 22 for lecanoric acid and 78 for atranorin by TLC developed in solvent system toluene: acetic acid (70:30).

2.1.5. Gas Chromatography –Mass Spectroscopy

Stojanovic *et al.* (2011) observed that GC-MS gave good results in analysis of volatile constituents of selected Parmeliaceae lichens *viz. Hypogymnia physodes*, *Evernia prunastri* and *Parmelia sulcata*. According to them, orcinol and atraric acid were the major components of the *Evernia prunastri* acetone extract; and orcinol derivatives *viz.* atranol and methyl haematommate were present in smaller quantities. The major compounds of *Parmelia sulcata* extract were atraric acid, α – tocopherol, atranol and oleic acid.

GC-MS analysis of petroleum ether, chloroform and acetone extracts of *Parmelia perlata* revealed the presence of 49 compounds in each. Usnic acid, 5-methyl-1,3-benzenediol, 5-pentyl-1,3-benzenediol, atranorin, methoxyolivetol and Z-10-tetradecen-1-ol were identified as the major compounds (Pratibha and Sharma, 2016).

Chahra *et al.* (2016) reported that the essential oil recovery from the lichen species *Evernia prunastri* and *Ramalina farinacea* was very low (0.01% v/w) and they identified 32 compounds in oil of *Evernia prunastri* and 34 compounds in that of *Ramalina farinacea*. The major compounds in oil of *Evernia prunastri* were n-octadecanol, n-tetradecanol, heptadecane, eicosene-1, n-hexadecanol and tridecanol acetate, whereas *Ramalina farinacea* oil was characterized mainly by manool, n-octadecanol, eicosene-1, n-tetradecanol, manool oxide, α-pinene and abietal.

2.2. Evaluation of antimicrobial property of lichen extracts

2.2.1. Food spoilage organisms

Food-borne fungi, both yeasts and moulds, cause serious spoilage of stored food. It is estimated that 5 - 10% of the world's food production is lost due to fungal deterioration (Pitt and Hocking, 1999). *Penicillium* and *Aspergillus* species are reported as spoilage organisms from a wide range of food and feeds (Schnurer and Magnusson, 2005).

Species of *Aspergillus* fungi have long been known to be common contaminants of human food and animal feeds (Gourama and Bullerman, 1995). The most common species are *Aspergillus niger* and *Aspergillus flavus*, followed by *Aspergillus parasiticus*, *Aspergillus ochraceus*, *Aspergillus carbonarius*, and *Aspergillus alliaceus*. They can contaminate agricultural products at different stages including pre-harvest, harvest, processing and handling. *Aspergillus* species cause food spoilage manifested as pigmentation, discoloration, rotting, development of off-

odors. Most of them are encountered as storage moulds on plant products (Perrone *et al.*, 2007). *Aspergillus* spp. are widely distributed among the spoilage fruit fungi and also secrete several plant cell wall degrading enzymes; and *Aspergillus oryzae* is found to be present in orange fruits (Al –Hindi, 2011). *Aspergillus niger* is a filamentous ascomycete fungus associated with post harvest decay of different substrates, and known as black mould (Gautam *et al.*, 2011). *Aspergillus niger* found in coffee beans and grapes produced fumonisins and ochratoxins (Salas *et al.*, 2017).

Bacillus spp. are important bacterial contaminants within various food-processing operations. *Bacillus cereus* is an important post pasteurization spoilage bacterium in dairy industry due to the production of extracellular enzymes. *Bacillus subtilis* strains are important spoilage bacteria, resulting in significant economic losses in the baking industry (Lindsay *et al.*, 2006).

Bacillus species are ever present in the terrestrial ecosystems and they can be introduced in food during processing. The genus *Bacillus* includes species like *Bacillus cereus, Bacillus licheniformis* or *Bacillus subtilis* that may be present in fresh and pasteurised food products due to their ability to generate heat-resistant spores under adverse environmental conditions (Fernandez-No *et al.*, 2011).

Mun *et al.* (2013) opined that the pathogen *Staphylococcus aureus* is the causative agent for a large number of human infections, including pneumonia and meningitis. *Staphylococcus aureus* is notorious for developing rapid resistance to antibiotics, caused by horizontal transfer of resistance genes (Chambers and De Leo, 2009).

Praphailong and Fleet (1997) reported that yeasts are significant as spoilage micro organisms, especially in foods of low pH, high sugar content and high salt

content. Food-spoilage yeasts and moulds can grow in food stuffs with low P^H and cause deterioration of fruit and vegetable juices, dried fruits, soft drinks, pickled vegetables and dairy products (Krisch *et al.*, 2011).

Spoilage yeasts cause the deterioration of plant-derived, weak acids (sorbic, acetic acid) preserved products, such as fruit and vegetable juices and concentrates, fruit purees, soft drinks or pickled vegetable. *Zygosaccharomyces bailii* is well known for its resistance to sorbate and benzoate preservatives. *Candida cruzei, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Zygosaccharomyces bailii, Zygosaccharomyces rouxii* as major spoilage yeasts (Loureiro and Ferreira, 2003; Ramesh, 2011). *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are the most significant spoilage yeasts for fruit juices and soft drinks (Tserennadmid *et al.*, 2011).

2.2.2. Antimicrobial susceptibility testing

In order to screen the antimicrobial activity of natural products, two different qualitative methods are suggested *viz.* disc diffusion test and well diffusion test (Valgas *et al.*, 2007).

2.2.2.2.. Disc diffusion test

The disc diffusion method of antimicrobial susceptibility testing is the most practical method, where the antimicrobial contained in a reservoir (filter paper disc) was allowed to diffuse out into the medium and interact with the test organism in a freshly inoculated plate. The Kirby-Bauer method is generally used for antibiotics susceptibility testing, which makes use of antibiotics discs which are placed on the plates inoculated with the test organism. After incubation for the required time, the zone of inhibition is measured for the test antibiotics (Bartner *et al.*, 1994).

Disc diffusion methods are widely used to investigate the antibacterial activities of natural antimicrobial substances and plant extracts. In these assays, filter paper discs as reservoirs containing the solution of substances are made use of (Sangeetha *et al.*, 2009). According to Vineetha *et al.*, (2015) the Kirby-Bauer method makes use of antibiotics discs which are placed on the plates which are inoculated with the test organism. After incubation for the required time, the zone of inhibition is measured for the test antibiotics. Depending on the diameter of the zone of inhibition, the organism can be said as sensitive or resistant to the antibiotics. Advantages of disc-diffusion assay include low cost, simplicity, the ability to test a wide range of microorganisms and antimicrobial substances, and the ease to interpret the results (Balouiri *et al.*, 2016).

2.2.3. Antimicrobial activity of plants and plant products

Plant products have attracted the interest of scientists in search for newer source of antimicrobials which are safe to human health and also biodegradable (Verma and Dubey, 1999). The antimicrobial activity of various plant essential oils *viz.* coriander, clove, cinnamon, thyme, sage, rosemary, garlic and onion against food-related microorganisms as well as their applications in food system have been investigated (Gill *et al.*, 2002; Burt, 2004; Holley and Patel, 2005).

Essential oils from anise, angelica, basil, carrot, celery, cardamom, coriander, dill, fennel, oregano, parsley, and rosemary were evaluated by Elgayyar *et al.* (2000) against *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli* O:157:H7, *Yersinia enterocolitica, Pseudomonas aeruginosa, Lactobacillus plantarum, Aspergillus niger, Geotrichum,* and *Rhodotorula*. Oregano essential oil showed the greatest inhibition (zone 70 - 80 mm) to *E. coli* O:157:H7 and to the other bacteria and fungi, followed by coriander and basil oils. They suggested that due to the

powerful antimicrobial activity of oregano against gram-negative pathogens, oregano oil may be useful in some food formulations as an antimicrobial. Oil of basil completely inhibited growth of *S. aureus, Y. enterocolitica,* and the fungi imperfecti (*A. niger* and *Rhodotorula*).

Baydar *et al*. (2004) tested the efficacy of grape seed and bagasse extracts for their antibacterial effects by using the disc diffusion method against some food spoilage and pathogenic bacteria including *Aeromonas hydrophila, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus*. All the bacteria, except *Bacillus amyloliquefaciens* were inhibited by grape seed extracts at 20% concentration.

Antimicrobial study of hexane and methanol extracts of 18 ethnomedicinal plants from Palani hills of India against nine bacterial strains (*Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Ervinia sp*, *Proteus vulgaris*) indicated that methanol extract had significant antimicrobial activity against the tested microorganisms at three different concentrations of 1.25, 2.5 and 5 mg/disc (Duraipandiyan *et al*., 2006).

Broad spectrum inhibition against all gram-positive bacteria, yeast and moulds including *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Saccharomyces cerevisiae* and *Aspergillus fumigatus* by the ethyl acetate extract of kaffir lime (*Citrus hystrix*) peel was observed in the study conducted by Chanthaphon *et al*. (2008).

According to Shin *et al*. (2007) fatty acids function as the key ingredients of antimicrobial food additives due to their inhibitory action on undesirable microorganisms. They evaluated eicosapentaenoic acid (EPA), a long-chain polyunsaturated fatty acid for its antimicrobial action against the range of food borne and food spoilage pathogens, using agar disc diffusion assay. The EPA exhibited antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* ATCC 19166, *Staphylococcus aureus* ATCC 6538, *S. aureus* KCTC 1916 and *Pseudomonas aeruginosa* KCTC 2004.

The essential oil of *Thymus vulgaris* exhibited high broad spectrum fungitoxic spectrum against different food contaminating fungi *viz. Fusarium oxysporum, Cladosporium herbarum, Curvularia lunata, Aspergillus terreus, Aspergillus niger, Aspergillus fumigatus, Alternaria alternata* and *Botryodiploidia theobromae* as per the reports of Kumar *et al*. (2008).

The essential oil from flowers and ethanolic leaf extracts of *Lonicera japonica* revealed a remarkable antibacterial effect against *Listeria monocytogenes* ATCC 19116, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* SCK 11, *Staphylococcus aureus* ATCC 6538 and KCTC 1916), *Salmonella enteritidis* KCTC 12021, *Salmonella typhimurium* KCTC 2515, *Enterobacter aerogenes* KCTC 2190 and *Escherichia coli* ATCC 8739 (Rahman and Kang, 2009).

Wang *et al*. (2009) reported that the curcumin microcapsule was studied for its antibacterial and antifungal activities against some food borne pathogens and spoilage microorganisms such as *Escherichia coli*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Aspergillus niger*, *Penicllium notatum* and *Saccharomyces cerevisiae*. It exhibited broad spectrum

inhibitory effect against all organisms and its antibacterial activity was more prominent against Gram-positive bacteria than Gram-negative bacteria.

Tyagi and Mallik (2011) observed the antimicrobial potential of eucalyptus oil against different bacterial strains *(Escherichia coli, Pseudomonas aeruginosa, Pseudomonas fluorescens, Bacillus subtilis and Staphylococcus aureus*) and fungal strains (*Penicillium digitatum, Aspergillus flavus, Aspergillus niger, Mucor spp., Rhizopus nigricans* and *Fusarium oxysporum*) and yeasts (*Candida albicans* and *Saccharomyces cerevisiae*), by well diffusion method. Gram positive bacterial strains were more sensitive than Gram negative strains; and fungi were more inhibited than bacteria by the same concentration of essential oil.

The anti-yeast activity of lemon grass oil was evaluated by disc diffusion against several food spoiling yeasts (*Saccharomyces cerevisiae, Zygosaccharomyces bailii, Candida diversa, Pichia fermentans, Pichia kluyveri, Pichia anomala* and *Hansenula polymorpha*). Complete growth inhibition for *S. cerevisiae, Z. bailii, P. anomala* and *P. fermentans* was noticed at 30 μl lemon grass oil (Tyagi *et al.*, 2014).

The antibacterial effects of parsley, lovage, basil, and thyme essential oils alone and in combination against Gram-positive and Gram-negative bacteria associated with food products *viz. Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli,* and *Salmonella typhimurium* were shown by Semeniuc *et al*, (2017). They concluded that thyme essential oil had the maximum antibacterial potential.

2.2.4. Antimicrobial activity of lichens

According to Behera *et al.* (2012), the extracts of *Usnea ghattensis* showed inhibitory action against *Bacillus licheniformis, Bacillus megaterium, Bacillus subtilis* and *Staphylococcus aureus*, when tested by disc diffusion method. The size of zone of inhibition increased as the concentration of extract increased indicating that the effect was concentration dependent. Rankovic *et al.* (2010) studied the antimicrobial activities of methanol extracts of lichens *Cetraria pinastri, Cladonia digitata, Cladonia fimbriata, Fulgenasia fulgens, Ochrolechia parella* and *Parmelia crinita* by disc diffusion method and found that *Cetraria pinastri* and *Parmelia crinita* showed strongest antibacterial and antifungal action against the tested microorganisms.

In vitro antifungal activity of acetone, methanol and chloroform extracts lichen *Parmotrema tinctorum* against ten plant pathogenic fungi was studied by Tiwari *et al.* (2011) and found that the extracts were very effective against *Fusarium solani, Fusarium roseum* and *Fusarium oxysporum*.

Candan *et al.* (2007), found antimicrobial activity of acetone, chloroform, diethylether and methanol extracts of *Parmelia sulcata* against *Aeromonas hydrophila, Bacillus cereus, Bacillus subtilis, Listeria monocytogenes, Proteus vulgaris, Yersinia enterocolitica, Staphylococcus aureus, Candida albicans, Candida glabrata, Aspergillus niger, A. fumigatus* and *Penicillium notatum*. Hydro-ethanolic (50%) and ethanolic (90%) extracts of lichen *Parmelia perlata* were studied for screening their antibiotic activity against various bacterial strains by well diffusion method. It was found that *Parmelia perlata* had significant antibiotic activity towards *Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Corynebacterium xerosis, Proteus vulgaris*, *Kebsiella pneumoniae* and *Escherichia coli* (Abdur *et al.*, 2013).

A study was undertaken by Kumar *et al.* (2014) to analyse antioxidant and antimicrobial activity of ethanolic extract of lichen species *viz. Parmelia caperata, Parmelia parletum, Parmelia saxatilis, Everniastrum cirrhatum, Parmelia pereoridisum, Parmoterma mesotropum, Parmoterma reticulatum,* and *Parmelia squarrosa* collected from different locations of Uttarakhand. All the species of lichen were effective against the *Staphylococcus aureus*. The extract did not show activity against fungi.

Antibacterial efficacy of three *Parmotrema* species collected from Western Ghats of India was evaluated by Vivek *et al.* (2014) and observed a marked dose dependent inhibition of test bacteria by lichen extracts and they concluded that lichens of the genus *Parmotrema* are potential antimicrobial agents. The results showed methanol extract of *Parmotrema tinctorum* producing inhibition zones 1.8 ± 0.1 cm and 2.4 ± 0.2 cm against *Staphylococcus aureus* and *Staphylococcus mutans* respectively.

Anjali *et al.* (2015) conducted the *in vitro* testing of antimicrobial activity of 2- propanol extract of *Parmotrema tinctorum* (Despr. ex Nyl.) Hale, against ten bacterial and eight fungal pathogens by disc diffusion method. The highest zones of inhibition for bacteria were noted against *Escherichia coli* (14.66 mm), *Bacillus subtilis* (13.0 mm), *Salmonella abony* (12.33 mm) and *Corynebacterium rubrum* (11.33 mm). In the case of fungi, the highest zones of inhibition were noted against *Aspergillus flavus* (10.0 mm) followed by *Colletotrichum falcatum*, *Fusarium oxysporum* and *Penicillium chrysogenum* (7.33 mm), *Trichoderma lignorum* (7.0 mm) and *Fusarium moniliformae* (6.0 mm).

2.3. Evaluation of *Parmotrema tinctorum* for preservation of processed products

2.3.1. Food preservatives

Food spoilage is a complex process and significant quantity of foods is lost due to microbial spoilage despite modern day preservation techniques. Microbial spoilage is by far the most common cause of spoilage and may manifest itself as visible growth, as textural changes or as off-odours. During production and storage, each and every food product harbours its own specific microflora. This microflora is a function of raw material flora, processing, preservation and storage conditions (Gram *et al*., 2002).

Modern techniques in food preservation lead to a reduction in the levels of preservatives and the antimicrobials of animal, vegetable or microbial origin are being explored for substituting synthetic ones (Cerrutti and Alzamora, 1996). One of the strategies for inhibiting undesirable microorganisms is the use of synthetic or natural substances, and the demand for non-toxic, natural preservatives has been rising with increased awareness of ill-effects of synthetic chemicals present in foods (Wang *et al*., 2009).

To reduce health hazards and economic losses due to food borne microorganisms, the use of natural products as antibacterial compounds seem to be an interesting way to control the presence of pathogenic bacteria and to extend the shelf life of processed foods. These compounds could be added during the food preparation process. Among these products, essential oils from spices, medicinal plants and herbs have been shown to possess antimicrobial action and could serve as a source of antimicrobial agents against food pathogens (Oussalah *et al*. 2007).

Microbiological quality has become an increasingly important factor in determining the final quality of food. Fruits due to their high acidity and sugar content are more susceptible to mould and yeast attack than vegetables (Johar and Anand, 2012).

The use of synthetic preservatives as antimicrobial agents to control fungal spoilage of food has been in practice for many years. However, this has led to a number of environmental and health problems because of the carcinogenicity, teratogenicity, high acute toxicity, and long degradation periods of the synthetic preservatives (Tian *et al.*, 2011). A growing number of consumers refuse the use of synthetic chemicals and preservatives in their daily food. Benzoic acid can be transformed by decarboxylation into benzene, one of the most carcinogenic substances. Yeasts and moulds are able to degrade sorbic acid to 1,3-pentadiene, causing a kerosene-like off-odour (Krisch *et al.*, 2011).

The organic acids acetic, lactic, propionic, sorbic and benzoic acids are used as food preservatives (Brul and Coote, 1999; FSSAI, 2016). According to Stratford *et al.*, (2006), weak acids have been approved for use in foods, some of which are legally designated as preservatives. These include sorbic acid (2,4-hexadienoic acid), benzoic acid, propionic acid, and sulphites. These, together with acetic acid are commonly referred as weak-acid preservatives.

Food preservation is often achieved with the use of chemical preservatives, among which benzoic and sorbic acids, and their respective sodium, potassium and calcium salts, are widely used. They are generally used to inhibit yeast and mold growth, and also effective against a wide range of bacteria. These compounds are most active in foods of low pH value and essentially ineffective in foods having neutral pH values (Tfouni and Toledo, 2002).

According to Bjorkroth and Korkeala (1997) in acidic foods like pickles, tomato ketchup, the spoilage microbes are usually found to be non spore forming bacteria (lactic acid bacteria), or yeast (*Candida* spp., *Saccharomyces* spp.) or molds (*Byssochlamus fulva*).

2.3.2. Hazards caused by chemical preservatives

Benzoic acid and sodium benzoate are used mainly as antifungal agents (Davidson, 2001). Although benzoic acid is generally recognized as safe (GRAS), adverse effects such as asthma, urticaria, metabolic acidosis and convulsions were observed in sensitive persons. *In vitro* assays had shown it's weak clastogenic activity (Yilmaz *et al*., 2009). Benzoic acid has been found to be the second best antifungal agent which produced 75 to 100% mycelial growth inhibition of eight food-associated fungi (Pundir and Jain, 2010).

The ability of parabens to penetrate human skin intact without breakdown by esterases and to be absorbed systemically has been demonstrated through both by *in vitro* and *in vivo* studies. The oestrogen agonist and androgen antagonist activity properties of parabens together with their common metabolite (*p*-hydroxybenzoic acid) have been extensively reported. Studies have also proved that parabens act as inhibitors of sulfotransferase enzymes and to possess genotoxic activity (Darbre and Harvey, 2008).

Zengin *et al.* (2011) had investigated the genotoxic effects of sodium benzoate and potassium benzoate in cultured human peripheral lymphocytes by incubating the preservatives in different concentrations; and studying the level of nuclear DNA damage using the comet assay. A significant increase was observed in chromosomal

aberrations and in sister chromatid exchange, apart from the significant decrease in mitotic index, indicating the mutagenic and cytotoxic effects of sodium benzoate and potassium benzoate.

2.3.3. Evaluation of preservatives and natural antimicrobials

Hashmi *et al.* (2007) evaluated the effect of chemical preservatives *viz.* sodium benzoate, potassium metabisulphite and potassium sorbate individually and in combination on the microbial and sensory activity of the mango pulp stored in ambient condition (30-36^{0}C). The mean score for colour, flavour and overall acceptability significantly decreased while the microbial growth increased during the storage.

Many plants contain compounds which have some antibacterial properties, which are collectively called as 'green chemicals'. Spices and herbs are well known to inhibit bacteria, yeast and mold; and traditionally found use in food preservation. Natural antimicrobials derived from microorganisms are referred as biopreservatives (Smid and Gorris, 1999). Phytochemicals, such as essential oils, are naturally occurring antimicrobials found in many plants that have been shown to be effective in inhibiting the growth of microorganisms. Plant essential oils exhibit antimicrobial properties which make them possible alternatives to antibiotics (Calo and Ricke, 2015).

Gould (1996) reported that nisin and pediocin, are collectively called as bacteriocins, and nisin is the widely used bacteriocin which is allowed in 50 countries and mainly used as antispore agent mainly in dairy products. Nisin and pediocin are produced by lactic acid bacteria. Ukuku and Fett (2004) reported that treatment of whole and freshcut cantaloupe and honeydew melon with nisin-EDTA significantly reduced the natural microflora and extended the shelf-life. Bacteriocins, particularly

inhibit target cells by forming pores in the membrane, depleting the transmembrane potential resulting in the leakage of cellular materials (Cleveland *et al.*, 2001).

The change in the total bacterial counts was measured during nine days of storage at 4°C, when perilla leaf (*Perilla frutescens*) was added to, the tomato sauce, no microorganisms were detected until 0~1 days of storage. After five days of storage, microorganisms were detected in the group with 2% perilla leaf. Flavour and taste of the 2% perilla leaf treatment group were highly appreciated at 6.1 and 6.6, respectively (Kim *et al.*, 2013).

2.4. Toxicology studies

Toxicology is the important aspect of pharmacology that deals with the adverse effects of bio active substances prior to the use as drug or chemical on living organisms (Aneela *et al.*, 2011). Toxicity refers to the expression of being poisonous, indicating the state of adverse effects led by the interaction between toxicants and cells (Meenu Krishnan and Murugan, 2016).

The initial screening step in the assessment and evaluation of the toxic characteristics of all compounds is the determination of acute oral toxicity (Akhila *et al.*, 2007). Objective of acute toxicity studies is to estimate minimum dose causing lethality and to identify a dose causing major adverse effects. Acute toxicity testing involves administration of a single dose or multiple doses in a period not exceeding 24 hours, up to a limit of 2000 mg/kg (Robinson *et al.*, 2007).

2.4.1. Toxicology studies on medicinal plants

Toxicological testing of plant extracts and active principles are reported by many research workers. The results of the acute toxicity testing of methanolic leaf

extract of *Plectranthus amboinicus* Lour. in mice revealed no mortality up to the maximum dose level of 2000 mg/kg body wt of the extract administered orally, which is the single high dose for testing acute toxicity recommended by OECD guidelines 423 (Pillai *et al.*, 2011).

Sathya *et al.* (2012) evaluated the acute and sub acute toxicity of the ethanolic extract of *Acalypha indica* on male wistar rats at the dose levels 100-500 mg/kg body weight at a rate of 1.0 ml/rat/day for 30 days. Results elucidated that the rats when treated with different concentration of *Acalypha indica* from the range of 5mg/kg body weight to 2000 mg/kg body weight did not produce mortality, signs of toxicity, and behavioural changes

Wills and Asha (2012) investigated the acute toxicity and sub acute toxicity of aqueous, ethanol and n-hexane extracts of *Lygodium flexuosum*, a medicinal fern used as indigenous medicine. The administration of extracts up to a high dose of 5 g/kg body weight did not show mortality or any change in behavior of the experimental animals, male Wistar rats. Liju *et al.* (2013) showed the administration of turmeric essential oil at different doses such as 1–5 g/kg body weight did not produce any mortality. The body weight, clinical signs and food and water consumption did not differ as compared to control group.

No mortality or adverse symptoms were observed with the oral administration of ethanolic extract of *Kaempferia rotunda* rhizome to Wistar rats in acute toxicity study period for 14 days, with a limit test dose of 2000 mg/kg, which indicates that LD_{50} values of the extracts were greater than 2000 mg/kg orally (Sini *et al.*, 2014).

The aqueous extract of stevioside from *Stevia rebaudiana* when administered to female Wistar rats, in different concentrations from 300mg/kg body weight to 2000mg/kg body weight, no abnormality was detected in gross necropsy in the test animals and all the organs were found physiologically normal at a higher dosage of 2000mg/kg bodyweight revealing the non-toxic nature of stevioside in rats (Rajab *et al.*, 2016).

2.4.2. Toxicology studies on mushroom and lichens

No mortality and no significant changes in general behaviour of rats were observed to the maximum dose level of 5,000 mg/kg body weight of orally administered mushroom *Pleurotus ostreatus* for 72 hr treatment. The body weight and food and water consumption of rats were found to be unaffected by the treatment indicating the LD_{50} to be greater than 5,000 mg/kg body wt (Krishnamoorthy and Shankaran, 2014).

Shanmugam *et al.* (2017) reported that bioactive compounds obtained from *Parmotrema reticulatum*, *Parmotrema hababianum* and *Rocella montagnei* exhibited a strong anti-cancerous property which was confirmed through cancer cell-line studies and animal model experiments.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present investigation on the 'Evaluation and utilisation of edible lichen *Parmotrema tinctorum* (Nyl.) Hale for food preservation' was carried out at the Department of Post Harvest Technology, College of Horticulture, Vellanikkara, Thrissur during 2014-2017.

The whole programme was divided into four major experiments

3.1. Collection and characterization of lichen *Parmotrema tinctorum*

3.2. Evaluation of antimicrobial property of lichen extracts

3.3. Evaluation of *Parmotrema tinctorum* for preservation of processed products

3.4. Toxicology studies in *Parmotrema tinctorum*

The materials used and the methods followed for the studies are summarized below:

3.1. Collection and characterization of lichen *Parmotrema tinctorum*

3.1.1. Collection

The lichen *Parmotrema tinctorum* was collected during December 2015 from Chembra, Meppady, Moolankavu and Ambalavayal areas of South Wayanad Forest Division, Wayanad district, Kerala, India (Fig.1 and Appendix I). Authorized permit for collection of lichen samples has been obtained from the Department of Forest and Wildlife, Govt. of Kerala (WL-10-39996/2015 dt.19.10.2015).

3.1.1.1. Habitat

The habitat of the lichen *Parmotrema tinctorum* was recorded.

Fig. 1. Areas of lichen sample collection

3.1.1.2. Habit

The habit of the lichen *Parmotrema tinctorum* was recorded.

3.1.1.3. Morphological characters

The thallus characters of the lichen were observed under the microscope and recorded. Colour spot tests were employed for identification of lichen (Orange *et al.*, 2001). The following tests were done by spotting the chemicals on the medulla of the thallus and observed the reaction.

1) K - test

KOH (10 %) solution was spotted on the medulla of the thallus. Positive reaction is yellow or red colouration.

2) C- test

Sodium hypochlorite (2%) solution was spotted on the medulla. Positive reaction is pink or red or orange colouration.

3) KC –test

KOH (10%) solution was spotted on medulla, followed by sodium hypochlorite on the same fragment. Positive reaction is pink or red or violet colouration.

4) PD test

Steiner's solution obtained by dissolving 1 g of para phenylene diamine, 10 g of sodium sulphite and 0.5 ml of detergent in 100 ml of water, was used for spotting. Positive reaction is yellow or orange or red colouration.

3.1.1.4. Documentation on use and coverage of edible lichen

The use and coverage of edible lichen with respect to commerce are documented from Wayanad district through field visits and survey.

3.1.2 Phytochemical screening of secondary metabolites

The lichen extracts were prepared by cold maceration method using organic solvents *viz.* petroleum benzine, ethyl acetate, acetone and methanol. Five gram lichen powder was soaked in 25 ml of organic solvent and kept at room temperature for three days. This solution was filtered using Whatman No. 1 filter paper. The filtrate was screened for the following phytochemical constituents (Raaman, 2006; Sazada *et al.*, 2009).

3.1.2.1. Tests for carbohydrates

Fehling's test: Dissolved a small portion of extract in water and heated with Fehling's solution. Reddish brown colour indicated the presence of carbohydrates.

3.1.2.2. Tests for phenols

To one ml of the extract, added 2 ml of 5 per cent ferric chloride solution along the sides of the test tube. A dark green colour indicated the presence of phenolic compounds.

3.1.2.3. Test for flavonoids

Lead acetate test: To 5 ml of the extract solution added 1 ml of lead acetate solution. Flocculent white precipitate indicated the presence of flavonoids.

3.1.2.4. Test for tannins

Braemer's test: To a 2-3 ml of extract, 10% of alcoholic ferric chloride solution was

added. Dark blue or greenish grey colouration of the solution indicated the presence of tannins.

3.1.2.5. Test for alkaloids

Wagner's test: To 3 ml of the extract, 2 ml of Wagner's reagent was added. Reddish brown precipitate indicated the presence of alkaloids.

3.1.2.6. Test for saponins

Foam test: One ml of the extract was added with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. Occurrence of one cm layer of foam indicated the presence of saponins.

3.1.2.7. Test for aminoacids

Ninhydrin test: Dissolved a small quantity of the extract in few ml of water and added 1 ml of Ninhydrin reagent. Purple colour indicated the presence of aminoacids.

3.1.2.8. Test for fixed oils and fats

A small quantity of the extract was pressed between two filter papers. Oil stains on the filter papers indicated the presence of fixed oils.

3.1.2.9. Test for terpenoids

One ml of chloroform was added to 2 ml of the extract followed by a few drops of

concentrated sulphuric acid. A reddish brown precipitate produced indicated the presence of terpenoids.

3.1.2.10. Test for steroids

One ml of the extract was added with one ml of acetic acid, one ml of chloroform followed by 0.5 ml H_2SO_4. Change of test solution to blue green colour, denoted the presence of steroids.

3.1.2.11. Test for quinones

One ml of the extract was treated with 5 ml concentrated H_2SO_4. A yellow coloured precipitate denoted the presence of quinones.

3.1.2.12. Test for coumarins

Two ml of the extract was treated with 3 ml of 10% NaOH. Yellow colour of the solution indicates the presence of coumarins.

3.1.3. Proximate composition of lichen *Parmotrema tinctorum*

The lichen thalli were cleaned of the substratum and thoroughly washed under distilled water to remove all the debris, dried under shade and powdered using pestle and mortar. Estimation of moisture content, total carbohydrate, total protein, crude fat, ash content, crude fibre, total phenol, ascorbic acid and total free amino acid were conducted as per the procedures described by AOAC (2000).

3.1.3.1. Moisture content

A known quantity (10 g) of powdered lichen was taken in a pre-weighed dish (W_1) and dried in hot air oven at 100°C until weight recorded became constant, cooled and weighed (W_2). The moisture content of lichen was calculated using the formula. Moisture content = ([W_2-W_1] / weight of sample taken) x 100

3.1.3.2. Crude fibre

In this method, two gram of powdered lichen was extracted with petroleum benzine to remove fat. After extraction, the dry, fat-free lichen powder was boiled with 200 ml of 1.25% H_2SO_4 for 30 minutes. The mixture was filtered using linen cloth and the residue was washed with boiling water until washings were no longer acidic. The residue was further boiled with 200 ml of 1.25% NaOH for 30 minutes. The content was filtered again through linen cloth, washed with 25 ml of boiling 1.25% H_2SO_4, and then with three 50 ml portions of water and finally with 25 ml alcohol. The residue was transferred to a pre-weighed silica crucible (W_1), dried for two hours at 130^{0}C in hot air oven. The crucible was cooled in a desiccator and weighed (W_2). It was heated in muffle furnace at 600°C for 30 minutes and the crucible was again cooled and weighed (W_3). Crude fibre content was calculated using the formula:

Crude fibre % =([W_2 -W_1]-[W_3 -W_1]) / weight of sample x100

3.1.3.3. Crude fat

Five gram dry, powdered lichen was extracted using petroleum benzine (boiling point 40-60°C) in a Soxhlet extraction apparatus for six hours without interruption. The apparatus was allowed to cool and the extraction flask was dismantled. The solvent containing fat was transferred into another pre-weighed (W_1)

container; and was evaporated on water bath until no odour of petroleum benzine was left. After cooling in a desiccator, the container was again weighed (W_2). Heating and weighing was continued until constant weight was recorded. The crude fat (%) in the sample was calculated using the formula:

Crude fat (%) = $[W_2-W_1]$ / Weight of sample taken) ×100

3.1.3.4. Total Protein

The total protein content of lichen was estimated using the micro Kjeldahl method. Two gram of oven-dried lichen powder in a Kjeldahl flask was added with 30 ml of concentrated H_2SO_4, 10 g potassium sulphate and 1 g copper sulphate. The mixture was heated gently first and then strongly. The colourless solution was heated for another hour, cooled and diluted with distilled water to 100 ml in a volumetric flask. Ten ml of aliquot of the digest was poured into the distillation apparatus followed by the addition of 15 ml of 40% NaOH which releases ammonia from the sample. A colour change from pink to green was noticed as the ammonia was being trapped into 2 % boric acid solution containing mixed indicator. The boric acid-ammonia solution obtained was titrated against 0.1N HCl. The % of nitrogen was calculated by using the formula:

% Nitrogen = (T_1-T_0) x N x 0.0014 x D x 100 / Weight of the sample x V;

T_1 = sample titration reading

T_0 = blank titration reading

N = normality of HCl

D = dilution of digested sample

V = volume of sample taken for distillation

0.0014 = milliequivalent weight of nitrogen

% total protein was calculated using the formula:

% total protein = % nitrogen X 6.25* where * is the correction factor.

3.1.3.5. Total ash

Ten gram of lichen powder was placed in a pre-weighed silica crucible (W_1). The crucible was heated over a low flame till the complete charring of lichen sample. It was followed by heating at 550-600°C in a muffle furnace for about 2-3 hours. The crucible was cooled in a desiccator and weighed (W_2). The crucible was heated again in the furnace for half an hour, cooled and weighed in order to ensure complete ashing. This was repeated consequently till the weight of ash becomes constant (ash became grayish white). Total ash content was calculated using the formula:
Total ash content = $[W_2-W_1]$ / weight of sample) x 100.

3.1.3.6. Total carbohydrates

The carbohydrate content was estimated by anthrone method. Fifty milligram lichen powder was hydrolysed by 2.5 N HCl over boiling water bath for three hours. The mixture was cooled and neutralized with Na_2CO_3 granules, then made upto 100 ml volume and filtered. This filtrate was pipetted into test tubes and made up the solution to one millilitre with distilled water. To the tubes, 4 ml anthrone reagent was added and heated in boiling water bath for eight minutes. The contents were cooled rapidly and the absorbance of green complex was measured at 630 nm in UV-visible spectrophotometer (UV- 1800 Shimadzu, Japan). Concentration of carbohydrates in the sample was found out by preparing a standard curve using different concentrations of glucose and expressed as g carbohydrate/100 g material.
Amount of carbohydrates present in 100 mg sample= mg of glucose/volme of test sample x100

3.1.3.7. Total phenols

Hundred milligram of the sample was ground in a pestle and mortar with 10 times volume of 80% ethanol and the mixture was centrifuged at 10000 rpm for 20 min. The residue was extracted again with five times the volume of 80% ethanol and centrifuged again. The supernatants were collected and evaporated to dryness. The residue was dissolved in a known volume of distilled water. To the different volumes of aliquots (0.2-2.0 ml) taken in test tubes, distilled water was added to make up the volume to 3 ml, and added 0.5 ml of Folin – Ciocalteau reagent. After 3 minutes, 2 ml of 20% Na_2CO_3 solution was added to each tube and mixed thoroughly. The tubes were placed in boiling water for exactly one minute, cooled and measured the absorbance of mixture at 650 nm against a reagent blank. Concentration of phenols in the lichen sample was found out by preparing a standard curve using different concentrations of catechol and expressed as mg phenols/100 g sample.

3.1.3.8. Total free amino acids

Hundred milligram of the sample was ground in a pestle and mortar with acid washed sand, followed by addition of 80% ethanol. The mixture was filtered centrifuged; the supernatant was collected and concentrated by evaporation. To different volumes of aliquot (0.2-1.0 ml), one ml of ninhydrin solution was added, made up to 2ml with distilled water and boiled for 20 minutes. To the mixture, added 5 ml of the diluent and mixed well. After 15 minutes, a dark bluish purple product was formed which is colourimetrically measured at 570 nm. Concentration of the total free amino acids was determined from the standard curve using different concentrations of leucine and expressed as mg total free amino acids/g sample.

3.1.3.9. Ascorbic acid

Five gram of lichen powder was taken and extracted with four per cent oxalic acid. Ascorbic acid was estimated by using standard indicator dye 2,6-dichlorophenol indophenol and expressed as mg /100g sample.

3.1.3.10. Mineral composition of lichen

The lichen thalli were cleaned of the substratum and thoroughly washed under distilled water to remove all the debris, dried under shade and powdered. Powdered lichen thalli (0.5 g) was digested using 8 ml of Con. HNO_3 (69%) on a microwave digestor (CEM-Mars 6). The digested sample was filtered and diluted to 50 ml with double distilled water. The filtrate was fed to flame photometer (ELICO Ltd, Hyderabad, India). for analyzing sodium and potassium content. Phosphorus content was determined by colourimetry. The filtrate was aspirated into Inductively Coupled Plasma Optical Emission Spectroscope ICP-OES (Optima 8000™ Perkin Elmer, Norwalk, USA) to estimate the remaining elements.

3.1.4. Antioxidant assays

3.1.4.1. DPPH method (Diphenyl picryl hydrazyl method)

The antioxidant activity of the lichen was estimated by the method suggested by Molyneux (2004) using DPPH (1,1-diphenyl-1-picryl hydrazyl). Methanolic solution containing DPPH radicals (0.2 mM) was added to various concentrations of the sample and shaken vigorously. The reaction mixture was kept for thirty minutes in dark at room temperature. The absorbance was measured at 517 nm against the test blanks after the incubation period. The percentage inhibition (scavenging activity) of DPPH free radical was calculated using the formula,

Scavenging Activity (%) = $[(A_0-A_1)/A_0] \times 100$, where A_0 is the absorbance of DPPH and A_1 is the absorbance of DPPH and extract/standard combination. The sample concentration providing 50 per cent inhibition (Inhibition $Concentration_{50} - IC_{50}$) was calculated from the graph of scavenging activity percentage against sample concentration. Ascorbic acid was used as the standard.

3.1.4.2. ABTS method (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid method)

ABTS assay was conducted according to Re *et al.* (1999). The ABTS radical cation (ABTS+•) was produced by mixing 7 mM stock solution of ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark for at least 16 h at room temperature before use. The absorbance of ABTS+• solution was adjusted to 0.7 ± 0.05 at 734 nm (UV-1700PC; Shimadzu, Japan). Absorbance was measured 7 min. after the initial mixing of different concentrations of the methanol, acetone, ethyl acetate extracts of *Parmotrema tinctorum* with one ml of ABTS+• solution at 734 nm. Trolox was used as the standard. The total antioxidant capacity was then expressed as percent inhibition (PI), according to the equation:

PI = $(A_{ABTS} - A_{sample})/A_{ABTS}) \times 100$; here A_{ABTS} denotes the initial absorbance of diluted ABTS, and A_{sample} denotes the absorbance of the sample. Inhibition concentration (IC_{50}) was calculated from the graph of scavenging activity percentage against sample concentration (in mg/ml) (Guedes *et al.*, 2013).

3.1.5 Thin Layer Chromatography

Ten grams of air dried lichen powder was extracted with organic solvents *viz.* hexane, methanol and acetone. The extract was filtered using Whatman filter paper

(No.1), evaporated and stored. The TLC was done using Merck silica gel 60 F_{254} TLC plates. Five micro litre of lichen extracts was spotted on the plate. The plate was developed in a chamber previously saturated with the solvent systems. Three solvent systems were selected according to procedure given by Jayaprakasha *et al*. (1998) *viz.*

1. Hexane –ethyl acetate [80:20]
2. Chloroform- methanol [98:02]
3. Benzene-ethyl acetate [95:05]

After developing, the plates were removed from the chamber and air dried. The spots on the developed TLC plates were detected under UV light. The R*f* values of the obtained spots were calculated. The probable group of compounds based on the colouration of the spots was found out.

3.1.6. Flavour profiling by Gas Chromatography –Mass Spectrometry

The flavour profiling of different extracts of *Parmotrema tinctorum* was carried out using Gas Chromatography- Mass Spectrometry (GC-MS). The GC-MS profiling was done with GC model GCMS-QP 2010S, equipped with the Rxi-5 Sil Ms capillary column (30 m X 0.25 mm ID X 0.25 μm thickness). Helium was used as the carrier gas and an injection volume of 1.0 μl was employed in the split mode (50:1) with a column flow rate of 1.00 ml/min. The column oven temp. was programmed from 100^0C (3 min.) to 300^0C at 5 min. Ion source temp. was 200^0C, and interface temp. was 280^0C.

The method of electron ionization was applied and the full scan mass spectra was collected with scan speed of 1000 within the scan range of 50 to 500 m/z. Various components of extracts were identified by different retention times detected by Mass spectrophotometer. The chromatogram was recorded by the software GC-

MS solutions attached to it. The components were identified by comparing with reference mass spectra in the existing software libraries *viz*. NIST 11 and WILEY 8.

3.2. Evaluation of antimicrobial property of lichen extracts

3.2.1. Extraction of lichen material

The *Parmotrema tinctorum* lichen thalli were washed to remove debris, air dried, pulverized to powder and stored in sterile glass bottles in refrigerator. Lichen powder (25 g) was added to 250 ml of different solvents *viz*. acetone, ethanol and chloroform and subjected to Soxhlet extraction. The extract was prepared by decanting, followed by filtration with Whatman No.1 filter paper to obtain a clear filtrate. The extracts were then evaporated to dryness and they were kept at 4^0C till use (Fig.2). The extracts were weighed and dissolved in dimethyl sulfoxide (DMSO) to get the required concentrations of about 5.0, 10, 20 and 40 mg/ml for antimicrobial screening. Whatman filter paper (No.1) was used to prepare discs of approximately 6 mm in diameter, which were placed in hot air oven for sterilization. The sterilized discs were loaded with 10 μl of lichen extracts.

3.2.2 Isolation and preparation of test organisms

One each of the major spoilage organisms identified among fungi, yeast and bacteria in spoilt processed product (pickle) was isolated and selected for antagonistic studies. Spoilage organisms were isolated from the spoilt product in the respective media *viz*. Rose Bengal Agar (RBA) for mould, Sabouraud's Dextrose Agar (SDA) for yeast and Nutrient Agar (NA) for bacteria (Appendix II). The fungi and yeast isolates were identified by National Centre for Fungal Taxonomy (NCFT), New Delhi. Bacterial cultures isolated were identified by Dept. of Microbiology, College of Horticulture, Vellanikkara. The yeast species *Zygosaccharomyces bailii* was

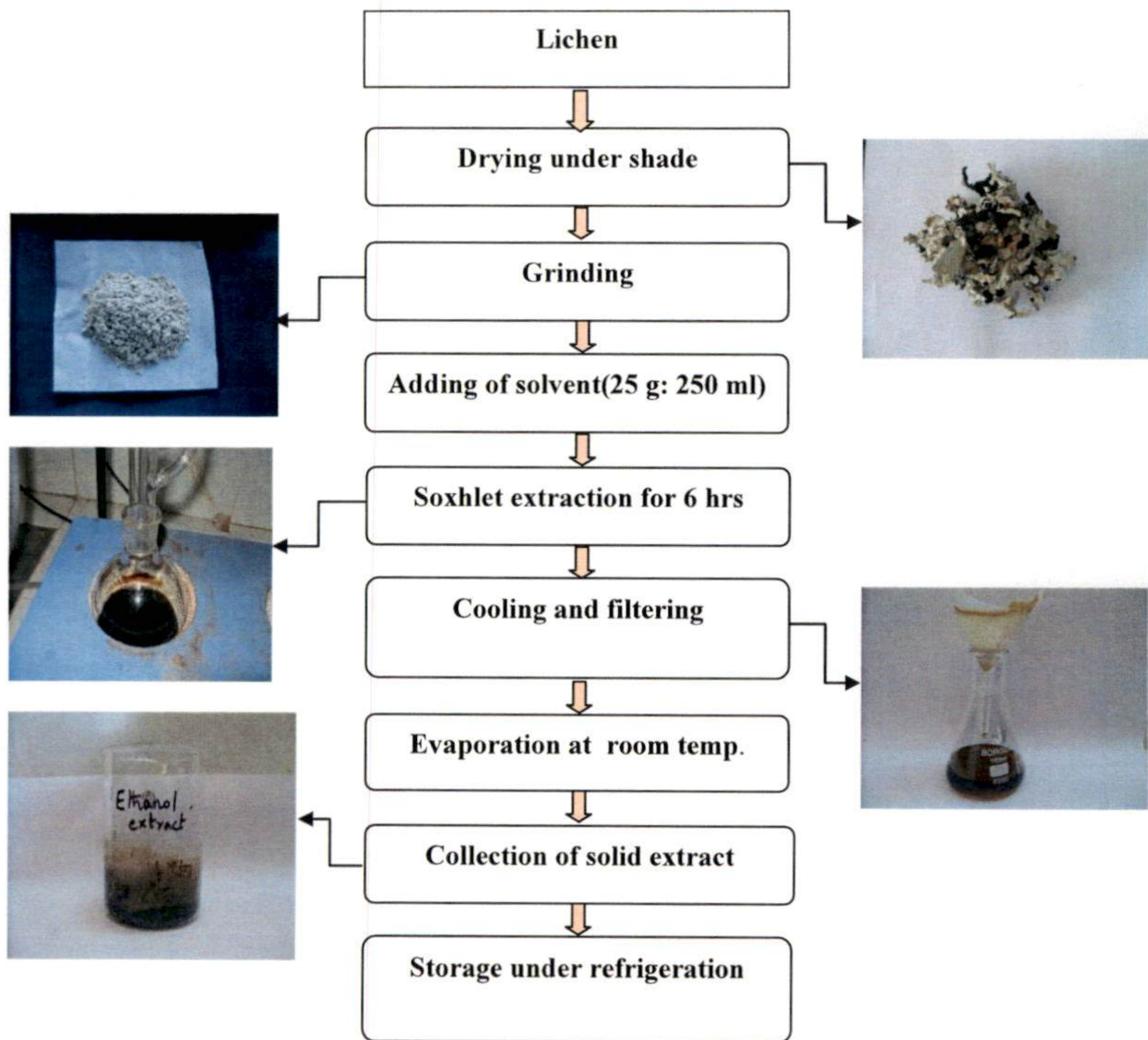


Fig. 2. Flowchart showing preparation of lichen extracts

procured from IMTECH (Institute of Microbial Technology), Chandigarh (code no. MTCC 257).

The microorganisms selected for antagonistic study were

1. Fungi - *Aspergillus niger*, *Aspergillus oryzae*

2. Yeast-*Saccharomyces cerevisiae, Zygosaccharomyces bailii* (MTCC 257)

3. Bacteria - *Bacillus subtilis, Staphylococcus aureus*

The inocula of fungi and bacteria were prepared in SDA and NA respectively and kept for incubation at 37^0C for 24 hrs.

3.2.3. Selection of reference antibiotic

To select the reference antibiotic against the isolated test micro organisms, antibiograms were performed using the available antibiotic discs purchased from Hi Media, Mumbai. The fungi *Aspergillus niger*, *Aspergillus oryzae*, *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* were screened in Sabouraud's Dextrose Agar (SDA) and bacterial strains *Bacillus subtilis* and *Staphylococcus aureus* were screened in Mueller Hinton Agar (MHA). The screening were conducted in triplicates, and the growth inhibition of test organisms by the antibiotic discs was measured as zone of inhibition in millimeters (mm).

3.2.4. Determination of antimicrobial activity

The antimicrobial activity of extracts against the test organisms were evaluated by disc diffusion method and by the well diffusion method (Baur *et al*, 1966). The tests against bacteria were performed on Nutrient Agar plates, and that against fungi were done on Sabouraud's Dextrose Agar plates. The antimicrobial

activity was determined by measuring the zone of inhibition produced by the disc and the experiments were done in triplicate.

3.2.4.1.Disc diffusion method

The media were prepared from a commercially dehydrated media (Hi Media, Mumbai) according to the manufacturer's instructions. Asceptically transfered about 25-30 ml of sterile medium into 100 mm diameter petri dishes, to a uniform depth of 4 mm. The test organisms were inoculated into their respective media and incubated for 24 hours at 37^0C. From this culture, inocula were prepared in sterile saline equivalent to 0.5 Mc Farland standard. The inocula were swabbed aseptically by sterile swab sticks on sterile media plates, by the standard method. The filter paper discs loaded with the lichen extracts at different concentrations were dispensed onto the surface of inoculated agar plates and pressed to ensure complete contact with the medium. The standard antibiotic was used as +ve control and DMSO was used as –ve control. The plates were incubated at 37^0C for 24 hours (for bacteria) and or 48 hours (for mould and yeast). The zone of inhibition was measured using a ruler. It was compared with zone formed around the positive control *ie* Itraconazole 10 mcg disc (Hi Media, Mumbai) for fungi and yeast and Ciprofloxacin 10 mcg disc (Hi Media, Mumbai) for bacteria.

3.2.4.2. Well diffusion method

The test organisms were inoculated into their respective media and incubated for 24 hours at 37^0C. From this culture, inocula were prepared in sterile saline equivalent to 0.5 Mc Farland standard. The inocula were swabbed aseptically on sterile media plates, by the standard method. Using a sterile cork borer, wells of 6 mm diameter were punched in the inoculated plates and 50 μl and 100 μl of lichen extract were transferred into the wells. The reference antibiotics *viz*. Itraconazle 10

mcg/Ciprofloxacin 10 mcg (Hi Media, Mumbai) discs were also placed on the microbial lawn. The plates were incubated at 37°C for 24 hours (for bacteria) and or 48 hours (for mould and yeast). The zones of inhibition formed around wells were measured using a ruler and were compared with that of the commercial antibiotic.

Design and Layout

Design : Completely Randomised Design

Treatments : 12

Extracts : 3

Antibiotic : 1

Test organisms: 3

Replications : 4

3.3. Evaluation of *Parmotrema tinctorum* for preservation of processed products.

The products (lime pickle, tomato sauce) were prepared by the standard procedure (Appendix III and IV). The evaluation of lichen species was done by two methods (i) addition in powder form at different levels. (2) Ethanol extract of the lichen after evaporation of solvent at different levels. Sodium benzoate was selected as the chemical preservative. The pickle was filled in sterile glass bottles, and sealed air tight with metal caps. Tomato sauce was filled in sterile glass bottles and sealed with crown corking machine and subjected to pasteurization at 85-90^{0}C, and one set of unpasteurized tomato sauce for all treatments was kept at ambient temperature. The products were stored at room temperature for a period of six months to evaluate the shelf life.

Treatments

1) Lime pickle

   T_1 – Control (No preservative added)

   T_2 – Sodium benzoate 250 ppm

   T_3 – *Parmotrema tinctorum* powder 0.1%

   T_4 – *Parmotrema tinctorum* powder 0.2%

   T_5 – *Parmotrema tinctorum* powder 0.3%

   T_6 – *Parmotrema tinctorum* extract 0.1%

   T_7 – *Parmotrema tinctorum* extract 0.2%

   T_8 – *Parmotrema tinctorum* extract 0.3%

2) Tomato sauce

   T_1 – Control (No preservative added)

   T_2 – Sodium benzoate 750 ppm

   T_3 – *Parmotrema tinctorum* powder 0.1%

   T_4 – *Parmotrema tinctorum* powder 0.2%

   T_5 – *Parmotrema tinctorum* powder 0.3%

   T_6 – *Parmotrema tinctorum* extract 0.025%

   T_7 – *Parmotrema tinctorum* extract 0.05%

   T_8 – *Parmotrema tinctorum* extract 0.1%

Design and Layout

Design : CRD

Treatments : 8

Replications : 3

3.3.1. Sensory evaluation

The prepared products were evaluated using a nine point hedonic scale to assess the appearance, colour, flavour, texture, odour, taste, after taste and overall acceptability of the products with help of 15 member semi trained panel having age from 20-50 (Appendix V). The products were stored for six months at ambient temperature and humidity (Appendix VI) and sensory evaluation was done at monthly intervals. For organoleptic test, Kendall's co-efficient of concordance was analysed and the mean rank scores were taken to differentiate the best product.

3.3.2. Enumeration of microbial load in the products

The estimation of microbial population present in the samples was carried out by serial dilution and plate count method as described by Agarwal and Hasija (1986). Ten gram sample was added to 90 ml sterile distilled water and shaken well to form a suspension. From this suspension, 1ml was transferred to a test tube containing 9 ml distilled water. This gave a dilution of 10^{-2}. Later 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were prepared from these serial dilutions.

The products were subjected to microbiological analysis initially and also at monthly intervals during their storage. The samples were analysed for the population of bacteria, fungi and yeast in standard plate count method in Nutrient Agar (NA), Rose Bengal Agar (RBA) and Sabouraud's Dextrose Agar (SDA) media, respectively and the results were expressed in CFU/g of sample.

