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In-vitro regeneration studies on *Paris polyphylla* - A threatened medicinal herb of Indian Himalayas

Dissertation Report

Submitted

By

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(Reg. No. 11512304)

In partial fulfillment of M.Sc. Botany (Hons.)

Under the supervision of

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DECLARATION

I hereby declare that the project entitled **"*In-vitro* regeneration studies on *Paris polyphylla* – A threatened medicinal herb of Indian Himalayas"** is an authentic record of our own work carried out at School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, for the partial fulfilment of the award of Masters of Science in Botany (Honours) under the guidance of Dr. Arbeen Ahmad Bhat, Assistant Professor, Lovely Professor University.

This work is my original work and has not been submitted for any degree/diploma in this or any other University. The information furnished in this report is genuine to the best of my knowledge and belief.

Place: Hom-Singli M

Date: Registration no. 11512304

CERTIFICATE

This is to certify that Hom-Singli M, Registration No. (11512304) have completed the project, entitled **"*In-vitro* regeneration studies on *Paris polyphylla* – A threatened medicinal herb of Indian Himalayas"** under my guidance and supervision. To the best of my knowledge, the present work is the result of her original investigation and study.

No part of the report has ever been submitted for any other degree at any University. The report is fit for submission and the partial fulfilment of the conditions for the award of Masters of Science in Botany (Honours).

Date: Supervisor

Signature

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ABBREVIATIONS

- 2, 4-D (2, 4-dichlorophenoxyacetic acid)
- *A. niger* (*Aspergillus niger*)
- Abs (Absorbance)
- BAP (6-Benzyl amino purine)
- C (Celsius scale temperature unit)
- cm (Centimeter)
- DPPH (2,2-diphenyl-1-picrylhydrazyl)
- *E. coli* (*Escherichia coli*)
- FRAP (Ferric reducing ability of plasma)
- g (gram)
- GAE (Gallic acid equivalent)
- IAA (Indole-3-acetic acid)
- IBA (Indole butyric acid
- IC (Inhibitory concentration)

- QE (Quercetin equivalent)
- *S. aureus* (*Staphylococcus aureus*)
- SD (Standard deviation)
- SE (Somatic embryogenesis)
- Sm. (Smith)
- Spp. (Species)
- *T. reesei* (*Trichoderma reesei*)
- UV (Ultra violet)
- µg (Microgram)
- IUCN (International Union for Conservation of Nature)
- IZE (Immature zygotic embryos)
- Lux