3.3.2.1. Estimation of bacterial population

Bacterial population was estimated using 10^{-6} dilution on Nutrient Agar medium. One ml of 10^{-6} dilution was pipetted into a sterile petridish using a micropipette. About 20 ml of the melted and cooled Nutrient Agar (NA) media was poured into the petridish and it was swirled. After solidification, it was kept for incubation at room temperature. Three petridishes were kept as replicate for each example. The petri dishes were incubated at room temperature for 48 hours. The colonies developed were counted and expressed as CFU/g of sample.

3.3.2.2. Estimation of fungal population

Fungal population was estimated using 10^{-3} dilution on Rose Bengal Agar medium. One ml of 10^{-3} dilution was pipetted into a sterile petridish using a micropipette. About 20 ml of the melted and cooled Rose Bengal Agar (RBA) media was poured into the petridish and it was swirled. After solidification, it was kept for incubation at room temperature. Three petridishes were kept as replicate for each example. The petridishes were incubated at room temperature for 48 to 72 hours. The colonies developed were counted and expressed as CFU/g of sample.

3.3.2.3.Estimation of yeast population

Yeast population was estimated using 10^{-3} dilution on Sabouraud's Dextrose Agar medium. One ml of 10^{-3} dilution was pipetted into a sterile petridish using a micropipette. About 20 ml of the melted and cooled Sabouraud's Dextrose Agar (SDA) media was poured into the petridish and it was swirled. After solidification, it was kept for incubation at room temperature. Three petridishes were kept as replicate

for each example. The petridishes were incubated at room temperature for 48 to 72 hours. The colonies developed were counted and expressed as CFU/g of sample.

3.4. Toxicology studies in *Parmotrema tinctorum*

3.4.1. Acute oral toxicity study

The acute oral toxicity study of ethanol extract of *Parmotrema tinctorum* was performed. In the test system, animal species used was rats of Wistar albino strain. The study was conducted at M/s CARe KERALAM Ltd., KINFRA Park, Koratty, Thrissur.

Five female nulliparous and non pregnant rats of weight 235-240 g and of age 8 to 12 weeks were used for the study. The animals were acclimatized for a minimum period of seven days to laboratory conditions and were observed for clinical signs daily. Veterinary examination of all the animals was recorded on the 1st and 7th day of acclimatization. Animals were housed under standard laboratory conditions: air-conditioned with adequate fresh air supply, room temperature 21.0 to 24.0^{0}C, relative humidity 57-65%, with 12 hours light and 12 hours dark cycle. The temperature and relative humidity were recorded daily. Single animal was housed in a standard polysulfone cage (Size: L 300 x B 170 x H 140 mm) having facilities for holding pelleted food and drinking water in water bottle fitted with stainless steel sipper tube.

The animals were fed *ad libitum* throughout the acclimatization and study period. Pelleted lab rodent food (Manufactured by Sai Durga Feeds and Foods, Bangalore) was provided. Water was provided *ad libitum* throughout the acclimatization and study period. Deep borewell water passed through activated charcoal filter and exposed to ultra violet rays in Aquaguard water filter cum purifier (Manufactured by Eureka Forbes Ltd., Mumbai) was provided in plastic water bottles with stainless steel sipper tubes.

3.4.1.1. Study design

The study was carried out following the OECD Guidelines for testing of Chemicals (No. 420, Section 4: Health Effects) on conduct of "Acute Oral Toxicity – Fixed dose procedure". The test item was administered as a single dose of 1 ml/100 g body weight of all animals and the actual volume of administration was calculated based on the most recent body weight of the animals. The limit test procedure was followed as the test material is likely to be non toxic, *i.e* having toxicity above regulatory limit doses. A sighting study at a starting dose of 2000 mg/kg body weight was conducted, followed by dosing of a further four animals at this level served as a limit test.

In sighting study, dosing was sequential, allowing at least 24 hours before dosing the next animal. In the main study, a total of five animals were used for each dose level. The five animals were made up of one animal from the sighting study dosed at the 2000mg/kg body weight dose level together with an additional four animals. The animals were fasted overnight (water provided *ad libitum*) prior to dosing. The test item was administered orally as a single dose, using animal feeding needle to each rat. The dosage volume was 1 ml/100 g body weight for all animals. Food was offered 3-4 hours followed by dosing. The time interval between dosing at each level was determined by the onset, duration, and severity of toxic signs. All animals were observed individually at least once during the first 30 minutes after dosing, then periodically during the first 24 hours (special attention given during the first four hours) and thereafter, daily for a total period of 14 days.

Observations were made including changes in skin and fur, eyes and mucous membranes, tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma and also respiratory, somatomotor activity and behaviour pattern in the animals. The body weight of each rat was recorded prior to treatment on day one, then weekly and at the

terminal sacrifice time. Individual body weights and body weight changes were calculated.

On 14th day, all animals were sacrificed under euthanasia condition (overdose of thiopental sodium injection intraperitoneal) and subjected to necropsy and gross pathological examination.

RESULTS

4. RESULTS

The results of the investigation on the 'Evaluation and utilisation of edible lichen *Parmotrema tinctorum* (Nyl.) Hale for food preservation ' carried out in the Department of Post Harvest Technology, College of Horticulture, Vellanikkara during 2014-17 are presented in this chapter under the following sections *viz*.

4.1. Collection and characterization of lichen *Parmotrema tinctorum*

4.2. Evaluation of antimicrobial property of lichen extracts

4.3. Evaluation of *Parmotrema tinctorum* for preservation of processed products

4.4. Toxicology studies in *Parmotrema tinctorum*

4.1. Collection and characterization of lichen *Parmotrema tinctorum*

4.1.1. Collection of *Parmotrema tinctorum*

The lichen *Parmotrema tinctorum* (Plate 1) was collected during December 2015 from Chembra, Meppady hill areas of Wayanad district, Kerala, India by detailed survey of South Wayanad Forest Division, Wayanad, Kerala. Wayanad district is at the Southern Western Ghats and located in the northeast part of Kerala between North latitude $11^0 26'$ to $12^0 00'$ and East longitude $75^0 75'$ to $76^0 56'$ (Volga *et al.*, 2013). The areas of collection are shown in Fig. (1). Authorized permit for collection of lichen samples has been obtained from the Department of Forest and Wildlife, Govt. of Kerala (WL-10-39996/2015 dt.19.10.2015). The lichen samples were collected from bark of the trees and from the surface of the rocks (Plate 2).

4.1.1.1 Habitat

Parmotrema tinctorum prefers moist or shady places in evergreen forests between an altitude of 736 – 2100 m.

Plate 1. ***Parmotrema tinctorum***

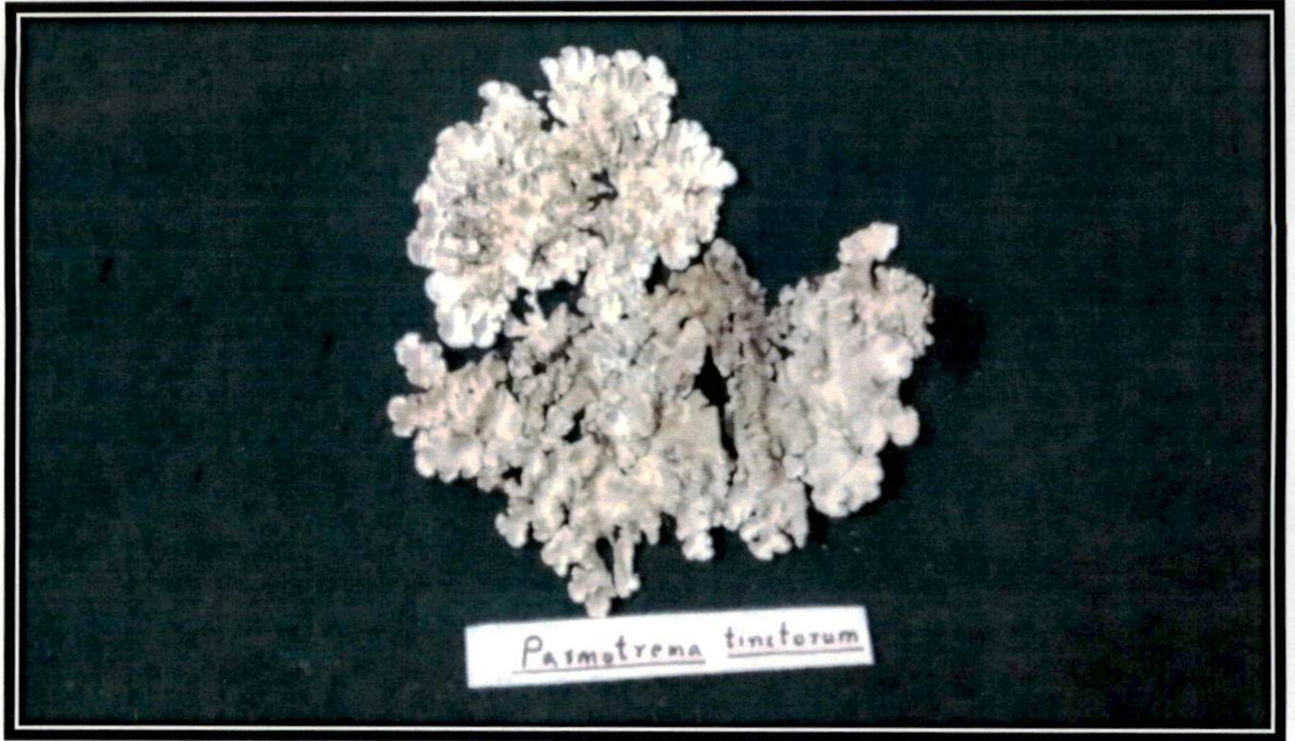
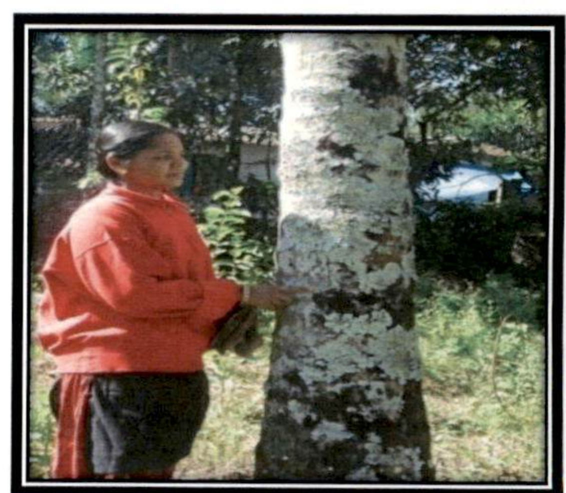
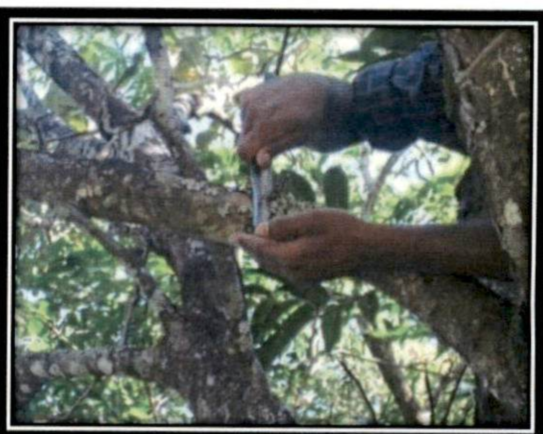


Plate 2. Lichen sample collection





4.1.1.2 Habit

The lichen *Parmotrema tinctorum* is mostly corticolous (growing on tree bark) and rarely saxicolous (growing on rock surfaces). In the present investigation, all the samples of *Parmotrema tinctorum* were collected from tree barks and no saxicolous specimens were collected.

4.1.1.3 Morphological characters

Morphological features and the colour spot tests prescribed by Orange *et al.* (2001) were employed for identification of lichen. The habit and morphology of the lichen *Parmotrema tinctorum* are shown in Plate 3. The lichen was identified according to the specific colour reactions, shown as in the Plate 4 and Table 1.

Table 1. Results of colour spot tests for *Parmotrema tinctorum*

Sl No	Spot test	Results
1	K	-
2	C	+(Red)
3	KC	+(Red)
4	Pd	-

The thalli found positive for the colour tests were sent to Jawaharlal Nehru Tropical Botanical Garden and Research Institute (JNTBGRI), Thiruvananthapuram for further confirmation of the identity of lichens. The lichen species was positively identified as *Parmotrema tinctorum*.

The morphological features of lichen *Parmotrema tinctorum* are as follows

Plate 3. Habit and morphology of *Parmotrema tinctorum*

a) Corticolous habit of lichen *P.tinctorum*

b) Upper surface of *P.tinctorum*

c) Lower surface of *P.tinctorum*

Plate 4. Colour spot tests –Key for identification of lichens

a) K test

b) C test

c) KC test

d) PD test

1. The lichen species is found to be heteromerous (upper surface and lower surface different)
2. Thallus is foliose, loosely adnate with irregular lobes
3. Upper surface is pale grey to green, shiny becoming dull towards the thallus centre
4. Lower surface is smooth, shiny black with a broad brown marginal zone
5. Medulla is white in colour
6. Rhizines are sparse, coarse, and short with 0.5-2.0 mm length.

4.1.1.4. Documentation on use and coverage of edible lichen

Lichen is one of the leading minor forest produce. Trading of lichen in Wayanad began since 2000. The details of co-operative societies and retail sellers involved in the trade of edible lichen in Wayanad were collected. Edible lichen species were collected by the tribal people, particularly Kattunaikan and Paniya tribes; which is approved by the Dept. of Forests and Wildlife. The picking season starts in October after the rains and it extends till March.

There are three co-operative societies which are engaged in lichen collection. The details of lichen trade by co-operative societies are presented in the Table 2. The societies are under the administrative control of Dept. of Co-operation, Kerala. The societies collect the lichen and store them in their godowns (Plate 5). The sale of lichen is effected through the Kerala State Federation of Scheduled Castes/Scheduled Tribes Development Co-operatives Limited (also called as SC/ST Federation). located at Kalpetta, Wayanad. Lichen is sold through the auctions conducted by the federation in the months of January, February and March, mainly to the sellers of Tamil Nadu, Karnataka, Maharashtra. Retail sellers of lichen in Kerala and Tamil

Plate 5.Lichen trading in Wayanad, Kerala

a) Lichen collected by tribes b) SulthanBathery Tribe Service Co-op. Soceity

c) Lichens stored in godowns of tribe society

d) Lichen bagged in polythene bags e) SC/ST federation, Kalpetta (auction centre)

Plate 6. Market samples of lichens

nadu were visited for collecting details on trade of lichen (Plate 6). The details of sale are presented in the Table 3.

4.1.2. Phytochemical screening of secondary metabolites

Phytochemical screening of lichen extracted with organic solvents *viz.* methanol, acetone, ethyl acetate and petroleum benzine was carried out (Plate 7) as per the procedure given by Raaman, 2006, and Sazada *et al.*, 2009. The results of preliminary phytochemical screening for secondary metabolites are presented in Table 4 and in Plate 8 and 9. Methanol extract contained maximum phytochemicals *viz.* carbohydrates, phenols, flavonoids, tannins, terpenoids, fixed oils and coumarins. In this study, extracts using medium polar solvents *viz.* acetone and ethyl acetate was found positive for carbohydrates, phenols, tannins, and terpenoids. Acetone extract also showed presence of fixed oils and quinones whereas ethyl acetate indicated the presence of flavonoids. Petroleum benzine extract did not show any phytochemicals except saponins. Presence of alkaloids and steroids was not detected in all the four extracts.

4.1.4. Proximate composition of lichen *Parmotrema tinctorum*

The proximate composition of *Parmotrema tinctorum* was evaluated and the results are shown in Table 5. The mean moisture content of *Parmotrema tinctorum* was found to be 8.09 %. Carbohydrate is the major component of lichen recording a value of 20.03g/100g. *Parmotrema tinctorum* also recorded high value for total ash (10.50 %), crude fibre (14.16 %) and total protein (15.70 %). Fat content was comparatively low (1.28 %) in this species. The content of ascorbic acid and total free amino acids in *Parmotrema tinctorum* were 4.66 mg/100g and 8.25 mg/g respectively. The total phenol content recorded was 322.66 mg/100g, indicating that *Parmotrema tinctorum* is rich in phenols.

Table 2. Trading of edible lichen in Wayanad district (2015-18)

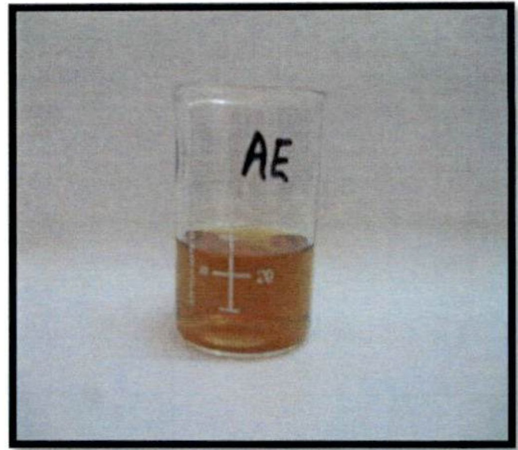
Sl. No.	Name of the society	Quantity Traded (kg)	Rate (Rs/kg)	Quantity Traded (kg)	Rate (Rs/kg)	Quantity Traded (kg)	Rate (Rs/kg)
		2015-16		2016-17		2017-18	
1	Sulthan Bathery Scheduled Tribe Service Co-operative Society Ltd.(Sulthan Bathery, Noolpuzha Panchayats)	3800	430	3105	410	3200	490
2	Pulpally Scheduled Tribe Service Co-operative Society Ltd.(Pulpally, Poothadi, Mullankolly Panchayats)	1458	325	1100	350	1232	397
3	Tirunelly Scheduled Tribe Service Co-operative Society Ltd.(Begur and Tolpetty Forest ranges)	1340	300	1230	320	1168	328

Table 3. Details of retail sellers of lichen

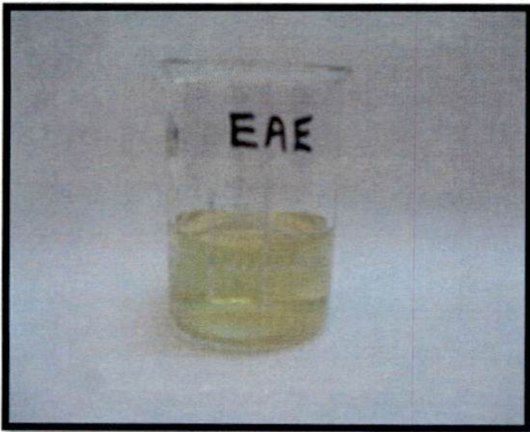
Sl.No.	Name of trader	Location	Sale price of lichen	Mode of sale
1	M/s Guru Spices	Munnar, Idukki district	Rs.40/50g	PP cover
2	M/s Krishna Spice supermarket	Munnar, Idukki district	Rs.30/50g	PP cover
3	M/s Spice 'N' Valley	Munnar, Idukki district	Rs.75/pack	Assorted pack of spices
4	M/s Vanila Shop	Munnar, Idukki district	Rs.150/pack	Assorted pack of spices
5	M/s Mani Traders	R.G. Street, Coimbatore, Tamil Nadu	Rs.40/100g	PP cover
6	M/sKannan Departmental Store	Gandhipuram Coimbatore, Tamil Nadu	Rs.52/ 100g	PP cover
8	M/sCoorg Spices Mall	Madikeri, Karnataka	Rs. 60/100g	PP cover

Plate 7. *P.tinctorum* extracts for phytochemical screening

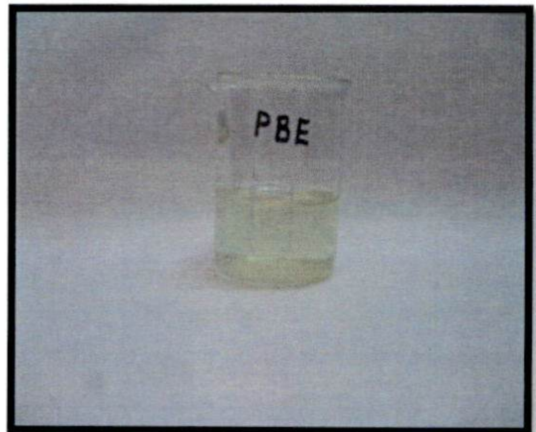
a) Methanol extract



b) Acetone extract



c) Ethyl acetate extract



d) Petroleum benzine extract

Table 4. Phytochemical screening of *Parmotrema tinctorum* extracts

Phytochemicals	Extracts			
	Methanol	Ethyl acetate	Acetone	Petroleum benzine
Carbohydrates	+	+	+	-
Phenols	+	+	+	-
Flavonoids	+	+	-	-
Tannins	+	+	+	-
Alkaloids	-	-	-	-
Saponins	-	-	-	+
Fixed oils and fats	+	-	+	-
Terpenoids	+	+	+	-
Steroids	-	-	-	-
Quinones	-	-	+	-
Coumarins	+	-	-	-

+ Presence of compound, - Absence of compound

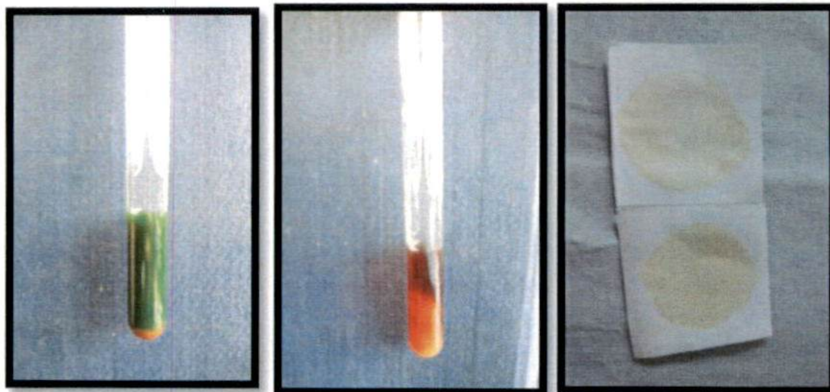
Table 5. Proximate composition of lichen *Parmotrema tinctorum*

Parameter	***Content**
Moisture (%)	8.09 ±0.64
Total carbohydrates (g/100g)	20.03±0.61
Total protein (%)	15.70±1.30
Crude fibre (%)	14.16±2.11
Total fat (%)	1.28±0.15
Total ash (%)	10.50 ±0.34
Total phenols (mg/100g)	322.66±16.10
Ascorbic acid(mg/100g)	4.66±0.230
Total free amino acids(mg/g)	8.25±0.025

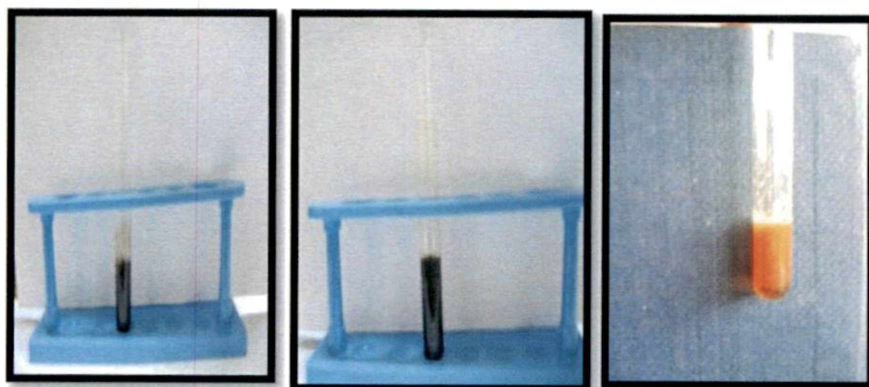
* value expressed as mean ±SD

Plate 8. Phytochemical screening of lichen extracts

a) **Methanol extract**

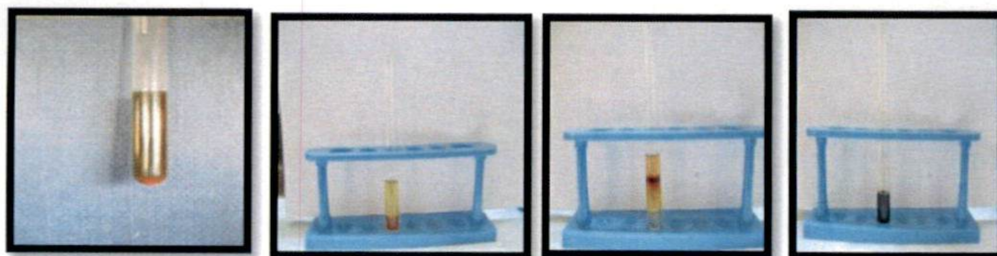


i) Carbohydrates ii) Flavonoids iii) Fats



iv) Phenols v) Tannins vi)) Coumarins

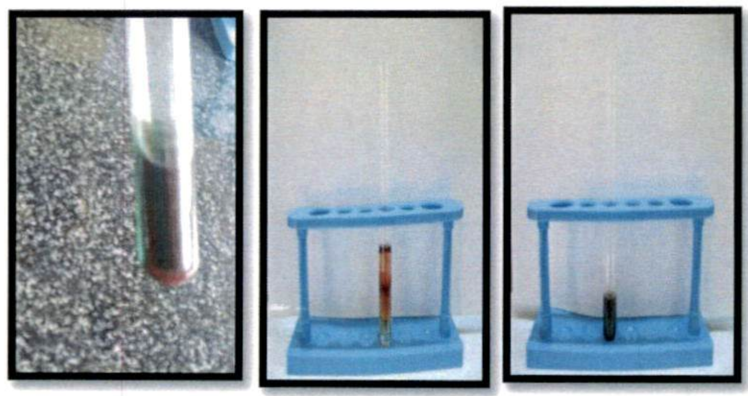
b)**Ethyl acetate extract**



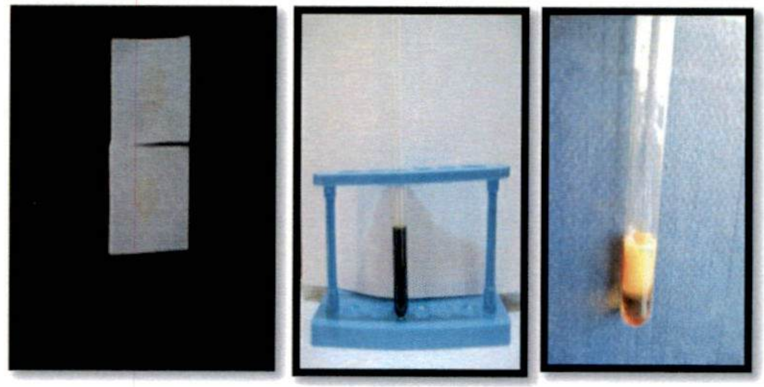
i) Carbohydrates ii) Flavonoids iii) Terpenoids iv)Tannins

Plate 9. Phytochemical screening of lichen extracts- Contd.

c)Acetone extract



i) Carbohydrates **ii) Terpenoids** **iii) Phenols**



iv)Fats **v) Tannins** **vi) Quinones**

d)Petroleum benzine extract

i) Saponins

4.1.5. Mineral composition of lichen

The mineral composition of *Parmotrema tinctorum* is presented in the Table 6. Content of eighteen elements were detected. In the case of major elements, calcium was observed at the highest level of 21970 mg/kg. The analysis has revealed high amount of magnesium (1781.66 mg/kg), potassium (2936.66 mg/kg) and iron (785.26 mg/kg) in *Parmotrema tinctorum*. The content of Cu and Zn detected were 8.46 mg/kg and 42.56 mg/kg respectively. The lichen *Parmotrema tinctorum* recorded very low values of heavy metals *viz*. arsenic (0.19 mg/100g), lead (0.36 mg/100g) and cadmium (0.19 mg/100g).

4.1.6. Antioxidant assays

In the present study, the antioxidant activity of the lichen extracts were measured by two assays *viz*. DPPH method and ABTS method.

4.1.6.1. DPPH method (1,1-Diphenyl 2-picryl hydrazyl method)

The methanol, acetone and ethyl acetate extract of *Parmotrema tinctorum* were analysed for DPPH radical scavenging activity according to the method described by Molyneux (2004), and the results of the screening as compared to ascorbic acid are shown in Table 7. Scavenging of DPPH free radicals by three extracts of *Parmotrema tinctorum* was concentration dependent *i.e.*, scavenging potential was found to increase with increase in the concentration of extracts. The sample concentration providing 50 per cent inhibition is described as IC_{50}, and lower value of IC_{50} indicates higher radical scavenging activity of the sample. Among the three extracts, methanol extract showed the maximum scavenging action against the DPPH free radicals. (IC_{50}1.47 mg/ml). The IC_{50} value of ethyl acetate extract and

Table 6. Mineral content of *Parmotrema tinctorum*

Minerals	***Content (mg/1000 g DW)**
Calcium (Ca)	21970.00±1496.22
Magnesium (Mg)	1781.66±556.83
Iron (Fe)	785.26±132.86
Copper (Cu)	8.46±1.48
Zinc (Zn)	42.56±12.08
Manganese (Mn)	44.00±12.95
Nickel (Ni)	1.86±0.55
Chromium (Cr)	2.63±0.68
Cobalt (Co)	0.53±0.15
Molybdenum (Mo)	3.30±0.69
Potassium (K)	2936.66±445.57
Sodium (Na)	1690.00±329.69
Phosphorus (P)	1000.33±2.51
Vanadium (V)	2.90±0.1
Aluminium (Al)	197.70±15.6
Arsenic (As)	0.19±0.01
Lead (Pb)	0.36±0.11
Cadmium (Cd)	0.19±0.01

* value expressed as mean ±SD

acetone extract were 2.34 mg/ml and 5.04 mg/ml respectively (Fig.3). Ascorbic acid, the standard antioxidant recorded an IC_{50} value of 0.004 mg/ml.

4.1.6.2. ABTS method (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid method)

The lichen extracts were found to scavenge the ABTS radicals in a dose dependent manner at concentration of 0.1–0.5 mg/ml. The positive control *viz.* Trolox at concentration of 0.1–0.5 mg/ml were also found to produce dose dependent inhibition of ABTS radical. The IC_{50} values of different solvent extracts and the positive control, Trolox were calculated in the present study and presented in Table 7. The methanol extract of *Parmotrema tinctorum* exhibited the highest ABTS radical scavenging capacity (IC_{50} 1.29 mg/ml). Ethyl acetate extract (EAE) recorded a similar IC_{50} value (1.27 mg/ml) which was significantly on par with that of methanol extract (Fig.4). Among the extracts, acetone extract recorded the least scavenging action (IC_{50}=3.16 mg/ml). The positive control Trolox recorded an IC_{50} value of 0.005 mg/ml.

Table 7. Scavenging effect of *P.tinctorum* extracts on DPPH and ABTS radicals

	Inhibition Concentration 50 (IC_{50}) (mg/ml)	
Lichen extracts/Standard	**DPPH assay**	**ABTS assay**
Acetone extract (AE)	5.04±0.77	3.16±0.06
Ethyl acetate extract (EAE)	2.34±0.47	1.27±0.52
Methanol extract (ME)	1.47±0.02	1.29±0.26
Ascorbic acid	0.004±0.10	-
Trolox	-	0.005±0.25
CD(0.05)	0.283	0.559

Value expressed as Mean± S.D of three experiments

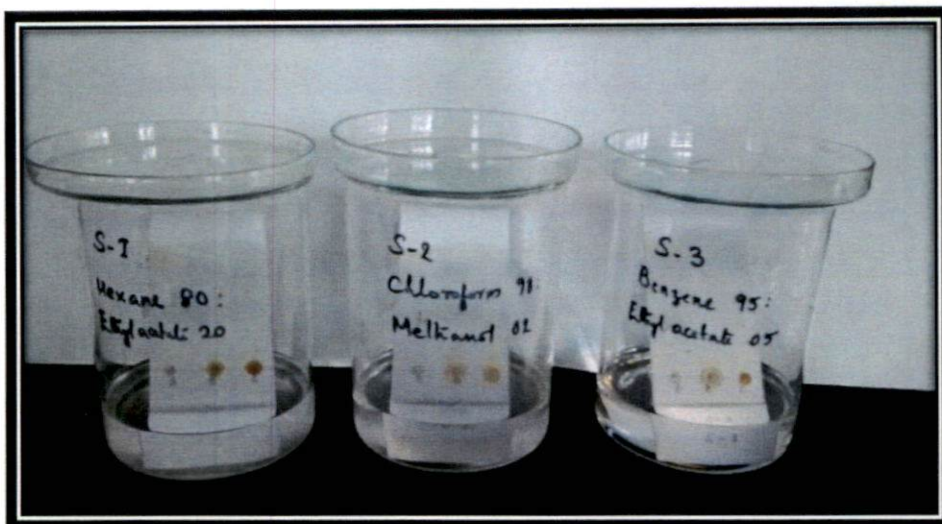
3.1.5 Thin Layer Chromatography

The Thin Layer Chromatography of three lichen extracts *viz.* hexane, methanol and acetone was done using Merck silica gel 60 F_{254} TLC plates. The plate was developed in three solvent systems *viz.* S1. hexane –ethyl acetate [80:20], S2. chloroform- methanol [98:02], and S3. benzene-ethyl acetate [95:05]. The spots developed on the TLC plates were detected under UV light. The R_f values of the spots were calculated.

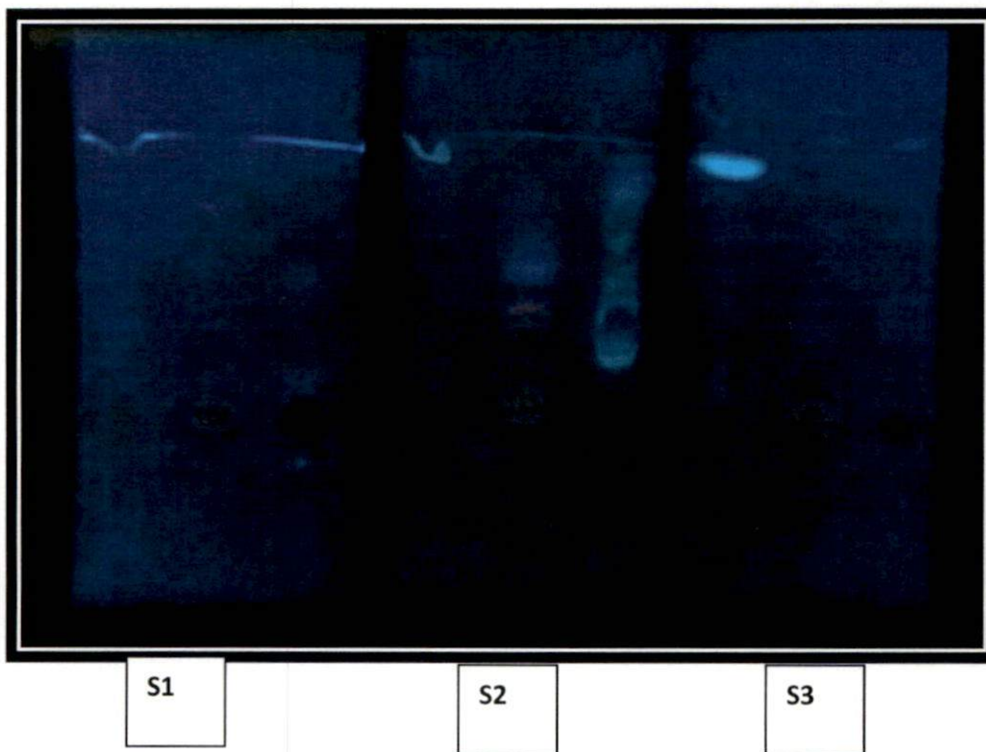
Hexane extract of *Parmotrema tinctorum* developed four spots in S1 (hexane-ethyl acetate) and in S3 (benzene-ethyl acetate), while no spots were found to be developed in S2. The Rf values of spots were 0.45, 0.58, 0.77 and 0.93 in S1, and 0.41, 0.53, 0.62 and 0.80 in S3.

All the three solvent systems S1, S2 and S3 were found to separate compounds from methanol extract of *Parmotrema tinctorum*, resulting in five spots in S1, four spots in S2 and four spots in S3 (Table 8 to 10). The Rf values were S1-0.27, 0.33, 0.54, 0.72, 0.43; S2- 0.31, 0.37, 0.52, 0.85; and S3- 0.16, 0.25, 0.60 and 1.04. When acetone extract was subjected to TLC in the three solvent systems, all the solvent systems produced spots, five in S1, four in S2 and five in S3. Retention factor (R_f) calculated for each spot under the three solvent systems are given in Table 8. The colouration of the spots and the possible group of compounds to which they belong are also shown. The TLC profiles of different extracts in different solvent systems are shown in Plate 10.

Plate 10. Thin layer chromatography of lichen extracts



a) Developing chromatogram in solvent systems



S1 **S2** **S3**

b) TLC profile of *Parmotrema tinctorum*

Lane 1 : hexane extract, Lane 2: methanol extract, Lane 3: acetone extract

TLC performed in different solvent systems and viewed under short wave UV

Table 8. Result of TLC of hexane extract of *Parmotrema tinctorum*

Solvent systems	No.of spots detected	R*f* values	Colouration of spot and possible group of compounds
Hexane –ethyl acetate [80:20]	4	0.45 0.58 0.77 0.93	Light yellow- Phenols Light blue- Phenols Green –Lipids Light blue- Phenols
Chloroform- methanol [98:02]	-	-	-
Benzene-ethyl acetate [95:05]	4	0.41 0.53 0.62 0.80	Light blue – Phenols No fluorescence detected No fluorescence detected Flourescent blue- Phenols

Table 9: Result of TLC of methanol extract of *Parmotrema tinctorum*

Solvent systems	No.of spots detected	R*f* values	Colouration of spot and possible group of compounds
Hexane –ethyl acetate [80:20]	5	0.27 0.33 0.54 0.72 0.93	Brown-Terpenoids Light orange-Phenols Light orange - Phenols Orange- Phenols Light orange –Phenols
Chloroform- methanol [98:02]	4	0.31 0.37 0.52 0.85	Brown- Terpenoids Orange- Phenols Light blue- Phenols Brown- Terpenoids
Benzene-ethyl acetate [95:05]	4	0.16 0.25 0.60 1.04	Orange- Phenols Brown- Terpenoids Light blue- Phenols Light orange- Phenols

Table 10. Result of TLC of acetone extract of *Parmotrema tinctorum*

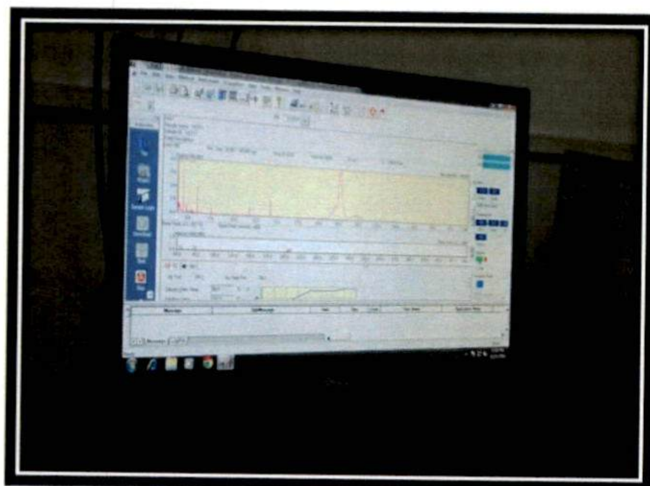
Solvent systems	No.of spots detected	R*f* values	Colouration of spot and possible group of compounds
Hexane–ethyl acetate [80:20]	5	0.41 0.50 0.66 0.75 0.87	Light yellow-Phenols Light orange-Phenols Brown- Terpenoids Light blue-Phenols Brown- Terpenoids
Chloroform-methanol [98:02]	4	0.20 0.37 0.62 1.00	Light blue- Phenols Brown- Terpenoids Light blue- Phenols Light green –Lipids
Benzene-ethyl acetate [95:05]	5	0.16 0.35 0.53 0.67 0.89	Brown- Terpenoids Brown- Terpenoids Brown- Terpenoids Brown- Terpenoids Light blue- Phenols

3.1.6. Flavour profiling by Gas Chromatography –Mass Spectrometry

The flavour profiling of different extracts of *Parmotrema tinctorum* was carried out (Plate 11) using Gas Chromatography-Mass Spectrometry (GC-MS). The GC-MS analysis was conducted using methanol, acetone, hexane, chloroform and ethanol extract of the lichen. The compounds detected, their retention time (RT), molecular formula, molecular weight, and nature of compounds are presented in Tables 11 to 15. The hexane extract showed maximum number (23) of compounds. Glyceryl trilaurate, a fatty acid ester was the major component (73.04%), followed by lauric acid, vinyl ester (4.90%) and decane (4.83%) (Fig.5). Methanol extract showed

Plate 11. GC-MS analysis of lichen extracts

a) GC- MS model -Shimadzu (QP 2010 S)



b) Developing gas chromatogram

five compounds *viz.* orcinol (37.75%), methyl orsellinate (49.35%), methyl haematommate (6.52%), atranorin (6.31%), hexadecanoic acid, 15- methyl-methyl ester (0.08%).(Fig.6) Chloroform extract contained seven compounds *viz.* phenol,2,4-bis(1,1-dimethylethyl)-, E-14-Hexadecenal, atraric acid, p-orsellinic acid, methyl ester, methyl haematommate, 9-eicosene,(E)-, and 2-nonadecene of which atraric acid recorded the maximum peak area (71.78%), followed by methyl haematommate (10.98%) which is an aromatic hydroxy ketone (Fig.7). Eight compounds were detected in the GC-MS profiling of ethanol extract *viz.* orcinol, chloroatranorin, ethyl orsellinate, methyl haematommate, campesterol, gamma-sitosterol, 14-.beta-H-Pregna and stigmasta-3,5-dien-7-one. Ethyl orsellinate was the predominant compound (47.24%), followed by gamma-sitosterol (29.10%) and by orcinol (15.51%). Ethanol extract also showed the presence of phytosterols, the major one being gamma-sitosterol. (Fig.8) In acetone extract, presence of 17 phytochemicals was detected. (Fig.9). *viz.* orcinol, 1,4-benzenediol,2,5-dimethyl-, orcinol mono acetate, 1-ethoxy-2-methoxy-4-methyl benzene, phenol,2,4-bis(1,1-dimethylethyl)-, orsellinaldehyde, methyl orsellinate, 1-butyl-2-propyl cyclopentane, nonadecane, atranorin, 2,6-octadiene, 4,5-dimethyl-, decane, 1-bromo-2-methyl-,3-heptadecanol, eicosane, benzopteridine, 2,4-diamino-6,7,8,9-tetrahydro-7-methyl-,hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, and myristic acid, 4-methoxyphenyl ester. The major compounds were orcinol (85.31%), atranorin (3.98%), and methyl orsellinate (2.20%).

3.2. Evaluation of antimicrobial property of lichen extracts

3.2.1. Isolation of test organisms

The spoilage microorganisms from the spoiled product (pickle), had been isolated and got identified (Plate 13). The fungal and yeast isolates were identified at the National Centre for Fungal Taxonomy (NCFT), New Delhi. The inoculation of spoiled product yielded two isolates of bacteria which were identified at the

Department of Agricultural Microbiology, College of Horticulture, KAU, Thrissur. For antagonistic studies, two each of fungi, yeast and bacteria were selected. Since the two yeast isolates identified were *Saccharomyces cerevisiae*, another yeast species *Zygosaccharomyces bailii* was procured from IMTECH, Chandigarh, with code no. MTCC 257. The microscopic view of bacteria and yeast were presented in Plate 14.

The microorganisms selected for antagonistic study were

Fungi - *Aspergillus niger*, *Aspergillus oryzae*

Yeast - *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii* (MTCC 257)

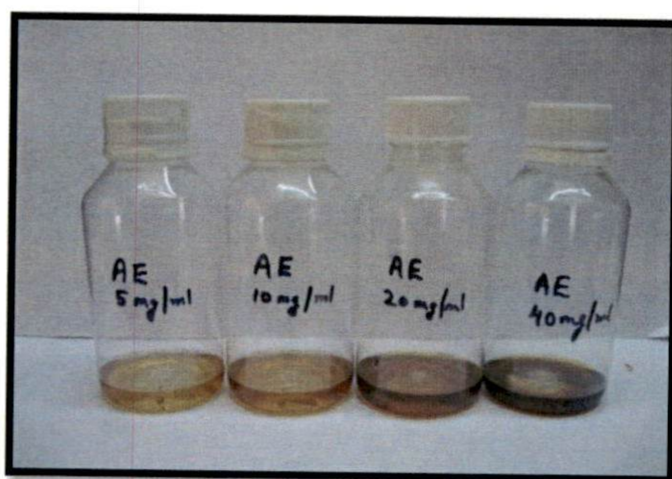
Bacteria - *Bacillus subtilis, Staphylococcus aureus*

The inocula of fungi and bacteria were prepared in SDA and NA respectively and kept for incubation at 37^0C for 24 hrs.

Plate 12. Lichen extracts for antimicrobial studies

a) **Ethanol extract**

b) **Chloroform extract**



c) Acetone extract

Table 11. Compounds detected in GC-MS analysis of hexane extract of *Parmotrema tinctorum*

Peak	Retention. time	Peak area %	Name of compound	Molecular formula	Molecular Weight	Nature of compound
1	4.100	2.09	1,1,2,3-tetramethylcyclohexane	$C_{10}H_{20}$	140.27	Cycloalkane
2	4.167	1.03	1,2,3-trimethylbenzene	C_9H_{12}	120.19	Aromatic hydrocarbon
3	4.250	0.19	s-methyl o-(2-methylcyclohexyl) dithiocarbonate	$C_9H_{16}OS_2$	204.34	Phenol
4	4.301	0.30	2-(3-hydroxy-2-nitro-cyclohexyl)-1-phenyl-ethanone	$C_8H_8O_2$	136.15	Aromatic ketone
5	4.529	4.83	Decane	$CH_3(CH_2)_8CH_3$	142.28	Alkane
6	4.592	0.11	1,1-dimethylcyclohexane	C_8H_{16}	112.21	Cycloalkane
7	4.849	0.95	4-methyldecane	$C_{11}H_{24}$	156.31	Alkane
8	4.930	0.60	1,3,5-trimethylbenzene/Mesitylene	C_9H_{12}	120.19	Aromatic benzene derivative
9	5.097	0.64	2-cyclohexylbutane	$C_{10}H_{20}$	140.27	Cycloalkane
10	5.335	0.56	3-benzoyl-4-benzyl-2-tert-butyl-4-methyl-1,3-oxazolidin-5-one	$C_{11}H_{16}O_2$	180.24	Phenol
11	5.419	0.60	2-methyl acetophenone	$CH_3C_6H_4COCH_3$	134.178	alkyl-phenylketone, flavouring component
12	5.478	0.33	2,6,10,14-tetramethylpentadecane	$C_{20}H_{42}$	282.55	Alkane

13	5.564	0.46	Stearaldehyde/Octadecanal	$C_{18}H_{36}O$	268.48	Long chain fatty aldehyde
14	5.842	0.26	p-cymene	$CH_3C_6H_4CH(CH_3)_2$	134.22	Monoterpene
15	6.023	3.11	Undecane	$CH_3(CH_2)_8CH_3$	156.31	Alkane
16	6.094	0.29	Spiro[3.6]deca-5,7-dien-1-one, 5,9,9-trimethyl	$C_{13}H_{18}O$	190.28	Alkene
17	6.400	0.28	4.beta.-p-mentha-2,5-dien-1.alpha.-yl-hydroperoxide	$C_{10}H_{16}O_2$	168.24	Ester
18	9.265	0.25	(z)-1-(methoxymethoxy)-2-methyl-2-pentene	$C_8H_{19}O_2$	112.16	Alkene
19	11.787	0.98	1-hydroxy-2, 4-ditert- butylbenzene	$C_{14}H_{22}O$	206.32	Phenol
20	14.218	3.73	Atranorin	$C_{19}H1_8O_8$	374.34	Poly phenol
21	22.701	73.04	Glyceryl trilaurate	$C_{39}H_{74}O_6$	639.01	Fatty acid triester
22	23.108	0.48	2-ethyl hexyl octanoate	$C_{16}H_{32}O_2$	256.43	Fatty acid ester
23	37.734	4.90	Lauric acid, vinyl ester	$C_{14}H_{26}O_2$	226.36	Fatty acid ester

Table 12. Compounds detected in GC-MS analysis of methanol extract of *Parmotrema tinctorum*

Peak	Retention time	Peak area %	Name of compound	Molecular formula	Molecular Weight	Nature of compound
1	7.105	37.75	Orcinol	$C_7H_8O_2$	124.13	Phenol
2	10.728	49.35	Methyl orsellinate	$C_9H_{10}O_4$	182.17	Ester
3	10.786	6.52	Methyl haematommate	$C_{10}H_{10}O_5$	210.18	Hydroxy benzoic acid derivative
4	11.239	6.31	Atranorin	$C_{19}H_{18}O_8$	374.34	Depside
5	13.799	0.08	Hexadecanoic acid, 15-methyl-, methyl ester	$C_{18}H_{36}O_2$	284.48	Monoacyl glyceride

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Table 13. Compounds detected in GC-MS analysis of chloroform extract of *Parmotrema tinctorum*

Peak	Retention. time	Peak area %	Name of compound	Molecular formula	Molecular Weight	Nature of compound
1	19.226	2.09	Phenol,2,4-bis(1,1-dimethylethyl)-	$C_{16}H_{25}NO$	247.38	Phenol
2	21.282	2.79	E-14-Hexadecenal	$C_{16}H_{30}O$	238.41	Aldehyde
3	23.905	71.78	Atraric acid	$C_{10}H_{12}O_4$	196.20	Depside
4	24.537	6.23	p-Orsellinic acid,methyl ester	$C_9H_{10}O_4$	182.17	Aromatic hydroxy ketone
5	24.651	10.98	Methyl haematommate	$C_{10}H_{10}O_5$	210.18	Hydroxy benzoic acid derivative
6	25.751	3.35	9-Eicosene,(E)-	$C_{20}H_4$	280.54	Alkene
7	30.084	2.77	2-Nonadecene	$C_{19}H_{38}$	266.51	Alkene

Table 14. Compounds detected in GC-MS analysis of ethanol extract of *Parmotrema tinctorum*

Peak	Retention time	Peak area %	Name of compound	Molecular formula	Molecular Weight	Nature of compound
1	9.949	15.51	Orcinol	$C_7H_8O_2$	124.13	Phenol
2	14.085	2.49	Chloratranorin	$C_{19}H_{17}ClO_8$	408.78	Phenol
3	14.479	47.24	Ethyl orsellinate	$C_9H_{10}O_4$	182.17	Phenol
4	14.541	3.36	Methyl haematommate	$C_{10}H_{10}O_5$	210.18	Hydroxy benzoic acid derivative
5	20.923	1.26	Campesterol	$C_{28}H_{48}O$	400.68	Phytosterol
6	25.149	29.10	Gamma-sitosterol	$C_{29}H_{50}O$	414.71	Phytosterol
7	25.936	0.94	14-.beta-H-Pregna	$C_{37}H_{42}O_6$	582.74	Phytosterol
8	27.918	0.09	Stigmasta-3,5-dien-7-one	$C_{29}H_{46}O$	410.68	Phytosterol

Table 15. Compounds detected in GC-MS analysis of acetone extract of *Parmotrema tinctorum*

Peak	Retention time	Peak area %	Name of compound	Molecular formula	Molecular Weight	Nature of compound
1	12.490	85.31	Orcinol	$C_7H_8O_2$	124.13	Phenol
2	12.825	1.28	1,4-Benzenediol,2,5-dimethyl-	$C_8H_{10}O_2$	138.16	Phenol
3	13.542	0.07	Orcinol, monoacetate	$C_9H_{10}O_3$	166.17	Phenol
4	13.720	0.48	1-ethoxy-2-methoxy-4-methyl benzene	$C_{10}H_{14}O_3$	166.21	Phenol
5	13.843	1.80	Phenol,2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206.32	Phenol
6	14.535	1.91	Orsellinaldehyde	$C_8H_8O_3$	152.14	Phenol
7	15.764	2.20	Methyl orsellinate	$C_9H_{10}O_4$	182.17	Ester
8	16.019	0.12	1-butyl-2-propyl cyclopentane	$C_{12}H_{24}$	168.32	Cycloalkane
9	16.113	0.05	Nonadecane	$C_{19}H_{38}$	266.51	Alkane
10	16.307	3.98	Atranorin	$C_{19}H1_{8}O_{8}$	374.34	Phenol
11	16.405	0.51	2,6-Octadiene, 4,5-dimethyl-	$C_{10}H_{18}$	138.25	Cycloalkene
12	16.600	0.09	Decane, 1-bromo-2-methyl-	$C_5H_{11}BrO$	167.04	Alkane
13	17.262	0.10	3-Heptadecanol	$C_{19}H_{36}O$	256.47	Alcohol
14	20.415	0.04	Eicosane	$C_{20}H_{42}$	282.54	Alkane
15	21.475	1.81	Benzopteridine, 2,4-diamino-6,7,8,9-tetrahydro-7-methyl-	$C_{10}H_6N_4$	366.80	Heterocyclic N compound
16	24.120	0.37	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{17}H_{38}O_4$	330.50	Ester
17	31.186	0.26	Myristic acid, 4-methoxyphenyl ester	$C_{13}H_{14}O_3$	218.25	Ester

Table 16. Antibiogram against test fungi

Sl. No.	Antibiotics tested	Code No.	Growth inhibition of test fungi [Diameter of inhibition zone(mm)][A]			
			Aspergillus niger	*Aspergillus oryzae*	*Saccharomyces cerevisiae*	*Zygosaccharomyces bailii*
1.	Nystatin 50 mcg	A	21.66	17.33	18.67	21.33
2	Miconazole 30 mcg	B	18.33	19.33	29.33	24.66
3	Fluconazole 10 mcg	C	0.00	0.00	37.33	8.00
4	Ketoconazole 10 mcg	D	8.33	11.33	35.66	15.00
5	Itraconazole 10 mcg	E	23.00	28.00	40.33	20.66
6	Itraconazole 30 mcg	F	23.33	28.66	40.00	25.66
7	Clindamycin 10 mcg	G	0.00+	0.00+	0.00+	0.00+
8	Amikacin 10 mcg	H	0.00+	0.00+	0.00+	0.00+
CD (0.05)			2.739	2.047	4.800	1.109

[+] Not included in the statistical analysis

[A] Diameter of inhibition zone (mm) including disc diameter of 6 mm. Diameter of inhibition zone values are presented as mean ± standard deviation of three replicates.

Table 17. Antibiogram against test bacteria

Sl. No.	Antibiotics tested	Code No.	Growth inhibition of test bacteria [Diameter of inhibition zone(mm)][A]	
			Bacillus subtilis	*Staphylococcus aureus*
1.	Amikacin 10 mcg	A	27.66	22.33
2	Amoxycillin 10 mcg	B	20.00	24.00
3	Gentamicin 50 mcg	C	35.33	30.33
4	Ciprofloxacin 10 mcg	D	41.66	38 66
5	Itraconazole 30 mcg	E	35.33	0.00+
6	Itraconazole 10 mcg	F	37.00	0.00+
7	Streptomycin 25 mcg	G	24.33	22.66
8	Clindamycin 10 mcg	H	30.00	36.00
9	Chloramphenicol 10 mcg	I	24.00	25.66
10	Vancomycin 10 mcg	J	22.00	18.66
CD (0.05)			5.377	2.370

[+] Not included in the statistical analysis

[A]Diameter of inhibition zone (mm) including disc diameter of 6 mm. Diameter of inhibition zone values are presented as mean ± standard deviation of three replicates.

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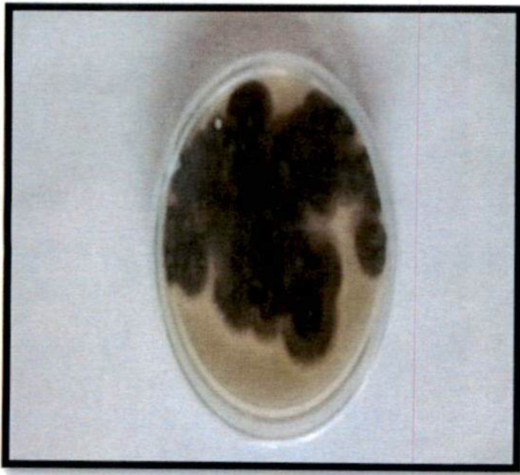
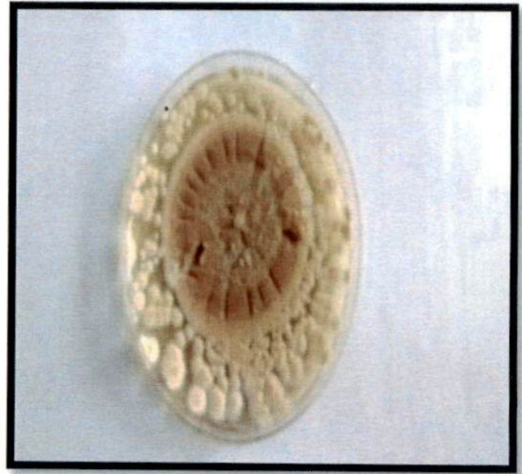
Plate 13. Cultures of test organisms

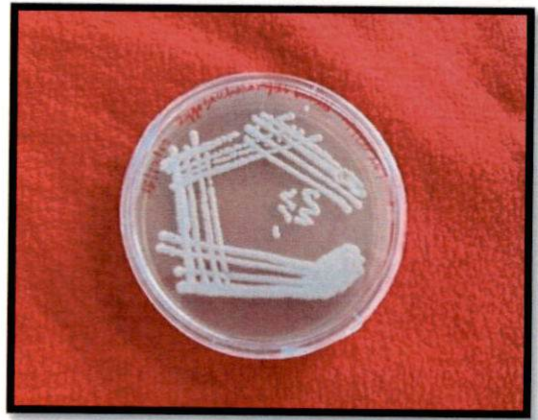


a) *Aspergillus niger*



b) *Aspergillus oryzae*

c) *Saccharomyces cerevisiae*

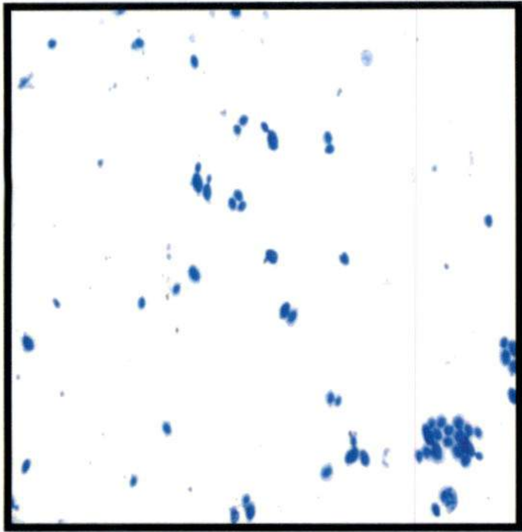


d) *Zygosaccharomyces bailii*

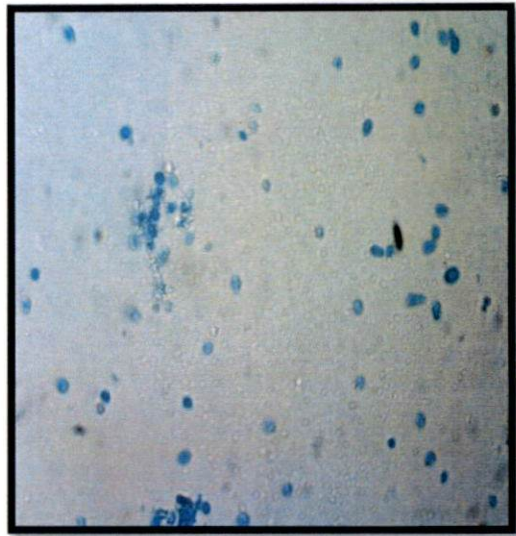
e) *Bacillus subtilis*

f) *Staphylococcus aureus*

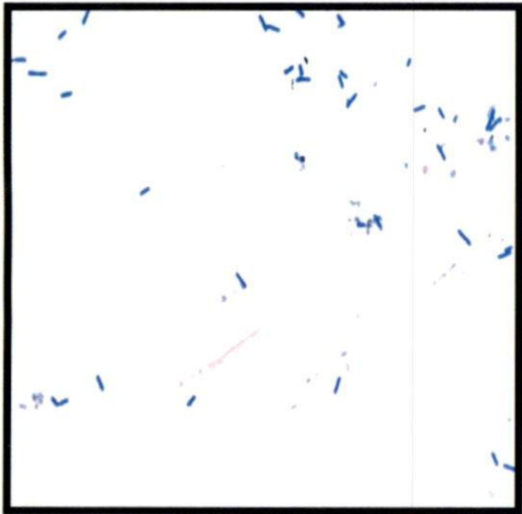
Plate 14. Test organisms- microscopic view



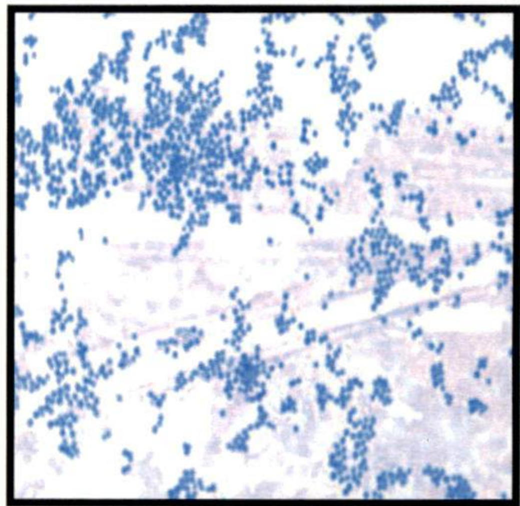
a)Saccharomyces cerevisiae



b) *Zygosaccharomyces bailii*



c) *Bacillus subtilis*



d) *Staphylococcus aureus*

3.2.2. Selection of reference antibiotic

Antibiograms were performed using the available antibiotic discs purchased from Hi Media Laboratories, India) (Table 16 to 17) to select the reference antibiotic against the test micro organisms. Among the antifungal antibiotic tested, Itraconazole 10 mcg produced the significantly highest zone of inhibition against the strains viz. *Aspergillus niger* (23.00 mm) *Aspergillus oryzae* (28.00 mm) *Saccharomyces cerevisiae* (40.33 mm) and *Zygosaccharomyces bailii* (20.66 mm) (Plate 15). Maximum inhibition of the test bacterial strains *Bacillus subtilis* and *Staphylococcus aureus* (41.66 mm and 38.66 mm respectively) was obtained for the antibacterial antibiotic Ciprofloxacin 10 mcg (Plate 16).

3.2.3. Determination of antimicrobial activity of lichen extracts

The antimicrobial activity of three extracts *viz*. acetone, chloroform and ethanol extracts (Plate 12) against the test organisms were evaluated by disc diffusion method and also by well diffusion method (Baur *et al.*, 1966). The zones of inhibition were compared with that of the selected commercial antibiotic, which was used as reference.

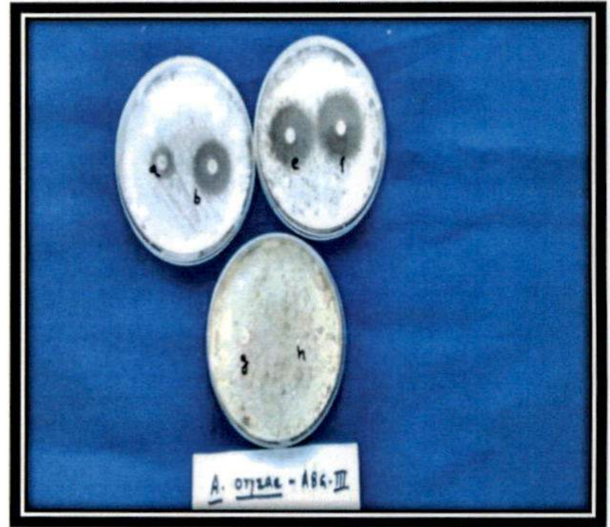
3.2.3.1. Disc diffusion assay

3.2.3.1.1.Antifungal activity of Parmotrema tinctorum

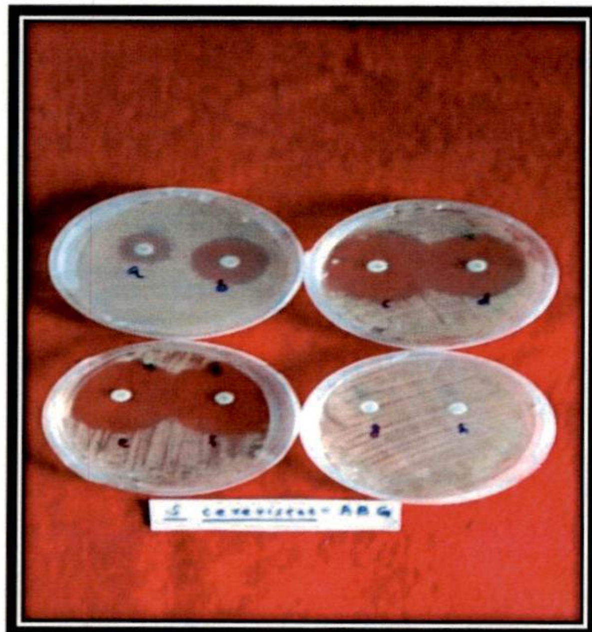
Disc diffusion assay of the lichen extracts displayed a dose dependent inhibitory action against the test organisms. The antifungal activity of different solvent extracts of *Parmotrema tinctorum viz*. acetone, ethanol and chloroform (AE, EE and CE) were recorded against two fungi species *Aspergillus niger* and *Aspergillus oryzae* (Table 18). EE at 40 mg/ml recorded the maximum zone of inhibition (20.50 mm) against *Aspergillus niger* which was significantly superior,

Plate 15. Antibiogram of test fungi

a) *Aspergillus niger*

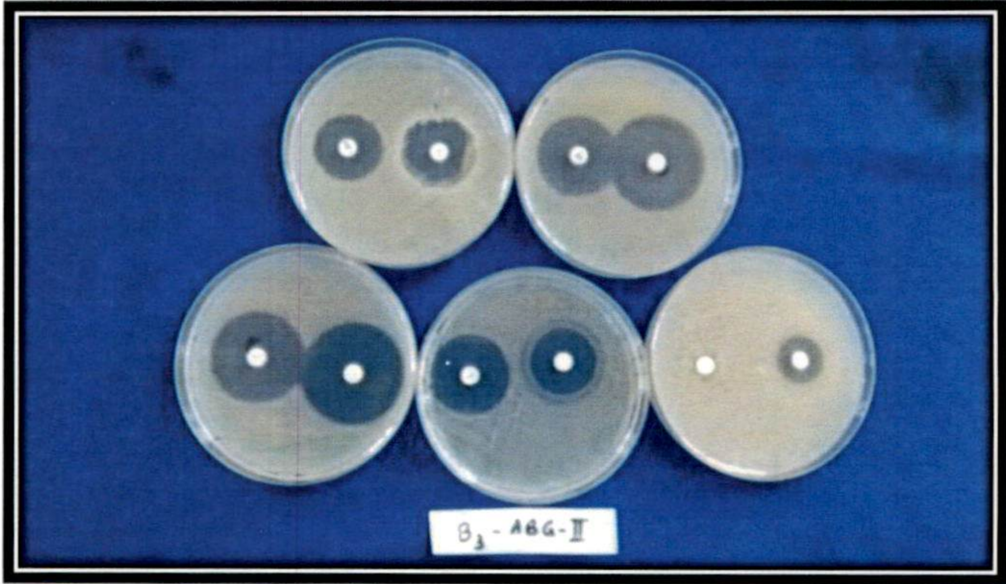


b) *Aspergillus oryzae*

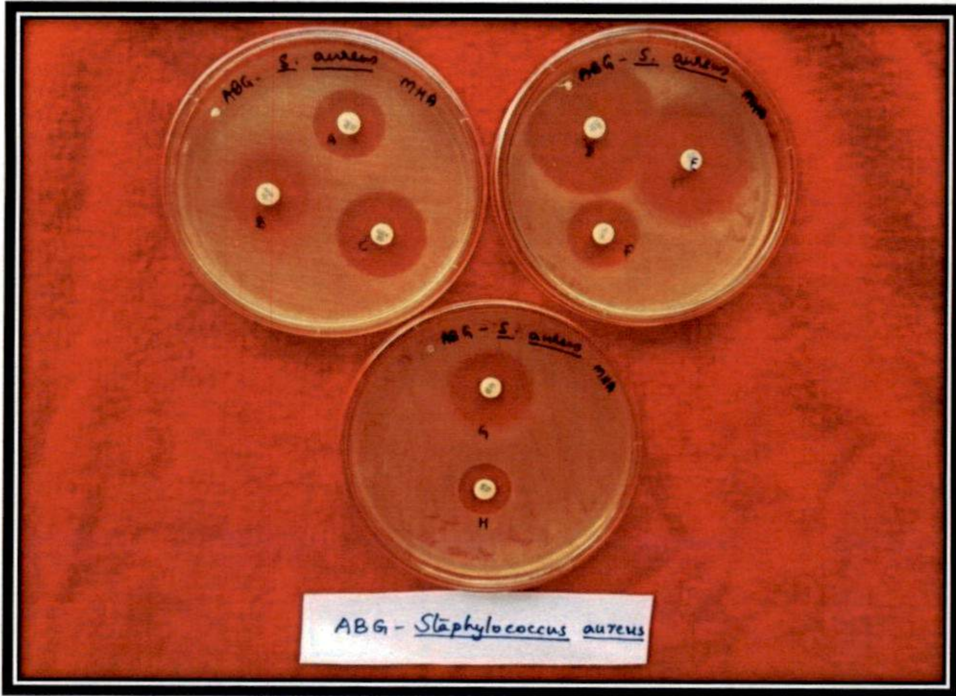


c) *Saccharomyces cerevisiae*

Plate 16. Antibiogram of test bacteria



a) *Bacillus subtilis*



b) *Staphylococcus aureus*

followed by EE at a concentration of 20 mg/ml (15 mm) and by CE at 40 mg/ml (15.75 mm) which were on par (Plate 17). Chloroform extract exhibited inhibitory action against *Aspergillus niger* at all the four concentrations. *Aspergillus oryzae* growth was significantly inhibited by chloroform both at 40 mg/ml (29.25 mm) and at 20 mg/ml (25.25 mm). Acetone extract had no activity against the fungal growth, whereas the ethanol extract had activity only at concentrations of 20 mg/ml and 40 mg/ml (Plate 19). The positive control Itraconazole 10 mcg showed the highest inhibition for the fungi *Aspergillus niger* and *Aspergillus oryzae* with 26 mm and 29 mm respectively.

3.2.3.1.2. Antiyeast activity of Parmotrema tinctorum

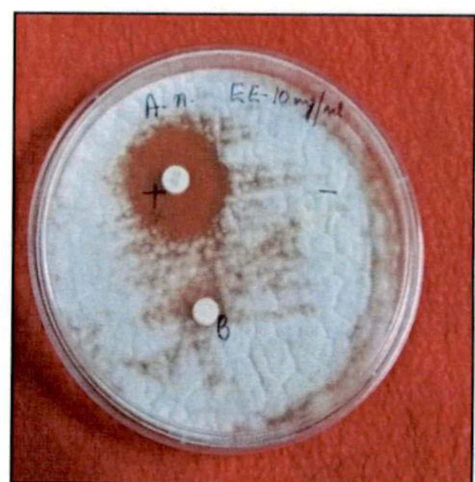
In the yeast species studied, *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*, acetone extract (AE) recorded no anti yeast activity. *S. cerevisiae* growth was inhibited significantly by CE forming a zone of 9.8 mm and 12.75 mm only at higher concentrations of 20 mg/ml and 40 mg/ml (Plate 21). Ethanol extract (EE) showed a similar trend with an inhibition zone of 11.75 mm and 14.25 mm at the concentrations of 20 mg/ml and 40 mg/ml. The antibiotic Itraconazole 10 mcg produced an inhibition zone of 40 mm. With regard to the growth of the yeast *Zygosaccharomyces bailii*, ethanol extract and chloroform extract could exhibit significant growth inhibition only at the higher concentration of 40 mg/ml, 9.00 mm and 8.25 mm respectively (Plate 23). The antibiotic Itraconazole 10 mcg, used as positive control exhibited highest inhibition of 20 mm (Table 19).

3.2.3.1.3.Antibacterial activity of Parmotrema tinctorum

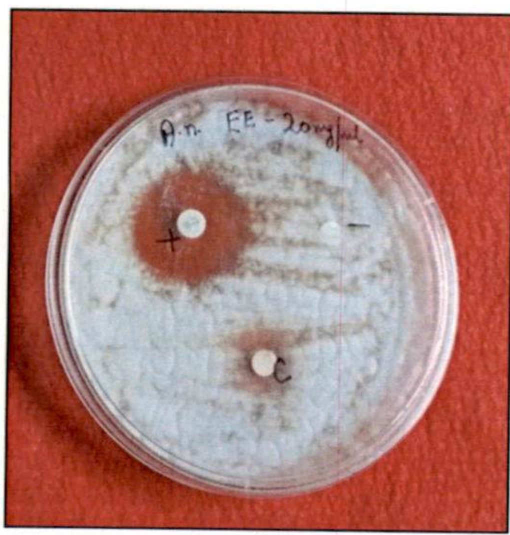
All the three extracts AE, CE and EE displayed significant inhibitory potential against the bacterial strains tested *viz. Bacillus subtilis* and *Staphylococcus aureus*. *Bacillus subtilis* was found to be inhibited maximum by AE (20.25 mm), followed by CE (16.75 mm) at the highest concentration of 40 mg/ml (Plate 25). Acetone extract was found to inhibit bacterial growth at all concentrations varying from 5

Plate. 17. Disc diffusion study of *Aspergillus niger* –Ethanol extract (EE)

A-EE at 5mg/ml



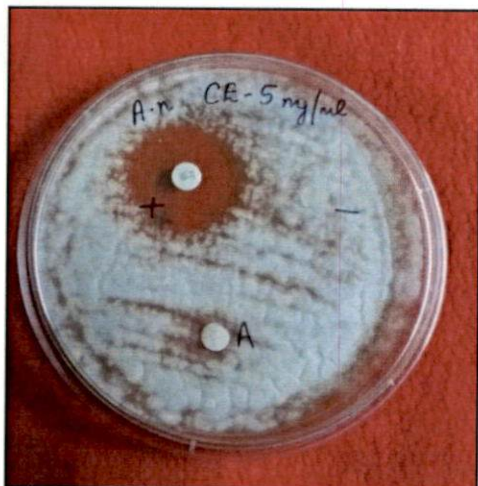
B-EE at 10mg/ml



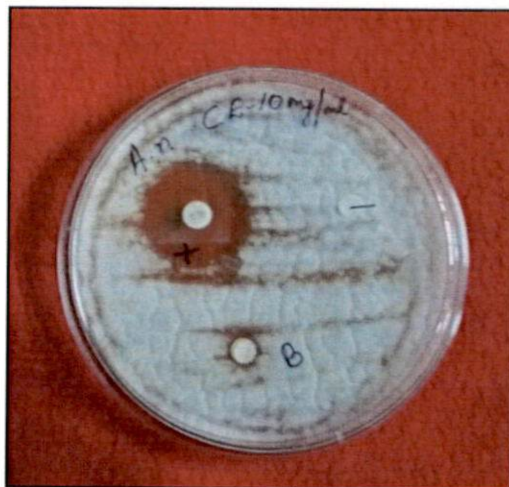
C-EE at 20mg/ml

D-EE at 40mg/ml

Plate. 18. Disc diffusion study of *Aspergillus niger* –Chloroform extract (CE)

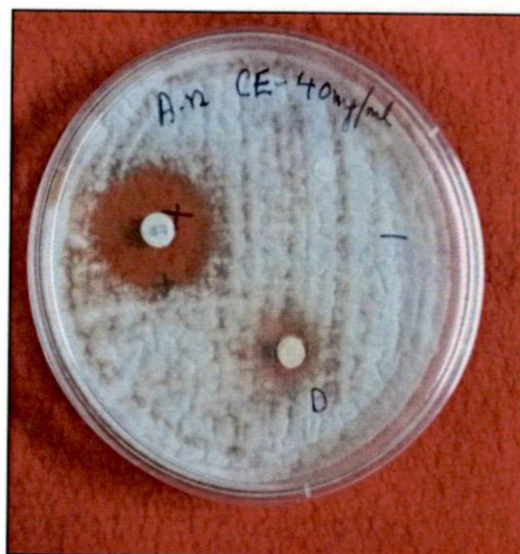


A-CE at 5mg/ml



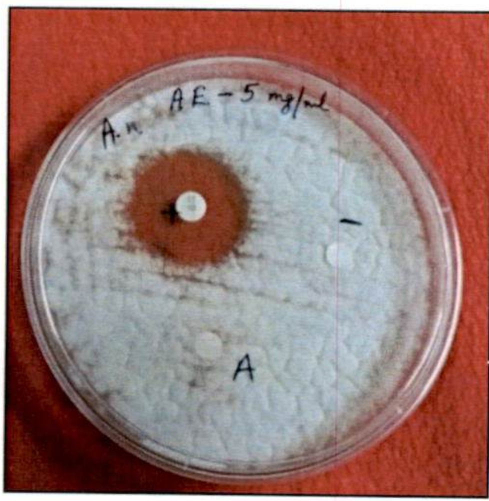
B-CE at 10mg/ml

C-CE at 20mg/ml



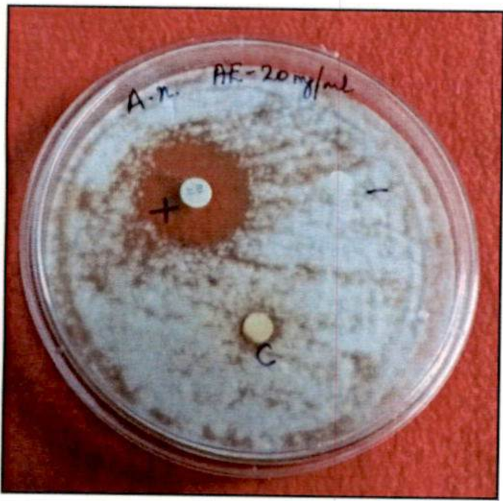
D-CE at 40mg/ml

Plate. 19. Disc diffusion study of *Aspergillus niger* – Acetone extract (AE)

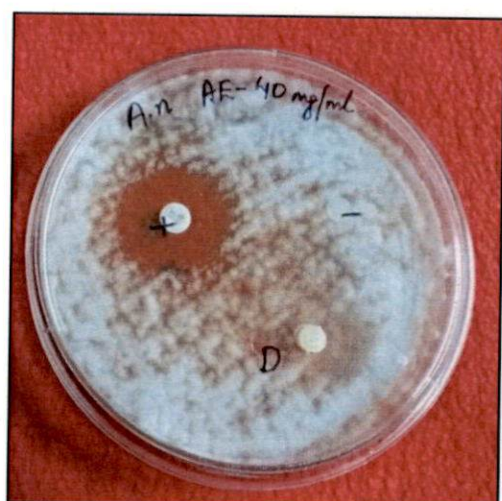


A-AE at 5mg/ml

B-AE at 10mg/ml



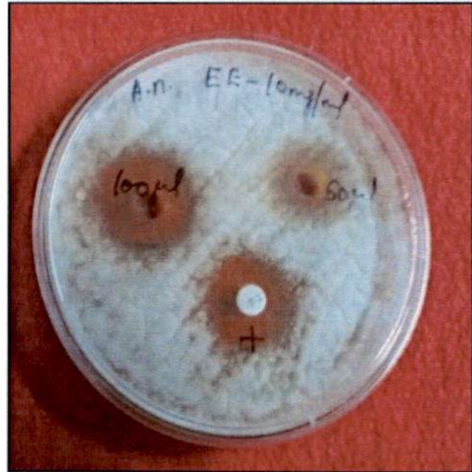
C-AE at 20mg/ml



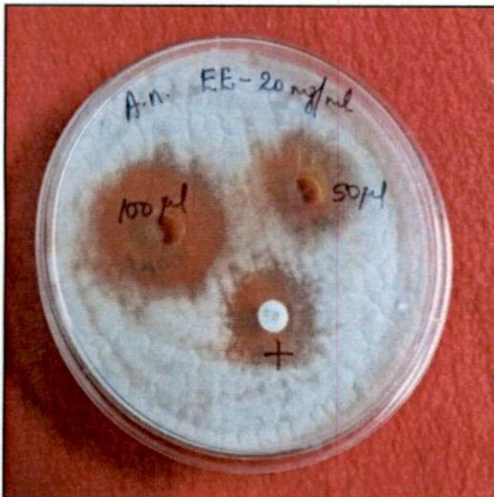
D-AE at 40mg/ml

Plate 20. Well diffusion study of *Aspergillus niger*- Ethanol extract (EE)

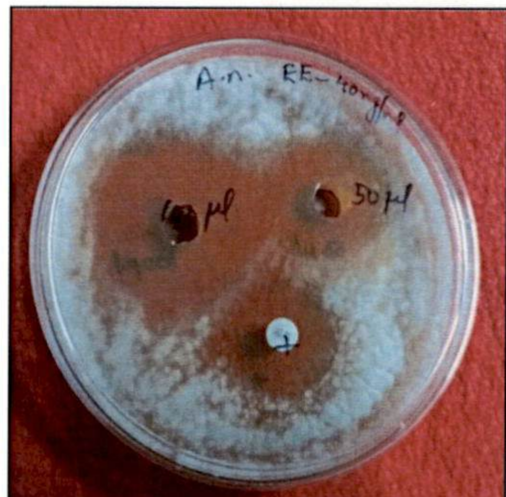
EE at 5mg/ml



EE at 10mg/ml

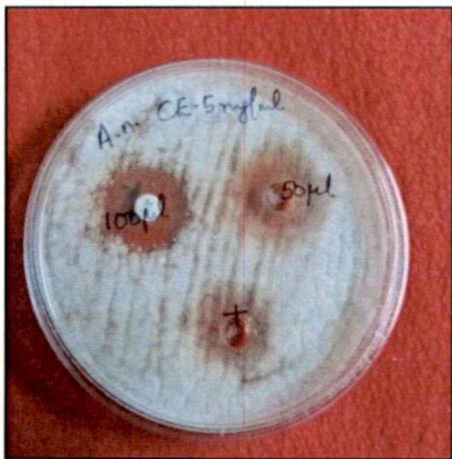


EE at 20mg/ml

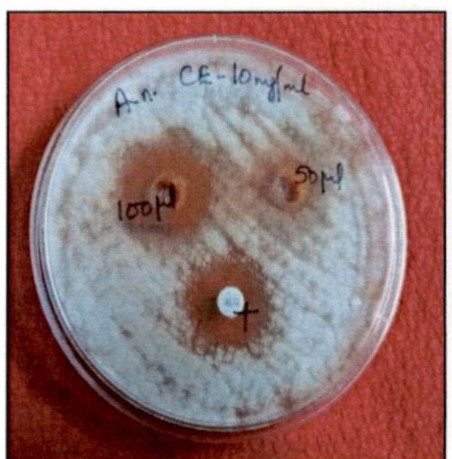


EE at 40mg/ml

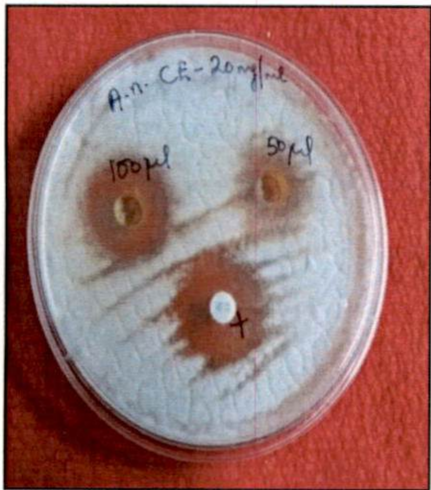
Plate 21. Well diffusion study of *Aspergillus niger*- Chloroform extract(CE)



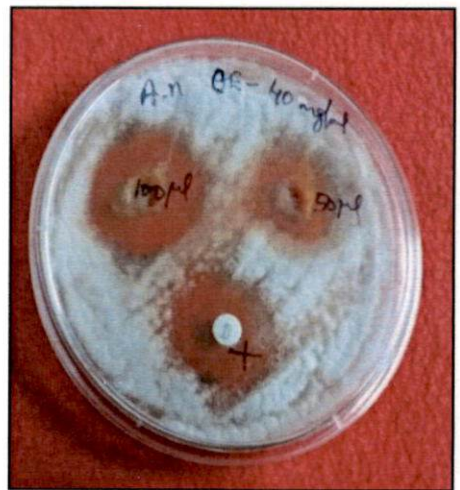
CE at 5mg/ml



CE at 10mg/ml

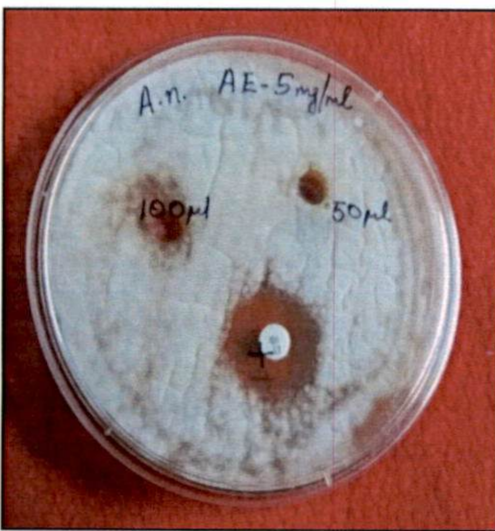


CE at 20mg/ml

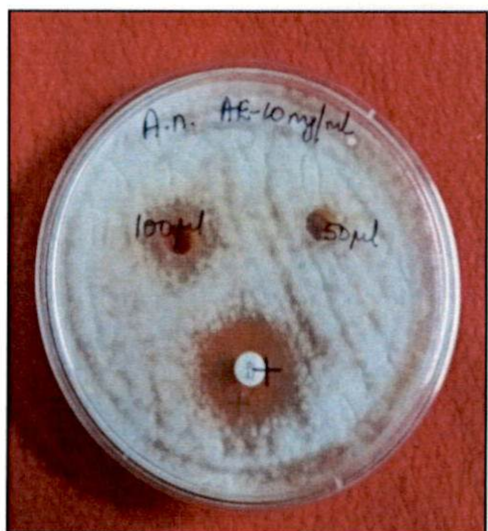


CE at 40mg/ml

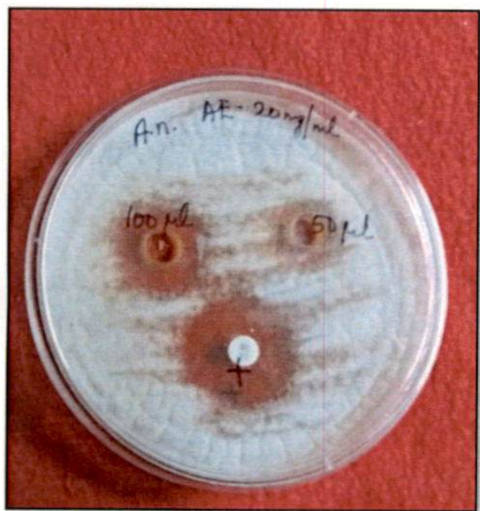
Plate 22. Well diffusion study of *Aspergillus niger*- Acetone extract (AE)



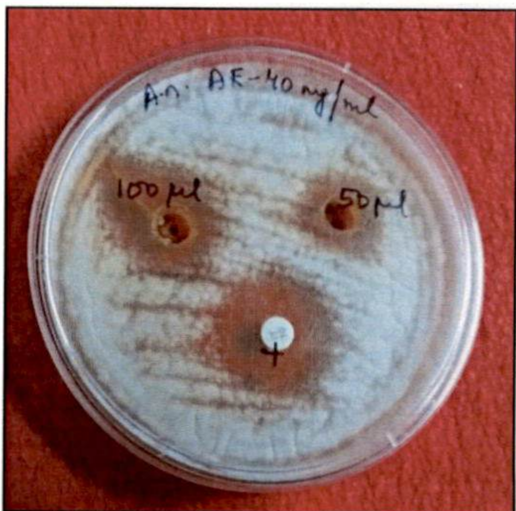
AE at 5mg/ml



AE at 10mg/ml



AE at 20mg/ml



AE at 40mg/ml

mg/ml (10.00mm) to 40 mg/ml (20.25 mm). In the case of *Staphylococcus aureus*, significantly highest inhibition was recorded by AE at 40 mg/ml (24.25 mm), followed by CE at 40 mg/ml (22.25 mm) and by AE at 20 mg/ml (21.75 mm) which were on par. (Plate 27). The reference antibiotic Ciprofloxacin 10 mcg had better inhibitory efficacy for both species of bacteria (41 mm and 40 mm respectively) as compared to the lichen extracts (Table 20).

In all the cases, DMSO (100%) used as negative control did not produce inhibition of any of the test organisms (not shown in tables).

3.2.3.2. Well Diffusion Assay

Well diffusion assay was performed with two volumes of lichen extracts *viz.* 50 μl and 100 μl.

3.2.3.2.1.Antifungal activity of Parmotrema tinctorum

All the lichen extracts (AE, EE and CE) inhibited the growth of fungi *Aspergillus niger* and *Aspergillus oryzae* with varying degree of sensitivity (Fig.16) When the well diffusion was done using 50 μl, the lower concentrations of AE (5 mg/ml and 10 mg/ml) did not show any activity against *Aspergillus niger*. The highest inhibition of 28.75 mm was displayed by ethanol extract at 40 mg/ml, which was significantly superior to all other treatments; even better than the reference antibiotic Itraconazole 10 mcg (26.00 mm). Growth inhibition at 40 mg/ml was on par for both acetone extract (26.50 mm) and chloroform extract (25.75 mm). Chloroform extract was found to be exhibiting inhibitory action at all the concentrations, having an inhibition zone of 14.25 mm, even at a concentration of 5 mg/ml (Table 21).

Plate. 23. Disc diffusion study of *Aspergillus oryzae* –Ethanol extract (EE)

A-EE at 5mg/ml

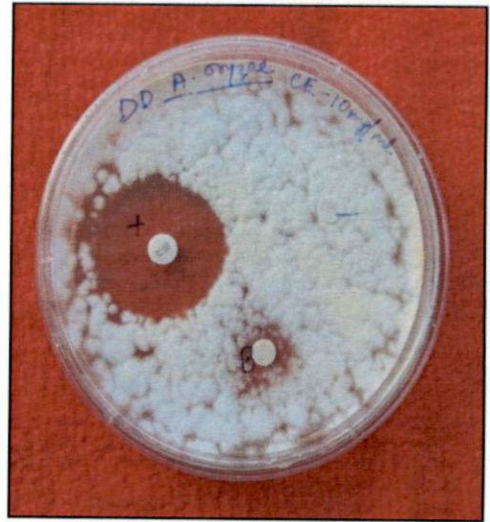
B-EE at 10mg/ml

C-EE at 20mg/ml

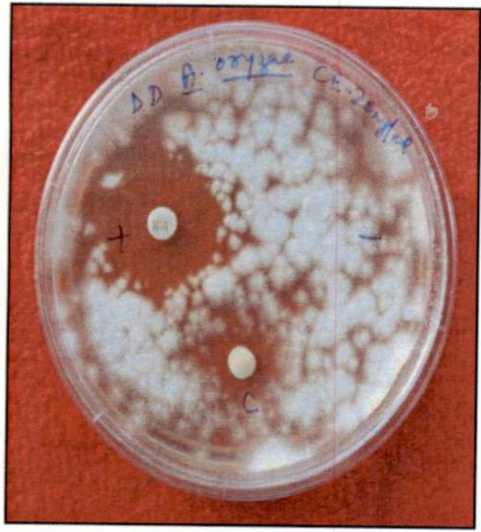
D-EE at 40mg/ml

Plate. 24. Disc diffusion study of *Aspergillus oryzae* –Chloroform extract (CE)

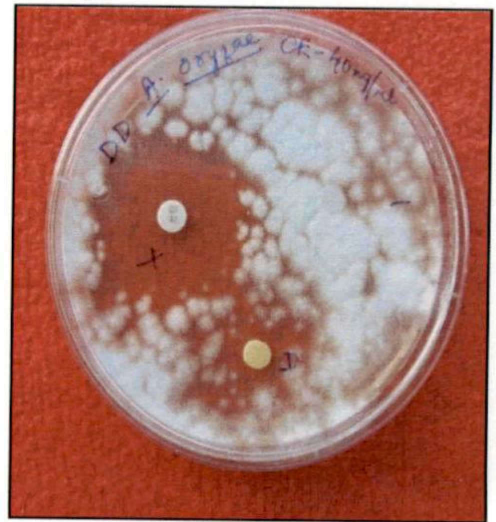
A-CE at 5mg/ml



B-CE at 10mg/ml



C-CE at 20mg/ml

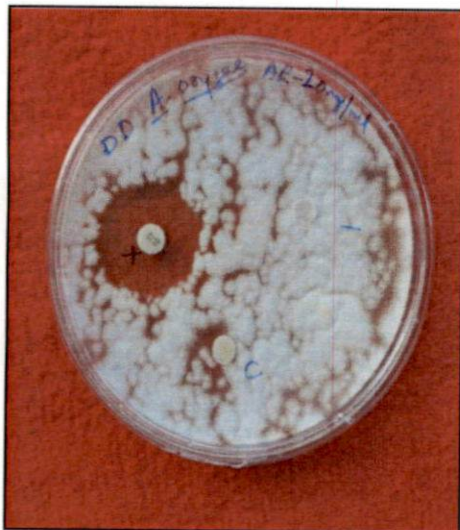


D-CE at 40mg/ml

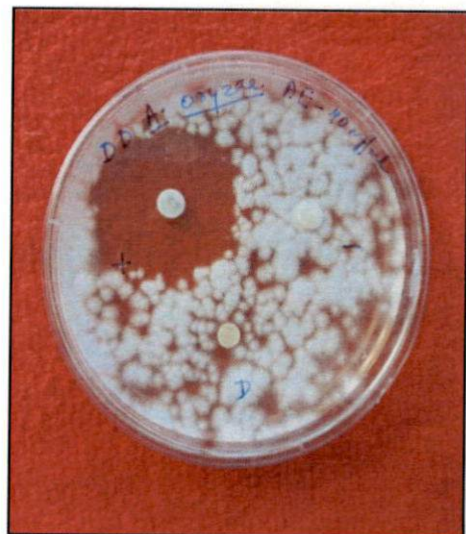
Plate. 25.. Disc diffusion study of *Aspergillus oryzae* –Acetone extract (AE)

A-AE at 5mg/ml

B-AE at 10mg/ml



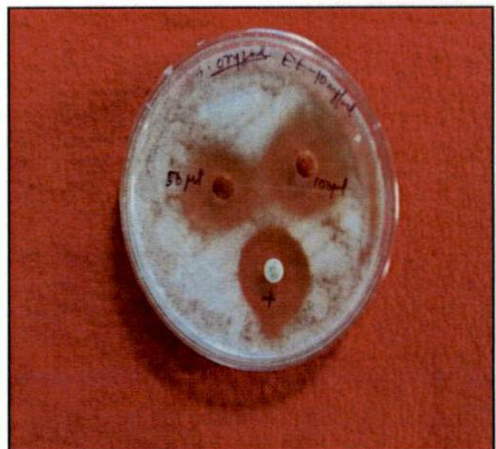
C-AE at 20mg/ml



D-AE at 40mg/ml

Plate 26.Well diffusion study of *Aspergillus oryzae*- Ethanol extract(EE)

EE at 5mg/ml

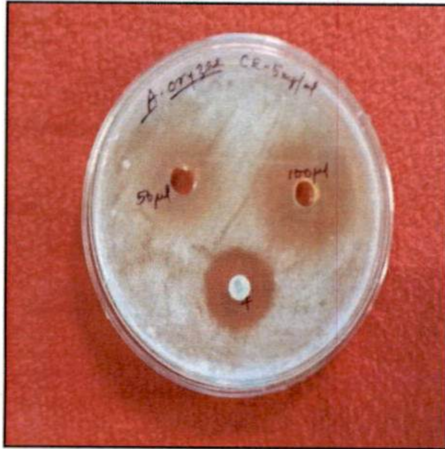


EE at 10mg/ml

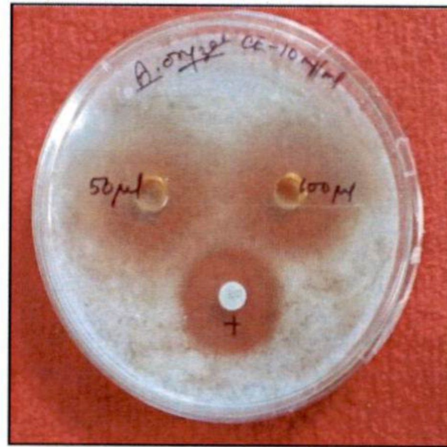
EE at 20mg/ml

EE at 40mg/ml

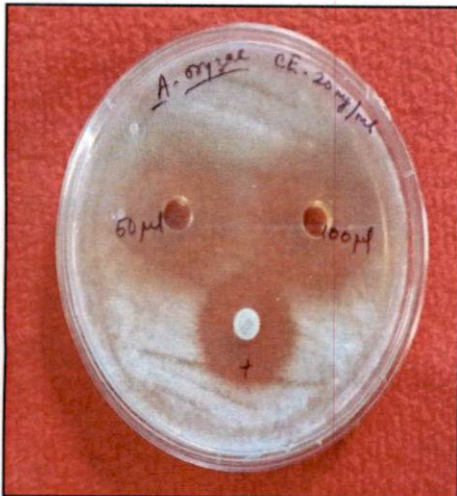
Plate 27. Well diffusion study of *Aspergillus oryzae*- Chloroform extract(CE)



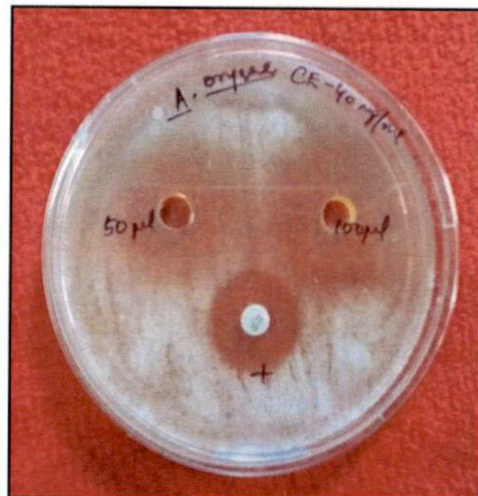
CE at 5mg/ml



CE at 10mg/ml

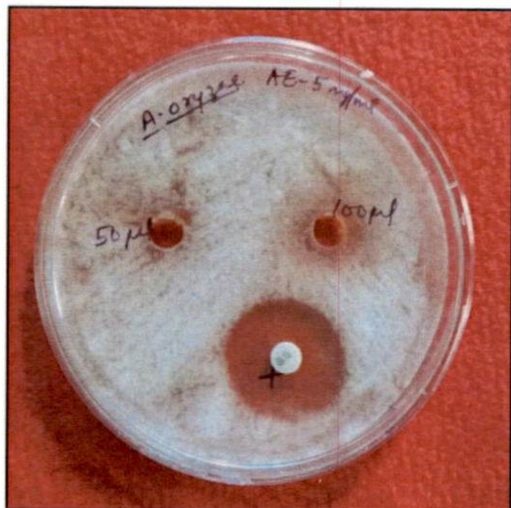


CE at 20mg/ml



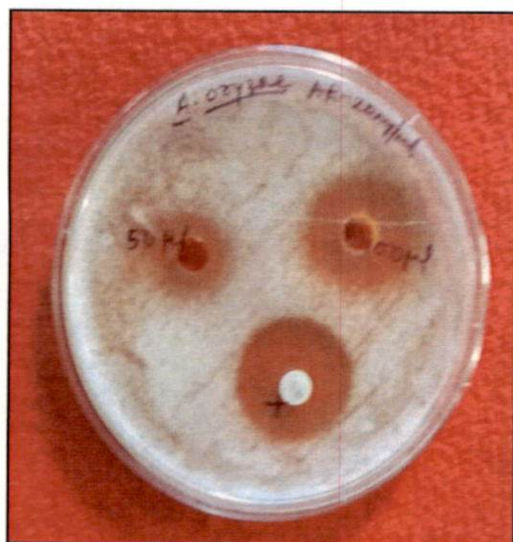
CE at 40mg/ml

Plate 28.Well diffusion study of *Aspergillus oryzae*- Acetone extract(AE)

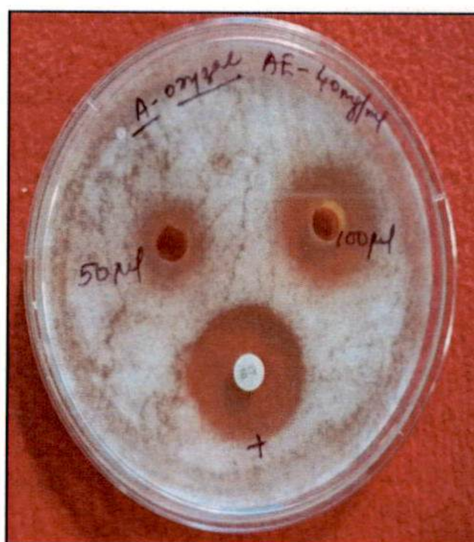


AE at 5mg/ml

AE at 10mg/ml



AE at 20mg/ml



AE at 40mg/ml

In the well diffusion with 100 µl, all the concentrations of the extracts except AE at 5 mg/ml were effective in inhibiting the fungal growth (Plate 18 and 20). The highest concentration of all the extracts tested (40 mg/ml) produced higher inhibition zone (AE-31.75 mm, EE-33.50 mm, CE- 32.75 mm) than the reference antibiotic Itraconazole 10 mcg (26.00 mm). Ethanol extract at 40 mg/ml had the significantly highest inhibitory action on the fungus (33.50 mm), which was on par with the chloroform extract at 40 mg/ml (32.75 mm). Ethanol and chloroform extracts produced remarkable inhibitory zones at all the concentrations tested.

Aspergillus oryzae growth was inhibited by all the concentrations of EE and CE, when tested in well diffusion assay with 50 µl. Ethanol extract (EE) caused maximum inhibition of *Aspergillus oryzae* at 40 mg/ml (35.50±0.57 mm), followed by EE at 20 mg/ml (32.00±0.0 mm) and by CE at 40 mg/ml (30.25 mm); whereas the antibiotic showed an inhibition zone of 26 mm. Acetone extract produced inhibitory effect only in higher concentrations of 20 mg/ml and 40 mg/ml, similar to that observed in the case of *Aspergillus niger*

The ethanol extract (EE) caused the maximum inhibition of *Aspergillus oryzae* as seen in well diffusion using 50 µl, at 40 mg/ml (41.75 mm), followed by EE at 20 mg/ml (36.50 mm) and by CE at 40 mg/ml (34.75 mm) in the well diffusion assay using 100 µl also. Inhibitory action against the fungus was noticed for all the extracts at all the concentrations. Chloroform extract produced notable inhibitory action against the fungus from 5 mg/ml (22.50 mm) to 40 mg/ml (34.75 mm).

Table 18. Antifungal activity of *Parmotrema tinctorum* extracts by disc diffusion method

Lichen Extracts/ Antibiotic	Concentration of extract taken (mg/ml)	[B]Final concentration of extract (µg/ml)	Growth inhibition of test fungi [Diameter of inhibition zone(mm)][A]	
			Aspergillus niger	*Aspergillus oryzae*
Acetone extract (AE)	5	50	0.00+	0.00+
	10	100	0.00+	0.00+
	20	200	0.00+	0.00+
	40	400	7.75±0.96	0.00+
Ethanol extract (EE)	5	50	0.00+	0.00+
	10	100	9.25±0.50	0.00+
	20	200	15.00±0.82	9.50±0.57
	40	400	20.50±1.73	15.8±0.50
Chloroform extract (CE)	5	50	8.25±0.50	0.00+
	10	100	9.50±0.58	13.00±0.81
	20	200	12.75±0.96	25.25±0.50
	40	400	15.75±0.50	29.25±0.50
Antibiotic [C] Itraconazole 10µg	-	-	26.00	29.00
CD (0.05)			1.324	0.891

+ Not included in the statistical analysis

Diameter of inhibition zone values are presented as mean ± standard deviation of four replicates.

Diameter of inhibition zone (mm) including disc diameter of 6 mm. [B]Vol. of extract applied is 10 µl. [C] Itraconazole disc (10µg) wa s used as positive reference standard for fungi.

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Table 19. Antiyeast activity of *Parmotrema tinctorum* extracts by disc diffusion method

Lichen Extracts/ Antibiotic	Concentration of extract taken (mg/ml)	[B]Final concentration of extract (μg/ml)	Growth inhibition of test yeast [Diameter of inhibition zone(mm)][A]	
			Saccharomyces cerevisiae	*Zygosaccharomyces bailii*
Acetone extract (AE)	5	50	0.00[+]	0.00[+]
	10	100	0.00[+]	0.00[+]
	20	200	0.00[+]	0.00[+]
	40	400	0.00[+]	0.00[+]
Ethanol extract (EE)	5	50	0.00[+]	0.00[+]
	10	100	0.00[+]	0.00[+]
	20	200	11.75±0.00	0.00[+]
	40	400	14.25±0.009	9.00±0.50
Chloroform extract (CE)	5	50	0.00[+]	0.00[+]
	10	100	0.00[+]	0.00[+]
	20	200	9.8±0.00	0.00[+]
	40	400	12.75±0.00	8.25±0.00
Antibiotic [C]Itraconazole (10μg)	-	-	40.00	20.00
CD (0.05)			0.995	0.612

[+] Not included in the statistical analysis
Diameter of inhibition zone values are presented as mean ± standard deviation of four replicates.
[A]Diameter of inhibition zone (mm) including disc diameter of 6 mm. [B]Vol. of extract applied is 10 μl. [C] Itraconazole disc (10μg) was used as positive reference standard for fungi.

Table 20. Antibacterial activity of *Parmotrema tinctorum* extracts by disc diffusion method

Lichen Extracts/ Antibiotic	Concentration of extract taken (mg/ml)	[B]Final concentration of extract (µg/ml)	Growth inhibition of test bacteria [Diameter of inhibition zone(mm)] [A]	
			Bacillus subtilis	*Staphylococcus aureus*
Acetone extract (AE)	5	50	10.00±0.81	15.50±0.57
	10	100	12.75±0.95	19.50±0.57
	20	200	15.25±1.25	21.75±0.50
	40	400	20.25±0.95	24.25±0.50
Ethanol extract (EE)	5	50	8.75±0.50	0.00[+]
	10	100	10.75±0.95	9.50±0.57
	20	200	12.75±0.95	15.50±0.57
	40	400	15.25±0.95	19.75±0.50
Chloroform extract (CE)	5	50	0.00[+]	15.50±0.57
	10	100	9.75±0.50	17.75±0.50
	20	200	11.25±0.95	20.25±0.50
	40	400	16.75±0.95	22.25±0.50
Antibiotic [C]Ciprofloxacin (10µg)	-	-	41.00	40.00
CD (0.05)			1.314	0.772

[+] Not included in the statistical analysis

Diameter of inhibition zone values are presented as mean ± standard deviation of four replicates.

[A]Diameter of inhibition zone (mm) including disc diameter of 6 mm. [B]Vol. of extract applied is 10 µl. [C] Ciprofloxacin disc (10µg) was used as positive reference standard for bacteria.

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Plate. 29. Disc diffusion study of *Saccharomyces cerevisiae*- Ethanol extract(EE)

A-EE at 5mg/ml

B-EE at 10mg/ml

C-EE at 20mg/ml

D-EE at 40mg/ml

Plate. 30. Disc diffusion study of *Saccharomyces cerevisiae*- Chloroform extract(CE)

A-CE at 5mg/ml

B-CE at 10mg/ml

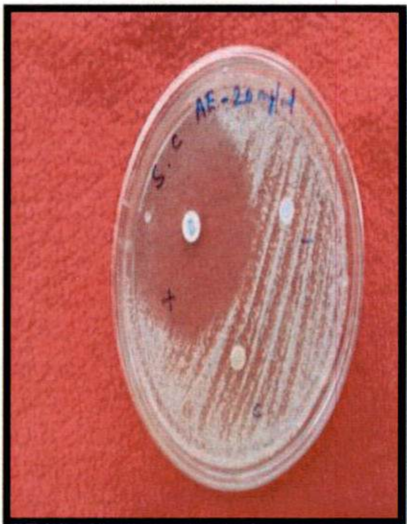
C-CE at 20mg/ml

D-EE at 40mg/ml

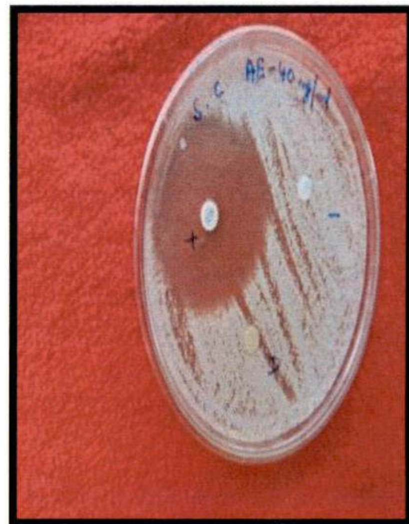
Plate. 31. Disc diffusion study of *Saccharomyces cerevisiae*- Acetone extract (AE)

-AE at 5mg/ml

B- AE at 10mg/ml

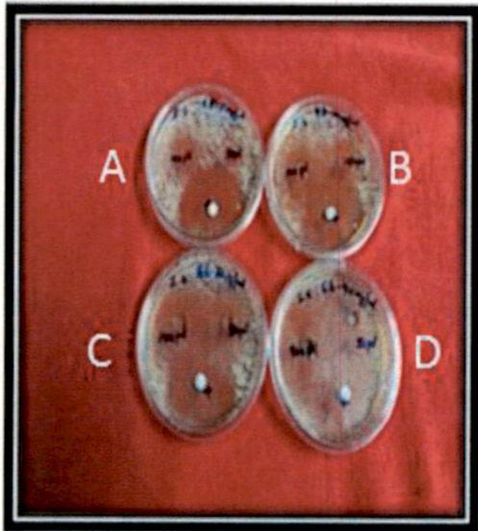


C-AE at 20mg/ml



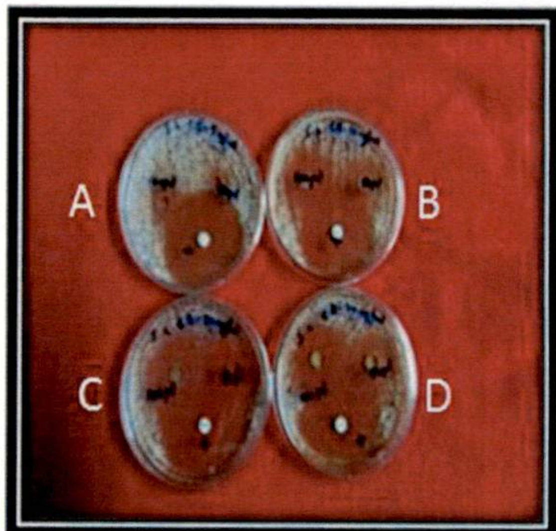
D- AE at 40mg/ml

Plate 32. Well diffusion study of *Saccharomyces cerevisiae*



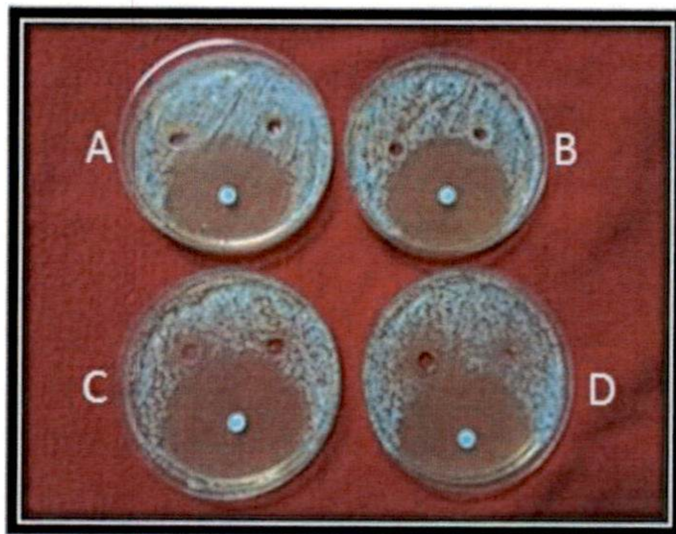
a) Ethanol extract

(A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)



b) Chloroform extract

(A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)



c) Acetone extract(A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)

3.2.3.2.2. Antiyeast activity of Parmotrema tinctorum

Among the three extracts of *Parmotrema tinctorum* studied, AE had no action on growth of *Saccharomyces cerevisiae* at all the concentrations. Ethanol extract and chloroform extract proved to be effective only at three concentrations, 10 mg/ml, 20 mg/ml and 40 mg/ml. The growth of yeast *Saccharomyces cerevisiae* was most suppressed by EE (26.00 mm), followed by CE (21.00) both at 40 mg/ml. The antibiotic Itraconazole 10 mcg produced maximum zone of inhibition for the yeast growth (40.00 mm).

When the extracts were tested using 100 µl, acetone extract could result in inhibition of microbial growth only at the highest concentration of 40 mg/ml (9.75 mm). The other two solvent extracts, EE and CE produced inhibition at all the concentrations tried. The most significant inhibition for *Saccharomyces cerevisiae* was noted for ethanol extract with an inhibition zone of 28.75 mm, followed by chloroform extract with a zone of 25.25 mm.

Similar result was noticed in disc diffusion (50 µl) for *Zygosaccharomyces bailii* where maximum inhibition occurred for EE (20.50 mm), followed by CE (19.50 mm) both at a concentration of 40 mg/ml; which were on par. Lowest concentration of both ethanol and chloroform extract (5 mg/ml) did not have an inhibitory effect. The growth of yeast *Zygosaccharomyces bailii* was found to be suppressed only at the highest concentration of 40 mg/ml (9.50 mm) of AE, producing no zones at concentrations of 5 mg/ml, 10 mg/ml and 20 mg/ml.

When disc diffusion was performed using 100 µl, inhibition of yeast growth was noticed for ethanol and chloroform extracts at all the concentrations. The most significant inhibition was observed for ethanol extract with a zone of 27.00 mm at the concentration of 40 mg/ml; which was followed by chloroform extract with a zone of 24.50 mm, both at a concentration of 40 mg/ml (Plate 22 and 24). The antibiotic

produced an inhibition zone of 22.00 mm. Acetone extract exhibited inhibitory effect only in the higher concentrations of 20 mg/ml and 40 mg/ml (Table 22).

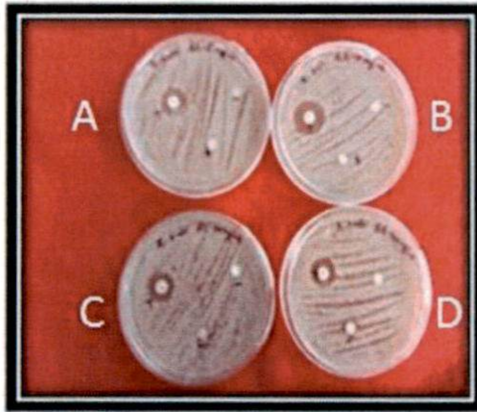
3.2.3.2.3. Antibacterial activity of Parmotrema tinctorum

The growth of *Bacillus subtilis* was remarkably inhibited by the acetone extract (AE) and by chloroform extract (CE) forming inhibition zones in all the concentrations tested, in well diffusion with 50 µl extract. The acetone extract (AE) at 40 mg/ml caused maximum inhibition of growth of *Bacillus subtilis*, resulting in a zone of 26.75 mm, followed by AE at 20 mg/ml with a zone size of 22.75 mm. Ethanol extract (EE) at 40 mg/ml and AE at 10 mg/ml displayed inhibition zones at 21.00 mm and 20.75 mm respectively which were on par. The inhibition zone diameter of reference antibiotic Ciprofloxacin 10 mcg was 38.00 mm (Table 23).

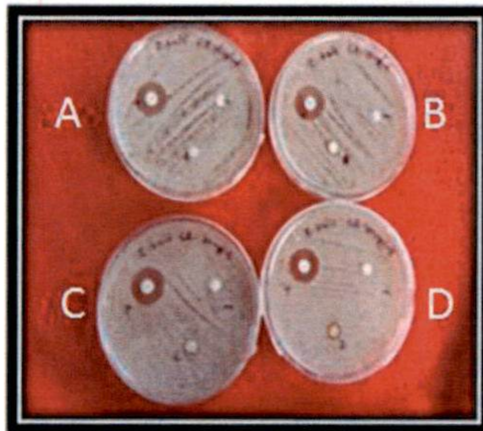
When the volume of extract was enhanced to 100 µl, all the concentrations of the lichen extracts resulted in the inhibition of the bacterium *Bacillus subtilis*. The most significant inhibition was observed for acetone extract at 40 mg/ml (29.50 mm), followed by AE at 20 mg/ml (26.50 mm). Comparison of efficacy of all the extracts revealed that least inhibitory effect was for the ethanol extract, whereas the standard antibiotic exhibited the largest inhibition zone of 38 mm (Plate 26).

In the well diffusion assay conducted with 50 µl extract, the growth of the bacterium *Staphylococcus aureus* was significantly inhibited by the acetone extract (AE) and by CE at all the given concentrations while the inhibitory activity of EE was observed only at higher concentrations of 10 mg/ml, 20 mg/ml and 40 mg/ml. The maximum zone of inhibition was measured for AE at 40 mg/ml (25.50 mm), followed by AE at 20 mg/ml (23.25 mm) and by EE at 40 mg/ml (20.00 mm); while the antibiotic Ciprofloxacin 10 mcg recorded an inhibition zone of 40 mm.

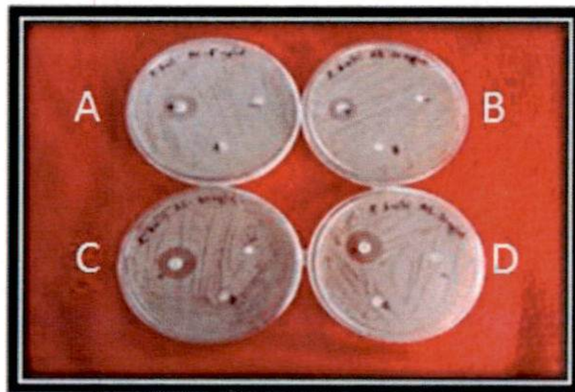
Plate 33.Disc diffusion study of *Zygosaccharomyces bailii*



a) Ethanol extract (A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)

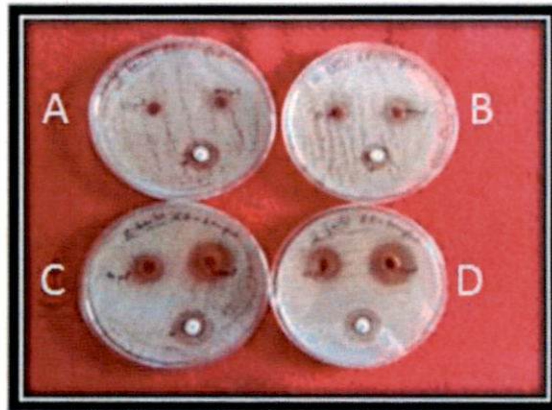


b) Chloroform extract (A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)

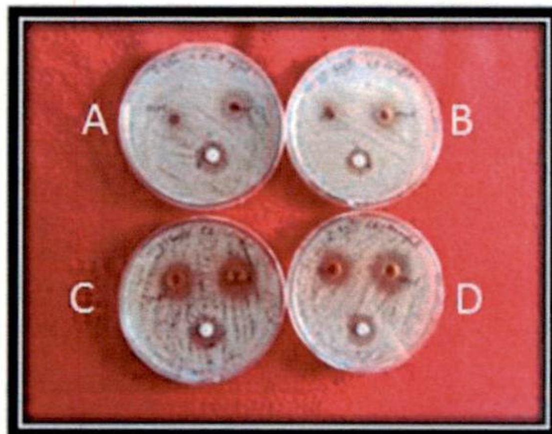


c) Acetone extract (A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)

Plate 34. Well diffusion study of *Zygosaccharomyces bailii*



a) Ethanol extract(A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)



b) Chloroform extract (A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)

c) Acetone extract (A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)

Table 21. Antifungal activity of *Parmotrema tinctorum* extracts by well diffusion method

Lichen Extracts/ Antibiotic	Concentration of extract taken (mg/ml)	Growth inhibition of test fungi species [Diameter of inhibition zone(mm)][A]			
		Aspergillus niger		*Aspergillus oryzae*	
		50µl	100µl	50µl	100µl
Acetone extract (AE)	5	0.00[+]	0.00[+]	0.00[+]	12.00±0.60
	10	0.00[+]	11.50±0.57	0.00[+]	14.50±0.50
	20	10.50±0.57	19.75±0.95	11.25±0.57	20.00±0.57
	40	26.50±1.29	31.75±1.70	15.25±0.50	25.25±0.80
Ethanol extract (EE)	5	0.00[+]	15.00±0.81	12.00±0.81	19.25±0.0
	10	17.00±0.81	22.25±1.25	20.50±0.57	27.75±0.57
	20	22.25±1.25	29.50±0.57	26.50±0.57	31.75±0.50
	40	28.75±0.95	33.50±1.29	32.00±0.0	34.75±0.0
Chloroform extract (CE)	5	14.25±0.95	19.75±0.95	14.75±0.5	22.50±0.81
	10	19.25±0.95	23.50±1.73	22.25±0.5	30.00±0.60
	20	22.0±0.81	26.75±1.70	30.25±0.5	36.50±0.50
	40	25.75±0.95	32.75±0.95	35.50±0.57	41.75±0.60
Antibiotic [B]Itraconazole/10µg	-	26.00	26.00	25.00	25.00
CD (0.05)		1.417	1.744	0.780	1.159

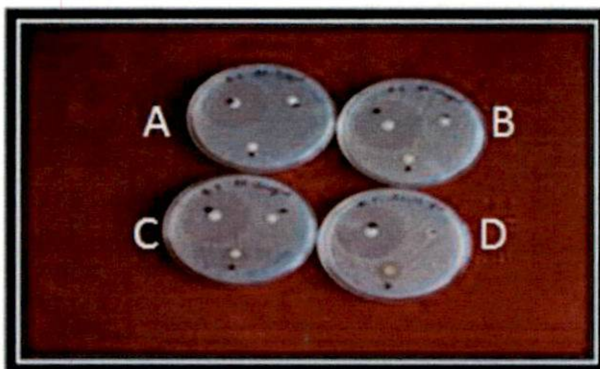
[+] Not included in the statistical analysis

Diameter of inhibition zone values are presented as mean ± standard deviation of four replicates.

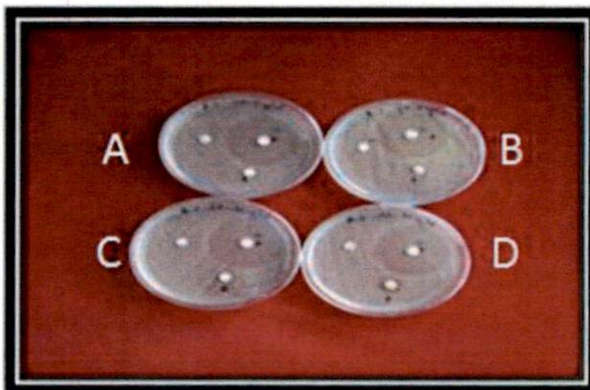
Diameter of inhibition zone (mm) including disc diameter of 6 mm. [B] Itraconazole disc (10µg) was used as positive reference standard for fungi.

Plate 35. Disc diffusion study of *Bacillus subtilis*

a) **Acetone extract** (**A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml**)

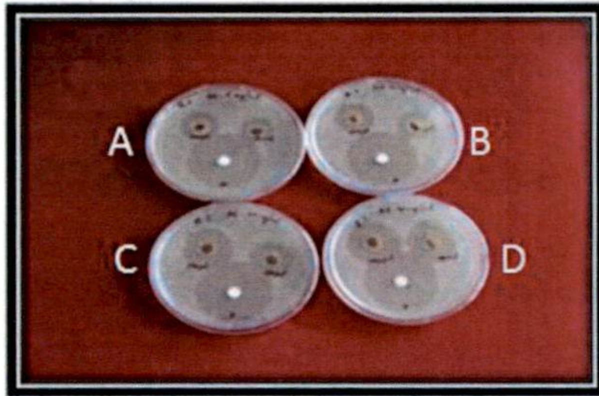


b) **Ethanol extract** (**A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml**)

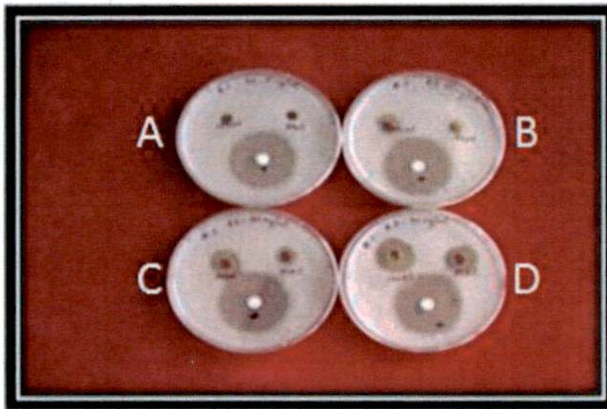


Chloroform extract (**A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml**)

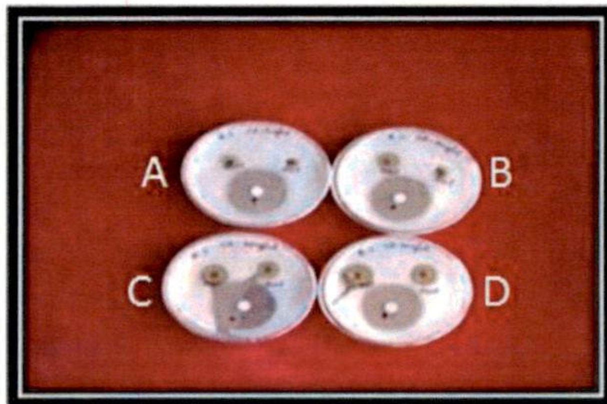
Plate 36. Well diffusion study of *Bacillus subtilis*



a) Acetone extract(A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)



b) Ethanol extract (A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)



c) Chloroform extract(A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)

Table 22. Antiyeast activity of *Parmotrema tinctorum* extracts by well diffusion method

Lichen Extracts/ Antibiotic	Concentration of extract taken (mg/ml)	Growth inhibition of test yeast species [Diameter of inhibition zone(mm)][A]			
		Saccharomyces cerevisiae		*Zygosaccharomyces bailii*	
		50µl	100µl	50µl	100µl
Acetone extract (AE)	5	0.00+	0.00+	0.00+	0.00+
	10	0.00+	0.00+	0.00+	0.00+
	20	0.00+	0.00+	0.00+	11.50±0.57
	40	0.00+	9.75±1.30	9.50±0.60	15.25±0.50
Ethanol extract (EE)	5	0.00+	12.00±0.50	0.00+	8.75±0.50
	10	11.25±0.50	20.00±0.50	9.00±0.0	15.50±0.57
	20	17.00±0.82	22.75±0.50	14.50±0.58	20.00±0.82
	40	26.00±1.40	28.75±1.30	20.50±0.60	27.00±0.80
Chloroform extract (CE)	5	0.00+	12.75±0.81	0.00+	13.75±0.50
	10	13.25±0.50	19.25±0.82	10.50±0.58	18.25±0.50
	20	18.75±0.50	23.25±0.96	13.00±1.41	21.50±0.58
	40	21.00±0.81	25.25±0.50	19.50±0.57	24.50±0.57
Antibiotic [B]Itraconazole/10µg	-	40.00	40.00	22.00	22.00
CD (0.05)		1.226	1.225	1.064	0.874

+ Not included in the statistical analysis

Diameter of inhibition zone values are presented as mean ± standard deviation of four replicates.

Diameter of inhibition zone (mm) including disc diameter of 6 mm. [B] Itraconazole disc (10µg) was used as positive reference standard for fungi.

Table 23. Antibacterial activity of *Parmotrema tinctorum* extracts by well diffusion method

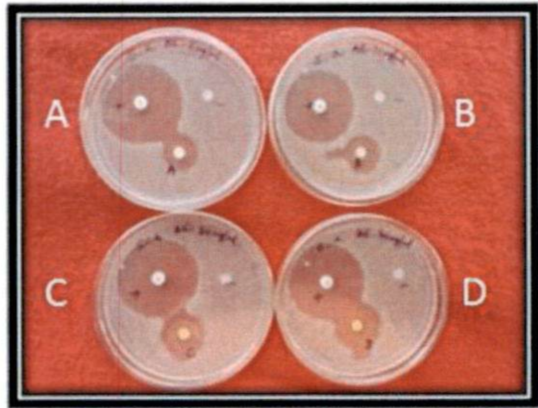
Lichen Extracts/ Antibiotic	Concentration of extract taken (mg/ml)	Growth inhibition of test bacteria species [Diameter of inhibition zone(mm)][A]			
		Bacillus subtilis		*Staphylococcus aureus*	
		50µl	100µl	50µl	100µl
Acetone extract (AE)	5	16.25±0.95	21.25±0.58	12.50±0.57	18.75±0.95
	10	20.75±0.95	22.75±0.50	17.75±0.50	23.50±0.57
	20	22.75±0.96	26.50±0.60	23.25±0.95	27.50±0.57
	40	26.75±0.95	29.50±0.50	25.50±0.57	29.25±0.50
Ethanol extract (EE)	5	0.00^{+}	8.75±0.81	0.00^{+}	0.00^{+}
	10	13.00±0.80	12.50±0.50	8.75±0.50	9.75±0.50
	20	17.00±0.82	17.00±0.58	15.50±0.57	19.75±0.50
	40	21.00±0.80	21.75±0.60	20.00±0.81	22.25±0.50
Chloroform extract (CE)	5	9.75±0.50	11.25±0.80	12.25±0.95	17.00±0.81
	10	12.50±0.60	14.25±0.50	15.00±0.81	20.25±0.5
	20	16.00±0.80	15.25±0.50	16,25±0,95	21.75±0.5
	40	18.75±0.95	19.00±0.57	17.50±0.57	23.50±0.57
Antibiotic [B] Ciprofloxacin 10µg	-	38.00	38.00	40.00	40.00
CD (0.05)		1.214	1.091	1.055	0.877

$^{+}$ Not included in the statistical analysis

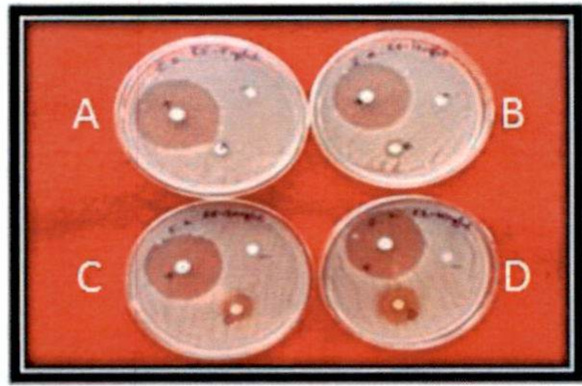
Diameter of inhibition zone values are presented as mean ± standard deviation of four replicates.

Diameter of inhibition zone (mm) including disc diameter of 6 mm. [B] Ciprofloxacin disc (10µg) was used as positive reference standard for bacteria.

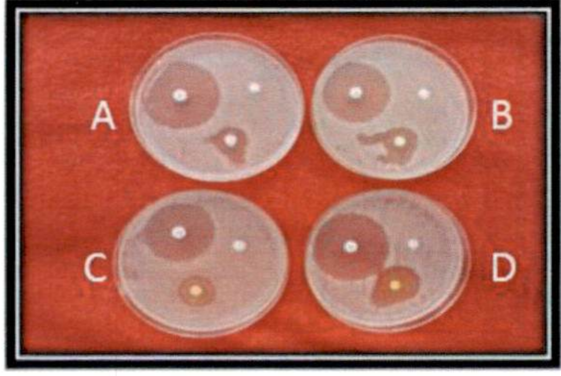
Plate 37. Disc diffusion study of *Staphylococcus aureus*



a) Acetone extract (A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)

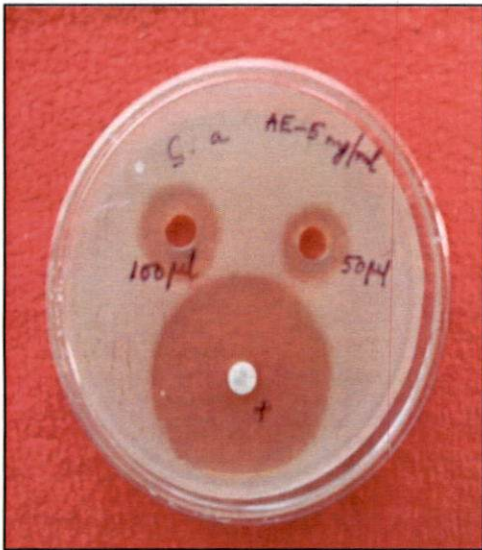


b) Ethanol extract (A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)

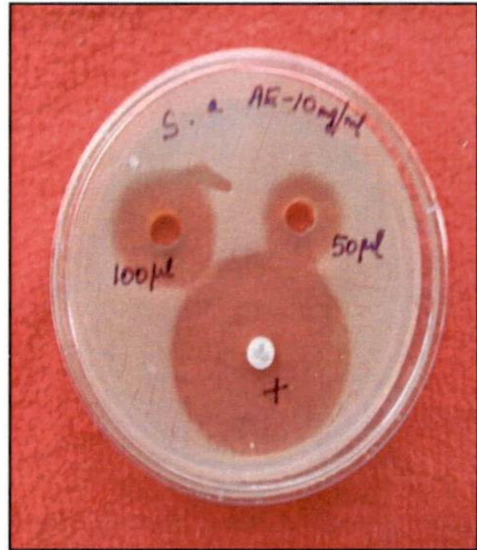


c) Chloroform extract (A-5mg/ml, B-10mg/ml, C-20 mg/ml, D-40 mg/ml)

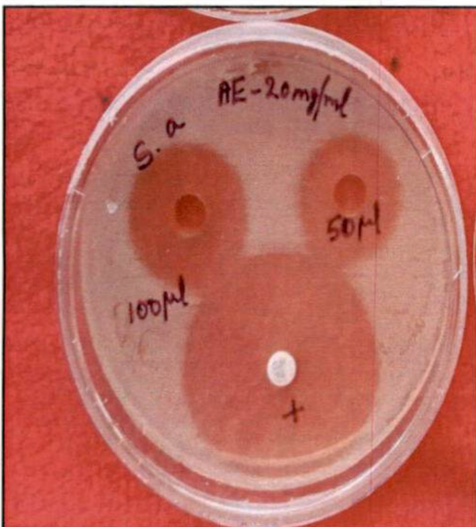
Plate 38..Well diffusion study of *Staphylococcus aureus*--Acetone extract(AE)



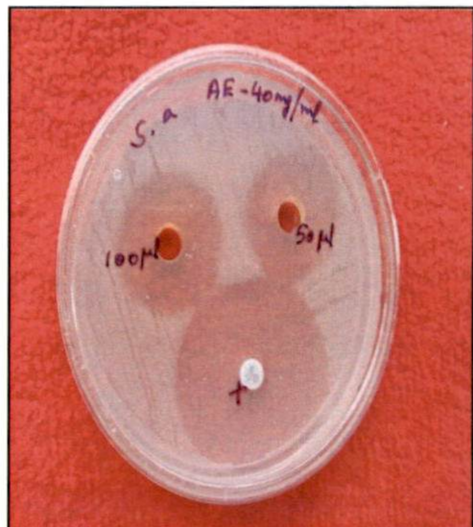
AE at 5mg/ml



AE at 10mg/ml

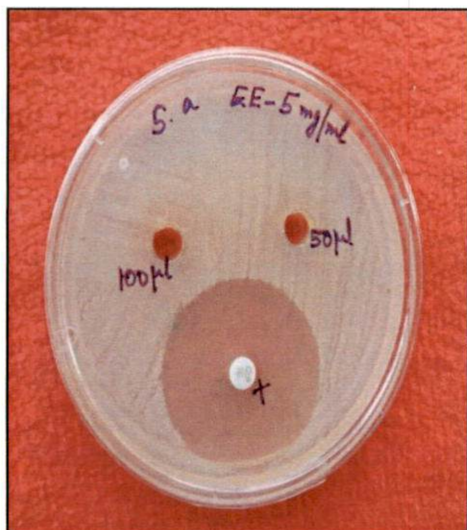


AE at 20mg/ml

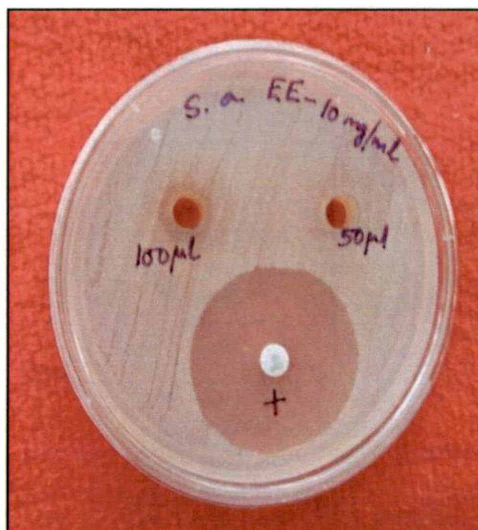


AE at 40mg/ml

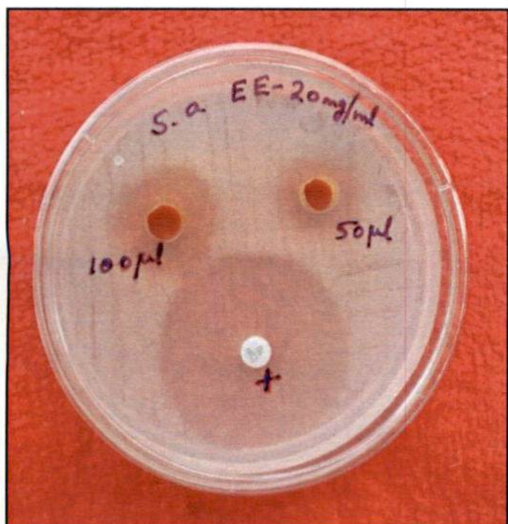
Plate 39..Well diffusion study of *Staphylococcus aureus*-- Ethanol extract(EE)



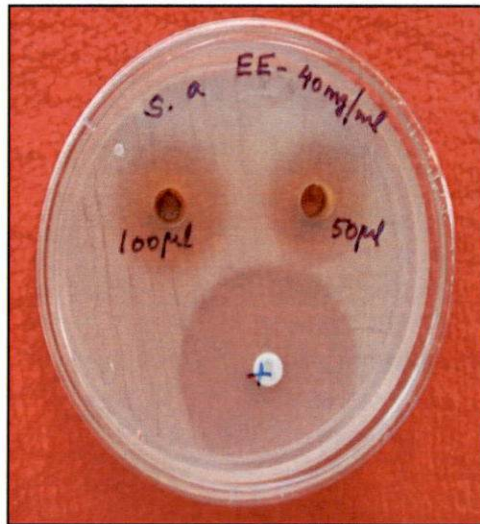
EE at 5mg/ml



EE at 10mg/ml

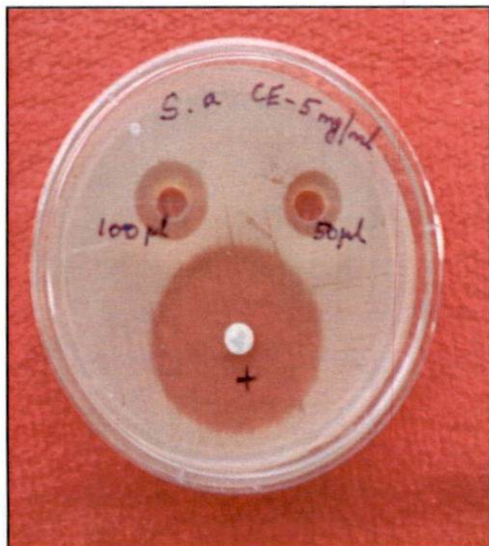


EE at 20mg/ml

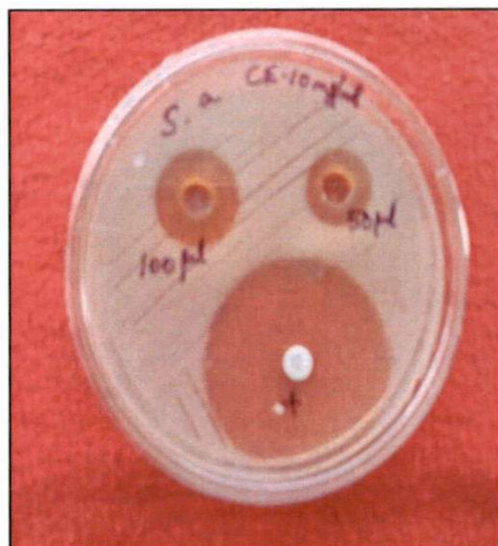


EE at 40mg/ml

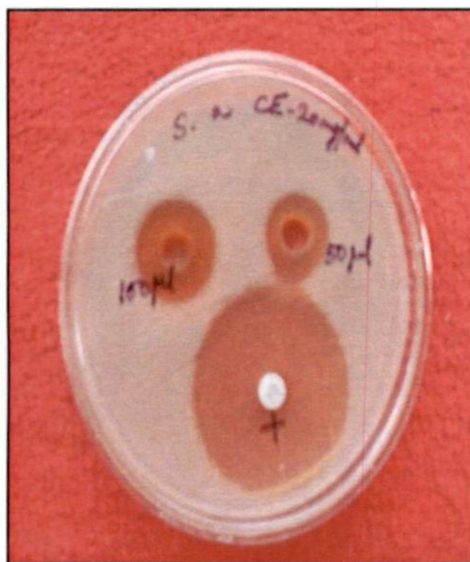
Plate 40.Well diffusion study of *Staphylococcus aureus*-- Chloroform extract(CE)



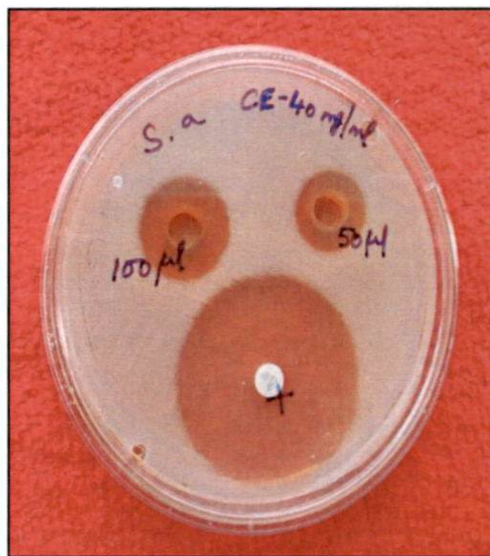
CE at 5mg/ml



CE at 10mg/ml



CE at 20mg/ml



CE at 40mg/ml

When the extract volume was increased to 100 µl, same trend was noticed for the inhibition of bacterial growth. The maximum inhibition for *Staphylococcus aureus* was observed for the reference antibiotic (40 mm). Among the extracts, highest zone of inhibition was noticed in AE at 40 mg/ml (29.25 mm), which was significantly superior. The next best treatment was AE at 20 mg/ml (27.50 mm), followed by AE at 10 mg/ml (23.50 mm) and by CE (23.50 mm) (Plate 28).

4.3. Evaluation of *Parmotrema tinctorum* for preservation of processed products

Evaluation of *Parmotrema tinctorum* for preservation of processed products was done by both by sensory evaluation and by enumeration of microorganisms in the products (Plate 29 and 30).

4.3.1. Sensory evaluation

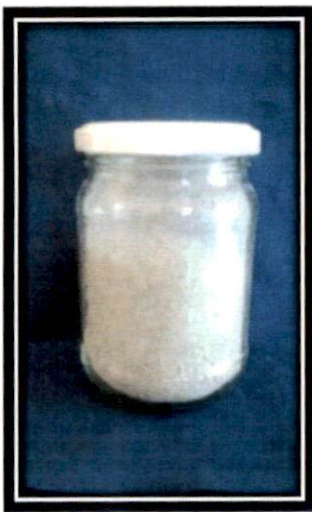
In the sensory evaluation of the two products *viz.* lime pickle and tomato sauce based on eight characters *viz.* appearance, colour, flavour, texture, odour, taste, aftertaste and overall acceptability, the characters were scored based on 9 point hedonic scale. Effect of storage on the different organoleptic parameters was evaluated at monthly intervals with a panel of 15 judges. Means of the scores of the judges were ranked for each of the eight characters at monthly intervals and results are given in Tables 24 to 37.

4.3.1.1. Lime pickle

With regard to appearance of the product lime pickle, a significant difference could be observed between the different treatments from fifth month of storage. The Kendall's coefficient of concordance was proved to be non significant upto fifth month of storage indicating that the judges were not in a position to differentiate the different treatments. However up to fifth month of storage most of the treatments recorded a score value of around seven, showing that the products were moderately liked. The treatment T_4 (4.75) registered the highest mean score for appearance, followed by T_2(4.56) and T_3(4.50). The judgement regarding parameters showed that the rank order as T_3(4.95), T_2(4.68), and T_1(4.44) at the sixth month of storage.

Plate 41. ***Parmotrema tinctorum*** **lichen for addition in food products**

a) Whole lichen



b) Lichen powder

c) Ethanol extract- before and after rotary evaporation

In the case of colour of lime pickle also, the Kendall's coefficient of concordance was found to be non significant upto fifth month of storage indicating that the judges were not in a position to differentiate the different treatments. Most of the treatments recorded a score value of around seven, showing that the products were moderately liked. The treatment T_6(4.30) attained the highest mean score for appearance, followed by T_2(4.26) and T_1(4.20). The judgement regarding parameters showed that the rank order as T_3(4.95), T_2(4.68), and T_1(4.44) at the sixth month of storage (Plate 30).

The mean rank score for flavour of lime pickle was highest for T_3 (4.60) and lowest for T_6 (4.56) initially. After six months of storage, the highest mean score for the flavour of lime pickle was recorded in T_6 with a score of 4.29, followed by T_5 (3.82); while the lowest score was recorded in treatment T_8(3.00). A decreasing trend was observed for this character also during the storage period.

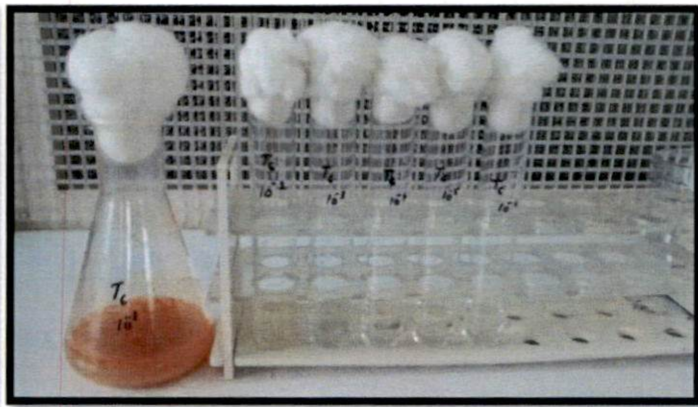
The Kendall's coefficient of concordance was found to be non significant for the character, texture of lime pickle upto three months of storage. The highest mean rank score was recorded by the treatment T_6 from the third to sixth month of storage.

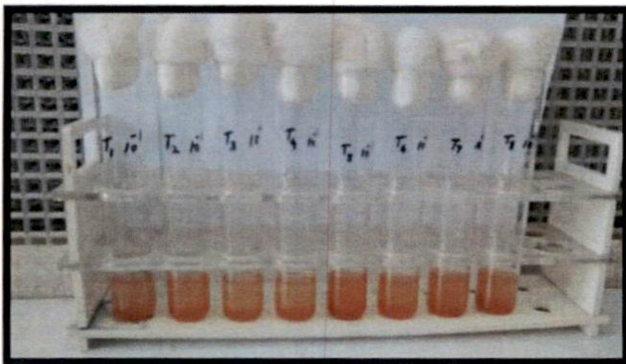
The mean rank scores for odour of lime pickle was found to be decreasing during storage in T_1 and T_2. In all other treatments, the rank score gradually increased upto three months, then decreased. The highest mean score for odour was recorded in T_2 (5.23) initially, followed by T_6 with a score of 4.90. After storage for six months, it was T_6 which recorded the highest score (5.20) followed by T_5 (4.56)

The mean rank scores recorded for the character taste was also found to be gradually increasing in the first three months of storage, but found to be decreasing thereafter for all the treatments. Taste recorded the highest rank score value for the treatment T_1 (4.80), followed by T_3 (4.77) and by T_2 (4.68). When the products were evaluated after storage period of six months, T_6 registered the highest with a score of 5.14, followed by T_3 (3.88). The lowest score for taste was obtained for T_5 (2.90).

Plate 42.Evaluation of the products

a) Sensory evaluation





b) Microbiological analysis of products

With respect to the character after taste of the product, the mean rank scores showed an increasing trend in the first three months of storage, then declined gradually. The highest score for after taste before the storage was recorded by T_1 with a score of 4.43, followed by T_3 (4.32) and T_3 (4.14). After storage of the product, T_1 recorded the highest score (4.30) for after taste, followed by T_2 (4.24).

The character overall acceptability increased gradually up to three months and then showed a decreasing pattern. Initially the highest mean score for overall acceptability was recorded in T_3 (4.89) followed by T_2 (4.77); and lowest in T_4 (4.00). After six months storage period, T_3 and T_6 recorded highest for the character, with mean rank scores of 4.05 and 3.60 respectively, while lowest mean score was observed for T_1 (3.30). All the treatments marked a mean score within the acceptable levels.

4.3.1.2. Tomato sauce (pasteurized)

When the parameter appearance of the product was analysed, the Kendall's coefficient of concordance was proved to be non significant up to fourth month of storage indicating that the judges were not in a position to differentiate the different treatments. But up to fourth month of storage most of the treatments recorded a score value of around seven, indicating that the products were moderately liked. At fifth month of storage, a differentiation could be judged between the different treatments with respect to appearance. It was observed that the treatment T_2 (5.75), had the highest mean score for appearance, followed by T_3 (5.70) and T_1(4.15). At the sixth month of storage, the judgement regarding parameters showed the rank order as T_2 (3.94), T_3 (3.90), and T_4 (3.80).

The mean rank scores for colour suggests that upto three months of storage, the judges were not able to distinguish between different treatments with respect to colour. From fourth month of storage, the treatments could be differentiated regarding colour of the product. (Plate 31). Among the treatments, the highest rank score was showed by T_1 (5.00) followed by T_2 (4.30),and T_6 (4.15). When analysed after storage period of six months, the highest rank score was observed in T_2 (4.00), followed by T_3 (3.68).

The mean rank scores recorded for flavour were found to be decreasing gradually throughout the storage period. Initially the highest mean score was registered by T_3 7.15(5.50),

followed by T_4(5.23) and the lowest by T_8 (3.75) After six months storage, the mean rank score was found to be highest for T_6 (4.60), followed by T_3 (4.35).

The mean rank scores of texture of the product was also found to be showing a declining pattern. The treatments could be differentiated by the judges with respect to texture only during fifth and sixth months of storage. The treatment T_5 (4.70) recorded the highest rank score for texture in sixth month of storage, followed by T_2 (3.90) and T_1.

The mean rank scores of odour for the treatments T_1 to T_4 gradually decreased throughout the storage, whereas in treatments T_5 to T_8, the mean scores increased upto third month of storage, then showed a decreasing trend. Before storage, the highest mean score was observed in T_3 (5.45), followed by T_4 (6.70). After the storage period, the mean score was found to be highest for T_4 (4.22), and lowest for T_8 (3.10).

The mean rank scores for taste of tomato sauce was recorded highest for T_1 (6.10), followed by T_3 (5.90) initially. The mean score was found to be increasing from 1 MAS to 3 MAS, which then decreased from 4 MAS onwards in treatments T_6 to T_8. Treatment T_3 scored the highest (4.33), followed by T_4 (4.00) and T_2 (4.00); while the treatment T_8 recorded the lowest mean score for taste (3.40), after storage for six months.

While analyzing the mean score for after taste of the product initially, it was found that T_1 had the highest mean score (5.80), followed by T_2 (5.55). The treatments T_3 (4.20) scored highest for after taste followed by T_1 (4.11) after six months. The mean scores for after taste for the treatments T_1 to T_5 were found to be gradually decreasing during storage. The mean scores for after taste initially recorded a lower value, then gradually increased upto 3 MAS, and then decreased thereafter for the treatments T_6 to T_8.

Table 24. Sensory attributes of lime pickle at the beginning of storage study

Treatments	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	8.00 (5.82)	8.00 (5.91)	7.00 (4.30)	7.10 (4.30)	7.10 (4.90)	7.10 (4.80)	6.63 (4.43)	6.36 (4.77)
T_2	7.90 (5.68)	7.80 (5.40)	7.10 (4.50)	7.43 (5.14)	6.90 (5.23)	6.82 (4.68)	6.36 (4.14)	7.00 (4.39)
T_3	7.70 (5.65)	7.70 (5.20)	7.40 (4.60)	7.14 (4.57)	6.70 (4.21)	7.00 (4.77)	5.90 (4.32)	6.63 (4.89)
T_4	7.71 (5.58)	7.70 (5.70)	7.60 (4.44)	7.13 (5.12)	6.80 (4.35)	6.91 (4.31)	5.81 (4.09)	6.39 (4.40)
T_5	7.63 (5.00)	7.71 (4.82)	7.14 (4.50)	7.00 (4.43)	6.88 (4.32)	6.81 (4.20)	5.32 (4.09)	7.00 (4.00)
T_6	7.90 (5.40)	7.71 (5.45)	7.00 (4.56)	7.70 (4.00)	6.86 (3.68)	7.07 (4.59)	5.90 (4.00)	6.74 (4.41)
T_7	8.00 (5.00)	7.64 (5.75)	7.05 (4.51)	7.10 (4.60)	6.70 (4.30)	6.91 (4.15)	5.81 (4.00)	6.55 (4.27)
T_8	7.70 (5.10)	7.70 (5.55)	7.00 (4.12)	7.07 (5.60)	6.90 (3.50)	6.75 (3.81)	5.45 (3.68)	6.50 (4.18)
Kendall's coefficient	0.757^{NS}	0.041^{NS}	0.055*	0.064^{NS}	0.062*	0.072**	0.057*	0.060*

Figures in parenthesis indicate mean rank scores. * Significant at 1% level, ** Significant at 5% level, NS-non significant. T_1 – Control(No preservative added), T_2 – Sodium benzoate 250 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.1%, T_7 – *P.tinctorum* extract 0.2%, T_8 – *P.tinctorum* extract 0.3%

Table 25. Sensory aributes of lime pickle one month after storage

Treatments	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	7.81 (5.23)	7.80 (5.86)	7.10 (4.50)	7.20 (3.60)	7.10 (4.86)	7.20 (4.95)	6.55 (4.76)	7.90 (4.80)
T_2	7.80 (5.44)	7.79 (5.04)	7.29 (4.61)	7.45 (5.77)	7.50 (5.20)	7.00 (4.80)	6.73 (4.65)	7.00 (4.65)
T_3	7.60 (5.50)	7.54 (4.82)	7.57 (5.11)	7.27 (3.82)	6.72 (4.32)	7.60 (5.05)	6.82 (4.35)	7.10 (4.90)
T_4	7.65 (5.40)	7.57 (4.94)	7.36 (4.95)	7.21 (4.36)	7.00 (4.59)	7.13 (4.70)	6.18 (4.18)	6.80 (4.70)
T_5	7.60 (4.96)	7.63 (4.45)	7.20 (4.70)	7.13 (4.81)	7.21 (4.69)	7.00 (4.55)	6.36 (4.14)	7.09 (4.80)
T_6	7.54 (5.31)	7.60 (4.86)	7.13 (4.95)	7.63 (4.73)	7.00 (4.91)	7.09 (4.24)	6.57 (4.54)	6.90 (4.50)
T_7	7.64 (5.21)	7.60 (4.65)	7.20 (4.60)	7.00 (4.50)	6.91 (4.55)	6.93 (4.65)	6.55 (4.32)	7.00 (4.40)
T_8	7.54 (4.55)	7.63 (4.70)	7.21 (4.12)	7.20 (3.95)	6.80 (4.45)	7.00 (4.85)	6.27 (4.15)	7.00 (4.90)
Kendall's coefficient	0.212^{NS}	0.248^{NS}	0.0490**	0.115^{NS}	0.273**	0.513*	0.116**	0.176*

Figures in parenthesis indicate mean rank scores. * Significant at 1% level, ** Significant at 5% level, NS-non significant
T_1 – Control (No preservative added), T_2 – Sodium benzoate 250 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.1%, T_7 – *P.tinctorum* extract 0.2%, T_8 – *P.tinctorum* extract 0.3%

Table 26. Sensory attributes of lime pickle two months after storage

Treatments	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	7.21 (5.00)	7.70 (5.35)	7.13 (4.65)	7.21 (4.61)	7.21 (4.61)	7.20 (5.00)	7.00 (4.90)	7.21 (4.95)
T_2	7.50 (5.36)	7.72 (4.82)	7.09 (5.36)	7.72 (4.86)	7.72 (4.86)	7.36 (4.96)	7.07 (4.77)	7.28 (4.82)
T_3	7.54 (5.30)	7.50 (4.80)	7.14 (5.10)	7.30 (4.05)	7.30 (4.05)	7.21 (5.47)	6.93 (4.43)	7.20 (5.10)
T_4	7.60 (5.40)	7.20 (4.85)	7.25 (5.25)	7.45 (4.23)	7.45 (4.23)	7.64 (5.05)	7.14 (4.25)	7.60 (4.90)
T_5	7.57 (4.32)	7.50 (3.55)	7.43 (5.50)	7.20 (4.80)	7.20 (4.80)	7.60 (4.94)	6.57 (4.94)	7.57 (4.96)
T_6	7.50 (5.25)	7.60 (4.82)	7.29 (5.45)	7.70 (5.25)	7.70 (5.25)	7.20 (5.25)	6.91 (4.81)	7.21 (5.50)
T_7	7.54 (4.36)	7.60 (4.54)	7.27 (4.81)	7.45 (4.27)	7.45 (4.27)	7.20 (4.96)	6.57 (4.95)	7.35 (4.64)
T_8	7.50 (4.55)	7.63 (4.70)	7.25 (4.89)	7.27 (3.82)	7.27 (3.82)	7.10 (4.90)	6.93 (4.75)	7.10 (5.05)
Kendall's coefficient	0.757^{NS}	0.404^{NS}	0.648*	0.981^{NS}	0.995**	0.633*	0.236*	0.964*

Figures in parenthesis indicate mean rank scores. * Significant at 1% level, ** Significant at 5% level, NS-non significant
T_1 – Control (No preservative added), T_2 – Sodium benzoate 250 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.1%, T_7 – *P.tinctorum* extract 0.2%, T_8 – *P.tinctorum* extract 0.3%

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Table 27. Sensory attributes of lime pickle three months after storage

Treatments	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	7.20 (4.88)	7.50 (5.20)	7.20 (5.05)	7.27 (5.27)	7.29 (4.36)	7.25 (5.75)	7.63 (5.00)	7.40 (5.69)
T_2	7.50 (5.20)	7.40 (4.45)	6.81 (4.84)	7.30 (3.70)	7.55 (4.67)	7.50 (5.23)	7.50 (4.82)	7.30 (5.05)
T_3	7.35 (4.95)	7.30 (4.32)	7.90 (5.42)	7.00 (4.55)	7.50 (4.96)	7.81 (5.75)	7.40 (5.36)	7.35 (5.14)
T_4	7.60 (5.38)	7.50 (4.40)	6.80 (5.31)	7.70 (5.05)	7.00 (4.77)	7.30 (5.05)	7.10 (5.09)	7.71 (5.18)
T_5	7.00 (3.95)	7.00 (3.60)	7.45 (5.77)	7.90 (5.75)	7.60 (5.55)	7.70 (5.45)	7.60 (5.05)	7.60 (5.23)
T_6	7.50 (5.21)	7.45 (4.55)	7.30 (5.81)	7.63 (6.19)	7.25 (5.69)	7.50 (5.30)	7.00 (5.10)	7.60 (5.81)
T_7	7.10 (4.45)	7.50 (4.30)	7.40 (5.00)	7.50 (4.65)	7.50 (5.94)	7.13 (5.25)	7.50 (5.06)	7.25 (5.65)
T_8	7.21 (4.32)	7.50 (4.59)	7.54 (5.15)	7.43 (5.07)	7.36 (5.04)	7.20 (5.20)	7.10 (4.93)	7.64 (5.21)
Kendalls coefficient	0.197^{NS}	0.058^{NS}	0.138**	0.169*	0.080*	0.169*	0.057*	0.097**

Figures in parenthesis indicate mean rank scores. * Significant at 1% level, ** Significant at 5% level, NS-non significant
T_1 – Control (No preservative added), T_2 – Sodium benzoate 250 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.1%, T_7 – *P.tinctorum* extract 0.2%, T_8 – *P.tinctorum* extract 0.3%

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Table 28. Sensory attributes of lime pickle four months after storage

	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	7.00 (4.44)	7.27 (4.85)	6.90 (4.30)	7.05 (4.81)	6.88 (4.10)	7.21 (4.98)	7.10 (4.84)	7.25 (4.50)
T_2	7.28 (4.68)	6.90 (4.40)	6.75 (3.90)	7.10 (4.65)	6.75 (4.45)	6.75 (3.94)	6.63 (4.50)	7.09 (4.10)
T_3	7.20 (4.85)	7.30 (4.11)	6.70 (3.80)	7.09 (4.59)	7.00 (5.05)	6.70 (5.20)	6.78 (4.25)	7.27 (5.03)
T_4	7.50 (5.20)	7.35 (4.32)	6.90 (4.76)	6.91 (4.55)	6.60 (4.40)	6.76 (3.86)	6.88 (4.0)	6.50 (4.56)
T_5	6.88 (3.90)	6.80 (3.60)	6.88 (4.25)	7.09 (4.55)	6.80 (4.60)	6.75 (4.50)	6.75 (5.09)	7.00 (4.69)
T_6	7.35 (4.74)	7.20 (4.44)	7.00 (4.50)	7.81 (5.14)	7.09 (4.73)	7.30 (5.75)	6.60 (4.18)	7.80 (5.35)
T_7	7.10 (4.40)	7.50 (4.04)	6.73 (4.30)	6.91 (4.14)	7.38 (4.65)	7.50 (4.65)	6.13 (4.22)	7.38 (4.95)
T_8	7.20 (4.25)	7.18 (4.05)	6.55 (4.00)	7.00 (4.25)	6.73 (4.23)	7.29 (4.14)	6.63 (4.02)	6.90 (4.25)
Kendall's coefficient	0.343^{NS}	0.337^{NS}	0.106**	0.155*	0.069*	0.099**	0.298*	0.111**

Figures in parenthesis indicate mean rank scores. * Significant at 1% level, ** Significant at 5% level, NS-non significant
T_1 – Control (No preservative added), T_2 – Sodium benzoate 250 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.1%, T_7 – *P.tinctorum* extract 0.2%, T_8 – *P.tinctorum* extract 0.3%

Table 29. Sensory attributes of lime pickle five months after storage

	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	6.91 (4.05)	7.25 (4.20)	6.82 (4.07)	6.95 (5.20)	6.20 (3.60)	6.91 (4.00)	7.00 (4.56)	7.10 (4.14)
T_2	7.13 (4.75)	6.75 (4.26)	6.70 (3.81)	6.75 (3.31)	6.70 (4.36)	6.70 (3.75)	6.60 (4.31)	6.70 (3.60)
T_3	7.13 (4.50)	6.84 (4.05)	6.54 (3.70)	6.75 (3.19)	7.00 (5.00)	6.25 (3.95)	6.50 (4.20)	7.00 (4.30)
T_4	6.91 (4.56)	7.13 (4.10)	6.72 (4.41)	6.90 (5.20)	6.54 (4.27)	6.30 (3.65)	6.70 (4.00)	6.64 (4.05)
T_5	6.50 (3.00)	6.75 (3.40)	6.44 (3.91)	6.60 (4.20)	6.60 (3.90)	6.50 (3.50)	6.60 (4.10)	6.80 (3.95)
T_6	7.00 (4.24)	7.00 (4.30)	6.91 (4.44)	7.00 (5.35)	6.90 (4.70)	7.00 (5.25)	6.90 (4.80)	7.20 (4.77)
T_7	6.75 (4.15)	7.00 (3.95)	6.72 (4.15)	6.75 (4.65)	6.54 (4.23)	6.70 (4.24)	6.70 (4.50)	6.91 (4.45)
T_8	7.00 (4.00)	7.10 (4.00)	6.50 (3.50)	6.90 (4.31)	6.50 (3.50)	6.55 (3.95)	7.00 (3.94)	6.88 (4.20)
Kendall's coefficient	0.119**	0.170**	0.198**	0.221*	0.247*	0.238*	0.080**	0.263**

Figures in parenthesis indicate mean rank scores. * Significant at 1% level, ** Significant at 5% level, NS-non significant
T_1 – Control (No preservative added), T_2 – Sodium benzoate 250 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.1%, T_7 – *P.tinctorum* extract 0.2%, T_8 – *P.tinctorum* extract 0.3%

Table 30. Sensory attributes of lime pickle six months after storage

	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	6.88 (3.90)	7.20 (3.60)	6.80 (3.40)	6.20 (3.80)	6.00 (3.59)	6.54 (3.60)	6.55 (4.30)	6.90 (3.30)
T_2	6.80 (4.38)	6.45 (4.12)	6.60 (3.75)	6.70 (3.92)	6.20 (3.95)	6.54 (3.70)	6.10 (4.24)	6.63 (3.50)
T_3	6.64 (4.14)	6.54 (4.00)	6.38 (3.65)	6.60 (4.15)	6.50 (4.56)	5.90 (3.88)	6.45 (4.15)	7.00 (4.05)
T_4	6.65 (3.45)	7.00 (3.59)	6.50 (3.60)	6.80 (3.95)	6.25 (4.19)	5.63 (3.52)	6.35 (4.10)	6.60 (3.43)
T_5	5.73 (2.18)	6.09 (3.00)	6.25 (3.82)	6.64 (3.36)	6.55 (3.32)	6.36 (2.90)	6.24 (3.86)	6.55 (3.07)
T_6	6.64 (4.30)	7.00 (4.10)	6.90 (4.29)	6.86 (4.45)	6.70 (5.20)	6.64 (5.14)	6.88 (4.11)	7.00 (3.60)
T_7	6.45 (3.59)	7.00 (3.50)	6.60 (3.47)	6.30 (3.15)	6.50 (4.00)	6.09 (4.11)	6.90 (4.00)	6.80 (3.52)
T_8	6.27 (3.45)	6.63 (3.44)	6.36 (3.00)	6.64 (3.77)	6.50 (3.50)	5.81 (3.92)	7.00 (3.73)	6.45 (3.45)
Kendalls coefficient	0.197*	0.161**	0.097**	0.211**	0.130*	0.187*	0.974*	0.123**

Figures in parenthesis indicate mean rank scores. * Significant at 1% level, ** Significant at 5% level, NS-non significant
T_1 – Control (No preservative added), T_2 – Sodium benzoate 250 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.1%, T_7 – *P.tinctorum* extract 0.2%, T_8 – *P.tinctorum* extract 0.3%

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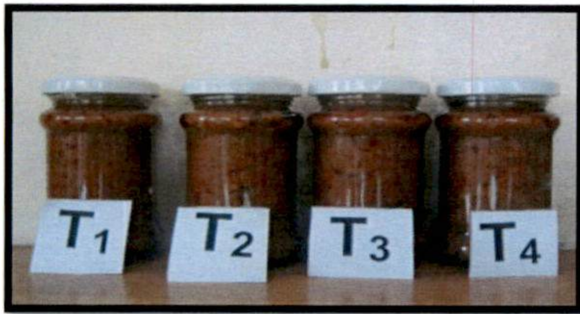
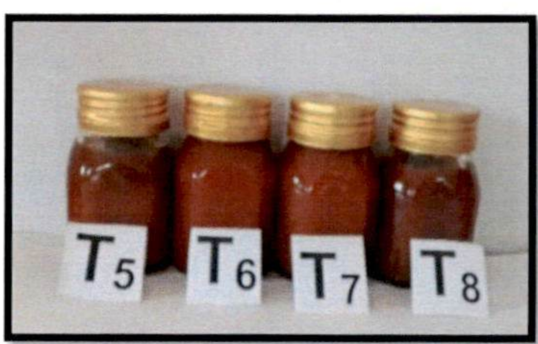
Plate 43. Storage study of processed products

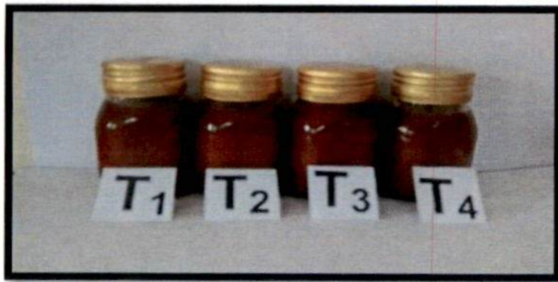
a) Lime pickle -initial



b)Lime pickle -6 MAS

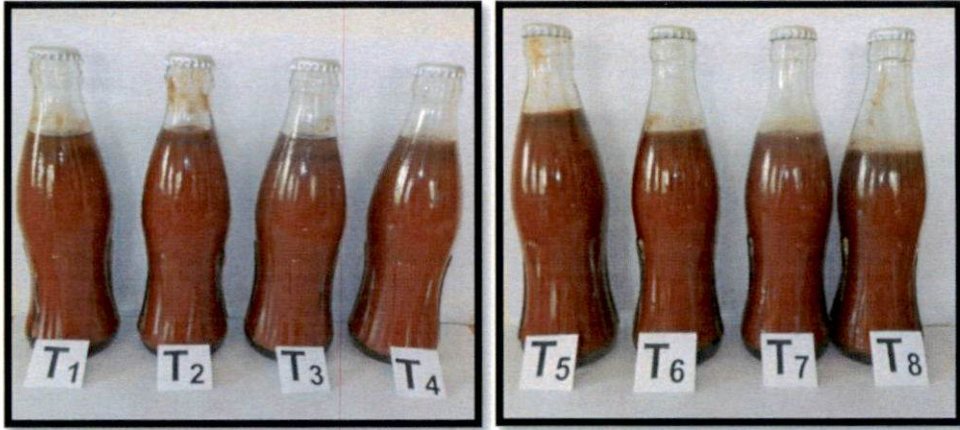


c) Unpasteurised tomato sauce- initial

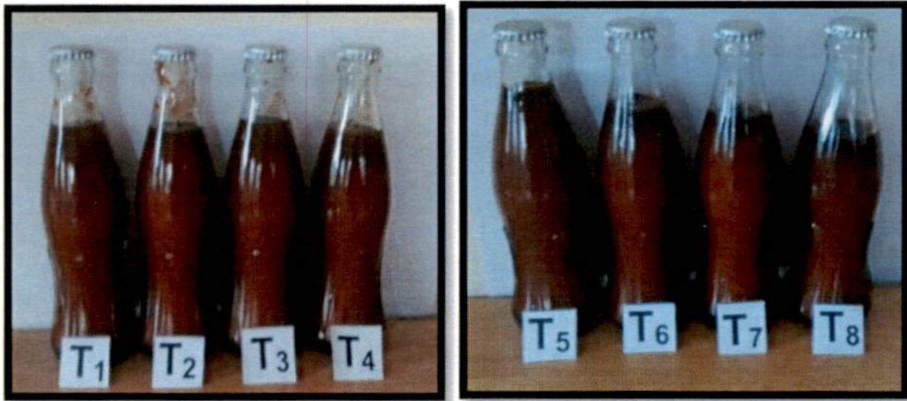


d) Unpasteurised tomato sauce- 6 MAS

Plate 44. Storage study of pasteurized tomato sauce



a) Pasteurised tomato sauce -initial



b) Pasteurised tomato sauce- 6 MAS

For the treatments, T_5 to T_8, the mean scores for overall acceptability showed an increasing trend first upto 3 MAS, then decreased gradually over the storage period of six months. But the mean scores for T_1 to T_4 showed a gradual decrease in overall acceptability of the product. The highest mean score was observed for T_3 (6.12) followed by T_1 7.20), while the treatments T_5 to T_8 recorded a lower mean score initially. The treatments T_2 (4.35) recorded the highest mean score after six months storage period; followed by T_1 (4.20).

4.3.2. Evaluation of microbial load in the products

The microbial populations of the stored products were assessed at monthly intervals for a period of six months as per the serial dilution and plate count method. Bacteria, fungi and yeast were the major groups of microorganisms observed in the samples.

4.3.2.1. Lime pickle

Bacterial population was noticed in the product throughout the storage period. Initially the population were very less but as the storage advanced, bacterial populations increased steadily in all the samples. After sixth month of storage T_5 and T_4 (77.7 X 10^6 CFU/g and 100.1 X10^6 CFU/g respectively) recorded the highest bacterial count. The population was found to be significantly lowest in T_8 (13.6 X 10^6 CFU/g) and in T_7 (16.6 X10^6 CFU/g). The control treatment recorded a bacterial count of 64.5 X10^6 CFU/g. (Table 38). In all the treatments, fungal colonies were detected after third month of storage, except in T_6, T_7 and T_8. After storage period of five months, fungi was noticed in all the treatments, lowest in T_8 (1.0 X 10^3 CFU/g) and highest in T_1 (1.4 X 10^3 CFU/g). This trend was noticed in the next month also, with the highest fungal load in T_1 (70.0 X 10^3 CFU/g) and lowest in T_8 ((1.0 X 10^3 CFU/g). Fungal growth was detected in the treatment T_2 after sixth month of storage (Table 39). Yeast colonies were observed from fourth month of storage, but the count was found to non significant among the treatments on analysis 4 MAS and 5 MAS. Significant yeast population was observed after sixth month of storage (Table 40). T_1 (control) showed the highest count of 7.0 X 10^3 CFU/g, followed by T_3 (5.0 X 10^3 CFU/g). Lowest count was observed in T_6 and in T_7 (1.0 X10^3 CFU/g)

Table 31. Sensory attributes of tomato sauce at the beginning of storage study

Treatments	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	7.60 (5.40)	7.70 (5.77)	7.06 (5.20)	7.20 (5.70)	7.00 (5.30)	7.50 (6.10)	7.33 (5.80)	7.20 (6.00)
T_2	7.53 (5.70)	7.66 (5.85)	7.13 (5.20)	7.33 (5.85)	7.13 (4.93)	7.06 (4.60)	6.93 (5.55)	7.20 (5.55)
T_3	7.60 (5.75)	7.80 (5.80)	7.15 (5.50)	7.26 (5.75)	7.20 (5.45)	7.30 (5.90)	7.06 (4.85)	7.10 (6.12)
T_4	7.53 (5.40)	7.50 (5.66)	7.13 (5.23)	7.20 (5.25)	7.23 (5.35)	7.13 (5.14)	7.00 (5.35)	7.00 (5.25)
T_5	7.66 (5.60)	7.20 (5.60)	7.06 (5.04)	7.33 (5.40)	6.80 (5.15)	6.60 (4.70)	6.40 (4.50)	6.90 (5.10)
T_6	7.70 (5.45)	7.80 (5.80)	7.00 (4.18)	7.46 (5.50)	6.70 (4.64)	6.80 (4.60)	6.50 (4.39)	6.66 (4.14)
T_7	7.70 (5.25)	7.80 (5.55)	6.50 (3.97)	7.33 (5.10)	6.70 (4.46)	6.70 (4.40)	6.20 (4.43)	6.80 (4.43)
T_8	7.45 (5.35)	7.30 (5.18)	6.30 (3.75)	7.26 (5.25)	6.60 (4.35)	6.50 (4.35)	6.00 (4.00)	6.60 (4.40)
Kendall's coefficient	0.206^{NS}	0.185^{NS}	0.046**	0.017^{NS}	0.031**	0.077**	0.569*	0.087**

Figures in parenthesis indicate mean rank scores * Significant at 1% level, ** Significant at 5% level,, NS-non significant
T_1 – Control (No preservative added), T_2 – Sodium benzoate 750 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.025%, T_7 – *P.tinctorum* extract 0.05%, T_8 – *P.tinctorum* extract 0.1%

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Table 32. Sensory attributes of tomato sauce one month after storage

Treatments	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	6.93 (5.30)	7.70 (6.00)	7.00 (4.95)	7.20 (5.45)	7.00 (5.05)	7.30 (5.65)	6.90 (5.50)	7.10 (5.95)
T_2	7.50 (5.05)	7.40 (5.57)	7.05 (5.10)	7.10 (4.96)	7.00 (4.85)	7.00 (4.50)	6.90 (5.25)	6.70 (5.45)
T_3	7.60 (4.55)	7.30 (4.45)	7.10 (5.35)	7.00 (4.75)	6.90 (4.65)	7.20 (5.21)	7.00 (4.65)	7.00 (5.45)
T_4	7.40 (4.60)	7.40 (4.85)	6.80 (4.55)	7.20 (4.70)	6.93 (5.00)	7.00 (5.10)	6.80 (5.11)	6.80 (5.11)
T_5	7.60 (5.55)	7.00 (4.75)	6.70 (4.75)	7.20 (5.36)	7.10 (4.95)	6.40 (4.65)	6.46 (4.65)	7.00 (5.20)
T_6	7.26 (4.80)	7.60 (4.95)	7.06 (4.80)	7.00 (4.61)	6.90 (4.90)	6.80 (4.80)	7.06 (4.45)	6.80 (4.39)
T_7	7.60 (5.15)	7.60 (4.95)	6.52 (4.30)	7.00 (4.64)	7.00 (4.95)	7.00 (4.90)	6.70 (4.60)	7.00 (4.90)
T_8	7.40 (4.79)	7.30 (5.15)	6.40 (3.97)	7.00 (5.25)	7.06 (4.90)	6.80 (4.70)	6.40 (4.00)	7.00 (4.05)
Kendall's coefficient	0.159^{NS}	0.106^{NS}	0.100**	0.225^{NS}	0.149**	0.040**	0.020**	0.92**

Figures in parenthesis indicate mean rank scores * Significant at 1% level, ** Significant at 5% level,, NS-non significant
T_1 – Control (No preservative added), T_2 – Sodium benzoate 750 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.025%, T_7 – *P.tinctorum* extract 0.05%, T_8 – *P.tinctorum* extract 0.1%

Table 33. Sensory attributes of tomato sauce two months after storage

Treatments	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	6.90 (5.30)	7.60 (5.65)	6.80 (4.90)	7.10 (4.90)	6.90 (4.65)	7.00 (5.35)	7.10 (5.50)	7.00 (5.75)
T_2	7.50 (4.80)	7.20 (4.95)	6.70 (4.95)	7.00 (4.90)	6.50 (4.83)	6.85 (4.50)	6.70 (5.00)	6.60 (5.15)
T_3	7.40 (4.55)	7.30 (4.25)	7.00 (4.85)	6.80 (4.60)	6.70 (4.54)	7.20 (5.20)	7.00 (5.32)	6.90 (4.55)
T_4	7.40 (4.55)	7.20 (4.70)	6.60 (4.45)	6.70 (4.65)	6.90 (5.00)	6.80 (4.50)	6.50 (4.80)	6.60 (4.80)
T_5	7.60 (5.00)	7.00 (3.90)	6.70 (4.50)	7.10 (5.40)	7.20 (4.90)	6.20 (4.55)	6.60 (4.80)	7.10 (4.70)
T_6	7.00 (4.50)	7.45 (4.82)	7.10 (4.85)	6.70 (4.60)	7.20 (5.25)	7.26 (5.36)	6.90 (4.60)	7.06 (4.60)
T_7	7.46 (5.10)	7.46 (4.54)	6.60 (4.40)	6.90 (4.60)	7.13 (5.05)	7.20 (5.03)	6.80 (4.85)	7.00 (4.60)
T_8	7.30 (4.20)	7.20 (4.85)	6.60 (4.75)	7.00 (4.32)	7.20 (5.55)	7.13 (4.50)	6.70 (4.87)	7.06 (4.39)
Kendall's coefficient	0.728^{NS}	0.025^{NS}	0.621**	0.160^{NS}	0.888**	0.124**	0.459**	0.128**

Figures in parenthesis indicate mean rank scores * Significant at 1% level, ** Significant at 5% level,, NS-non significant
T_1 – Control (No preservative added), T_2 – Sodium benzoate 750 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.025%, T_7 – *P.tinctorum* extract 0.05%, T_8 – *P.tinctorum* extract 0.1%

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Table 34. Sensory attributes of tomato sauce three months after storage

Treatments	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	6.80 (4.50)	7.30 (5.40)	6.70 (4.60)	7.00 (4.14)	6.80 (4.61)	6.93 (5.30)	6.70 (5.15)	6.80 (5.35)
T_2	7.10 (4.75)	7.20 (4.40)	6.70 (4.80)	7.00 (4.50)	6.40 (4.70)	6.20 (4.36)	6.70 (5.00)	6.50 (5.00)
T_3	7.00 (4.35)	7.13 (4.14)	7.00 (4.83)	6.50 (4.55)	6.50 (4.40)	7.00 (5.15)	6.90 (4.50)	7.33 (5.17)
T_4	7.00 (4.20)	7.00 (4.20)	6.60 (4.40)	6.30 (4.43)	6.70 (4.65)	6.80 (4.40)	6.40 (4.40)	6.70 (4.50)
T_5	7.40 (4.55)	7.00 (3.35)	6.40 (3.91)	6.90 (5.15)	7.00 (4.75)	6.20 (4.50)	6.30 (4.40)	6.50 (4.45)
T_6	6.90 (4.50)	7.30 (4.10)	7.10 (5.40)	6.70 (4.45)	7.00 (4.95)	6.60 (4.71)	7.20 (4.95)	7.20 (4.75)
T_7	7.10 (5.00)	7.00 (4.10)	7.00 (4.89)	6.80 (4.55)	6.60 (4.35)	6.90 (4.85)	6.90 (5.00)	7.10 (5.75)
T_8	7.06 (3.80)	7.10 (4.23)	6.90 (4.77)	6.30 (3.65)	6.70 (4.35)	6.90 (4.80)	6.80 (4.25)	7.10 (4.65)
Kendall's coefficient	0.405^{NS}	0.004^{NS}	0.836**	0.567^{NS}	0.736*	0.343**	0.682**	0.482*

Figures in parenthesis indicate mean rank scores * Significant at 1% level, ** Significant at 5% level,, NS-non significant
T_1 – Control (No preservative added), T_2 – Sodium benzoate 750 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.025%, T_7 – *P.tinctorum* extract 0.05%, T_8 – *P.tinctorum* extract 0.1%

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Table 35. Sensory attributes of tomato sauce four months after storage

Treatments	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	6.70 (4.40)	7.20 (5.05)	6.40 (4.15)	6.50 (4.10)	6.60 (4.20)	6.80 (5.10)	6.70 (5.05)	6.70 (4.90)
T_2	6.70 (4.50)	7.10 (4.30)	6.60 (4.83)	6.80 (4.40)	6.33 (4.41)	6.10 (4.35)	6.00 (4.35)	6.40 (4.95)
T_3	6.60 (4.30)	6.90 (4.00)	6.90 (4.70)	6.20 (4.54)	6.40 (4.04)	6.90 (4.70)	6.90 (5.40)	6.40 (4.45)
T_4	6.90 (4.00)	6.90 (3.70)	6.30 (4.30)	6.20 (3.75)	6.70 (4.50)	6.50 (4.43)	6.30 (4.15)	6.40 (4.50)
T_5	7.00 (4.45)	6.90 (3.10)	6.30 (3.70)	6.80 (5.05)	6.90 (4.71)	6.10 (4.50)	6.10 (4.00)	6.60 (4.70)
T_6	6.90 (4.25)	6.80 (4.15)	6.80 (4.95)	6.50 (4.30)	6.50 (4.05)	6.40 (4.31)	6.50 (4.10)	6.90 (4.53)
T_7	6.80 (4.61)	6.70 (3.90)	6.30 (4.45)	6.70 (4.22)	6.40 (4.15)	6.60 (4.30)	6.30 (4.12)	6.30 (4.25)
T_8	6.50 (3.75)	7.00 (3.75)	6.00 (3.30)	6.20 (3.65)	6.20 (3.70)	6.10 (4.00)	6.00 (3.75)	6.20 (3.70)
Kendall's coefficient	0.073^{NS}	0.076**	0.106**	0.118^{NS}	0.190**	0.099**	0.084**	0.176**

Figures in parenthesis indicate mean rank scores * Significant at 1% level, ** Significant at 5% level,, NS-non significant
T_1 – Control (No preservative added), T_2 – Sodium benzoate 750 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.025%, T_7 – *P.tinctorum* extract 0.05%, T_8 – *P.tinctorum* extract 0.1%

Table 36. Sensory attributes of tomato sauce five months after storage

Treatments	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	6.70 (4.15)	6.93 (4.07)	6.20 (4.00)	6.30 (4.05)	6.40 (4.20)	6.50 (4.32)	6.50 (4.55)	6.50 (4.50)
T_2	6.50 (4.30)	6.90 (4.10)	6.50 (4.66)	6.40 (4.12)	6.20 (4.30)	6.00 (4.10)	5.90 (4.25)	6.30 (4.40)
	6.50 (4.20)	6.50 (4.00)	6.30 (4.50)	6.10 (4.25)	6.20 (4.00)	6.30 (4.42)	6.50 (5.10)	6.30 (4.15)
T_4	6.70 (3.84)	6.70 (3.45)	6.20 (4.30)	6.20 (3.70)	6.50 (4.50)	6.40 (4.35)	6.30 (4.05)	6.30 (4.01)
T_5	6.40 (4.05)	6.80 (2.85)	6.20 (3.70)	6.40 (4.70)	6.50 (4.60)	6.10 (3.80)	6.00 (3.95)	6.50 (4.23)
T_6	6.60 (4.10)	6.50 (3.25)	6.50 (4.80)	6.00 (4.00)	6.00 (4.00)	6.20 (3.85)	6.10 (3.45)	6.60 (4.00)
T_7	6.60 (4.00)	6.20 (3.42)	6.00 (4.25)	6.00 (3.90)	6.00 (4.10)	5.70 (3.70)	6.00 (3.40)	6.10 (4.20)
T_8	6.20 (3.40)	6.00 (3.40)	5.80 (3.85)	5.80 (2.95)	6.10 (3.45)	6.00 (3.45)	6.00 (3.10)	6.00 (3.60)
Kendall's coefficient	0.144**	0.457**	0.091**	0.134**	0.123**	0.143**	0.141**	0.134**

Figures in parenthesis indicate mean rank scores * Significant at 1% level, ** Significant at 5% level,, NS-non significant

T_1 – Control (No preservative added), T_2 – Sodium benzoate 750 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.025%, T_7 – *P.tinctorum* extract 0.05%, T_8 – *P.tinctorum* extract 0.1%

Table 37. Sensory attributes of tomato sauce six months after storage

Treatments	Appearance	Colour	Flavour	Texture	Odour	Taste	After taste	Overall acceptability
T_1	6.20 (3.31)	6.90 (3.65)	6.20 (3.80)	5.80 (3.75)	6.00 (4.00)	6.20 (3.95)	6.30 (4.11)	6.10 (4.20)
T_2	6.70 (3.94)	6.80 (4.00)	6.40 (4.30)	6.00 (3.90)	6.00 (4.15)	5.90 (4.00)	5.80 (4.10)	6.00 (4.35)
T_3	6.40 (3.90)	6.30 (3.68)	6.00 (4.35)	6.10 (3.40)	6.10 (4.00)	6.70 (4.33)	6.30 (4.20)	6.20 (3.89)
T_4	6.40 (3.80)	6.40 (3.30)	6.20 (4.05)	6.10 (3.05)	6.20 (4.22)	6.10 (4.00)	6.00 (4.00)	6.30 (3.55)
T_5	6.00 (3.70)	5.70 (2.82)	6.00 (3.50)	6.40 (4.36)	6.00 (5.80)	6.20 (3.35)	5.90 (3.89)	6.00 (3.95)
T_6	6.50 (3.75)	6.10 (3.10)	6.40 (4.60)	6.00 (3.85)	6.00 (3.50)	6.00 (3.65)	5.80 (3.30)	6.50 (3.60)
T_7	6.10 (3.15)	6.00 (3.05)	5.90 (3.85)	5.80 (3.70)	5.90 (4.00)	5.60 (3.55)	5.70 (3.20)	6.00 (3.54)
T_8	6.00 (3.20)	5.50 (3.35)	5.40 (3.25)	5.60 (2.90)	5.50 (3.10)	5.50 (3.40)	5.50 (3.00)	5.50 (3.14)
Kendall's coefficient	0.042**	0.304**	0.116**	0.276**	0.203**	0.139**	0.091**	0.168**

Figures in parenthesis indicate mean rank scores * Significant at 1% level, ** Significant at 5% level,, NS-non significant
T_1 – Control (No preservative added), T_2 – Sodium benzoate 750 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.025%, T_7 – *P.tinctorum* extract 0.05%, T_8 – *P.tinctorum* extract 0.1%

4.3.2.2. Tomato sauce (Un pasteurised)

Initially, all the samples pertaining to different treatments showed no bacterial population (Table 41), but recorded bacterial growth after first month of storage. The treatment T_8 recorded no bacterial growth, upto fifth month of storage. After the six months storage period, T_8 recorded significantly least bacterial count (5.2X10^6 CFU/g), in which ethanol extract @ 0.1% was added, followed by T_6 (17.0 X10^6 CFU/g) and by T_7 (17.2 X 10^6 CFU/g). The highest bacterial load was seen in T_4 (99.3 X 10^6 CFU/g), followed by T_3 (89.1 X10^6 CFU/g) and T_1 (32.5 X10^6 CFU/g). When the unpasteurized sauce was analysed soon after the preparation, no fungal growth was observed in all the treatments. Fungal colonies were detected after the first month of storage, except in T_2, T_7 and T_8, and growth gradually increased throughout the storage period, in most of the treatments (Table 42). In the sample where T_2 was applied, the fungal growth was observed only after fifth month of storage onwards with a count of 6.4 X 10^3 CFU/g, which increased to 15.6 X10^3 CFU/g. At the end of six months, lowest fungal load was shown by T_2 (15.6 X 10^3 CFU/g) followed by T_8 (20.5 X 10^3 CFU/g) and T_7 (34.0 X10^3 CFU/g); while highest population was recorded in T_1 (89.2 X10^3 CFU/g). The treatments where *Parmotrema tinctorum* powder was added showed high fungal load *viz*. T_3 (33.1 X10^3 CFU/g), T_4 (48.3 X10^3 CFU/g) and T_5 (43.2 X10^3 CFU/g). The yeast population was detected after fourth month of storage, in all the treatments. Highest yeast count was detected in T_1 (27.9 X 10^3 CFU/g), and lowest was in T_8 (0.8X10^3 CFU/g). No growth was shown in T_2 throughout the storage period. The treatments T_6 and T_7 in which the lower levels of lichen extract were added also recorded low yeast counts (2.3 X 10^3 CFU/g) and (1.9 X10^3 CFU/g). Significant difference in yeast population was observed among the treatments only after sixth month of storage (Table 43).

Table 38. Effect of treatments on bacterial population in lime pickle during storage

Treatments	Bacteria (10^6 CFU/g)						
	Initial	**1 MAS**	**2MAS**	**3MAS**	**4MAS**	**5MAS**	**6 MAS**
T_1	1.0 (1.00)	1.0 (1.00)	0.7 (0.83)	0.67 (0.77)	2.67 (1.62)	18.0 (4.19)	64.5 (8.02)
T_2	N.D	N.D.	N.D	1.0 (1.00)	1.0 (1.00)	1.0 (1.00)	25.1 (4.99)
T_3	1.0 (1.00)	1.0 (1.00)	0.3 (0.57)	0.67 (0.83)	1.3 (1.13)	88.0 (8.83)	77.7 (9.38)
T_4	0.3 (0.57)	0.3 (0.57)	0.7 (0.83)	1.33 (1.13)	1.0 (1.00)	65.6 (8.12)	100.1 (10.03)
T_5	0.6 (0.79)	0.7 (0.87)	1.3 (1.13)	1.0 (1.00)	2.0 (1.88)	29.0 (5.41)	19.2 (6.24)
T_6	1.0 (1.00)	1.0 (1.00)	0.6 (0.75)	0.9 (0.89)	2.0 (1.38)	3.5 (1.90)	43.4 (6.58)
T_7	1.0 (1.00)	1.0 (1.00)	0.3 (0.54)	0.7 (0.83)	1.3 (1.13)	3.0 (1.71)	16.6 (4.12)
T_8	0.3 (0.57)	0.3 (0.57)	0.7 (0.87)	1.0 (1.00)	0.9 (0.89)	2.0 (1.38)	13.6 (3.74)
CD(0.05)	0.05	0.05	0.21	0.19	0.34	0.39	0.18

Figures in parenthesis are transformed values(SQRT)

N.D. –Not detected; NS-Non significant; CFU-Colony forming units; MAS- Months after storage
T_1 – Control (No preservative added), T_2 – Sodium benzoate 250 ppm, T_3 – *P.tinctorum* powder 0.1% , T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.1%, T_7 – *P.tinctorum* extract 0.2%, T_8 – *P.tinctorum* extract 0.3%

Table 39. Effect of treatments on fungal population in lime pickle during storage

Treatments	Fungi (10^3CFU/g)						
	Initial	**1 MAS**	**2MAS**	**3MAS**	**4MAS**	**5MAS**	**6 MAS**
T_1	N.D	N.D	N.D	2.6 (1.62)	3.4 (1.79)	14.3 (3.74)	70.0 (8.30)
T_2	N.D	N.D	N.D	N.D	N.D	N.D.	0.6 (0.77)
T_3	N.D	N.D	N.D	1.0 (1.00)	0.3 (0.57)	1.7 (1.27)	3.0 (1.71)
T_4	N.D	N.D	N.D	N.D	0.3 (0.57)	0.6 (0.79)	3.0 (1.71)
T_5	N.D	N.D	N.D	1.0 (1.00)	1.0 (1.00)	1.3 (1.13)	5.0 (2.13)
T_6	N.D	N.D	N.D	N.D	N.D	1.0 (1.00)	4.0 (1.95)
T_7	N.D	N.D	N.D	N.D	N.D	1.0 (1.00)	2.0 (1.41)
T_8	N.D	N.D	N.D	N.D	N.D	1.0 (1.00)	1.0 (1.00)
CD(0.05)	-	-	-	0.05	0.39	0.22	0.64

Figures in parenthesis are transformed values (SQRT)

N.D. –Not detected; NS-Non significant; CFU-Colony forming units; MAS- Months after storage

T_1 – Control (No preservative added), T_2 – Sodium benzoate 250 ppm, T_3 – *P.tinctorum* powder 0.1%

T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.1%,

T_7 – *P.tinctorum* extract 0.2%, T_8 – *P.tinctorum* extract 0.3%

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Table 40. Effect of treatments on yeast population in lime pickle during storage

Treatments	Yeast (10^3 CFU/g)						
	Initial	**1 MAS**	**2MAS**	**3MAS**	**4MAS**	**5MAS**	**6 MAS**
T_1	N.D	N.D	N.D	N.D	1.3 (1.13)	6.0 (2.44)	7.0 (2.67)
T_2	N.D	N.D	N.D	N.D	N.D	N.D.	0.3 (0.57)
T_3	N.D	N.D	N.D	N.D	0.3 (0.57)	4.0 (2.09)	5.0 (2.33)
T_4	N.D	N.D	N.D	N.D	2.0 (1.41)	2.0 (1.41)	3.0 (1.85)
T_5	N.D	N.D	N.D	N.D	1.0 (1.00)	1.0 (1.00)	N.D
T_6	N.D	N.D	N.D	N.D	0.7 (0.83)	2.0 (1.41)	1.0 (1.00)
T_7	N.D	N.D	N.D	N.D	N.D.	N.D	1.0 (1.00)
T_8	N.D	N.D	N.D	N.D	N.D.	N.D.	N.D.
CD(0.05)	-	-	-	-	NS	NS	0.41

Figures in parenthesis are transformed values(SQRT)

N.D. –Not detected; NS-Non significant; CFU-Colony forming units; MAS- Months after storage

T_1 – Control (No preservative added), T_2 – Sodium benzoate 250 ppm, T_3 – *P.tinctorum* powder 0.1%
T_4 –*P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.1%,
T_7 – *P.tinctorum* extract 0.2%, T_8 – *P.tinctorum* extract 0.3%

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Table 41. Effect of treatments on bacterial population in unpasteurized tomato sauce during storage

Treatments	Bacteria (10^6 CFU/g)						
	Initial	**1 MAS**	**2MAS**	**3MAS**	**4MAS**	**5MAS**	**6 MAS**
T_1	N.D	10 (3.16)	7.0 (2.64)	9.0 (3.0)	.14.9 (3.86)	17.9 (4.23)	32.5 (5.70)
T_2	N.D	N.D	0.3 (0.54)	1.3 (1.14)	10.4 (3.72)	16.0 (4.00)	14.13 (3.75)
T_3	N.D	6.8 (2.60)	10.3 (3.20)	14.5 (3.80)	12.1 (3.47)	13.4 (3.66)	89.1 (9.43)
T_4	N.D	N.D	2.30 (1.51)	11.7 (3.42)	62.6 (7.91)	4.1 (2.02)	99.3 (9.96)
T_5	N.D	N.D	0.1 (0.31)	4.0 (2.0)	9.8 (3.13)	5.0 (2.23)	18.7 (4.42)
T_6	N.D	N.D	N.D	N.D	N.D	2.8 (1.67)	17.0 (4.12)
T_7	N.D	N.D	N.D	N.D	N.D	0.9 (0.94)	17.2 (4.14)
T_8	N.D	N.D	N.D	N.D	N.D	N.D.	5.2 (2.28)
CD(0.05)	-	0.19	0.38	0.32	0.18	0.19	0.21

Figures in parenthesis are transformed values(SQRT)

N.D. –Not detected; NS-Non significant; CFU-Colony forming units; MAS- Months after storage

T_1 – Control (No preservative added), T_2 – Sodium benzoate 750 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.025%, T_7 – *P.tinctorum* extract 0.05%, T_8 – *P.tinctorum* extract 0.1%

Table 42. Effect of treatments on fungal population in unpasteurized tomato sauce during storage

Treatments	Fungi (10^3 CFU/g)						
	initial	1 MAS	2MAS	3MAS	4MAS	5MAS	6 MAS
T_1	N.D.	4.0 (2.00)	3.0 (1.73)	11.4 (3.37)	29.1 (5.39)	31.0 (5.56)	38.4 (6.19)
T_2	N.D.	N.D.	N.D.	N.D.	0.3 (0.54)	1.0 (1.00)	12.0 (3.46)
T_3	N.D.	8.0 (2.82)	1.0 (1.00)	12.3 (3.57)	15.7 (3.96)	25.0 (5.0)	33.1 (5.75)
T_4	N.D.	8.0 (2.82)	28 (5.29)	68.1 (8.25)	52.0 (7.21)	41.5 (6.44)	48.3 (6.94)
T_5	N.D.	3.0 (1.73)	6.2 (2.48)	7.0 (2.64)	7.3 (2.70)	22.0 (4.69)	43.2 (6.57)
T_6	N.D.	1.0 (1.00)	1.0 (1.00)	6.0 (2.44)	7.0 (2.64)	20.1 (4.48)	47.0 (6.85)
T_7	N.D.	N.D.	N.D.	1.9 (1.37)	3.0 (1.73)	6.4 (2.52)	15.6 (3.91)
T_8	N.D.	N.D.	N.D.	0.2 (0.44)	0.8 (0.89)	4.00 (2.00)	12.0 (3.46)
CD(0.05)	-	0.69	0.30	0.25	0.23	0.22	1.06

Figures in parenthesis are transformed values(SQRT)

N.D. –Not detected; NS-Non significant; CFU-Colony forming units; MAS- Months after storage

T_1 – Control (No preservative added), T_2 – Sodium benzoate 750 ppm, T_3 – *P.tinctorum* powder, 0.1%
T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.025%,
T_7 – *P.tinctorum* extract 0.05%, T_8 – *P.tinctorum* extract 0.1%

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Table 43. Effect of treatments on yeast population in unpasteurized tomato sauce during storage

Treatments	Yeast (10^3CFU/g)						
	initial	**1 MAS**	**2MAS**	**3MAS**	**4MAS**	**5MAS**	**6 MAS**
T_1	N.D.	N.D.	N.D.	N.D.	N.D.	2.4 (1.54)	27.9 (5.28)
T_2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
T_3	N.D.	N.D.	N.D.	N.D.	N.D.	0.3 (0.57)	7.2 (2.68)
T_4	N.D.	N.D.	N.D.	N.D.	N.D.	0.9 (0.89)	8.5 (2.91)
T_5	N.D.	N.D.	N.D.	N.D.	N.D.	0.6 (0.77)	31.8 (5.63)
T_6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	2.3 (1.51)
T_7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.9 (1.37)
T_8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.8 (0.89)
CD(0.05)	-	-	-	-	-	NS	0.42

Figures in parenthesis are transformed values(SQRT)

N.D. –Not detected; NS-Non significant; CFU-Colony forming units; MAS- Months after storage

T_1 – Control (No preservative added), T_2 – Sodium benzoate 750 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.025%, T_7 – *P.tinctorum* extract 0.05%, T_8 – *P.tinctorum* extract 0.1%

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Table 44. Effect of treatments on bacterial population in pasteurized tomato sauce on storage

Treatments	Bacteria (10^6 CFU/g)						
	Initial	1 MAS	2MAS	3MAS	4MAS	5MAS	6 MAS
T_1	N.D	N.D	N.D	N.D	1.0 (1.00)	1.0 (1.00)	1.0 (1.00)
T_2	N.D	N.D	N.D	N.D	N.D	N.D	N.D
T_3	N.D	N.D	N.D	N.D	0.6 (0.77)	0.6 (0.77)	0.6 (0.77)
T_4	N.D	N.D	N.D	N.D	N.D	1.0 (1.00)	0.3 (0.54)
T_5	N.D	N.D	N.D	N.D	N.D	0.6 (0.77)	1.0 (1.00)
T_6	N.D	N.D	N.D	N.D	N.D	1.0 (1.00)	0.6 (0.77)
T_7	N.D	N.D	N.D	N.D	N.D	1.0 (1.00)	0.6 (0.77)
T_8	N.D	N.D	N.D	N.D	N.D	N.D.	0.3 (0.54)
CD(0.05)	-	-	-	NS	NS	NS	NS

Figures in parenthesis are transformed values(SQRT)

N.D. –Not detected; NS-Non significant; CFU-Colony forming units; MAS- Months after storage

T_1 – Control (No preservative added), T_2 – Sodium benzoate 750 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.025%, T_7 – *P.tinctorum* extract 0.05%, T_8 – *P.tinctorum* extract 0.1%

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Table 45. Effect of treatments on fungal population in pasteurized tomato sauce during storage

Treatments	Fungi (10^3CFU/g)						
	initial	1 MAS	2MAS	3MAS	4MAS	5MAS	6 MAS
T_1	N.D.	N.D.	N.D.	N.D.	1.0 (1.00)	0.6 (0.77)	1.6 (1.38)
T_2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
T_3	N.D.	N.D.	N.D.	N.D.	0.3 (0.57)	0.3 (0.57)	1.0 (1.00)
T_4	N.D.	N.D.	N.D.	N.D.	N.D.	0.6 (0.77)	0.3 (0.57)
T_5	N.D.	N.D.	N.D.	N.D.	0.3 (0.57)	0.3 (0.57)	1.3 (1.13)
T_6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.0 (1.00)
T_7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.3 (0.57)
T_8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D
CD(0.05)	-	-	-	-	NS	NS	NS

Figures in parenthesis are transformed values(SQRT)

N.D. –Not detected; NS-Non significant; CFU-Colony forming units; MAS- Months after storage

T_1 – Control (No preservative added), T_2 – Sodium benzoate 750 ppm, T_3 – *P.tinctorum* powder 0.1%
T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.025%,
T_7 – *P.tinctorum*extract 0.05%, T_8 – *P.tinctorum* extract 0.1%

Table 46. Effect of treatments on yeast population in pasteurized tomato sauce on storage

Treatments	Yeast (10^3CFU/g)						
	initial	**1 MAS**	**2MAS**	**3MAS**	**4MAS**	**5MAS**	**6 MAS**
T_1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.6 (0.77)
T_2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D
T_3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.3 (0.57)
T_4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.3 (0.57)
T_5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.6 (0.77)
T_6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.6 (0.77)
T_7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.3 (0.57)
T_8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
CD(0.05)	-	-	-	-	-	-	NS

Figures in parenthesis are transformed values(SQRT)

N.D. –Not detected; NS-Non significant; CFU-Colony forming units; MAS- Months after storage

T_1 – Control (No preservative added), T_2 – Sodium benzoate 750 ppm, T_3 – *P.tinctorum* powder 0.1% T_4 – *P.tinctorum* powder 0.2%, T_5 – *P.tinctorum* powder 0.3%, T_6 – *P.tinctorum* extract 0.025%, T_7 – *P.tinctorum* extract 0.05%, T_8 – *P.tinctorum* extract 0.1%

4.3.2.3. Tomato sauce (Pasteurised)

Bacterial colonies were detected only after five months of storage except in T_1 (1.0 X 10^6 CFU/g) and T_3 (0.6 X 10^6 CFU/g) (Table 44). The treatment T_2 where sodium benzoate 750 ppm was added did not record bacterial growth on storage. After six months storage period, bacterial colonies were detected in all the treatments which ranged from 0.3 X10^6 CFU/g in T_4 and T_8 to 1.0 X10^6 CFU/g in T_1, but found to be non significant. Regarding the fungal population, it was observed from fourth month after storage. When observed after fourth month of storage, highest fungal count was noted in T_1-control (1.0 X 10^3 CFU/g), followed by T_3 and T_5 (0.3X10^3CFU/g). The fungal count was highest in T_1 (1.6 10^3 CFU/g), followed by T_5 (1.3 X10^3 CFU/g) six months after storage. The treatments in which ethanol extract of *Parmotrema tinctorum* was added revealed lower counts of fungi. The treatment T_2 showed no fungal growth at the end of storage period.(Table 45). Similarly, no yeast colonies were detected in the samples upto six months of storage, while T_2 and T_8 were devoid of yeast growth throughout the storage period. The variation observed in yeast population among different treatments was not significant towards the end of the storage. The highest population was noted in T_1 (1.3 X 10^3 CFU/g) and lowest in T_3, T_4, T_7 (0.3 X10^3 CFU/g) (Table 46).

4.4. Toxicology studies in *Parmotrema tinctorum*

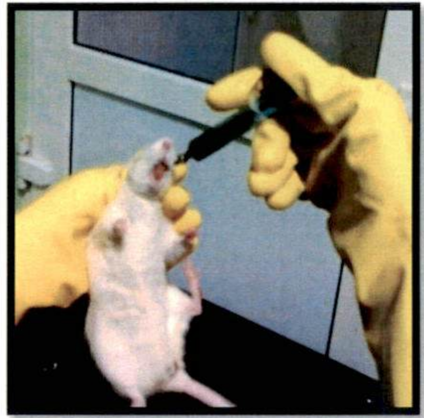
The acute oral toxicity study of ethanol extract of *Parmotrema tinctorum* (EEPT) was performed using rats of Wistar albino strain, as test animals (Plate 33). Observations were taken for mortality, toxicity signs, body weight changes, food and water intake for a total period of 14 days. On the 14th day, all animals were sacrificed and subjected to necropsy and gross pathological examination. The results of acute oral toxicity testing are given below.

Plate 45. Acute toxicity study

a) Test animal- Wistar rats

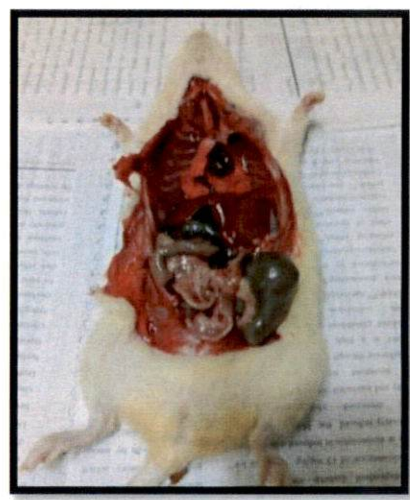
b) Test animals in polysulfone cage

c) Test substance-EEPT



d) Oral administrattion of EEPT

e) Periodic inspection of test animals



f)Test animal after necropsy

Table 47. Individual animal clinical signs and mortality record

Dose (mg/kg body weight)	Animal No.	Sex	Study day 1					Study days												
			30-40 min.	1 hr	2 hr	3 hr	4 hr	2	3	4	5	6	7	8	9	10	11	12	13	14
2000	1	F	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2000	2	F	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	3	F	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	4	F	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	5	F	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

N-Normal, F-Female

Table 48. Summary of clinical signs and mortality

Dose (mg/kg body weight)	No.of animals	Sex	Clinical signs	Mortality
2000 (sighting study)	1	F	N	0/1
2000	4	F	N	0/4

F-Female, N-Normal

Table 49. Changes in body weight during the study period

Dose (mg/kg body weight)	No. of animals	Sex	Average body weight at weekly intervals (g)			Body weight gain (%)	
			1 to 7	7 to 14	1 to 14	1 to 7	1 to 14
2000	1	F	240.0±0.0	242.5±3.5	241.3±2.5	0.0±0.0	2.1±0.0
2000	4	F	238.1±2.4	240.0±4.6	239.1±3.3	0.5±2.7	1.1±1.2

F-Female, Values are expressed as mean±SD

Table 50. Individual animal food intake during study period

Dose (mg/kg body weight)	Animal No.	Sex	Food intake on days (g)													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
2000	1	F	15	15	15	15	15	20	20	15	15	20	10	15	15	15
2000	2	F	10	20	15	15	20	20	20	15	15	15	20	15	15	20
2000	3	F	20	20	15	15	15	20	15	15	15	15	20	15	15	15
2000	4	F	15	15	20	15	10	15	20	15	15	20	15	15	15	20
2000	5	F	10	15	15	15	20	20	20	20	25	30	20	20	20	15

Table 51. Individual animal water intake during study period

Dose (mg/kg body weight)	Animal No.	Sex	Water intake on days (ml)													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
2000	1	F	25	26	40	23	25	48	35	40	25	30	37	30	40	36
2000	2	F	28	23	28	40	20	34	30	30	24	28	33	33	44	36
2000	3	F	25	30	30	50	26	40	40	47	33	30	39	29	38	40
2000	4	F	20	20	26	32	20	28	30	26	30	20	27	30	32	29
2000	5	F	22	25	29	33	24	35	38	30	34	36	40	35	30	35

F-Female

Table 52. Summary of pathological findings

Dose (mg/kg body weight)	No.of animals	Sex	Gross pathological findings	
			External	Internal
2000	1	F	NAD	NAD
2000	4	F	NAD	NAD

F-Female

NAD-No abnormalities detected

4.4.1. Clinical signs and mortality

The ethanol extract of *Parmotrema tinctorum* at the dose of 2000 mg/kg body weight orally produced no clinical signs of toxicity and mortalities in the tested animals (Table 47 and 48).

4.4.2. Body weight

There were no treatment related changes in body weight and percent body weight during the study period at the tested dose of 2000 mg/kg body weight in EEPT treated animals (Table 49).

4.4.3. Food and Water intake

There were no treatment related changes in food and water intake during the study period at the tested dose of 2000 mg/kg body weight in EEPT treated animals (Table 50 and 51).

4.4.4. Pathology

There were no gross pathological changes in any of the five animals sacrificed at the end of the study. The results of pathological findings are summarized in Table 52.

DISCUSSION

5. DISCUSSION

Lichens are nonvascular cryptogams adapted to all possible environmental habitats in the world. They constitute over as much as 8% of earth's surface thus, become the dominant life forms on earth (Ahmadjian, 1995). In Indian context, lichen flora are more prominent in mountainous ranges. The diverse climate, vegetation, asymmetrical topography and unlimited availability of substratum make India one of the lichen rich countries, standing fifth in the world (Negi and Gadgil, 1996). Taxonomic studies on Indian lichens have revealed the occurrence of more than 2300 species from India (Joshi *et al.,* 2011).

Western Ghats and Eastern Himalaya are the two main centers of lichen diversity in India. The Western Ghats runs through different states of southwestern India and covers various types of vegetation including subtropical deciduous forests and grasslands, stretching to a distance of 1600 km, and in width of 100 km. Kumar (2000) conducted detailed survey on macrolichens of Kerala and reported the occurrence of 254 lichen species showing the diversity of the macrolichen flora in the Kerala part of the Western Ghats. The Western Ghats harbor a large number of lichens, in it's various habitats, which comes to around 45 % of the total lichen population in India (Upreti *et al*.,2005).

Lichens have been used as medicine, food, and for production of dyes, alcohol since ancient times worldwide. The lichens have been household items in India since ancient times as medicines and as spice in foods (Kaushal and Upreti, 2001; Mohaptra *et al*., 2014). The Sanskrit synonyms of lichens are '*Shila Pushp*' and '*Shailaya*' (Shila = rock, Pushp = flower). The whole lichen or lichen powder is a major ingredient in common condiments used in food dishes the '*Meat Masala'*, '*Garam Masala'*, '*Sambar Masala*'. An indigenous perfume named 'Otto' (the Hina

Attar) in Uttar Pradesh, which has been famous for the past 800 years for its fragrance is derived from lichen. *Parmelia* spp. are used for wound healing in parts of Eastern Ghats of India. *Hypotrachyna cirrhata* (Fr.).and *Flavoparmelia caperata* (L) Hale are used by the folks of Sikkim and Tamil Nadu against wound infections, bite and burn (Pathak *et al*., 2016).

Kumar *et al*. (2009) reported that *Parmotrema tinctorum* occurs at an altitude of 750-2300 m in moist places in the evergreen/subtropical forests and in Kerala, the major localities identified are Silent valley, Munnar, Nelliampathy, Parambikulam, Siruvani and Wayanad.

Despite the large scale collection of this lichen by the tribal people from Wayanad district, Kerala the documentation on this lichen as a food additive is meager. Recently the antioxidant and antiglycation properties of this lichen was reported by Raj *et al*. (2014). No detailed study on the antimicrobial activity, and toxicological aspects of the edible lichen *Parmotrema tinctorum* from the forests of Kerala, has been reported so far. Keeping in mind the potential properties (antimicrobial, antioxidant and medicinal) of lichen in the preparation of food and for pharmaceutical purposes, the present study was taken up to evaluate and utilize the lichen *Parmotrema tinctorum* (Nyl.) Hale (Parmeliaceae) for food preservation.

The major findings obtained from the project entitled "Evaluation and utilisation of edible lichen *Parmotrema tinctorum* (Nyl.) Hale for food preservation" are discussed in this chapter under the following heads.

5.1. Collection and characterization of lichen *Parmotrema tinctorum*

5.2. Evaluation of antimicrobial property of lichen extracts

5.3. Evaluation of *Parmotrema tinctorum* for preservation of processed products

5.4. Toxicology studies in *Parmotrema tinctorum*

5.1. Collection and characterization of lichen *Parmotrema tinctorum*

5.1.1.Collection of *Parmotrema tinctorum* (Nyl.) Hale

Detailed explorations were done for the collection of the lichen *Parmotrema tinctorum* during December 2015 in the South Wayanad Forest Division of Wayanad district, Kerala, India (Fig.1), with the authorized permit from the Department of Forest and Wildlife, Govt. of Kerala (WL-10-39996/2015 dt. 19.10.2015). Wayanad district is at the Southern Western Ghats and located in the northeast part of Kerala between North latitude $11^{0}26'$ to $12^{0}00'$ and East longitude $75^{0}75'$ to $76^{0}56'$ (Volga *et al.*, 2013).

The details of lichen collection and trade by the tribal co-operative societies in Wayanad,were studied in the present study. The lichen, a non timber product assures a stable livelihood to the tribes of Wayanad. Lichen collection is mainly done by the *Kattunaikkan* and Paniya tribes. The collection is approved by the Department of Forests and Wildlife. The picking season is from October to March. According to the data collected during the surveys, the edible lichens are traded at the rate of Rs. 328-490/kg by the tribal co-operative societies. The collected lichen is sold through auctions to different parts of India, especially Karnataka, Tamil nadu and Maharashtra. Since the lichen is a vital ingradient in *Goda Masala*, *Chettinad Masala*, it is mostly consumed by the people of North India, Western India and Tamil Nadu. The retail selling rate of lichens ranges from Rs. 25 -70 per 100 g.

Lichen collection and marketing had also been reported from plains of Himalaya. An average of 800 metric tons of lichens is collected from Assam, Sikkim and Himachal Pradesh and out of which about 50-80 tons are exported (Shah, 1997).

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Kumar (2009a) reported that the commercially traded lichens from Garhwal Himalaya are Parmelioid lichens belonging to *Bulbothrix, Parmotrema, Everniastrum, Cetrariopsis, Hypotrachyna, Ramalina and Usnea* which are collected by the tribals. Uttarakhand hills stand first in the collection and marketing of lichens with a trade of 750 metric tons. Kumar (2009b) studied the lichen harvesting practices and the marketing strategy in Uttarakhand and reported that the lichen collection season lasts about six months in a year, and usually lichens are collected from about 15-20 primary collectors. Quantity of lichen traded on an average is about 301 quintal per season per trader.

5.1.2 Phyto chemical screening of secondary metabolites

Phytochmicals the natural bioactive compounds found in plants, act as a defense system to protect against biotic and abiotic stresses. Phytochemicals are divided into primary and secondary constituents; based on their functions. Primary constituents include carbohydrates, proteins and amino acids, whereas secondary constituents consist of phenols, terpenoids, flavonoids and alkaloids (Krishnaiah *et al*., 2009). Saponins are glycosides of triterpenes and sterols and are used as expectorant and emulsifying agent (Belewu *et al*, 2009). Flavonoids protect biological systems against the oxidation effects of macromolecules, mainly proteins, nucleic acids and lipids. Thus they have been reported to possess properties, such as antimicrobial, anti-allergic, antioxidant, and cytotoxic antitumor activity (Saxena *et al*., 2013). According to the study by Rashmi and Rajkumar (2014) saponins were detected only in one lichen species *Usnea subflorida*, not in *Parmotrema tinctorum.* But in the present study, petroleum benzine extract had shown the presence of saponins.

The results of preliminary phytochemical screening for secondary metabolites are presented in Table 1. Methanol extract contained maximum phytochemicals *viz.* carbohydrates, phenols, flavonoids, tannins, terpenoids, fixed oils and coumarins. Methanol being highly polar dissolves most of the secondary metabolites of *Parmotrema tinctorum* as reported by Rashmi and Rajkumar (2014). In this study, extracts using medium polar solvents *viz.* acetone and ethyl acetate was found positive for carbohydrates, phenols, tannins, and terpenoids, which were not reported in the study conducted by Rashmi and Rajkumar (2014). Acetone extract also showed presence of fixed oils; whereas ethyl acetate indicated the presence of flavonoids. Petroleum ether extract did not show any phytochemicals except saponins. Alkaloids, steroids and quinones were not detected in all the four extracts.

Due to slow growth and often harsh living conditions, it becomes a necessity to lichens to produce unique secondary metabolites which are protective in nature, as many metabolites are reported to be antimicrobial or antiherbivore agents (Prashith *et al.*, 2013).

Lichen substances are extracted using organic solvents since they are insoluble in water (Tiwari *et al.*, 2011). Most lichen substances with antibiotic activity are phenolic compounds. The phenolic compounds *viz.* lecanoric acid and atranorin are the main secondary metabolites detected in *Parmotrema tinctorum* which show biological activities (Hale, 1983; Din *et al.*, 2010; Sebastian *et al.*, 2014).

5.1.3. Proximate composition of lichen *Parmotrema tinctorum*

The mean moisture content of *Parmotrema tinctorum* lichen was found to be 8.09 % on wet weight basis. A moisture content of 9-12 % in *Parmotrema tinctorum* was reported by Kambar *et al.* (2014) and a content of 9.13% was observed by Raj *et*

al. (2014). Lichens are organisms which are able to survive long periods of dry conditions in a dormant stage. Due to lack of stomata, cuticle and any water storage system, water vapour is lost readily from the whole surface of lichens (Lumbsch, 2008). This may be the reason for low moisture content of lichen. Low moisture food materials are less susceptible to microbial damage (Ramesh, 2011).

Carbohydrates function as source of energy and as structural components (Chavan and Patil, 2015). It is the major component of lichen species *viz*., *Parmotrema tinctorum* (72.13%) *Ramalina conduplicans* (79.80%), *Ramalina hossei* (59.90%) and *Parmotrema pseudotinctorum* (53.20%) as reported by Kambar *et al.* (2014) by difference method (carbohydrate % =100-ash+moisture+fat+protein %). But the mean carbohydrate content estimated by UV-vis spectrophotometry in the present study, which is a specific method, was found to be 20.03g /100g. This is in concordance with the report by Lal and Rao (1956) who observed the carbohydrate content of *Parmotrema tinctorum* to be 25.0g/100g. Raj *et al*. (2014) also reported 32.33 % carbohydrate in *Parmotrema tinctorum*.

Crude fibre mainly consists of cellulose and lignin; and subjecting the dry, fat-free sample with dilute acid and subsequent with dilute alkali to bring about oxidative hydrolytic degradation of cellulose and lignin. Cellulose and lignin form the major part (97%) of crude fibre. Insoluble fibre passes through the intestines undigested, absorbing water and organic toxins (Chavan and Patil, 2015). The present study revealed high crude fibre content for *Parmotrema tinctorum* (14.16%). A similar crude fibre content of 16.36% was observed by Kambar *et al*. (2014) in *Parmotrema tinctorum*.

Ash is the inorganic residue left after ignition of a decarbonised material in a muffle furnace at 550^0C-600^0C for 2-3 hours. The soluble portion of ash comprises mineral matter and insoluble portion is consisting of silica. The ash content represents

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the mineral content of organic samples (Ooi *et al.*, 2012). The mean ash content of *Parmotrema tinctorum* lichen was found to be 10.50 %. A high ash value suggests the high percentage of mineral matter in the lichen. Earlier reports of Lal and Rao (1956) also showed a high ash value for this lichen to the tune of 12.6%. Also the result is in concordance with the study conducted by Raj *et al.* (2014) in which they reported an ash content of 11.65 % for *Parmotrema tinctorum.* Another edible lichen *Ramalina conduplicans* also recorded a significant ash content of 10.0% (Vinayaka *et al.*, 2009).

Parmotrema tinctorum recorded a high crude protein content of 15.70 % in the present study. Lal and Rao (1956) reported similar protein content for *Parmotrema tinctorum* (13.8%). Kambar *et al.* (2014) also observed a higher protein content of 11.3 % in *Parmotrema tinctorum.* According to Behadur *et al.* (2015) certain lichen species recorded high crude protein content and thus have good food value *viz.*, *Dermatocarpon moulnsii* (20.0%), *Lobaria isidiosa* (20.0%), *Roccella montagnei* (14.0%) and *Parmotrema tinctorum* (14.0%).

Phenols, the aromatic compounds with hydroxyl group are widespread in plant kingdom. Phenols are the largest group of phytochemicals, widespread in plant kingdom; more than 4000 phenolic compounds had been recognised which vary from <1 mg/kg to 3000 mg/kg in foods (King and Young, 1999). Total phenol content on analysis of *Parmotrema tinctorum* lichen powder recorded a significantly higher value of 322mg/100g. Majority of lichen substances with antibiotic activity are phenolic compounds (Manojlovic *et al.*, 2010). The correlation of the antioxidant activity with phenol content in the lichens have been reported (Stanly *et al.*, 2011; Kosanic and Rankovic, 2011). This results indicate that the lichen *Parmotrema tinctorum* can be further explored for developing natural antioxidants.

The amino acids are ionic compounds that form the basic building blocks of proteins. They also exists in the free form in many tissues and are known as free amino acids. The measurement of the total free amino acids gives the physiological and health status of the plants (Sadasivam and Manickam, 1992). The analysis for free amino acids have revealed that the content varied from 8.25 mg/g dry weight. Free amino acids may contribute to the taste of lichens, as in the case of mushrooms as reported by Mau *et al.* (2001) who studied the total free amino acid contents of speciality mushrooms *viz. Dictyophora indusiata*, *Grifola frondosa*, *Hericium erinaceus* and *Tricholoma giganteum* and it ranged from 7.41 to 12.3 mg/g dry weight. Aspartic acid and glutamic acid are monosodium glutamate like components which gave the most typical mushroom taste. Free amino acids are produced in cyanobacteria, algae and in lichens in reaction to metal accumulation from the environment (Backor *et al.*, 2004).

5.1.3.8. Mineral composition

In the present study, the content of 18 elements including three heavy metals in acid digested *Parmotrema tinctorum* lichen powder was estimated by ICP-OES technique, colourimetry and flame photometry. ICP-OES technique provides simultaneous estimation of a number of elements, hence, it is the most commonly employed for elemental analysis and several studies have been performed for element analysis of large variety of samples using this technique (Barnes and Debrah, 1997; Lachman *et al.*, 2007; Naozuka *et al.*, 2011; Kumaravel and Alagasundaram, 2014).

Minerals are inorganic substances, present in all body fluids and tissues. Although they do not yield energy, their presence is crucial for the maintenance of essential life processes (Malhotra, 1998). Bones are made up mainly of calcium, magnesium and phosphorus, and iron is a component of blood. Minerals like zinc,

molybdenum, copper, manganese, and magnesium are either structural part or part of enzyme systems. Sodium and potassium are vital for water and acid base balance of cellular fluids (Gopalan *et al*., 2002). These elements are classified generally into major and minor elements based on their daily requirement. The major elements are required in amounts greater than 100 mg/day and the minor elements are required in amounts less than 100 mg/day (Soetan *et al*., 2010).

i) *Major elements*

In the case of major elements, calcium was detected at the highest level of 21970 mg/kg. and phosphorus was at 1000.33 mg/kg. Similar calcium content for *Parmotrema tinctorum* (1780 mg/100g) and phosphorus (118mg/100g) was reported by Lal and Rao (1956). Other species of *Parmelia* also recorded significant calcium content; 3.1% was detected in *Parmelia sulcata* (Larry *et al*., 1988). Vivek *et al*. (2014) reported significant calcium content both in two lichens of *Parmotrema* genus *viz*. *P.grayanum* (3546.33 ppm) and *P.praesorediosum* (2965.35 ppm)

ii) *Minor elements*

The analysis revealed high amount of magnesium (1781.66 mg/kg), potassium (2936.66 mg/kg) and iron (785.26 mg/kg) in *Parmotrema tinctorum*. In the case of potassium, *Parmotrema grayanum* (2568 ppm) and *Parmotrema praesorediosum* (3805 ppm) were also good sources (Vivek *et al*., 2014). Kambar *et al*. (2014) observed high content of iron in *Parmotrema tinctorum* (8250.52 ppm) collected from Shivamogga district, Karnataka. It could be a good source for dietary iron.

Copper is involved in the iron absorption, neurotransmission and lipid metabolism and zinc is a co-factor for a number of enzymes (Amaradivakara *et al*., 2015). In the present study, the copper and zinc content were estimated to be 8.46

mg/kg and 42.56 mg/kg respectively. Chromium is required for normal sugar and fat metabolism. Molybdenum is involved in the uric acid metabolism (Prasanth *et al.*, 2015). Manganese, nickel, chromium, cobalt, molybdenum, vanadium were found at the concentration of 44.0 mg/ kg, 1.86 mg/ kg, 2.63 mg/ kg, 0.53 mg/ kg, 3.3 mg/ kg and 2.9 mg/ kg respectively in the present study.

Heavy metals are elements having high atomic weight and high density. The prescribed limits for heavy metals in food materials by FAO are As- 0.1 ppm, Cd-0.2 ppm, Hg-0.5 ppm, and Pb-10 ppm (Tchounwou, 2012). Heavy metals can be toxic for humans as they are not metabolized by the body and get accumulated in the biological tissues (Gautam, 2012). The lichen *Parmotrema tinctorum* showed very low levels of these heavy metals on analysis. The arsenic, lead and cadmium contents were observed as 0.19 mg/ kg, 0.36 mg/ kg and 0.19 mg/ kg respectively on mineral analysis.

The analysis of elements in the food stuffs is inevitable to ensure its safety and quality. Mineral composition of lichen *Parmotrema tinctorum* shows that it's use in the preparation of foods is nutritionally significant. The lichen *Parmotrema tinctorum* was found to be rich in minerals *viz.* calcium, magnesium, potassium and iron, and has immense potential as an ingredient for development of nutraceuticals. The heavy metals *viz.* arsenic, lead and cadmium were detected within the acceptable limits.

5.1.4.2. Antioxidant assays

Reactive Oxygen Species (ROS) are formed inside the living organisms as natural byproducts of the regular metabolism of oxygen and from exogenous sources like environmental pollutants and UV radiation. These include hypochlorous acid

(HClO), hydrogen peroxide (H_2O_2) and free radicals such as the hydroxyl radical (▪OH), superoxide anion (O2 ▪-), alkoxy and peroxy radicals (RO▪ and ROO▪), and peroxynitrite (ONOO-). Due to the presence of unpaired electron in their outer shell, these ROS are highly unstable and reactive; and they react with various bio molecules including DNA, proteins, lipids, fatty acids leading to DNA damage, decrease in membrane fluidity, and damage to membrane proteins and lipids, ultimately leading to cell death. The human body avoids the so-called oxidative stress by defense mechanisms made of endogenous antioxidants. When these defense mechanisms are insufficient, the cellular damage occurs which will be expressed as metabolic disorders, cancer, aging-related disorders, and cardio vascular diseases (Pinto *et al.*, 2005; Paudel *et al.*, 2012; Behera *et al.*, 2012).

The development of rancidity in foods is caused by free radicals leading to development of off-flavors (Horton *et al.*, 1987). The use of natural antioxidants in food is limited due to lack of knowledge about their molecular compositions, the content of active compounds and the relevant toxicological data. Hence, evaluation of the antioxidative activity of naturally occurring substances has been of interest in recent years (Amarowicz *et al.*, 1996). Antioxidants are compounds that terminate the propagation of oxidizing chain reactions thus delay or inhibit oxidation of bio molecules (Chatterjee *et al.*, 2007). Antioxidant capacity is a widely used parameter to characterize different substances which have the ability of scavenging free radicals; which is related to the presence of compounds capable of protecting a biological system against harmful oxidation. A number of substances occurring in the natural world exhibit antioxidant properties, the best example being plant components (Sini and Devi, 2004; Dawidowicz and Olszowy, 2013).

DPPH assay is considered as a rapid and widely used method for evaluation of antioxidant activity of the plant extracts. Most antioxidant activity assays have

been performed on methanol extracts since methanol is the most efficient and suitable solvent for extraction of bioactive compounds (El-Syed *et al.*, 2008). In the present study, scavenging of DPPH free radicals by three extracts of *Parmotrema tinctorum* was found to be concentration dependent. The IC_{50}, concentration of sample giving 50 per cent inhibition, for ethyl acetate extract and acetone extract were 2.34 and 5.04 mg/ml respectively. Methanol extract showed the maximum scavenging action against the DPPH free radicals (IC_{50}1.47 mg/ml), as lower value of IC_{50} denotes higher radical scavenging activity of the sample (Fig.3).

The antioxidant properties have been reported for lichen extracts due to their phenolic content. Phenolic constituents *viz.* methyl orsellinate, orsellinic acid, atranorin and lecanoric acid derived from the lichen *Parmotrema stuppeum* (Nyl.) Hale (Parmeliaceae) showed moderate antioxidant activity (Jayapraksha and Rao, 2000). Bhattarai *et al.* (2008) reported stronger antioxidant activities in extracts from Antarctic lichens than from lichens native to temperate or tropical regions. Secondary metabolites of lichen especially polysaccharides and phenolic compounds are known to exhibit such properties. The lichens *Peltigera canina, Peltigera praetextata, Sticta nylanderiana, Ramalina conduplicans, Usnea ghttensis* and *Parmotrema pseudotinctorum* were reported to have high DPPH scavenging activity (>85%). The methanol extracts of lichens showed the highest activities of reducing power, superoxide radical scavenging and lipid peroxidation inhibition.

Vivek *et al.* (2014) analysed the radical scavenging action of Parmeliaceae lichens, and among three species, *Parmotrema grayanum* (IC_{50} 148.39 µg/ml) showed higher scavenging potential followed by *Parmotrema praesorediosum* (IC_{50} 179.81 µg/ml) and *Parmotrema tinctorum* (IC_{50} 439.06 µg/ml). However, the radical scavenging efficacy of extracts was lesser than that of ascorbic acid (IC_{50} 2.3 µg/ ml). Antioxidant activity of ethyl acetate extract of *Parmotrema tinctorum* was reported

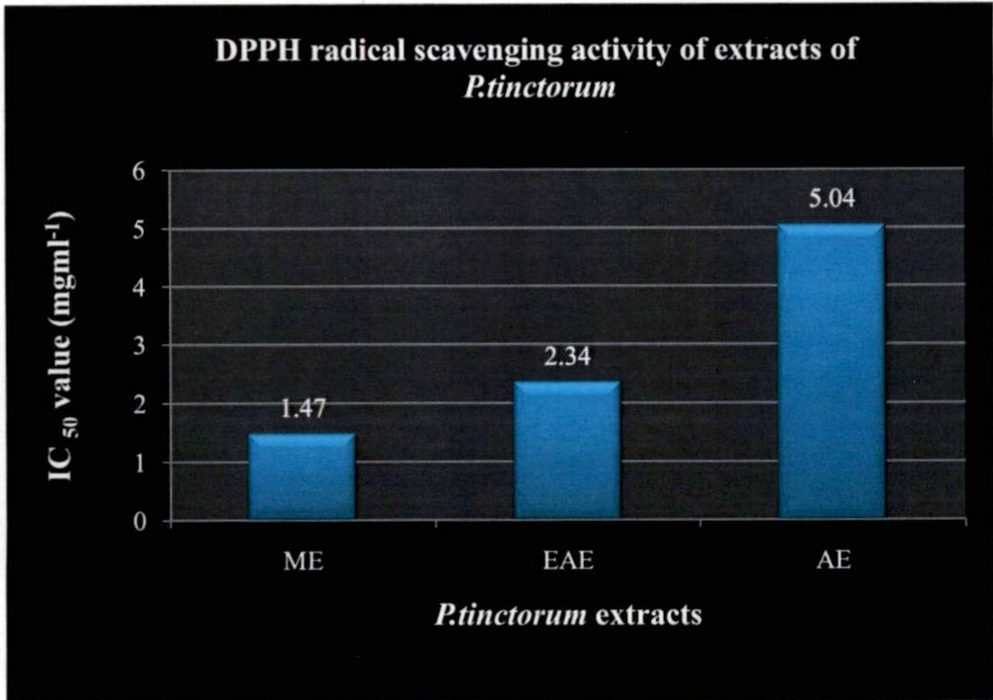

Fig. 3. IC_{50} of *P.tinctorum* solvent extracts on scavenging DPPH radicals

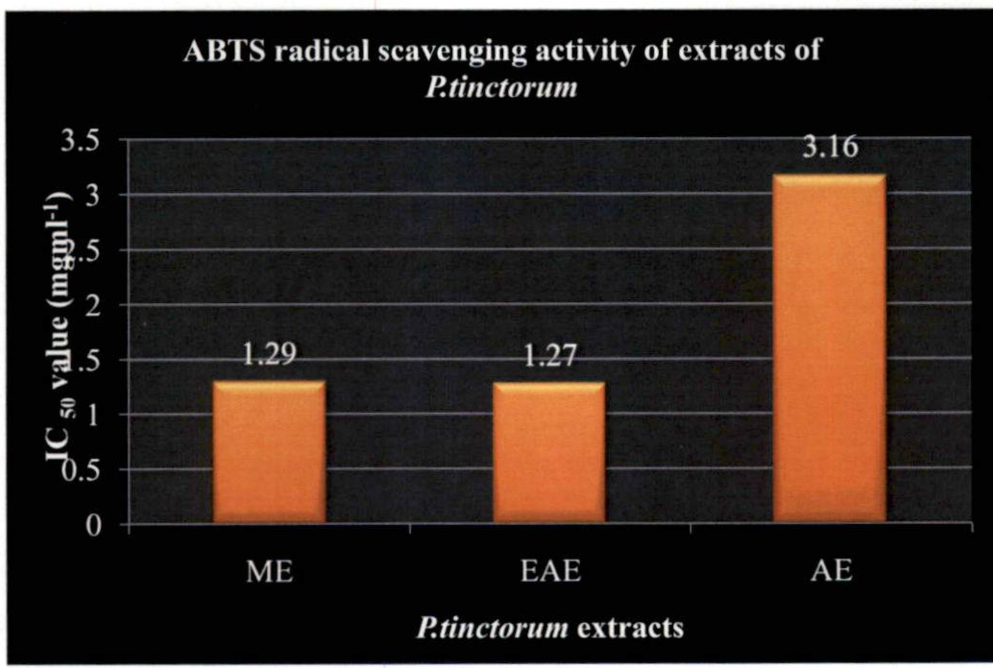

Fig. 4. IC_{50} of *P.tinctorum* solvent extracts on scavenging ABTS radicals

ME-Methanol extract. EAE- Ethyl acetate extract, AE-Acetone extract

by Raj *et al.* (2014) against DPPH (IC_{50} 396.83 μg/ml), ABTS (IC_{50} 151.34 μg/ml), superoxide (IC_{50} 30.29 μg/ml) and hydroxyl (IC_{50} 35.42 μg/ml) radicals. *Parmotrema tinctorum* shows very good potential as a functional food/nutraceutical for diabetic patients, as evident from the significant antioxidant activity and inhibitory potential against carbohydrate digestive enzymes.

Plant polyphenols are considered to be antimicrobial agents, and they are regarded as potential food natural preservatives (Proestos *et al.*, 2006). Generally, antioxidant compounds like phenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide *etc.* and thus inhibit the oxidative mechanisms (Jothy *et al.*, 2012). The lichens which contain higher content of phenols exert stronger antioxidant activity which means that phenols are important antioxidants (Behera *et al.*, 2012; Kosanic *et al.*, 2012; Manojlovic *et al.*, 2012). Pratibha and Sharma (2016) reported the antioxidant activity for lichen compounds *viz.*lecanoric acid, salazinic acid, stictic acid, usnic acid. Acetone extract demonstrated a very potent activity with IC_{50} value of 28 μg/ml as compared to petroleum ether (31 μg/ml) and chloroform (48 μg/ml) extracts. Shanmugam *et al.* (2017) observed that the methanol extract of *Parmotrema tinctorum* and *Parmotrema austrosinense* showed greater radical scavenging activity with IC_{50} value of 1059.7μg/ml and 1101.7μg/ml respectively, and reported that *Parmotrema austrosinense* can be used as natural antioxidant sources.

The presence of the phenolic groups in the lichen metabolites is considered to be a key factor for the antioxidative efficiency. Phenolic antioxidants are products of secondary metabolism, and their redox properties and chemical structure, play a vital role in chelating transitional metals, inhibiting lipoxygenase and scavenging free radicals leading to antioxidant potential (Decker, 1997). A positive correlation between phenolic composition and antioxidant activity was also reported by Manojlovic *et al.* (2012) while studying the strong antioxidant activity of lichen

Umbilicaria cylindrica. They found that the antioxidant property of the lichen could be attributed to the significant amount of depsidones, especially salazinic acid.

ABTS assay involves the generation of $ABTS^{+}\bullet$ chromophore by the oxidation of ABTS with potassium persulfate (Re *et al*. 1999). The antioxidant potential is measured by reduction of the pre-formed ABTS mono cation radical (ABTS•+) to ABTS, in the presence of hydrogen donator antioxidant substances, with a concomitant decrease in the absorbance of the mixture at 734 nm. (Pinto *et al*., 2005; Johnston *et al*., 2006). This assay is relatively quick compared to other total antioxidant assays, and does not require sophisticated analytical equipment. Both hydrophilic and lipophilic antioxidant compounds can be measured by ABTS assay and also it can determine antioxidant potential at different pHs (Labrinea and Georgiou, 2004; Johnston *et al*., 2006). Microalgae and cyanobacteria are potential sources of antioxidants as observed by Guedes *et al*. (2013) by ABTS assay and hence suitable as food/feed ingredients.

In ABTS assay, the methanol extract of *Parmotrema tinctorum* showed the highest ABTS radical scavenging capacity (IC_{50} 1.29 mg/ml). The activity of ethyl acetate extract recorded (EAE) was significantly on par with that of methanol extract with an IC_{50} value 1.27 mg/ml (Fig.4). In both assays, acetone extract showed the least scavenging action (IC_{50} 3.16 mg/ml). Similar results were obtained in the study conducted by Kumar *et al*. (2014) among fourteen saxicolous lichens from Trans Himalayan ladakh region where the methanol extract of lichen *Xanthoparmelia stenophylla* showed the highest ABTS radical scavenging capacity (IC_{50} 1.88±0.09 mg/ml).The maximum ABTS radical scavenging capacity in n-hexane and water extracts was rendered by *Acarospora badiofusca* (IC_{50} 6.55 mg/ml) and *Umbilicaria vellea* (IC_{50} 3.45 mg/ml). ABTS radical scavenging capacity of methanol extracts was significantly higher for all the lichen species. On the basis of the results obtained in the present analysis, it can be suggested that the extract of lichens *Parmotrema*

tinctorum could be used as a source of natural antioxidants for possible use in food or pharmaceutical products.

5.1.5. Thin layer chromatography

Thin layer chromatography (TLC) is a basic chromatographic technique used for separation and identification of organic compounds and is used to separate mainly non-volatile compounds (Sadasivam and Manickam, 1992; Harborne, 1998). The solvent that is used for the separation is used as the mobile phase whereas the absorbent materials are used as the stationary phase. The separation occurs based on the polarity, since both phases have different polarity (Raaman, 2006).

The separation of the active compounds by TLC and the resultant spots are highly dependent on the solvent systems used. Mixture of solvents with in different ratio having variable polarity can be used for separation of pure compounds from plant extract. The term retention factor (R_f), is commonly used to describe the chromatographic behaviour of sample solutes. The R_f value for each solute is the distance it has moved divided by the distance the solvent front has moved. The extent of the surface of the spot is a measure for the quantity of the material present (Banu and Nagarajan, 2014).

According to Liu *et al.* (2014), the techniques such as Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Gas Chromatography-Mass Spectrometry (GC-MS) are widely used for analyzing the chemical components from lichen species. Cuberson (1972) had described chromatographic data and Rf classes for 149 lichen products in three standard solvent systems.

In the present study TLC had been performed as per the procedure given by Jayaprakasha *et al.* (1998). Chromatograms were observed in three solvent systems

viz. hexane-ethyl acetate 80:20, chloroform-methanol 98:02 and benzene-ethyl acetate 95:05 for TLC of *Parmotrema tinctorum* acetone extract. They identified the major compounds as atranorin, methyl orsellinate, orsellinic acid and lecanoric acid, by Nuclear Magnetic Resonance spectrascopy. Vinayaka *et al*. (2009) observed the presence of atranorin and lecanoric acid in TLC in *Parmotrema pseudotinctorum* collected from the forests of Bhadra Wildlife Sanctuary, Karnataka.

Results of TLC profiling of the three lichen extracts showed the presence of number of phytochemicals. Each phytochemical gives different Rf values in different solvent system, based on their polarity. This information of their polarity will help in selection of appropriate solvent system for separation of pure compounds by column chromatography. Maobe *et al*. (2012) described that each spot is presumably due to a pure natural product or phytochemical; each also has a specific Rf value. The large Rf value indicates the low polarity of phytochemical.

5.1.6. Flavour profiling by Gas chromatography –Mass Spectrometry

The literature on GC-MS profiling conducted on lichen species are scarce as till recently high-pressure liquid chromatography (HPLC) methods have been used most widely for the analysis of the secondary metabolites in lichens. HPLC methods, however, need standards for compound identification or isolation of extract constituents and also are very expensive HPLC grade solvents. Moreover the analysis is a lengthy procedure and the identification of volatile part of the extracts by HPLC could be difficult. The MS libraries (Wiley 9 and Massfinder 10, NIST) enable constituent identification without standards.

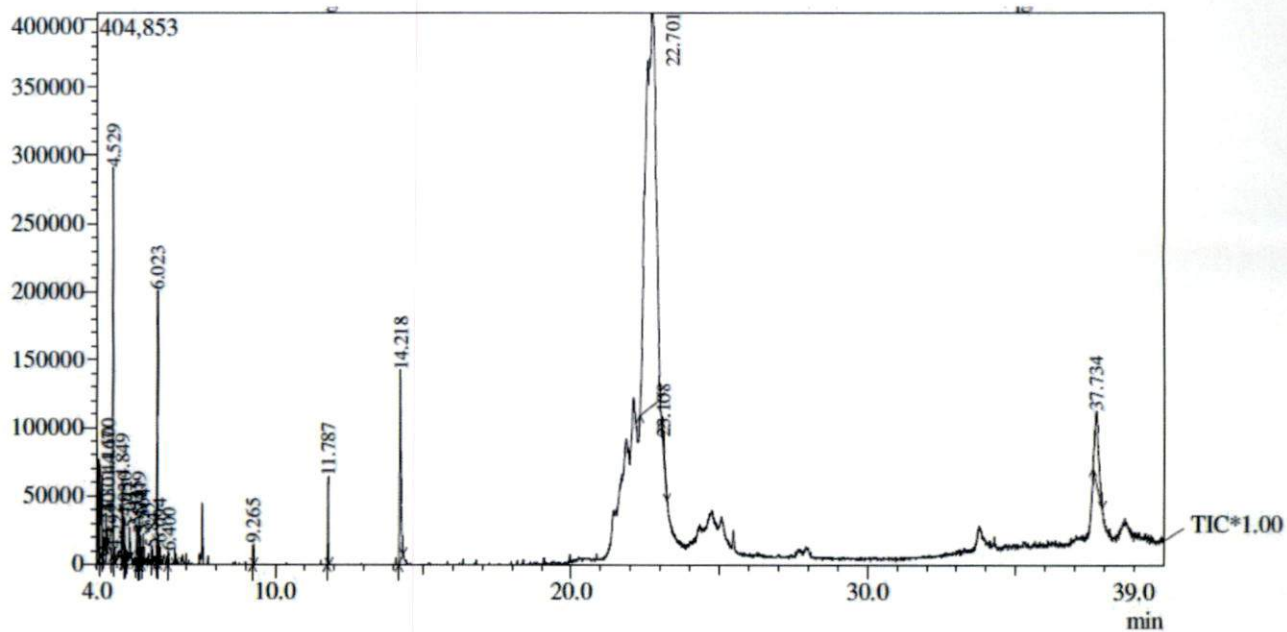

Fig.5. GC-MS profile of hexane extract of *Parmotrema tinctorum*

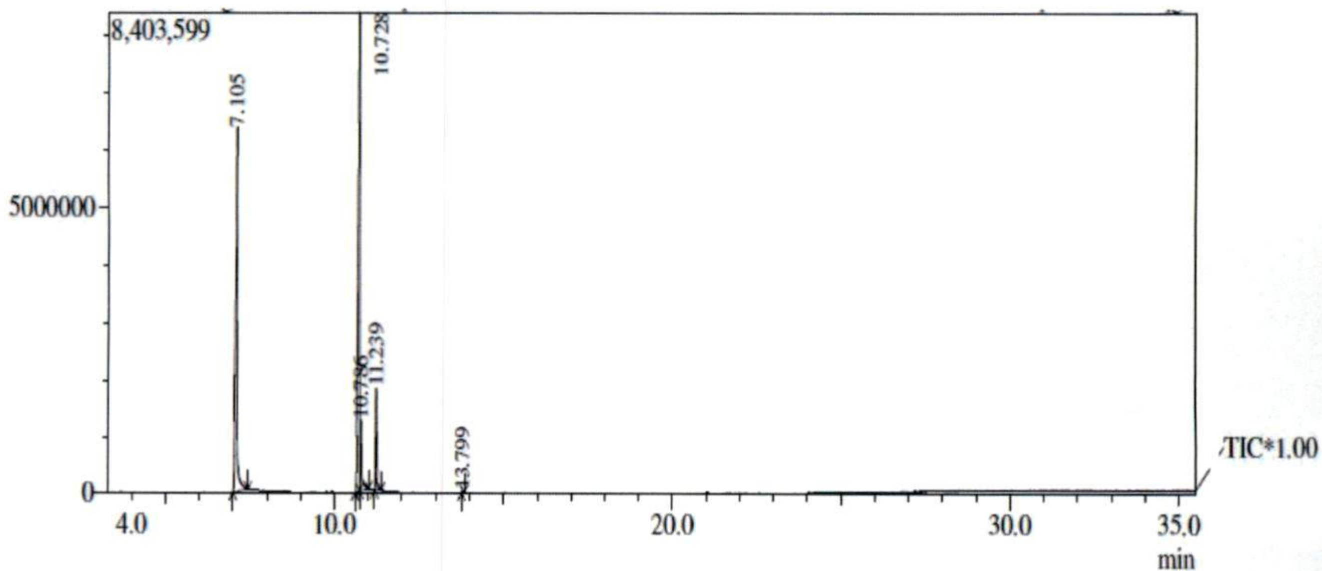

Fig.6. GC-MS profile of methanol extract of *Parmotrema tinctorum*

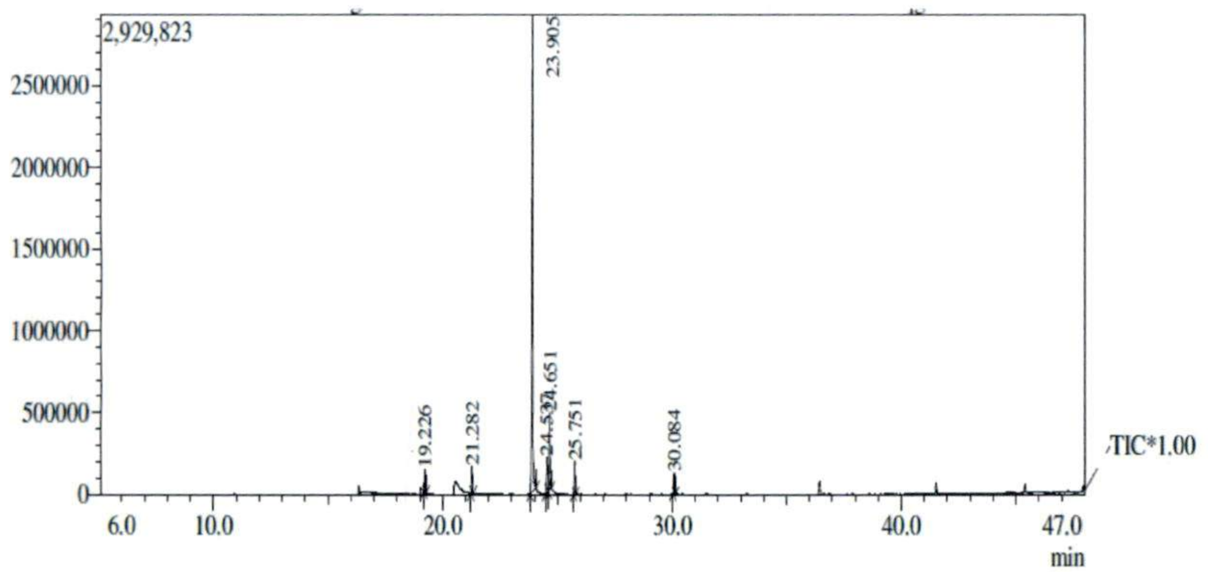

Fig.7. GC-MS profile of chloroform extract of *Parmotrema tinctorum*

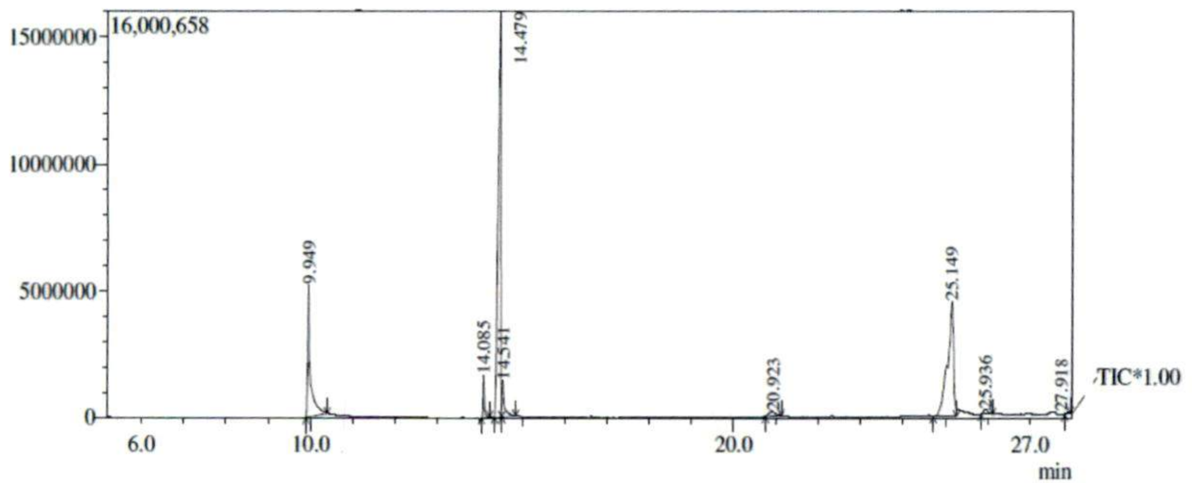

Fig.8. GC-MS profile of ethanol extract of *Parmotrema tinctorum*

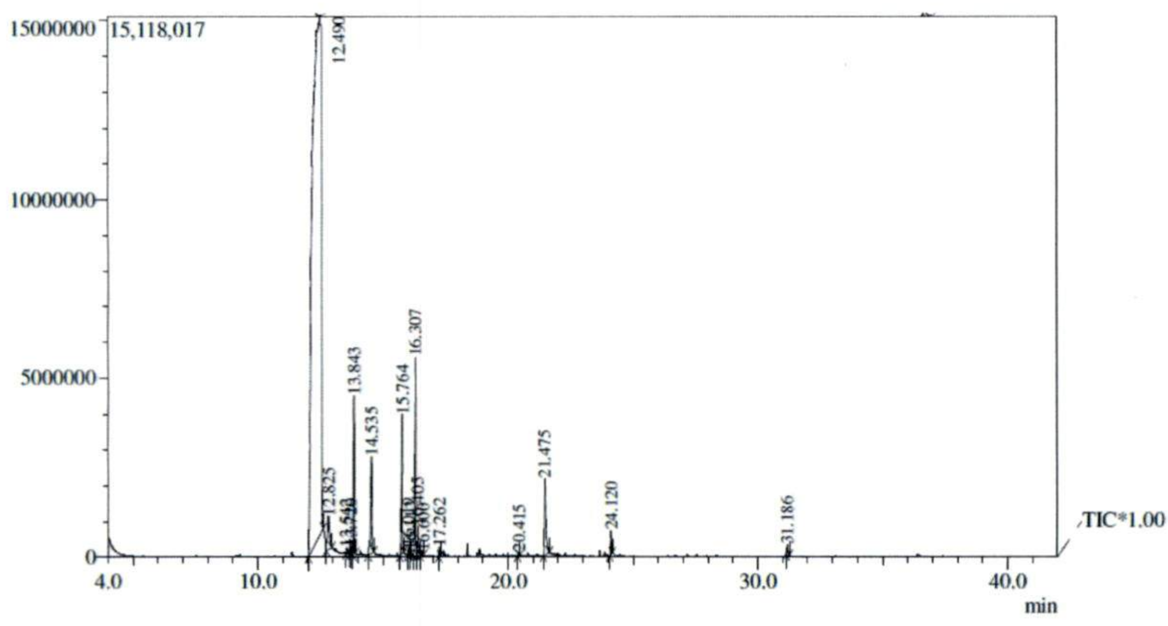

Fig.9. GC-MS profile of acetone extract of *Parmotrema tinctorum*

The GC-MS analysis was conducted using methanol, acetone, hexane, chloroform and ethanol extract of the lichen *Parmotrema tinctorum* in the present study. In the present study, maximum number of compounds (23) were observed in hexane extract, in which glyceryl trilaurate, a fatty acid ester was the major component (19.50%). Lauric acid is the most inhibitory saturated fatty acid against gram-positive organisms (Kabara *et al.*, 1972). Orcinol, the major phenolic compound specific to lichens was present in acetone, ethanol and methanol extracts of *Parmotrema tinctorum*.

Stojanovic *et al.* (2011) conducted the GC-MS analysis of methanol extracts of Parmeliaceae lichens *viz. Evernia prunastri, Hypogymnia physodes* and *Parmelia sulcata*. Olivetol (33.5 %), atraric acid (17.2 %) and olivetonide (15.7 %), were the major components of the *H. physodes* extract. Atraric acid was the principal compound in (30.0%) both in *E. prunastri* and *P. sulcata* extracts. *E. prunastri* extract was analysed to have high content of orcinol (25.0 %). In the present study also, methanol extract expressed similar content of orcinol (35.77%). Parmelioid lichens contain phenolic compounds such as depsides, depsidones which are formed by joining two or three phenolic units derived from acetate- polymelonate pathway (Shukla *et al.*, 2014).

The acetone, chloroform and petroleum ether of lichen *Parmelia perlata* were analysed by Pratibha and Sharma (2016). They found that compounds *viz.* pyrimido[1,2-a]azepine, 2,3,4,6,7,8,9,10-octahydro-, *o*-orsellinic acid, ethyl ester, methyl haematommate, 5,7-dihydroxy-4- methylcoumarin, linoleic acid, methyl ester, benzoic acid, 2,4-dihydroxy-6-(2-oxoheptyl)-, 4- carboxy-3-hydroxy-5-pentylphenyl ester were detected only in chloroform extract, while some metabolites were identified only in acetone extract like dodec-1-ene, butanal, 3-methyl-, orcinol, *o*-orsellinaldehyde, pentadecane, 1,13-tetradecadiene, benzoic acid, 2,4- dihydroxy-6-

methyl-, methyl ester, hexadecane, Z-10-tetradecen-1-ol acetate, octacosane, lauraldehyde 2,4- dinitrophenylhydrazone and tetracosyl heptafluorobutyrate. In the petroleum ether extract, usnic acid, D:A-friedooleanan-3-one, 5-methyl-1,3-benzendiol, 5-pentyl-1,3-benzenediol, atranorin, methoxyolivetol and Z-10-tetradecen-1-ol were identified as major compounds. According to Khadem and Marles (2010), orsellinic acid (2,4-dihydroxy-6-methylbenzoic acid) is present in some lichens such as *Roccella*, *Lecanora*, and *Lobaria* and are having antimicrobial activity. Some derivatives of orsellinic acid are found *viz*. 2-O-β-Dglucopyranoside in cloves (*Syzygium aromaticum*), and the 4-methyl ether (everninic acid) in the honey mushroom *Armillaria mellea*.

In the present study, phytosterols *viz*. campesterol, gamma sitosterol, stigmasta-3,5-dien-7-one were identified only in the ethanol extract. Sitosterol was the third most abundant component (10.6%) in the *Parmelia sulcata* extract subjected to GC-MS as analysed by Stojanovic *et al*. (2011). 14-Beta-H-Pregna is the major component detected in the essential oil of round garlic *Allium rotundatum* flower (Dehpour *et al*., 2012).

Joulain and Tabacchi (2009), analysed lichen 'oakmoss' (*Evernia prunastri* (L.) Arch. (Parmeliaceae) extract for it's component chemicals and found that there are 170 compounds. Fifteen depsides and dibenzofuran compounds were identified *viz*. evernic acid, barbatic acid, atranorin, chloroatranorin, lecanorin, lecanoric acid, evernin, prunastric acid, methyl 3'methyl lecanorate and usnic acid. Mono aromatic compounds such as orcinol, β-orcinol, atranol, orcinol monomethyl ether, orsellinic acid, ethyl orsellinate, methyl orsellinate, methyl orcinol carboxylate, ethyl orcinol carboxylate, haematommic acid, methyl haematommate, ethyl haematommate, are also present in the extracts.

Terpenoids (monoterpenes and sesquiterpenes) and oxygenated compounds (acids, alcohols, aldehydes, ketones, lactones, ethers, phenols and esters) are described as responsible for the characteristic odours and flavours of essential oils and extracts. Cymene is the precursor of carvacrol which forms the main component of thyme essential oil, and cymene was observed in hexane extract of *Parmotrema tinctorum*. Carvacrol has been regarded as a biocidal, resulting in bacterial membrane perturbations that lead to leakage of cellular fluids leading to cell death (Vernet *et al.*, 1986).

In *Parmotrema tinctorum* lichen extracts also, some of the above compounds were detected *viz.* orcinol, atranorin, chloroatranorin, atraric acid, methyl haematommate *etc.* which may be responsible for the flavour of lichen. Along with these compounds, benzoic acid derivatives, fatty acid esters, aldehydes, alkenes, and sterols also show their presence in the extracts, contributing to the flavour profile of lichen (Fig.5 to Fig.9).

5.2. Evaluation of antimicrobial property of lichen extracts

The contamination of food by microorganisms is a public health concern and is one of the major reason of trade problems internationally. Microbial activity is a primary mode of spoilage of many foods and is often results in loss of quality and safety (Baydar *et al.*, 2004). Concern over pathogenic and spoilage microorganisms in foods is increasing due to the increase in outbreaks of food-borne diseases. Resistance build up against antibiotics and their possbile side effects encourage researchers the investigation on antimicrobials from natural sources (Oussalah *et al.*, 2007; Rahman and Kang, 2009).

Lichens are well known as exceptional sources of phytochemicals that act against pathogens including drug resistant strains (Oksanen, 2006; Chauhan and

Abraham, 2013; Javeria *et al.*, 2013). Since ancient times, lichens have been in use for many purposes, such as food, drugs and perfumery. Also, the rising consumer demand and the emerging interest in food industry for effective, safe, natural products means that quantitative data on antimicrobials like plant oils and extracts are required. Lichens, especially the foliose and fruticose types are well known for their lichen acids, which exhibit exceptionally good antibacterial and antifungal efficacy (Shivanna and Garampalli, 2015). Lichens are reported to produce for more than 1000 secondary metabolites of which 80% are unique to lichens only and are not reported yet from any other natural source (Jha *et al.*, 2017).

The results of antimicrobial testing in the present study showed that all the three extracts of the lichen *Parmotrema tinctorum viz.* acetone, ethanol and chloroform have potential activity against the selected food spoilage organisms. The different solvent extracts (acetone, ethanol and chloroform) of lichen *Parmotrema tinctorum*, acetone, ethanol and chloroform have shown remarkable antibacterial activity against the test bacteria viz. *Bacillus subtilis* and *Staphylococcus aureus* at all the four levels of concentrations studied (Fig.14 and 15). *Bacillus subtilis* was found to be inhibited maximum by AE (20.25 mm), followed by EE (16.75 mm) at the highest concentration of 40 mg/ml. In the case of *Staphylococcus aureus*, significantly highest inhibition was shown by AE at 40 mg/ml (24.25 mm). While performing well diffusion with 50 μl and 100 μl, the growth of both the bacteria *Bacillus subtilis* and *Staphylococcus aureus* were remarkably inhibited by the acetone extract (AE) and by chloroform extract (CE) forming zones of inhibition at all the concentrations tested (Fig.24, 25, 26 and 27). In all the cases, the standard antibiotic Ciprofloxacin 10 mcg produced the highest inhibition of bacterial growth.

Staphylococcus aureus is the causative agent for a number of human infections, including pneumonia, and meningitis. This bacterial pathogen is notorious

for acquiring rapid resistance to antibiotics, caused primarily by the horizontal transfer of resistance genes (Chambers and De Leo 2009; Mun *et al*., 2013). *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Penicillium chrysogenum* are among the important drug resistant pathogens (Anjali *et al*., 2014). *Bacillus* spp. are important bacterial contaminants within various food-processing operations. *Bacillus cereus* is an important post pasteurization spoilage bacterium in dairy products due to the production of extracellular enzymes (Lindsay *et al*., 2006). The genus *Bacillus* includes species like *Bacillus cereus*, *Bacillus licheniformis* and *Bacillus subtilis* which are found in fresh and pasteurised food products due to their ability to produce heat-resistant spores.

The antibacterial effects of other Parmeliaceae lichens were reported by some workers. Abdur *et al*. (2013) analysed the antibiotic activity of the hydro-ethanolic and ethanolic extracts of lichen *Parmelia perlata* by screening against different bacterial strains using agar well diffusion method. *Parmelia perlata* had significant antibiotic activity towards *Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Corynebacterium xerosis, Proteus vulgaris*, *Kebsiella pneumonia* and *Escherichia coli*. The antimicrobial activity of ethanolic extract of different lichen species belonging to genus Parmeliaceae *viz*. *Parmelia perlata, Parmelia pereoridisum, Parmoterma mesotropum, Parmoterma reticulatum,* and *Parmoterma perlatum*, from Uttarakhand was reported by Kumar *et al*. (2014). All the species of lichen were effective against the *Staphylococcus aureus*, while no activity was shown against fungi. In a study conducted by Vivek *et al*. (2014), the extract was active against *Staphylococcus aureus* and *Streptococcus mutans*.

Wang *et al*. (2009) opined that the Gram-positive bacteria is more sensitive than the Gram-negative bacteria, due to their differences in cell membrane constituents and structure, while reporting the antimicrobial effect of curcumin, against *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*. Gram-positive

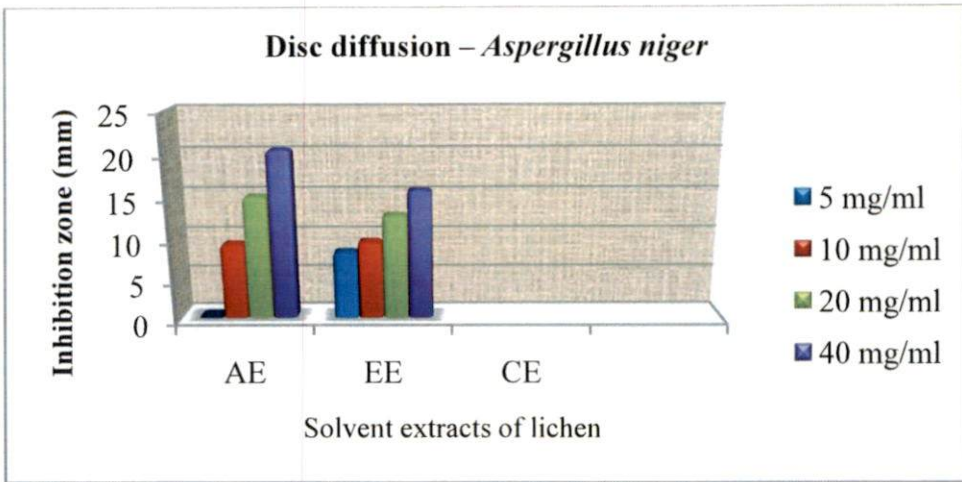

Fig.10. Effect of lichen extracts on growth of *Aspergilus niger* in disc diffusion

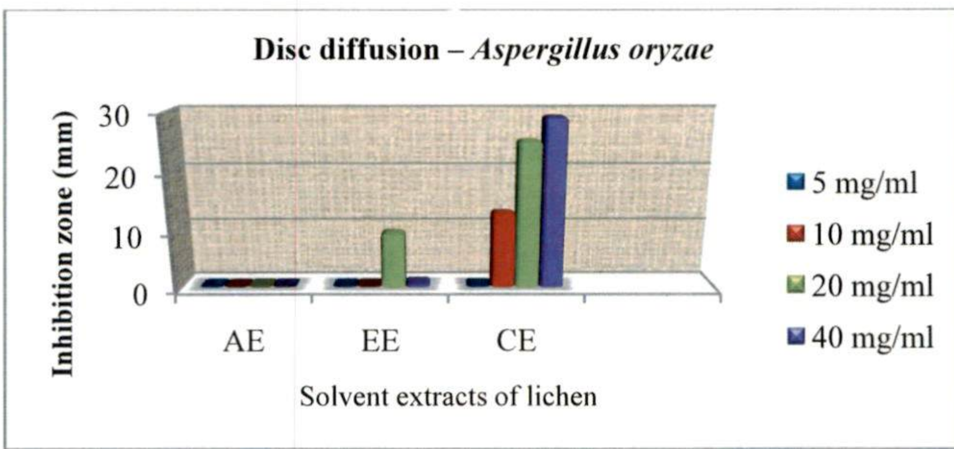

Fig.11. Effect of lichen extracts on growth of *Aspergillus oryzae* in disc diffusion

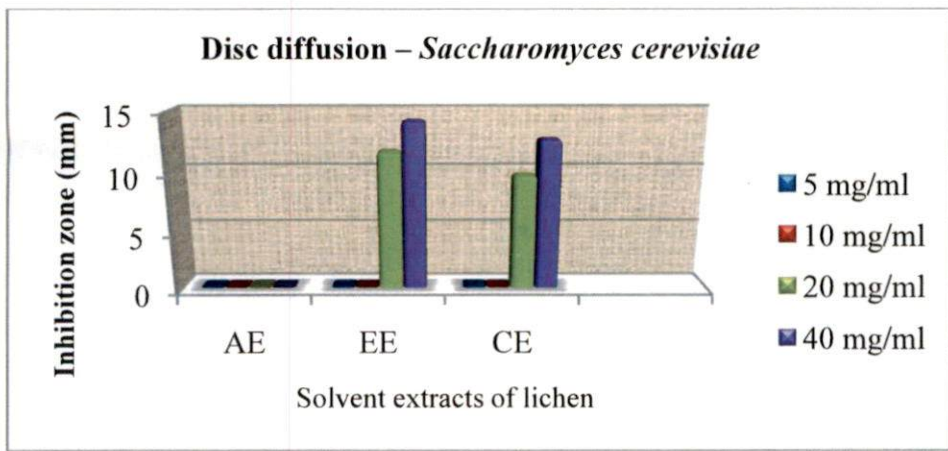

Fig.12. Effect of lichen extracts on growth of *Saccharomyces cerevisiae* in disc diffusion

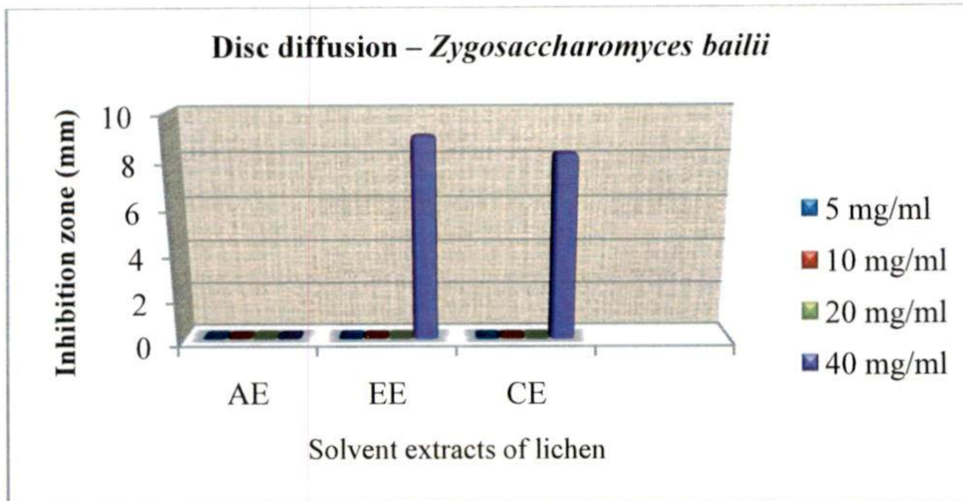

Fig.13.Effect of lichen extracts on growth of *Zygosaccharomyces bailii* in disc diffusion

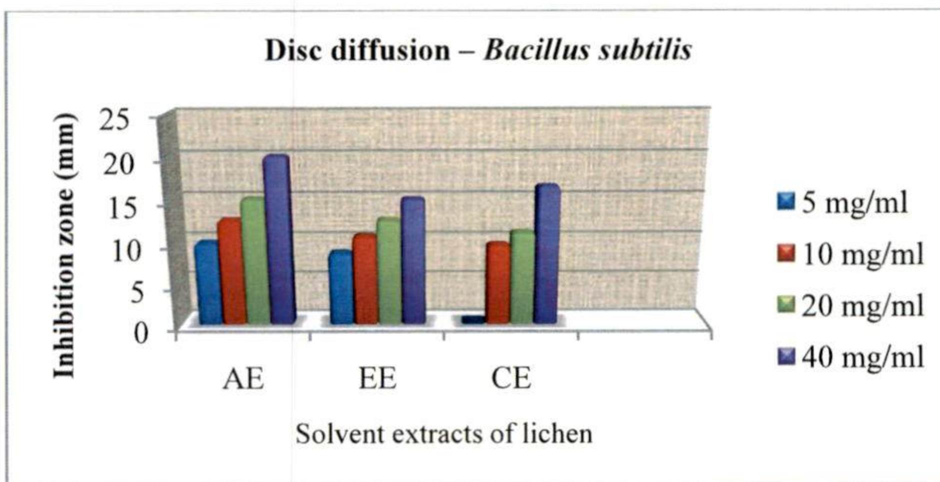

Fig.14. Effect of lichen extracts on growth of *Bacillus subtilis* in disc diffusion

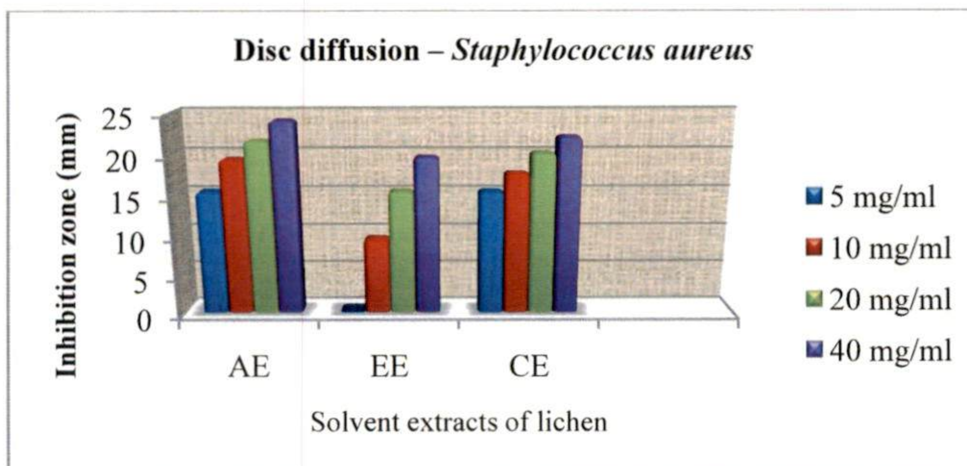

Fig.15. Effect of lichen extracts on growth of *Staphylococcus aureus* in disc diffusion

bacteria contain an outer peptidoglycan layer, which is an ineffective permeability barrier. In the present study also, the Gram positive bacteria were remarkably inhibited by the *Parmotrema tinctorum* extracts.

Lichens are rich and unique source of secondary metabolites with significant biological activities and among the lichen metabolites, phenols constitute the major group. Polyphenols are reported to be antimicrobial agents, and hence proposed as potential food natural preservatives, and the antimicrobial activity of phenolic compounds has been established (Tassou and Nychas, 1994; Nychas, 1995). According to Proestos *et al.* (2006) plant-derived polyphenols receive substantial interest because of their potential antioxidant and antimicrobial properties. Stanly *et al.* (2011) compared the antioxidant and total phenolic content of four lichen genera belonging to *Ramalina, Parmotrema, Bulbothrix* and *Cladia* from Malaysia and reported that the total phenolic content and the percentage of yield in *Parmotrema tinctorum* is high in acetone and methanol extract as compared to the other three lichen species.

The ethanol extract was found to contain compounds *viz.* orcinol, chloratranorin, ethyl orsellinate, methyl haematommate, campesterol, gamma-sitosterol, 14-.beta-H-pregna, stigmasta-3,5-dien-7-one. Orcinol, chloratranorin, ethyl orsellinate are phenol compounds, while methyl haematommate is a hydroxyl benzoic acid derivative. The better antimicrobial activity of the *Parmoterma tinctorum* was attributed to the presence of some of its major components orcinol, atranorin (antibacterial), methyl orsellinate and methyl haematommate (antifungal). The inhibitory effects of the extracts against microorganisms may be attributed to the disruption of bacterial and fungal membrane integrity (Pratibha and Sharma, 2016).

Phenolic compounds *viz.* depsides, depsidones, dibenzofurans and pulvinic acid derivatives are secondary metabolites of lichen samples. These compounds have gained attention of investigators because of their antiviral, antibiotic, and antitumor activities (Nishitoba *et al.*, 1987; Huneck, 1999; Honda and Vilegas, 1999). According to Dorman and Deans (2000), essential oils containing phenolic compounds possess the strongest antimicrobial activities.

Atranorin is the main compound from the lichen, which belongs to depside group and is present in most of the lichen species (Kristmundsdottir *et al.*, 2005). Studies on bioactive properties of extracts containing atranorin have exhibited antibiotic and antimicrobial activity (Ingolfsdottir *et al.*, 1998; Honda *et al.*, 2010; Yilmaz *et al.*, 2004), antiinflammatory properties (Bugni *et al.*, 2009).

In the present study, antifungal activity of the extracts of lichen *Parmotrema tinctorum* was also investigated. The test organisms were *Aspergillus niger* and *Aspergillus oryzae*. According to Schnurer and Magnusson (2005), *Aspergillus* and *Penicillium* species are reported as spoilage organisms in a broad range of food substances. Species of *Aspergillus* fungi can contaminate agricultural products at pre-harvest, harvest, processing and handling stages. According to Gautam *et al.* (2011) *Aspergillus niger* is known as black mould, and causes post harvest decay of different substrates. *Aspergillus* spp. are widely distributed as important spoilage fruit fungi which act by secreting plant cell wall degrading enzymes; and *Aspergillus oryzae* is found to be present in orange fruits (Al–Hindi *et al.*, 2011).

In disc diffusion assay, ethanol extract 40 mg/ml have exhibited the maximum inhibition (20.50 mm) against *Aspergillus. niger*, whereas chloroform extract at 40 mg/ml had the maximum inhibition against *Aspergillus oryzae*. (29.25 mm) (Fig.10 and 11). The results of well diffusion using 100 μl revealed that ethanol extract at 40

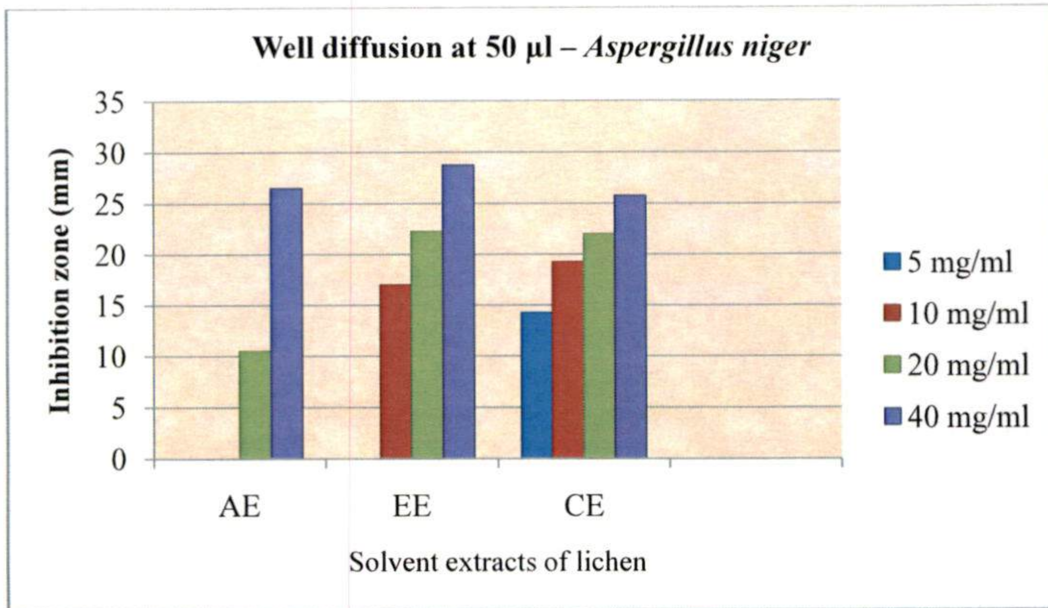

Fig.16. Effect of lichen extracts on growth of *A.niger* in well diffusion (50 µl)

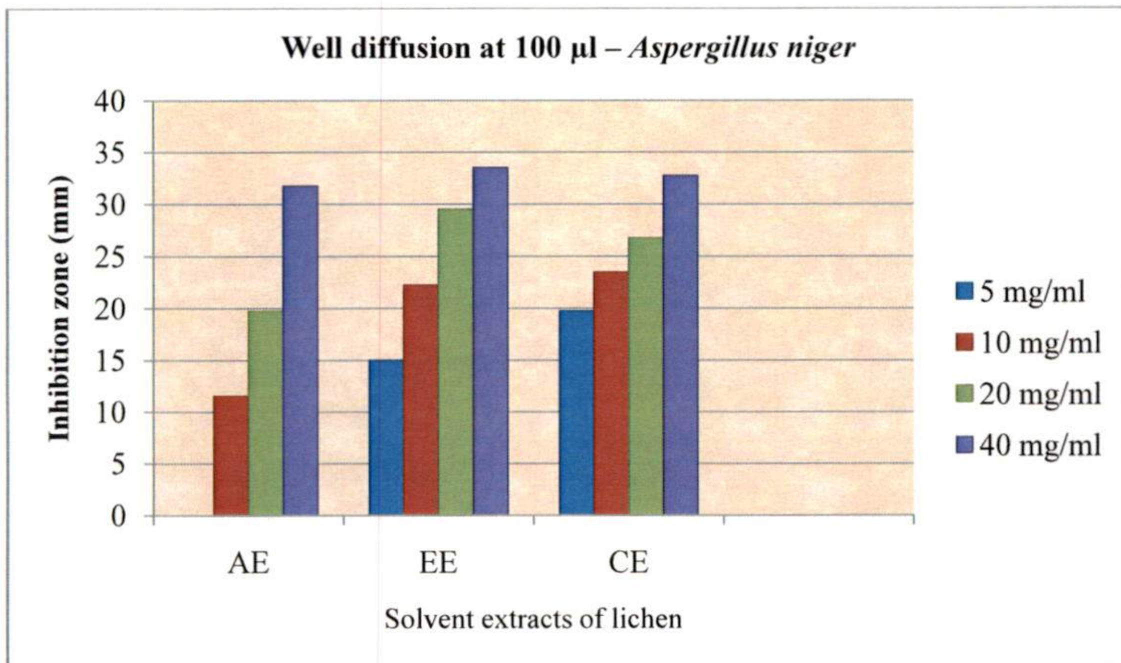

Fig.17. Effect of lichen extracts on growth of *A.niger* in well diffusion (100 µl)

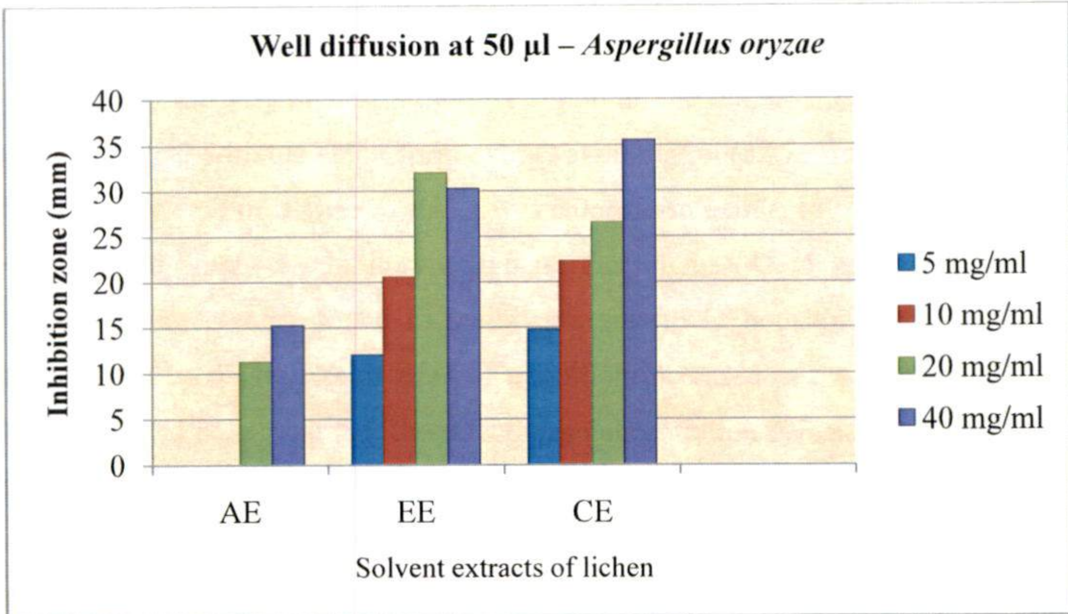

Fig.18. Effect of lichen extracts on growth of *A.oryzae* in well diffusion (50 µl)

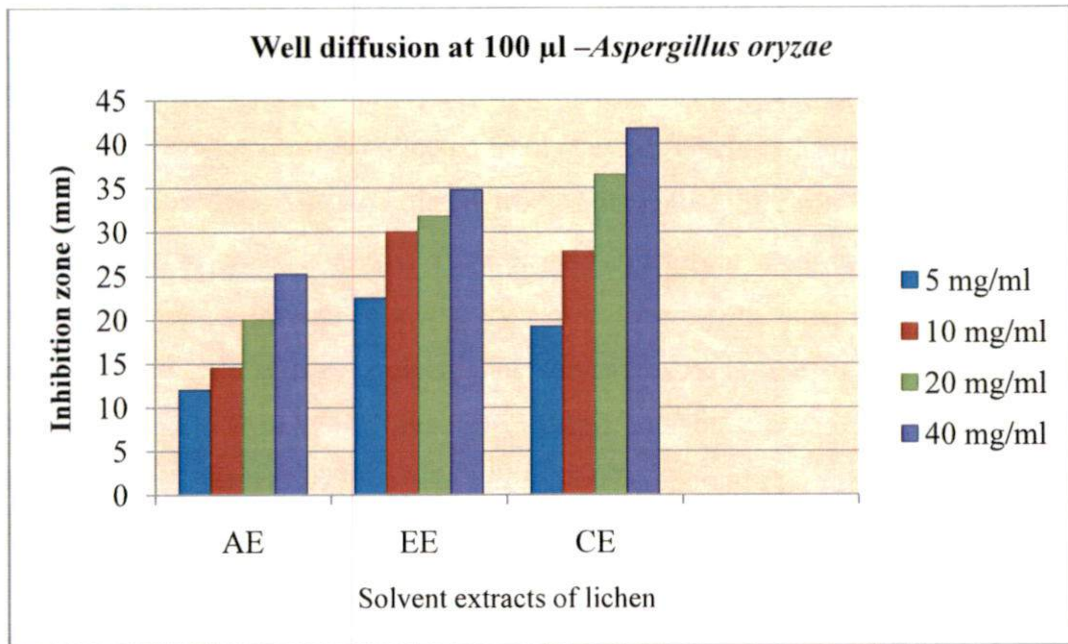

Fig.19. Effect of lichen extracts on growth of *A.oryzae* in well diffusion (100 µl)

mg/ml had significantly highest inhibitory action on the fungus *A.niger* (33.50 mm), which was on par with the chloroform extract at 40 mg/ml (32.75 mm) (Fig.16,17, 18 and 19). In the case of *A. oryzae* also it was chloroform extract (40 mg/ml) exhibited the significantly highest inhibitory action on the fungus (41.75 mm). Ethanol and chloroform extracts were found to inhibit the growth of both the fungi at all the concentrations tried The size of zone of inhibition increased as the concentration of extract increased indicating that the effect was concentration dependent. *In vitro* anti fungal activity of acetone, methanol and chloroform extracts of *Parmoterma tinctorum* was reported by Tiwari *et al.* (2011) against ten plant pathogenic fungi in which methanol extract was effective against all the fungal pathogens that were taken for the study. The inhibition zones against *Aspergillus flavus, Aspergillus niger* and *Fusarium oxysporum* were more with methanol, than with acetone and chloroform extracts.

Prashith *et al.*, (2013), showed similar results wherein the inhibitory action of three Parmeliaceae lichen *viz. Parmotrema tinctorum*, *Parmoterma grayanum*, and *Parmoterma praesorediosum* collected from Western Ghats of Karnataka against the fungus *Colletotrichum capsici* isolated from anthracnose of chilli was investigated and according to their analysis, *Parmotrema tinctorum* was more effective in inhibiting the fungal growth followed by *Parmoterma grayanum*, and *Parmoterma praesorediosum*. It has been opined that solvent extracts and purified secondary metabolites from *Parmotrema tinctorum* exhibit antagonistic activity against plant pathogenic fungi. Recently, Vinayaka *et al*. (2009) reported that methanolic extracts of *P. tinctorum* exhibited inhibition of amylase activity of the fungus, The antifungal action of 2-propanol extract of *Parmotrema tinctorum* was investigated by Anjali *et al*. (2015), and found that the action was significant against *Aspergillus flavus*.

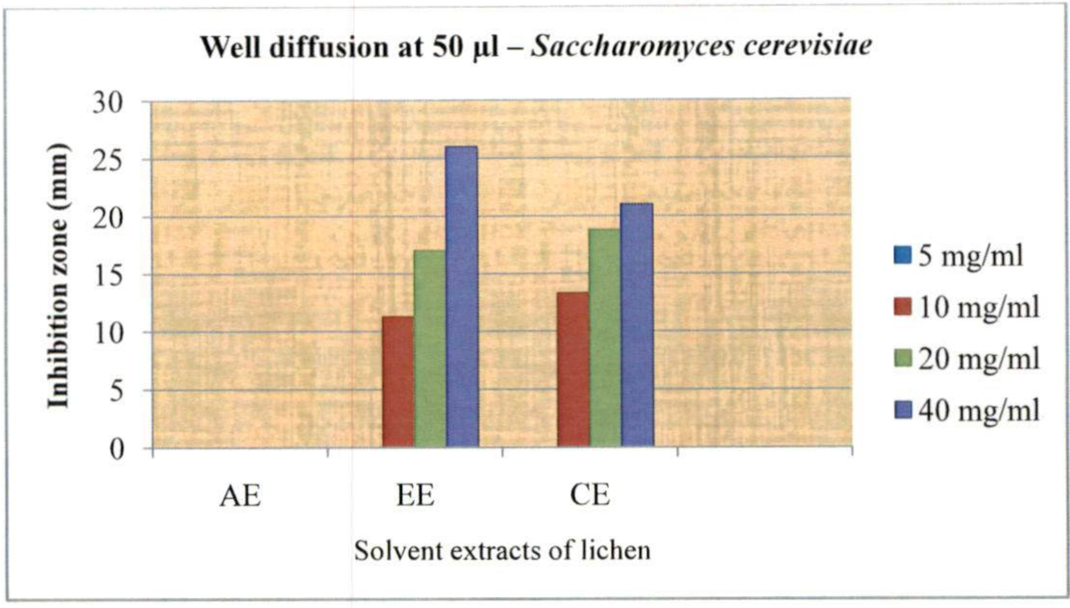

Fig.20. Effect of lichen extracts on growth of *S. cerevisiae* in well diffusion (50 µl)

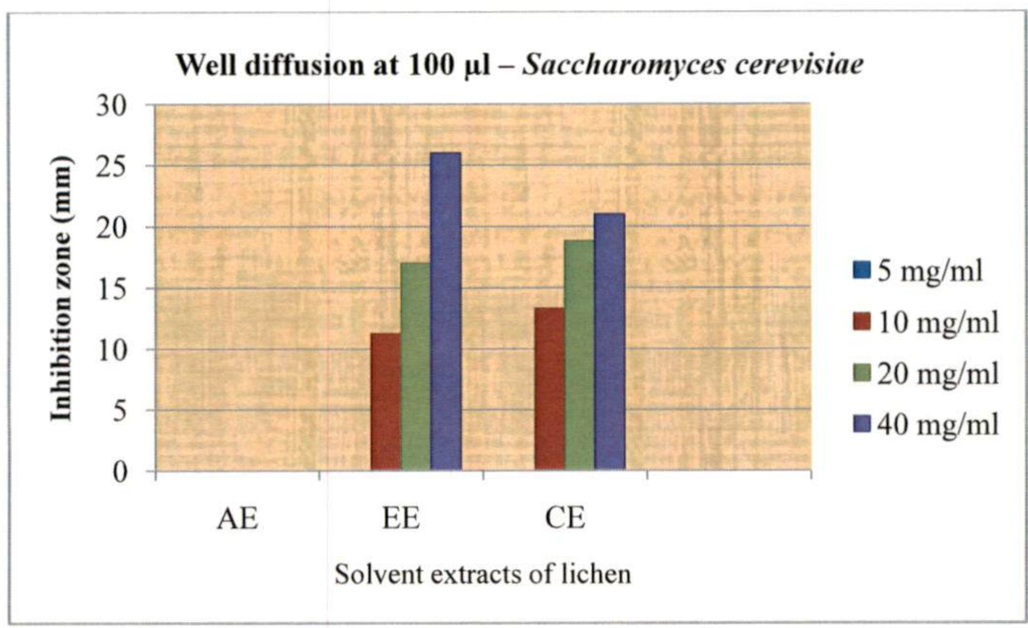

Fig.21. Effect of lichen extracts on growth of *S. cerevisiae* in well diffusion (100 µl)

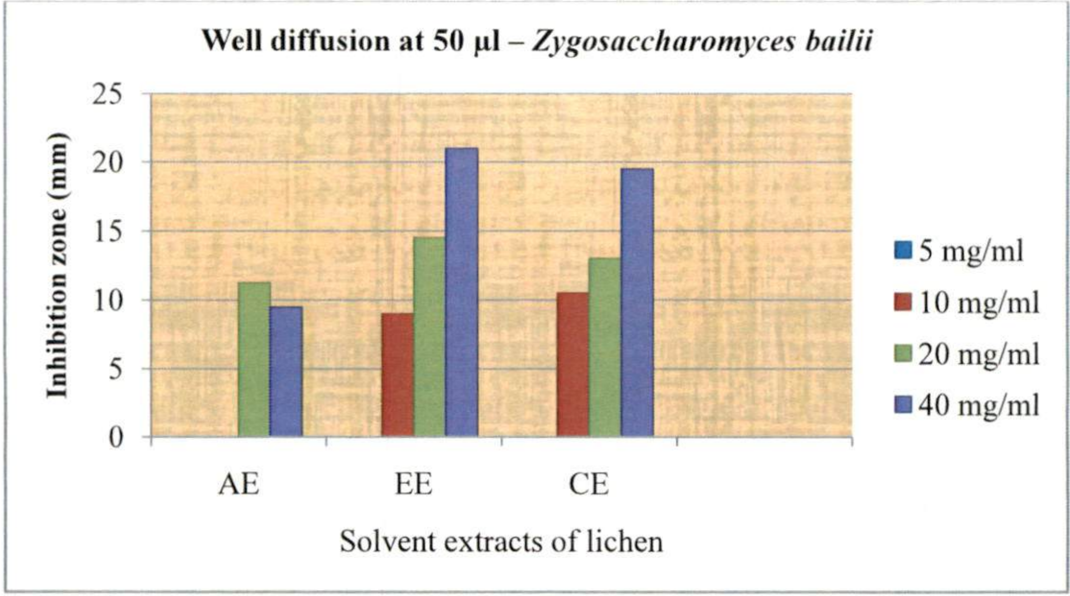

Fig.22. Effect of lichen extracts on growth of *Z.bailii* in well diffusion (50 µl)

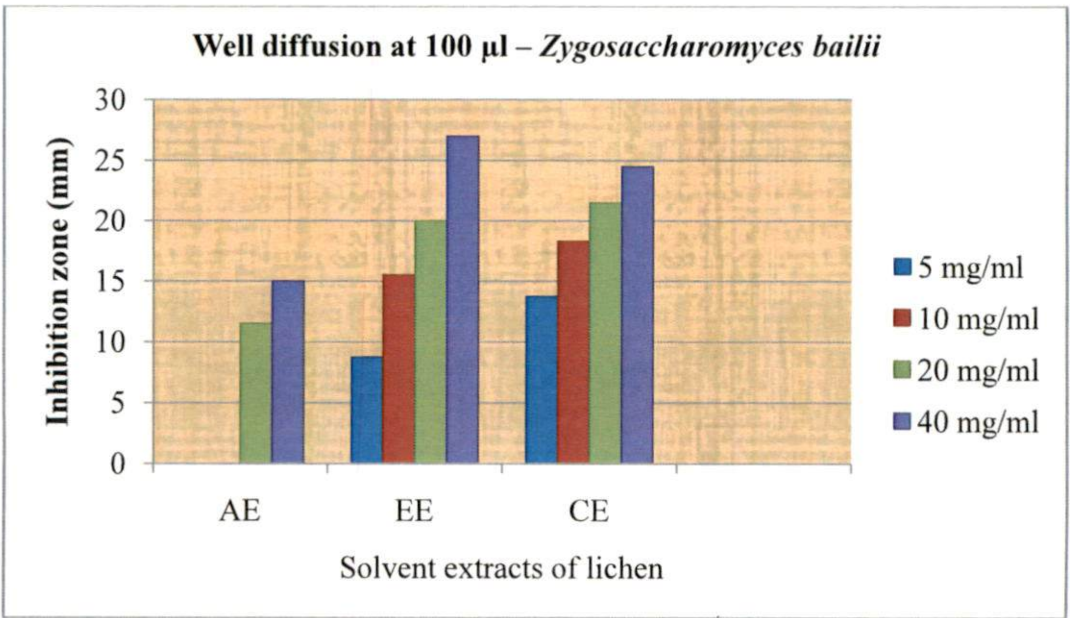

Fig.23. Effect of lichen extracts on growth of *Z.bailii* in well diffusion (100 µl)

Food spoilage yeasts have gained, an increasing importance in recent years being responsible for significant economic losses (Loureiro and Querol, 1999). Loureiro and Ferreira (2003) described *Candida cruzei*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe, Zygosaccharomyces bailii, Zygosaccharomyces rouxii* as major spoilage yeasts.

Anti yeast activity of the lichen extracts was also investigated. The reference spoilage organisms selected were *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. According to Rodrigues *et al*. (2001), *Zygosaccharomyces bailii* is an important yeast species which causes spoilage of wines and other food products; producing undesirable effects like off flavors, hazing in foods, and vigorous alcoholic fermentation. *Zygosaccharomyces bailii* is found to exhibit high tolerance to common food preservatives, low pH, high concentrations of sugars, ethanol, and organic acids. *Saccharomyces cerevisiae* is one of the major spoilage yeasts for fruit juices and soft drinks (Tserennadmid *et al*., 2011).

In the present study, the growth of both yeast species, *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*, was found to be inhibited by both the ethanol and chloroform extract of the lichen *Parmotrema tinctorum* (Fig.12 and 13). The acetone extract could not bring any activity at the lower concentrations. In well diffusion assay (100 µl), the growth of *Zygosaccharomyces bailii* was most significantly inhibited by the ethanol extract at 40 mg/ml producing a zone of 27.00 mm followed by the chloroform extract at the same concentrations with a zone of 24.50 mm; whereas *Saccharomyces cerevisiae* was inhibited significantly at 40 mg/ml of ethanol extract (35.25 mm) (Fig.20, 21, 22 and 23). The very low volume (10 µl) employed in disc diffusion method was found to be not effective for inhibiting the yeast growth of both *S. cerevisiae* and *Zygosaccharomyces bailii*. Inhibition of yeast species in food by active principles from plant sources was reported by some

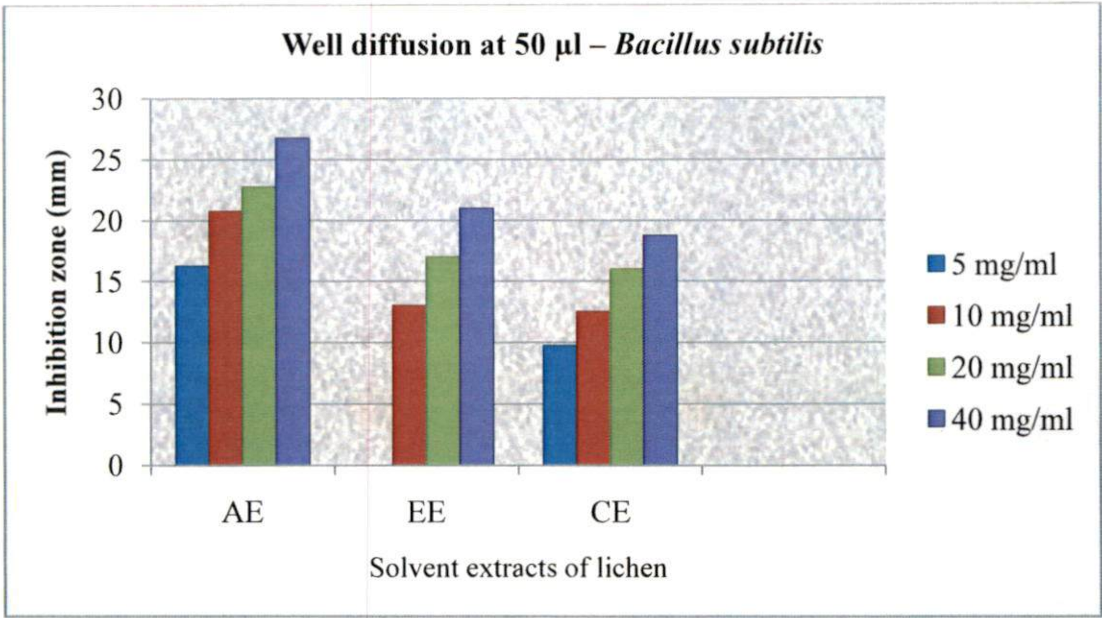

Fig.24. Effect of lichen extracts on growth of *B.subtilis* in well diffusion (50 µl)

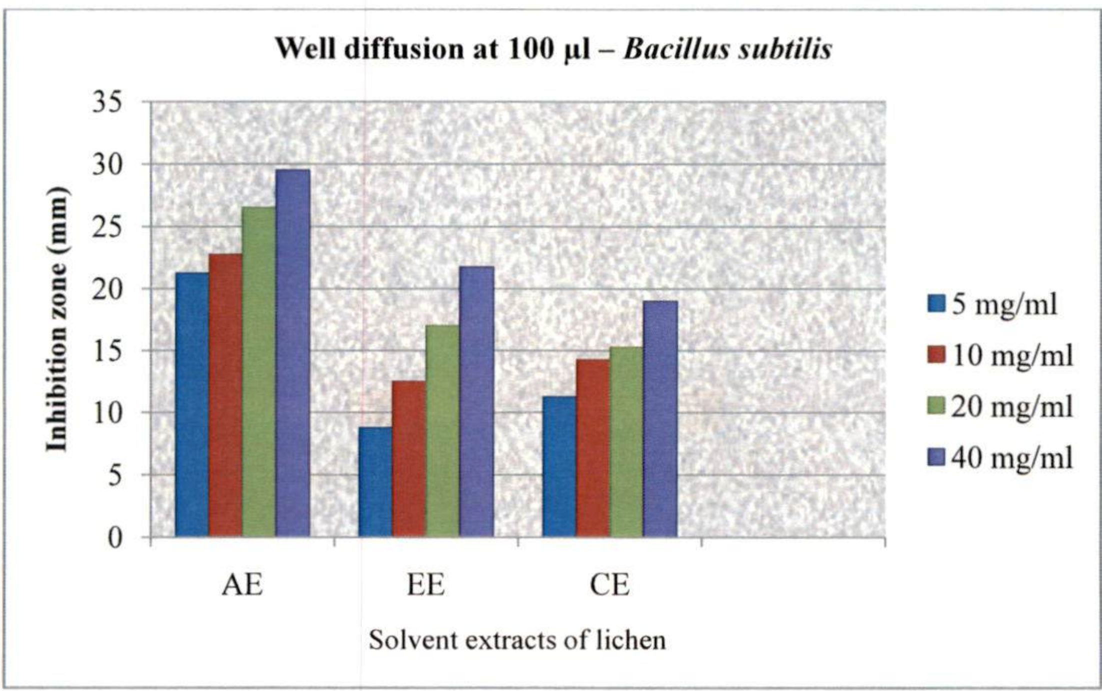

Fig.25. Effect of lichen extracts on growth of *B.subtilis* in well diffusion (100 µl)

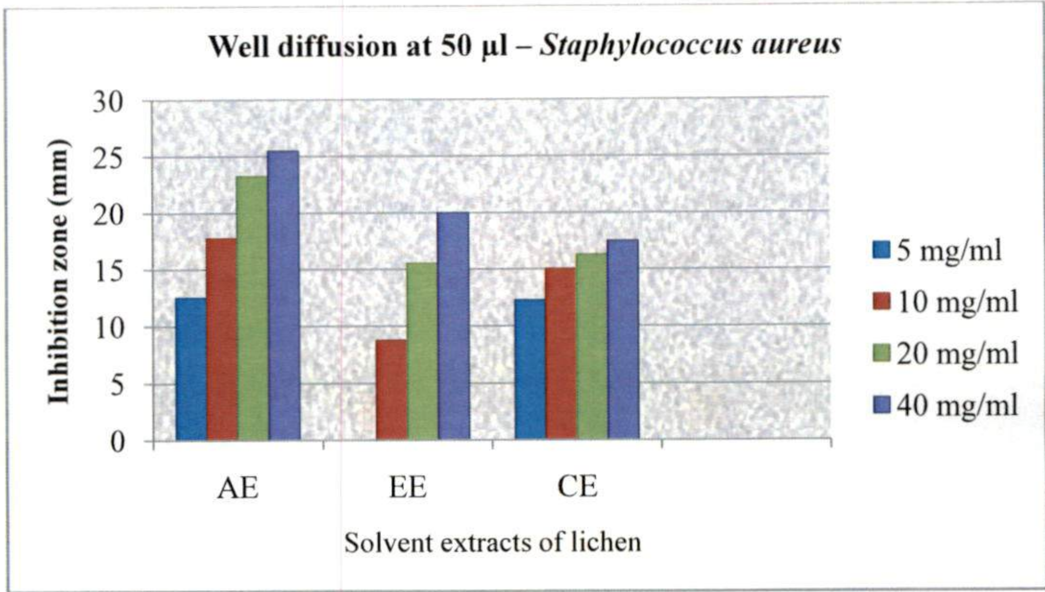

Fig.26. Effect of lichen extracts on growth of *S.aureus* in well diffusion (50 µl)

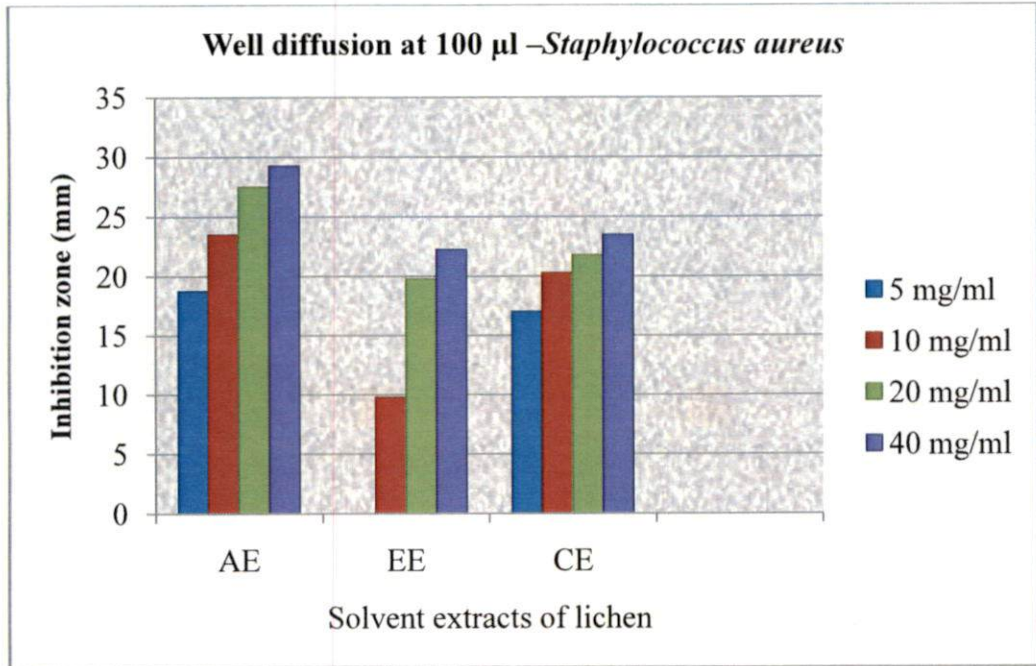

Fig.27. Effect of lichen extracts on growth of *S.aureus* in well diffusion (100 µl)

workers. Fitzgerald *et al.* (2003) reported that vanillin at the concentration of 13 mM inhibited the growth of *Saccharomyces cerevisiae, Zygosaccharomyces bailii, Debaryomyces hansenii* and *Zygosaccharomyces rouxii* in culture medium and apple puree for 40 days.

GC-MS profiling of acetone extract in the present study showed the presence of 17 compounds in which the major compounds were orcinol, atranorin, phenol, 2,4-bis (1,1-dimethyl ethyl), methyl orsellinate and orsellinaldehyde. The antimicrobial activity of orcinol, atranorin, methyl orsellinate are having antimicrobial properties (De melo *et al.*, 2011; Prathibha and Sharma, 2016). The chloroform extract indicated the presence of atraric acid, methyl haematommate, p-orsellinic acid methyl ester, 9-eicosene,(E)-, and 2-nonadecene. The antimicrobial activity of the lichen extracts may be due to the activity of these compounds.

The antimicrobial activity of lichen *Parmotrema tinctorum*, *Staphylococcus aureus,* and *Bacillus subtilis* and the antifungal action against spoilage fungi suggests that it's extracts have potential as natural antimicrobial agent in foods. Since the aroma of the lichen is quite strong, its usefulness may be limited to foods where the spicy flavour would be desirable. Preethi *et al.* (2010), conducted a study on the antimicrobial activity of different plant extracts against *Aspergillus spp.*, *Salmonella typhi, Pseudomonas spp* and *Bacillus subtilis* and observed that plant extracts can be used as preservatives against the food borne microorganisms during food processing and to design suitable drug.

5.3. Evaluation of *Parmotrema tinctorum* for preservation of processed products

Food spoilage is a complex process and considerable quantity of foods is lost due to microbial spoilage in spite of modern preservation techniques. Microbial spoilage is by far the most common cause of food spoilage, manifested as visible

growth, as textural changes and as off-odours. Each and every food product harbours its own specific microflora during production and storage. This microflora is a function of raw material flora, processing, preservation and storage conditions (Gram *et al.*, 2002).

According to Stratford (2007), some weak acids approved for use in foods, are legally designated as preservatives. These include sorbic acid (2,4-hexadienoic acid), benzoic acid, propionic acid, and sulphites. These, together with acetic acid are commonly referred as weak-acid preservatives. There is a rising interest to replace synthetic chemicals and preservatives by natural compounds having the same inhibitory action (Krisch *et al.*, 2011). To reduce the health hazards and economic losses due to food borne microorganisms, the use of natural products as antibacterial compounds seem to be an interesting way to control the presence of pathogenic bacteria and to extend the shelf life of processed food. Microbial analysis of the lime pickle revealed that there was an increase in bacterial population as the storage period increased upto six months. Bacteria were detected in all the stages as the product is in fermented state. The fermentation ensures the presence of beneficial bacteria. After the storage period, the treatment T_4 exhibited the highest bacterial load of 100.1 $X10^6$ CFU/g. In most cases, ingredients of pickles act as carriers of microbial contaminants (Rhyall and Pentizer, 1974). The treatments T_7 and T_8 in which the *P. tinctorum* extract was added at 0.2 % and 0.3 % concentration recorded the low bacterial counts 16.6 $X10^6$ CFU/g and 16.3 $X10^6$ CFU/g respectively. This clearly indicates the antibacterial effect of the lichen extract. Fungal population was not detected in treatments containing the ethanol extract at different concentrations upto fifth month of storage, indicating the antifungal action of extract. This was further proved by the lowest fungal load (1.0 $X10^3$ CFU/g) in treatment T_8 which contained the ethanol extract at 0.1%. Antifungal action of the lichen *P. tinctorum* was reported by many workers. It was also revealed by the *in vitro* studies conducted in the present

investigation. The product in which the sodium benzoate was added showed the yeast colonies five months after storage. This may be due to the presence of yeast species which are reported to exhibit resistance to the action of sodium benzoate. With change in microbial count during storage, the sensory quality of the products was not much affected, except for the color which turned slightly dark (Fig. 28 to Fig. 34).

5.3.3.1. Tomato sauce

In the case of pasteurized tomato sauce, bacteria population was detected after fourth month of storage, only in T_1 (control) and T_3 (*Parmotrema tinctorum* powder at 0.1%). After the storage period, bacterial colonies were detected in all the treatments but the count was found to be non significant. Yeast and fungi load were also found to be non significant among the treatments, excluding T_2 and T_8 in which no yeast and fungi were observed after the storage period. The result clearly indicates the effectiveness of ethanol extract of the lichen in reducing the microbial population. Pasteurization of the product and storing in air tight bottles served to have a predominant effect in reducing the microbial spoilage.

In the case of unpasteurized tomato sauce samples, total bacteria, fungi and yeast count increased during storage period of six months. No microbial colonies were detected initially, when analysed on the date of preparation. Low pH, and heating of tomato sauce samples of the mixture during the manufacturing process contribute to the destruction of microbes (Fatima *et al.*, 2015; Anandsynal *et al.*, 2018). The samples in which *Parmotrema tinctorum* powder at 0.2% were added, recorded the highest bacterial load (99.3 $X10^6$ CFU/g). Yeast and mould content increased on storage. The sample containing 750 ppm sodium benzoate, was found to record fungal growth only after the fifth month of storage, indicating the antifungal effect of sodium benzoate. Benzoic acid has been found to be the second best

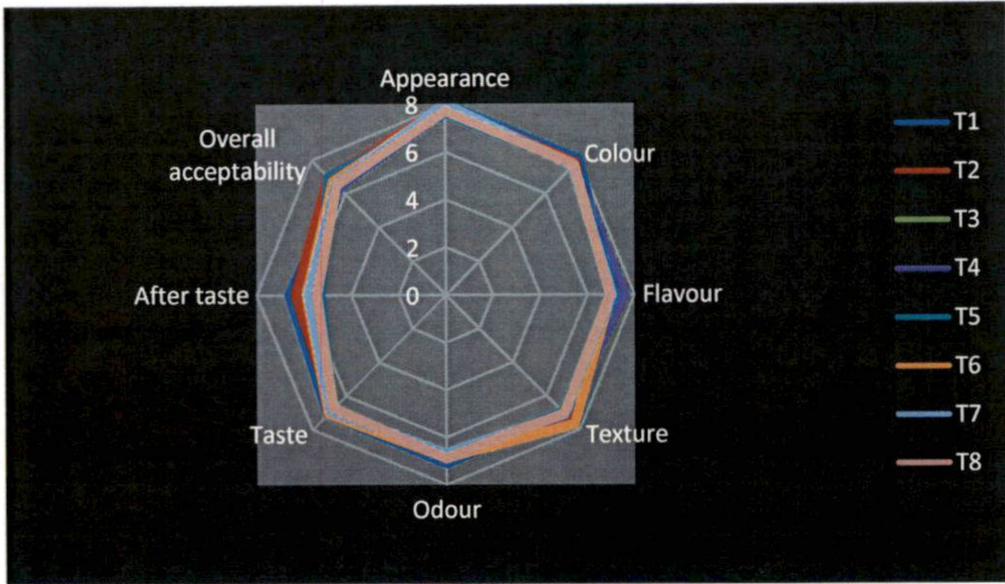

Fig. 28. Sensory attributes of lime pickle -Initial

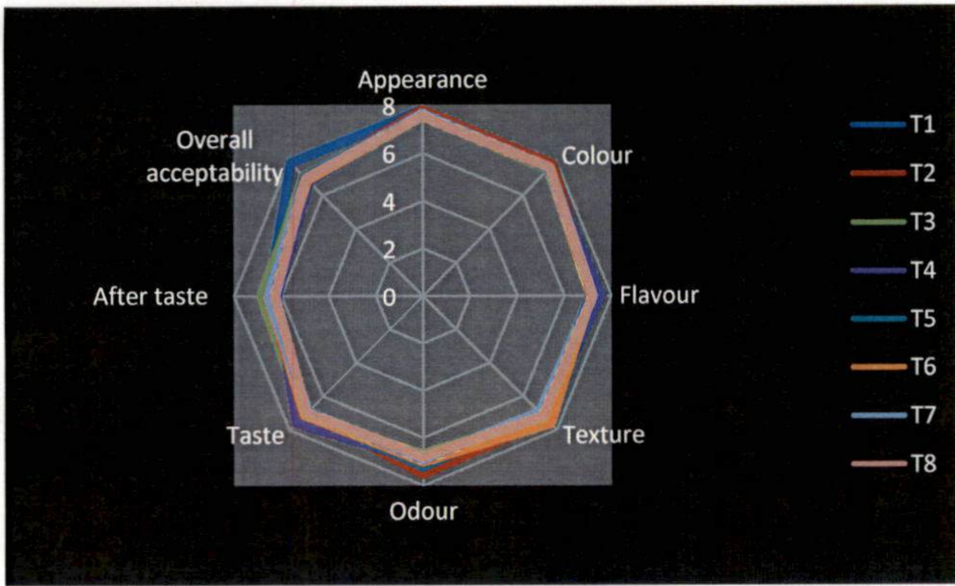

Fig.29. Sensory attributes of lime pickle -1 MAS

T1 – Control (No preservative added), T2 – Sodium benzoate 250 ppm, T3 – *P.tinctorum* powder 0.1% T4 – *P.tinctorum* powder 0.2%, T5 – *P.tinctorum* powder 0.3%, T6 – *P.tinctorum* extract 0.1%, T7 – *P.tinctorum* extract 0.2%, T8 – *P.tinctorum* extract 0.3%

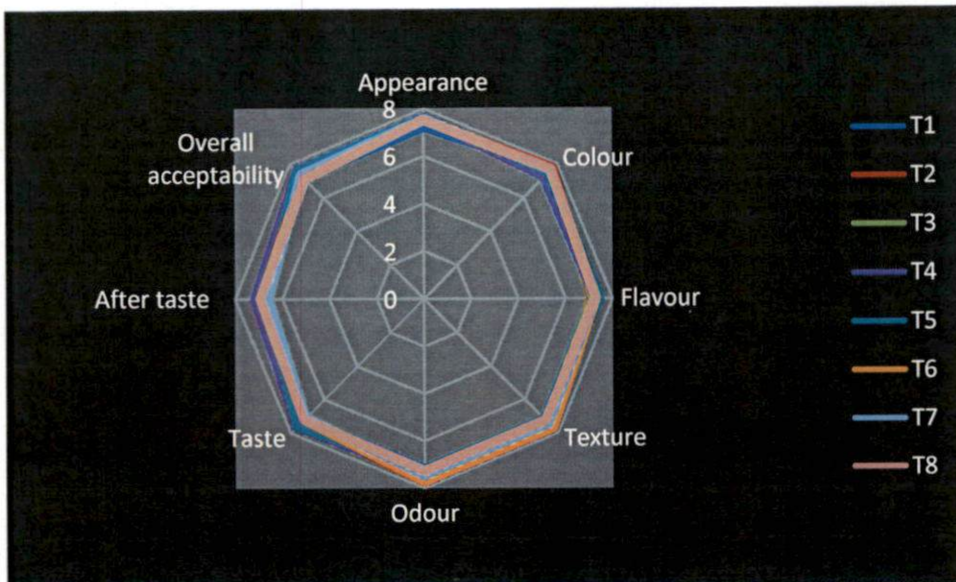

Fig. 30. Sensory attributes of lime pickle -2 MAS

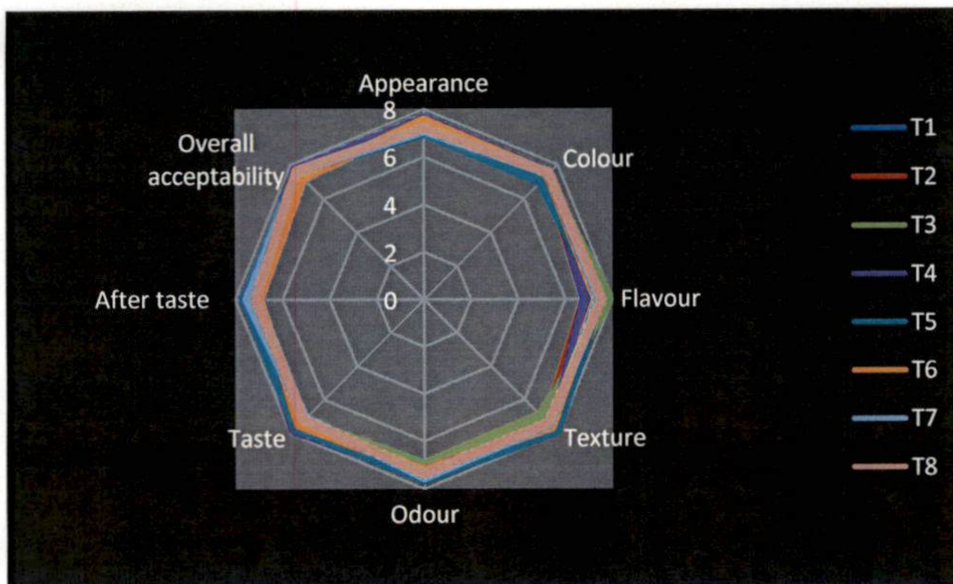

Fig.31. Sensory attributes of lime pickle -3 MAS

T1 – Control (No preservative added), T2 – Sodium benzoate 250 ppm, T3 – *P.tinctorum* powder 0.1% T4 – *P.tinctorum* powder 0.2%, T5 – *P.tinctorum* powder 0.3%, T6 – *P.tinctorum* extract 0.1%, T7 – *P.tinctorum* extract 0.2%, T8 – *P.tinctorum* extract 0.3%

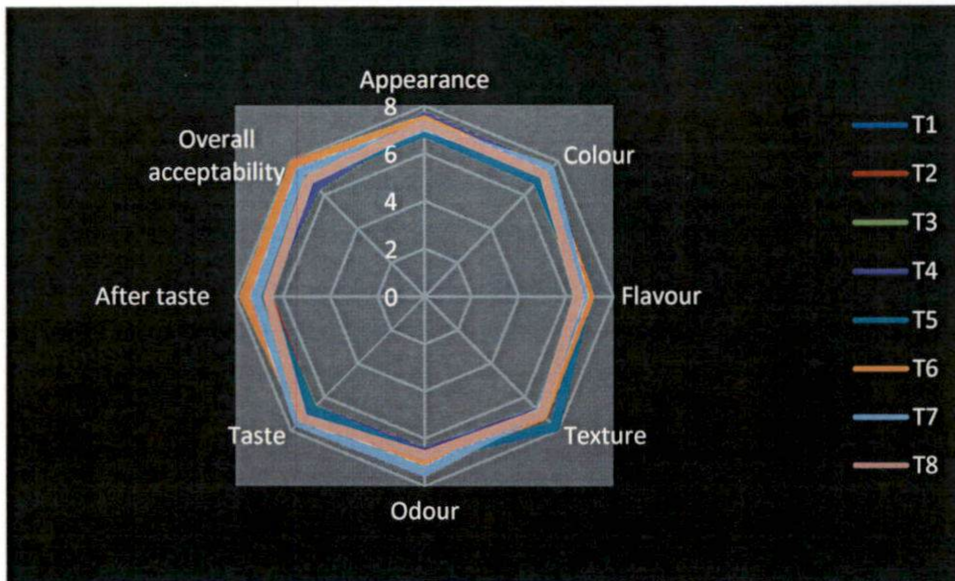

Fig. 32. Sensory attributes of lime pickle -4MAS

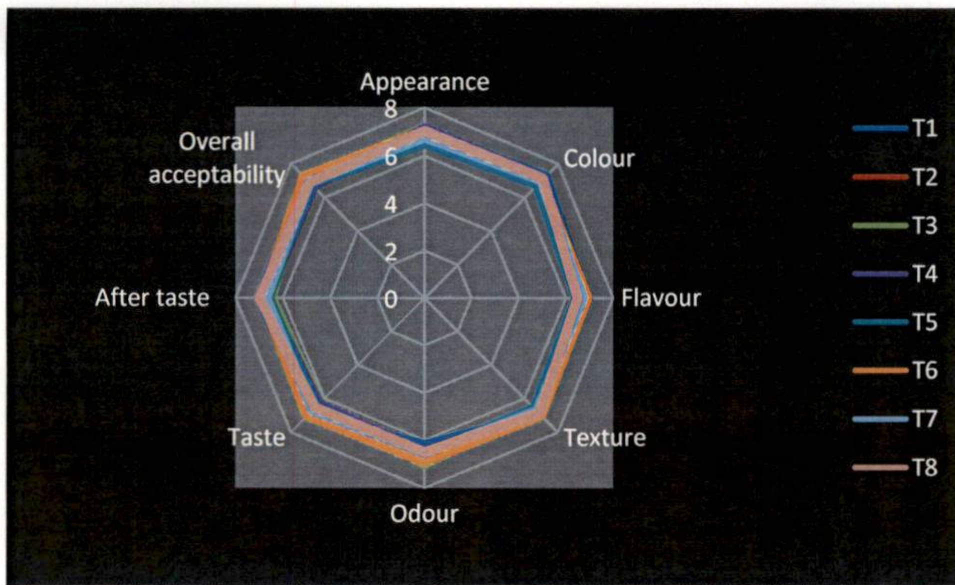

Fig. 33. Sensory attributes of lime pickle -5 MAS

T1 – Control (No preservative added), T2 – Sodium benzoate 250 ppm, T3 – *P.tinctorum* powder 0.1% T4 – *P.tinctorum* powder 0.2%, T5 – *P.tinctorum* powder 0.3%, T6 – *P.tinctorum* extract 0.1%, T7 – *P.tinctorum* extract 0.2%, T8 – *P.tinctorum* extract 0.3%

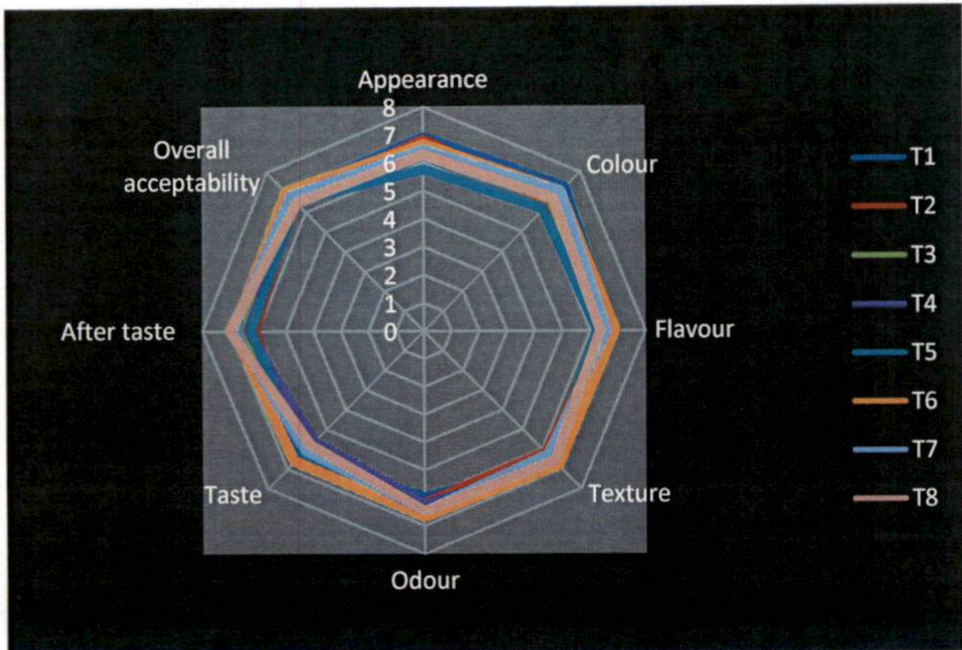

Fig. 34. Sensory attributes of lime pickle -6 MAS

T1 – Control (No preservative added), T2 – Sodium benzoate 250 ppm, T3 – *P.tinctorum* powder 0.1% T4 – *P.tinctorum* powder 0.2%, T5 – *P.tinctorum* powder 0.3%, T6 – *P.tinctorum* extract 0.1%, T7 – *P.tinctorum* extract 0.2%, T8 – *P.tinctorum* extract 0.3%

antifungal agent which caused 75 to 100% mycelial growth inhibition of food-associated fungi (Pundir and Jain, 2010). After the six months storage period, the data on mould count indicated that the treatment T_8 which contain ethanol extract of *Parmotrema tinctorum* at 0.1% was the next best treatment in resisting the fungal growth (20.5X 10^3 CFU/g), after T_2 (750 ppm sodium benzoate). In the case of unpasteurized tomato sauce, the treatments in which sodium benzoate was added also registered to contain mould and yeast growth 5 MAS and 6 MAS. Khan *et al.* (2005) reported that certain fungi such as *Penicillium* and *Mucor* exhibited the resistance to 0.5% sodium benzoate. Benzoates are used primarily as antifungals, as they remain best in the undissociated state, which is the predominant form at low P^H in high acid foods, and fungi are the primary spoilage microorganisms in acidic foods. Yeasts species *viz. Schizosaccharomyces pombe*, *Zygosaccharomyces bailii* have been observed to grow in the presence of benzoic acid. Other yeast species *Byssochlamys nivea* and *Pichia membranefaciens* are also known to be resistant to benzoates (Ramesh, 2011).

Significant differences in the yeast population were recorded by the treatments only after six months of storage. The treatments containing ethanol extract of *Parmotrema tinctorum* at various levels were effective in controlling the yeast population in the samples, recording a yeast population of 1.9X 10^3CFU/g in T_6 (EE at 0.025%) to 0.0X 10^3CFU/g, in T_8 (EE at 0.05%).

Recent literatures indicates that the acceptable limit of Total Viable Count, Yeast and Mould count for sauces and ketchups are $<10^4$ CFU/g, and $<10^4$ CFU/g respectively (Anandsynal *et al.*, 2018). Results of storage study of tomato sauce show that in the treatments T_7 and T_8 no mould and yeast colonies were detected only after two months of storage. Even though the mould and yeast population increased thereafter upto six months (T_7- 1.9 X10^3 CFU/g to 4.0X10^3 CFU/g; T_8- 0.2X10^3

CFU/g), the samples did not exceed the acceptable limit of yeast and mould count of 10^4 CFU/g. With respect to the bacterial load, T_8 was acceptable even after 6 MAS. So on analysis, it can be observed that the treatments which are added with ethanol extract at the concentrations of 0.05% and 0.1% (T_7 and T_8) were acceptable upto six months of storage. The treatment T_6 was also acceptable upto five months of storage (7.0 X10^3 CFU/g), in spite of recording fungal colonies from one month (1.0 X10^3 CFU/g) after storage. The treatments in which *Parmotrema tinctorum* was added in powder form at different concentrations of 0.1 to 0.3%, were safe only upto three months of storage, comparable to the control T_1.

It can be observed that the secondary metabolites especially phenols and monoterpenes are involved in the action against the cell membrane of the micro organisms (Proestos *et al.*, 2006). The chromatography analysis of the present study clearly shows the abundance of phenolic compounds and terpenes in the lichen *Parmotrema tinctorum*. *In vitro* assays of antimicrobial activity also clearly indicated the significant antagonistic action of different solvent extracts of this lichen. Ethanol extract contains phenol compounds *viz*. orcinol, chloratranorin, beta-resourylic acid, 6-methyl-ethyl ester, and hydroxyl benzoic acid derivatives such as methyl haematommate. The antimicrobial activity of the *Parmotrema tinctorum* was due to the presence of some of its major components orcinol, atranorin (antibacterial), methyl orsellinate (antifungal) and methyl haematommate (antifungal). Pratibha and Sharma (2016) reported that methyl orsellinate and methyl haematommate are having antifungal action and atranorin is having antibacterial action.

Organoleptic quality of the product added with ethanol extract was found to be decreasing on increasing the concentration of the extract. The strong aroma of the lichen extract may slightly affect the organoleptic properties of the foods, but the flavour was found to mellow on storage improving the acceptability of the products in which it was added (Fig,35 to Fig.41).

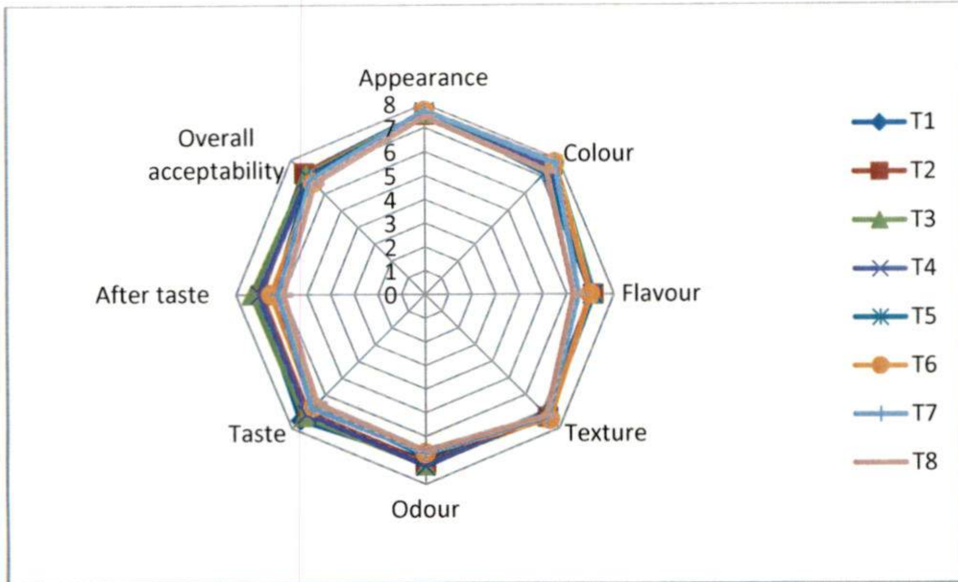

Fig. 35. Sensory attributes of tomato sauce -Initial

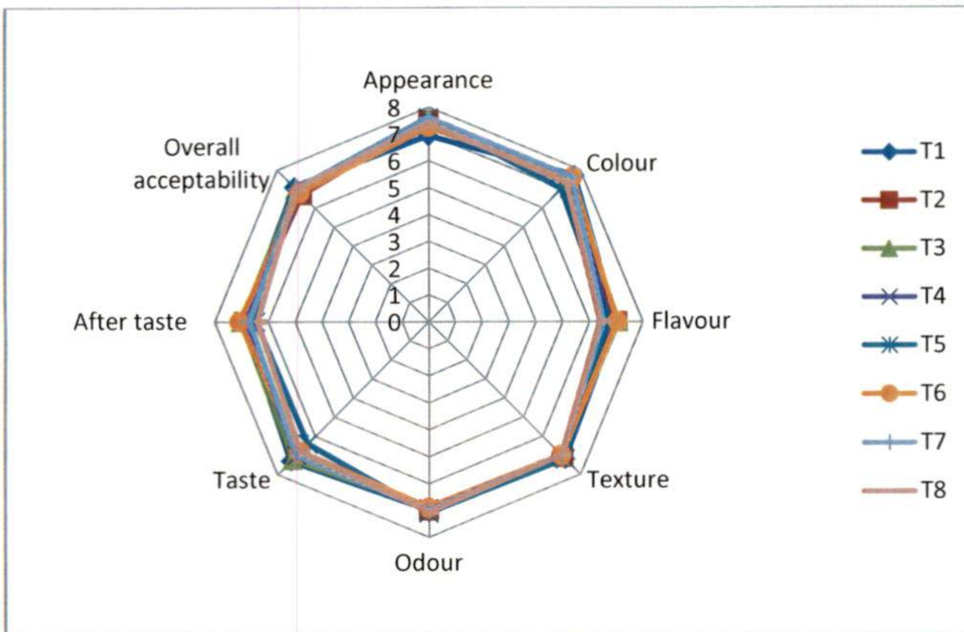

Fig. 36. Sensory attributes of tomato sauce -1 MAS

T1 – Control (No preservative added), T2 – Sodium benzoate 750 ppm, T3 – *P.tinctorum* powder 0.1% T4 – *P.tinctorum* powder 0.2%, T5 – *P.tinctorum* powder 0.3%, T6 – *P.tinctorum* extract 0.025%, T7 – *P.tinctorum* extract 0.05%, T8 – *P.tinctorum* extract 0.1%

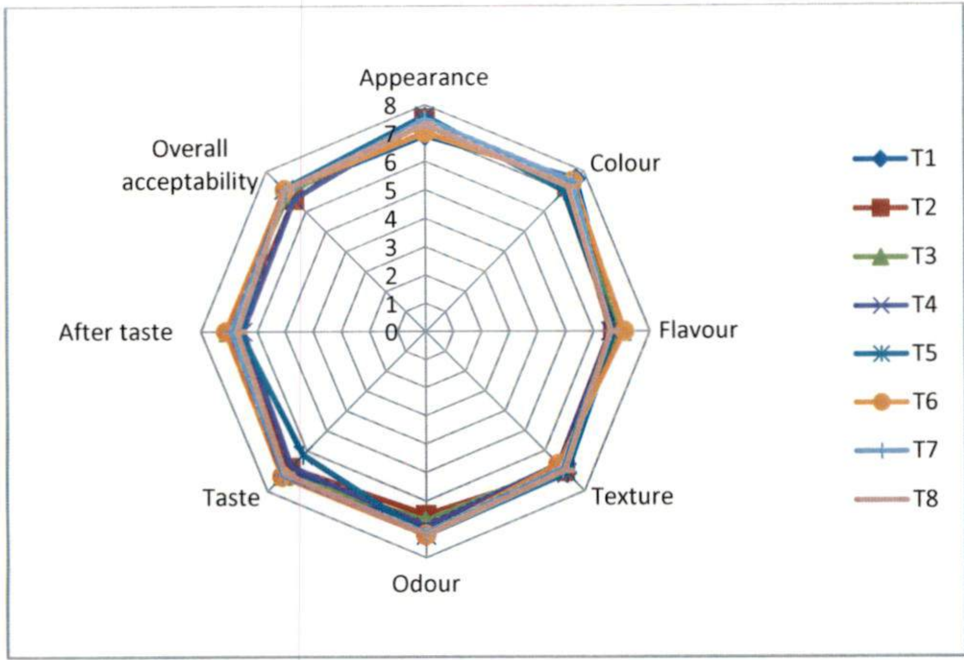

Fig.37. Sensory attributes of tomato sauce -2 MAS

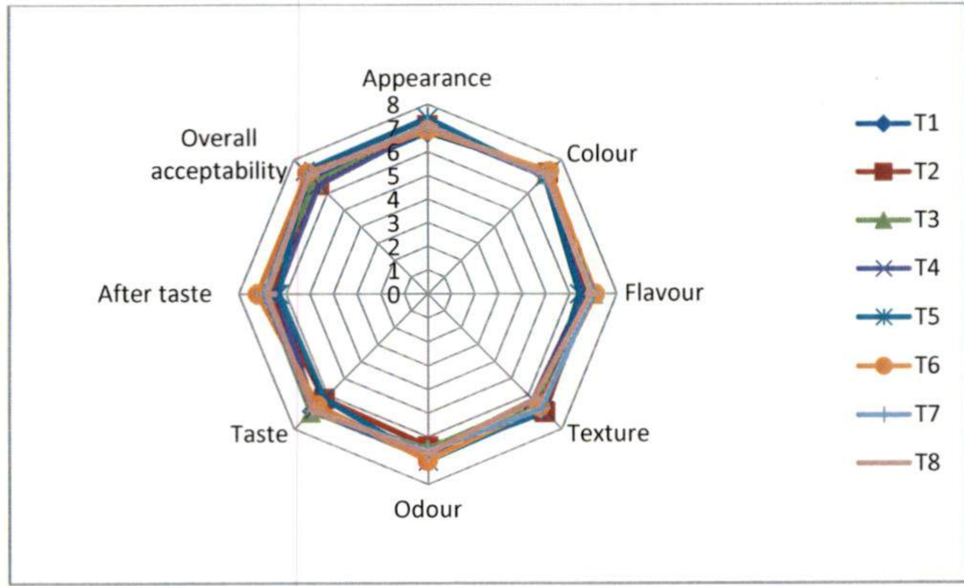

Fig. 38. Sensory attributes of tomato sauce -3 MAS

T1 – Control (No preservative added), T2 – Sodium benzoate 750 ppm, T3 – *P.tinctorum* powder 0.1% T4 – *P.tinctorum* powder 0.2%, T5 – *P.tinctorum* powder 0.3%, T6 – *P.tinctorum* extract 0.025%, T7 – *P.tinctorum* extract 0.05%, T8 – *P.tinctorum* extract 0.1%

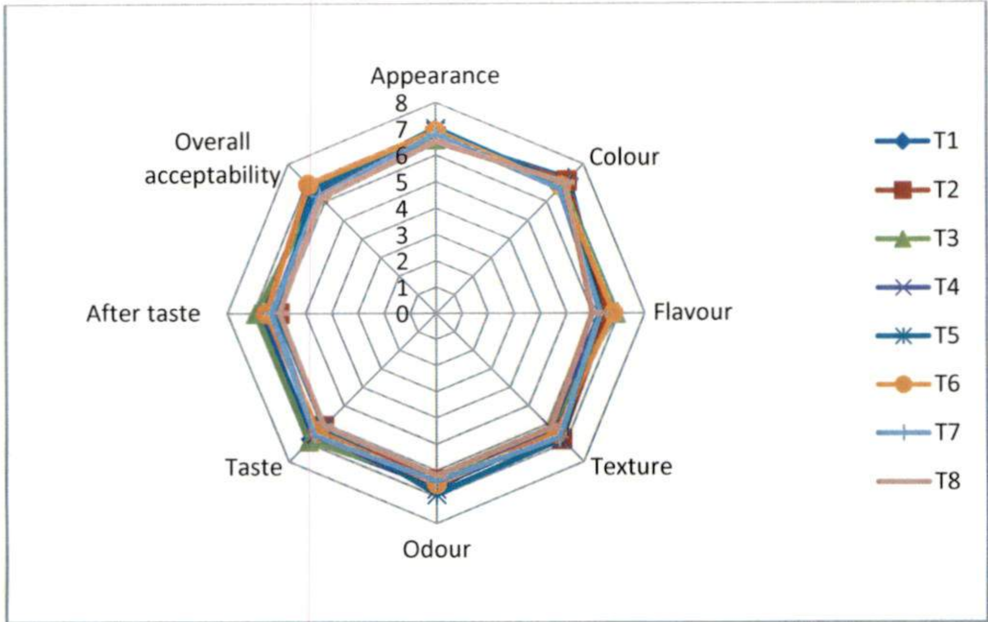

Fig. 39. Sensory attributes of tomato sauce -4MAS

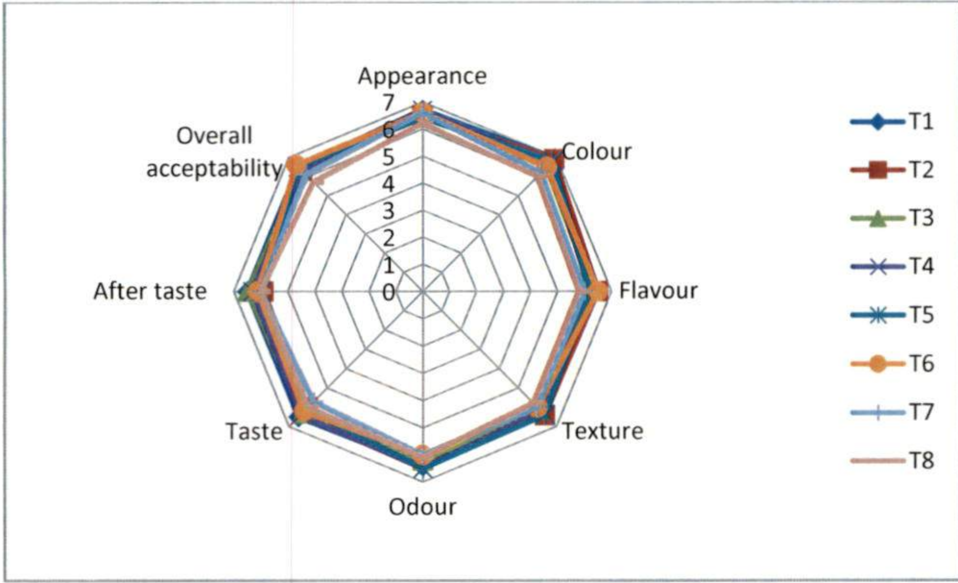

Fig. 40. Sensory attributes of tomato sauce -5 MAS

T1 – Control (No preservative added), T2 – Sodium benzoate 750 ppm, T3 – *P.tinctorum* powder 0.1% T4 – *P.tinctorum* powder 0.2%, T5 – *P.tinctorum* powder 0.3%, T6 – *P.tinctorum* extract 0.025%, T7 – *P.tinctorum* extract 0.05%, T8 – *P.tinctorum* extract 0.1%

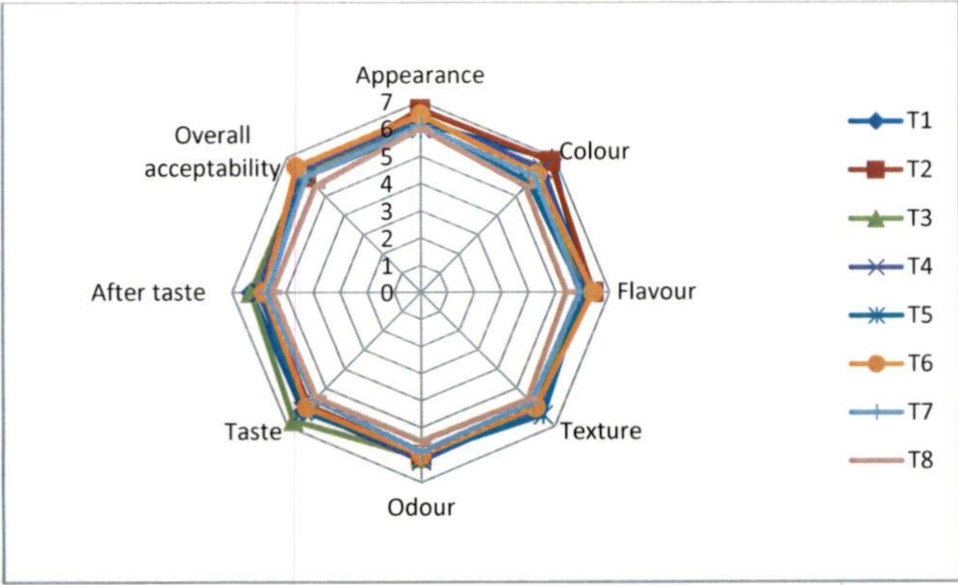

Fig.41. Sensory attributes of tomato sauce -6 MAS

T1 – Control (No preservative added), T2 – Sodium benzoate 750 ppm, T3 – *P.tinctorum* powder 0.1% T4 – *P.tinctorum* powder 0.2%, T5 – *P.tinctorum* powder 0.3%, T6 – *P.tinctorum* extract 0.025%, T7 – *P.tinctorum* extract 0.05%, T8 – *P.tinctorum* extract 0.1%

5. 4. Toxicological studies in *Parmotrema tinctorum*

Toxicology is the important aspect of pharmacology that deals with the adverse effects of bio active substances prior to the use as drug or chemical on living organisms (Aneela *et al.*, 2011). Acute oral toxicity testing is an initial screening step in evaluation of the toxic characteristics of compounds with an objective to identify a dose causing major adverse effects and an estimation of the minimum dose causing lethality. Acute toxicity is produced after administration of a single dose or multiple doses in a period not exceeding 24 hours, up to a limit of 2000 mg/kg (Akhila *et al.*, 2007; Robinson *et al.*, 2007).

The active principles from natural sources have contributed remarkably to the development of novel drugs from plants, lichens, and mushrooms for the treatment of various ailments, but there is inadequate scientific evidence about the safety and efficacy of application of these remedies. Although the use of medicinal plants or their active principles in the treatment of diseases is based on the experience of traditional systems of medicine gathered from different ethnic societies, their use in modern systems of medicine is limited due to lack of scientific evidence. Toxicology studies are essential part of evaluation of active substances from medicinal plants, as natural origin may not imply that they are safe (Ahmed *et al.*, 2012). Toxicological studies were reported in many plants and plant products *viz. Acalypha indica*, *Plectranthus amboinicus*, *Lygodium flexuosum*, *Kaempferia rotunda*, *Curcuma longa*, *Stevia rebaudiana etc.* (Pillai *et al.*, 2011; Sathya *et al.*, 2012; Liju *et al.*, 2013., Sini *et al.*, 2014., Rajab *et al.*, 2016).

Current toxicology testing procedures have evolved significantly over the past three decades. The Organisation for Economic Cooperation and Development had developed the guidelines of toxicological testing and has thus significantly reduced

the number of animals used for certain standard procedures (OECD, 2001). The preferred rodent species is the rat, although other rodent species may be used. Normally female animals are used, because usually there is little difference in sensitivity between the sexes, but in those cases where differences are noticed, females are generally slightly more sensitive (Lipnick *et al.*, 1995; OECD, 2001).

Lichens are one of the abundant sources of biologically active compounds, which have gained attention due to their significant antimicrobial activity (Chauhan and Abraham, 2013). Lichens have been used as drugs, perfumes, dyes, and food ingredients (Ingolfsdottir, 1998; Choudhary and Jalil, 2005).

Parmotrema tinctorum (Nyle.) Hale, an edible lichen, belonging to the genus *Parmotrema* and family Parmeliaceae and used for flavouring food items and for extraction of dyes (Abo-Khatwa *et al.*, 1997; Upreti *et al.*, 2005). Studies have shown that *Parmotrema tinctorum* is a rich source of secondary metabolites such as atranorin, methyl haematommate, which contribute to effects like antimicrobial (Tiwari *et al.*, 2011; Vivek *et al.*, 2014; Anjali *et al.*, 2015), antioxidant (Sharma and Kalikoty, 2012; Raj *et al.*, 2014) and antiglycemic (Sebastian *et al.*, 2014) activity, revealing it's potential as a drug/nutraceutical. Inspite of being widely used as a flavouring agent by the food industry, toxicological studies to ensure the safety of it's use in foods has not been reported so far. Hence the investigation was undertaken on acute toxicity of ethanol extract of *Parmotrema tinctorum* (EEPT) was conducted as per OECD guideline No. 420, Section 4: Health Effects. Acute oral toxicity refers to those adverse effects occurring following oral administration of a single dose of a substance, or multiple doses given within 24 hours (OECD, 2001).

The ethanol extract of *Parmotrema tinctorum* (EEPT) at the dose of 2000 mg/ kg body weight was administered orally to five female Wistar rats of weight 235-240

g and of age 8 to 12 weeks. No mortality and clinical symptoms of toxicity were shown by the test animals during the study period of 14 days. Observations like changes in eyes, skin and fur, and mucous membranes, convulsions, salivation, diarrhoea, sleep and coma and also respiratory, and behaviour pattern in the test animals were recorded. When the body weight and food intake of test animals were analysed, no treatment related changes were noticed at the dose of 2000 mg/kg body weight in the test animals.

The gross pathological changes were observed after animals were sacrificed under euthanasia condition and subjected to necropsy. It was found that there were no pathological changes showing the toxicity symptoms. Similar results were obtained by Krishnamoorthy and Shankaran (2014) in Sprague Dawley rats, while testing oyster mushroom *Pleurotus ostreatus* extract where the body weight, food and water intake of rats were found to be unaffected by the treatment. When *Pleurotus ostreatus* extract was orally administered for 72 hour at a dose level of 5000 mg/kg body weight, no mortality and no significant changes in the behavior of rats were observed showing the LD_{50} is > 5000 mg/kg body wt. Shanmugam *et al*. (2017) reported strong anti-cancerous property of bioactive compounds obtained from lichens *Parmotrema reticulatum*, *Parmotrema hababianum* and *Rocella montagnei* by conducting *in vivo* cancer cell-line studies and animal model experiments in Albino Wistar rats. They have identified that benzoic acid, 2, 4 dihydroxy, 6 methyl-methyl ester as the potent molecule from *Rocella montagnei.*

De Melo *et al*. (2011) had reported the anti-inflammatory and toxicity effects of atranorin, extracted from lichen *Cladina kalbii* in male and female Wistar rats. The results showed that atranorin (100 mg/ kg and 200 mg/ kg) exhibited significant anti-inflammatory activities. In the acute toxicity test, doses of 5 g/kg of atranorin caused

no deaths in rats during a 14-day observation period. The rats showed no toxicity signs or changes in general behavior..

The results revealed in the acute toxicity testing provides the very important preliminary information on the safety of ethanol extract of *Parmotrema tinctorum* in foods. The data will be very useful for future *in vivo* and clinical studies on this edible lichen. These results showed that the single exposure of ethanol extract of *Parmotrema tinctorum* upto a dose of 2000 mg/kg body weight orally was found to be safe in Wistar rats. As determination of acute oral toxicity is usually provides an initial screening of the toxic characteristics of compounds (Akhila *et al.*, 2007), studies on chronic toxicity are further necessary to support the safe use of lichen extracts.

From the investigation it can be concluded that the edible lichen *Parmotrema tinctorum* is nutritionally significant as it contains appreciable quantities of crude fibre, total protein, total ash, phenol content and minerals. Solvent extracts of *Parmotrema tinctorum* also showed good antioxidant properties. Chromatographic studies indicated a variety of organic compounds in *Parmotrema tinctorum*, of which many have antimicrobial action. Microbial analysis of products added with lichen extract showed low bacterial and fungal population indicating it's potential as natural antimicrobial in extending the shelf life. Sensory analysis revealed that the products added with ethanol extract were acceptable and the acute toxicity testing proved the safety of ethanol extract of *Parmotrema tinctorum.*

SUMMARY

6. SUMMARY

The project entitled "Evaluation and utilisation of edible lichen *Parmotrema tinctorum* (Nyl.) Hale for food preservation" was carried out in the Department of Post Harvest Technology during 2014-17. The main objectives of the study were to evaluate the biochemical constituents, proximate composition, antimicrobial activity and feasibility for food preservation, and to study the toxicological effect of the lichen *Parmotrema tinctorum*.

The lichen samples were collected from different places in South Wayanad Forest Division of Wayanad district, Kerala *viz*. Chembra, Meppadi, Moolankavu and Ambalavayal with proper sanction of the Department of Forest and Wildlife, Kerala. The edible lichen was identified as *Parmotrema tinctorum* by the colour spot tests. The samples read K-, C+, KC+ and Pd- for the lichen *Parmotrema tinctorum*. The habitat of lichen was found to be the shady places of the evergreen forests at 736m - 2100m above MSL. *Parmotrema tinctorum* is corticolous (growing on the surface of trees) in habit. Thallus of the lichen is foliose, loosely attached, lobes irregular, margins entire, upper surface grey, smooth, shining; lower surface black and marginal area brown.

Proximate analysis of *Parmotrema tinctorum* revealed a high content of total protein (15.70 %), crude fibre (14.16%), ash (10.50%) and total phenols (322 mg/100g). *Parmotrema tinctorum* contained total carbohydrate (20.03 g/100g), crude fat (1.28%), ascorbic acid (4.66 mg/100g) and total free amino acids (8.25 mg/g). Elemental analysis of the lichen revealed presence of eighteen elements in *Parmotrema tinctorum.* Analysis of elemental composition recorded high content of calcium (21970 mg/kg), magnesium (1781.66 mg/kg), potassium (2936.66 mg/kg) and iron (785.26 mg/kg) in *Parmotrema tinctorum*.

Antooxidant activity of lichen *Parmotrema tinctorum* was assessed using methanol, ethyl acetate and acetone extracts. In DPPH assay, methanol extract

showed the maximum scavenging action against the DPPH free radicals (IC_{50}1.47 mg/ml). The IC_{50}, concentration of sample giving 50 per cent inhibition, for ethyl acetate extract and acetone extract were 2.34 and 5.04 mg/ml respectively. In ABTS assay, the methanol extract of *Parmotrema tinctorum* showed the highest ABTS radical scavenging capacity (IC_{50} 1.29 mg/ml). Ethyl acetate extract recorded (EAE) an IC_{50} value 1.27 mg/ml which was significantly on par with that of methanol extract. In both assays, acetone extract showed the least scavenging action (IC_{50} 3.16 mg/ml). The antioxidant properties of the lichen can be attributed by the high phenol content in the lichen.

Preliminary phytochemical screening of *Parmotrema tinctorum* revealed maximum phytochemicals in methanol extract *viz.* carbohydrates, phenols, flavonoids, tannins, terpenoids, fixed oils and coumarins. The ethyl acetate extract indicated the presence of carbohydrates, phenols, flavonoids, tannins, and terpenoids whereas the acetone extract was positive for carbohydrates, phenols, tannins, fixed oils and fats, terpenoids and quinones. The petroleum benzine extract indicated the presence of saponins only.

The Thin Layer Chromatography (TLC) profiling of lichen extracts (hexane, methanol and acetone) was conducted using three solvent systems S1 hexane:ethyl acetate, S2.chloroform:methanol and S3.benzene:ethyl acetate. TLC profiles showed maximum detection of compounds in acetone extract, followed by methanol extract and hexane extract. The flouroscence of the spots indicated mainly the presence of phenols and terpenoids in the lichen extracts.

A range of volatile compounds were observed when the lichen extracts *viz.* methanol, hexane, acetone, chloroform and ethanol were subjected to Gas Chromatography-Mass Spectrometry analysis. The hexane extract showed maximum number (23) of compounds. Glyceryl trilaurate, a fatty acid ester was the major component (73.04%), followed by lauric acid, vinyl ester (4.90%) and decane

(4.83%). Methanol extract showed five compounds *viz.* orcinol (37.75%), methyl orsellinate (49.35%), methyl haematommate (6.52%), atranorin (6.31%), and hexadecanoic acid, 15- methyl-methyl ester (0.08%). Chloroform extract contained seven compounds *viz.* phenol, 2,4-bis(1,1-dimethylethyl)-, E-14-Hexadecenal, atraric acid, p-orsellinic acid, methyl ester, methyl haematommate, 9-eicosene,(E)-, and 2-nonadecene of which atraric acid recorded the maximum peak area (71.78%), followed by methyl haematommate (10.98%). Eight compounds were detected in the GC-MS profiling of ethanol extract *viz.* orcinol, chloroatranorin, ethyl orsellinate, methyl haematommate, campesterol, gamma-sitosterol, 14-.beta-H-Pregna and stigmasta-3,5-dien-7-one. Ethyl orsellinate was the predominant compound (47.24%), followed by gamma-sitosterol (29.10%) and by orcinol (15.51%). In acetone extract, presence of 17 phytochemicals was detected. *viz.* orcinol, 1,4-benzenediol,2,5-dimethyl-, orcinol mono acetate, 1-ethoxy-2-methoxy-4-methyl benzene, phenol,2,4-bis(1,1-dimethylethyl)-, orsellinaldehyde, methyl orsellinate, 1-butyl-2-propyl cyclopentane, nonadecane, atranorin, 2,6-octadiene, 4,5-dimethyl-, decane, 1-bromo-2-methyl-,3-heptadecanol, eicosane, benzopteridine, 2,4-diamino-6,7,8,9-tetrahydro-7-methyl-,hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, and myristic acid, 4-methoxyphenyl ester. The major compounds were orcinol (85.31%), atranorin (3.98%), and methyl orsellinate (2.20%). These compounds identified in the extracts were having the antimicrobial properties of this lichen and can attribute to the flavour of lichen.

In vitro testing of antimicrobial activity of acetone, ethanol and chloroform extracts of *Parmotrema tinctorum* using disc diffusion and well diffusion methods revealed their inhibitory action against the selected food spoilage organisms *viz.* *Aspergillus niger*, *Aspergillus oryzae*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Bacillus subtilis* and *Staphylococcus aureus*. Ethanol extract (EE) of *Parmotrema tinctorum* produced maximum inhibition of *Aspergillus*

niger, while chloroform extract (CE) produced maximum inhibition of *Aspergillus oryzae*. The growth of both yeast species, *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*, was found to be inhibited maximum by the ethanol extract followed by chloroform extract of the lichen *Parmotrema tinctorum*. In both disc diffusion and well diffusion methods, the growth of *Bacillus subtilis* and *Staphylococcus aureus* were remarkably inhibited by the acetone extract (AE) followed by chloroform extract forming zones of inhibition at all the concentrations tested. All the extracts produced a dose dependant action againt the test organisms and the presence of antimicrobial compounds in the extracts establish their inhibitory effect on the micro organisms.

The feasibility of utilizing *Parmotrema tinctorum* for food preservation was evaluated by adding in powder and ethanol extract form in two processed products *viz*. lime pickle and tomato sauce. In lime pickle, bacterial count was found to be least in treatments T_8 and T_7 (added with 0.3% and 0.2% ethanol extracts respectively). Product preserved with 250 ppm sodium benzoate (T_2) revealed least fungal count ($0.6X10^3$ CFU/g), which was on par with that containing 0.3% ethanol extract ($1.0X10^3$ CFU/g). Yeast population was absent in T_2 during the storage period, while T_8 (0.8 $X10^3$ CFU/g) and T_7 (1.9 $X10^3$ CFU/g) showed lower yeast count. A significantly lower bacterial count (5.2 $X10^6$ CFU/g) was detected in unpasteurised tomato sauce in which 0.1% ethanol extract (T_8) was added, followed by T_2 (product preserved with 750 ppm sodium benzoate). The product preserved with 750 ppm sodium benzoate recorded the lowest fungal count of 12.0 $X10^3$ CFU/g, and yeast population was not detected in it throughout the storage. The products added with 0.05% and 0.1% ethanol extract of the lichen also recorded lower fungal and yeast counts. When pasteurized tomato sauce was analysed, there was no significant difference between the treatments with respect to microbial count. According to the microbial standards of FSSAI, the shelf life of lime pickle treated with T_2 was estimated to be six months, while that of T_8 was found to be five months. The

microbial analysis of products showed the significance of ethanol extract of *Parmotrema tinctorum* as an alternative to sodium benzoate in preventing the spoilage of foods. Sensory analysis using 9 point hedonic scale revealed that lichen extract added products were acceptable for consumption. Organoleptic quality of the product added with ethanol extract was found to be decreasing on increasing the concentration of the extract. The strong aroma of the lichen extract was found to mellow on storage improving the acceptability of the products in which it was added.

Acute oral toxicity study of the ethanol extract of *Parmotrema tinctorum* was conducted using Wistar albino rats as test animals. The test animals did not exhibit clinical signs of toxicity and mortalities, treatment related changes in body weight during the study period of 14 days. The gross pathological changes were studied during necropsy after sacrificing the animals under euthanasia condition. It was found that there were no pathological changes showing the toxicity symptoms. The results proved that single dosing of ethanol extract of *Parmotrema tinctorum* upto a dose of 2000 mg/kg body weight orally was found to be safe in Wistar rats.

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APPENDICES

Appendix - I

List of places of lichen collection in Wayanad

Sl.No.	Name of places	Altitude (m)
1	Ambalavayal	974
2	Chembra	2100
3	Chembra	2100
4	Chembothara	792
5	Vithukadu	792
6	Chembothara	792
7	Meppadi	792
8	Meppadi	792
9	Ambalavayal	974
10	Meppadi	792
11	Moolankavu	736
12	Moolankavu	736
13	Meppadi	792

Appendix - II

Media for growth and enumeration of microbial population

1.NUTRIENT AGAR MEDIA

Beef extract	3g
Peptone	5g
Sodium chloride	5g
Agar	18g
Distilled water	1000ml
pH	6.8-7.2

2.ROSE BENGAL AGAR MEDIA

Papaic digest of soyabean meal	5g
Dextrose	10g
Monopotassium phosphate	1g
Magnesium sulphate	0.50g
Rose Bengal	0.05g
Agar	15g
pH	5.6

3.SABAURAUD DEXTROSE AGAR

Mycological peptone	10g
Dextrose	40g
Agar	15g
Distilled water	1000ml
pH	5.6

4. MUELLER HINTON AGAR

Beef extract	2g
Acid hydrolysate of Caesin	17.5g
Agar	17g
Starch	1.5g
Distilled water	1000ml
pH	7.3

Appendix - III

Method of preparation of lime pickle

INGREDIENTS

Ingredient	Quantity
Lime slices (cured with 10% salt)	1kg
Ginger	50g
Garlic	20g
Green chilli	10g
Chilli powder	75g
Mustard (ground)	5g
Turmeric powder	3g
Fenugreek (ground)	4g
Asafoetida	10g
Curry leaves	5g
Vinegar (5%)	80ml
Mustard	5g
Gingelly oil	100ml
Sodium benzoate	250mg

1. Fry asafoetida in a little quantity of oil and powder it.
2. Sauté ginger, green chillies, garlic separately in oil till they turn brown and make it into a paste with vinegar.
3. Heat gingelly oil, add mustard and curry leaves.
4. Reduce the flame and add chilli powder, turmeric powder, ground mustard and spice paste. Add this mixture to cured lime pieces*
5. Finally vinegar is added and mixed well.
6. Fill the pickle into sterile glass jars.
7. Pour gingelly oil as topping over the pickle for better keeping quality.
8. Cover the mouth of the jar air tight with screw caps.

* *Parmotrema tinctorum* lichen powder and ethanol extract were added to the product after sauting in hot oil.

Appendix - IV

Method of preparation of tomato sauce

INGREDIENTS

Tomato juice	1kg
Sugar	60g
Salt	10g
Onion(Large)	1No.
Garlic	4-5 cloves
Cumin	3g
Cardamom	3-4Nos.
Clove	1g
Black pepper	0.6g
Nutmeg mace	Few pieces
Chilli powder	1g
Acetic acid	3-5ml
Sodium benzoate	0.75g

METHOD OF PREPARATION

1. Take fully ripe, red colored tomatoes. Wash thoroughly.
2. Cut the fruits, crush, and boil the mass for 5 minutes.
3. Strain the boiled mass through a fine nylon net or 1mm stainless steel sieve by rubbing. Press out juice or pulp completely.
4. Add 1/3 quantity of sugar and salt to the juice
5. Prepare spice bag by tying in muslin cloth the ground spices and chopped pieces of onion and garlic*
6. Hang the bag in the juice.
7. Boil till the volume reduces to one third.
8. Remove the spice bag after squeezing to get all the extract.
9. Add the remaining quantity of salt and sugar, mix well without allowing to char.
10. Finally add acetic acid and sodium benzoate to the product.**
11. Boil for 2-3 minutes and fill in clean, sterilized bottles keeping them on a wooden plank.
12. Close the bottle and seal.

* *Parmotrema tinctorum* lichen powder was added in the spice bag along with other spices

**Ethanol extract of lichen was added by dissolving in the acetic acid

Appendix - V

Score card for organoleptic evaluation of products

Name of the judge:

Date: Signature:

Characteristics	Score							
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8
Appearance								
Colour								
Flavour								
Texture								
Odour								
Taste								
After taste								
Overall acceptability								

9 point Hedonic scale

Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

Appendix – VI

Temperature and Relative humidity during storage period of products

	Monthly data - August 2016 to August 2017 - Vellanikkara					
Months	**Maximum Temperature (^{0}C)**	**Minimum Temperature (^{0}C)**	**Mean temperature (^{0}C)**	**Forenoon Relative Humidity (%)**	**Afternoon Relative Humidity (%)**	**Mean Relative Humidity (%)**
Aug-16	30.4	23.2	26.8	95	71	83.0
Sept-16	30.3	23.6	27.0	95	69	82.0
Oct-16	31.5	22.7	27.1	93	68	80.5
Nov-16	32.9	22.2	27.6	83	54	68.5
Dec-16	32.4	22.3	27.4	85	52	68.5
Jan-17	34.1	22.9	28.5	68	37	52.5
Feb-17	36.0	23.2	29.6	70	31	50.5
Mar-17	36.1	24.7	30.4	85	48	66.5
Apr-17	35.7	26.0	30.9	85	55	70.0
May-17	34.6	24.9	29.8	84	60	72.0
Jun-17	30.4	23.5	27.0	95	78	86.5
July-17	30.8	22.8	26.8	95	74	84.5
Aug-17	30.1	23.3	26.7	96	78	87.0

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EVALUATION AND UTILISATION OF EDIBLE LICHEN *Parmotrema tinctorum* (Nyl.) Hale FOR FOOD PRESERVATION

By

ANUPAMA.T .V

(2014 - 22-102)

ABSTRACT OF THE THESIS

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COLLEGE OF HORTICULTURE

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ABSTRACT

Investigation on "Evaluation and utilisation of edible lichen *Parmotrema tinctorum* (Nyl.) Hale for food preservation" was carried out in the Department of Post Harvest Technology, College of Horticulture ,Vellanikkara during 2014-2017. The main objectives of the study were to evaluate the biochemical constituents, proximate composition, antimicrobial activity, feasibility for food preservation and to study the toxicological effect of the lichen *Parmotrema tinctorum*.

The lichen samples were collected from Chembra, Meppadi, Moolankavu and Ambalavayal areas in Wayanad district, and they were identified as *Parmotrema tinctorum* by colour spot tests. The samples read K-, C+, KC+ and Pd- for the lichen *Parmotrema tinctorum*. The habitat of lichen was found to be the shady places of the evergreen forests at 736m -2100m above MSL. *Parmotrema tinctorum* is found to be corticolous (growing on the surface of trees) in habit. Thallus of the lichen is foliose, loosely attached, lobes irregular, margins entire, upper surface grey, smooth, shining; lower surface black and marginal area brown.

Proximate analysis of *Parmotrema tinctorum* revealed a high content of total protein (15.70 %), crude fibre (14.16%), ash (10.50%) and total phenols (322 mg/100g). *Parmotrema tinctorum* also contained total carbohydrate (20.03 g/100g), crude fat (1.28%), ascorbic acid (4.66 mg/100g) and total free amino acids (8.25 mg/g). High content of calcium, magnesium, potassium and iron were found in the mineral analysis of *Parmotrema tinctorum.* Methanol, ethyl acetate and acetone extracts of *Parmotrema tinctorum* were analysed for antioxidant activity by DPPH and ABTS assays, and the highest scavenging action was detected in the methanol extract against the DPPH free radicals (IC_{50}-1.47 mg/ml) and the ABTS radicals (IC_{50}-1.27 mg/ml).

Preliminary phytochemical screening of *Parmotrema tinctorum* revealed maximum phytochemicals in methanol extract *viz.* carbohydrates, phenols,

flavonoids, tannins, terpenoids, fixed oils and coumarins. The TLC profiling of lichen extracts (hexane, methanol and acetone) showed maximum compounds in acetone extract, and the spots indicated the presence of phenols and terpenoids. A range of volatile compounds were observed when the lichen extracts (methanol, hexane, acetone, chloroform and ethanol) were subjected to GC-MS analysis.Volatile compounds with antimicrobial properties identified were orcinol, methyl orsellinate, atraric acid, atranorin, methyl haematommate, glyceryl trilaurate, lauric acid vinyl ester and gamma-sitosterol.

In vitro testing of antimicrobial activity of acetone, ethanol and chloroform extracts of *Parmotrema tinctorum* using disc and well diffusion methods revealed their inhibitory action against the selected food spoilage organisms. Ethanol extract (EE) of *Parmotrema tinctorum* produced maximum inhibition of *Aspergillus niger,* while chloroform extract (CE) produced maximum inhibition of *Aspergillus oryzae*. The growth of both yeast species, *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*, was found to be inhibited maximum by the ethanol extract followed by the chloroform extract of the lichen *Parmotrema tinctorum*. In both disc diffusion and well diffusion methods, the growth of *Bacillus subtilis* and *Staphylococcus aureus* were remarkably inhibited by the acetone extract (AE) followed by chloroform extract (CE) forming zones of inhibition at all the concentrations tested.

Feasibility of utilizing *Parmotrema tinctorum* for food preservation was evaluated by adding in powder and in ethanol extract form in two processed products *viz.* lime pickle and tomato sauce. In lime pickle, bacterial count was least in treatments T_8 and T_7 (added with 0.3% and 0.2% ethanol extracts respectively). Product preserved with 250 ppm sodium benzoate (T_2) revealed least fungal count (0.6×10^3 CFU/g), which was on par with that containing 0.3% ethanol extract (1.0×10^3 CFU/g). Lowest yeast count was observed in T_8, followed by T_2 (0.3×10^3 CFU/g). The shelf life of lime pickle treated with T_2 (product preserved with 250 ppm sodium benzoate) was estimated to be six months, while that of T_8 (product

treated with 0.3% ethanol extract) was found to be five months. Unpasteurised tomato sauce in which 0.1% ethanol extract was added had significantly lower bacterial count (5.2×10^6 CFU/g). The tomato sauce preserved with 750 ppm sodium benzoate recorded lowest fungal and yeast count. The products added with 0.05% and 0.1% ethanol extract of lichen also recorded lower fungal and yeast counts. Microbial analysis of products showed the relevance of ethanol extract as an alternative to sodium benzoate in preventing the microbial spoilage of foods. Sensory analysis revealed that lichen extract added products were acceptable for consumption.

Acute oral toxicity study of the ethanol extract of *Parmotrema tinctorum* conducted in Wistar rats revealed the absence of clinical signs of toxicity and mortalities. There were no treatment related changes in body weight and gross pathological changes in the test animals. Single dosing of ethanol extract of *Parmotrema tinctorum* upto a dose of 2000 mg/kg body weight orally was found to be safe in Wistar rats.

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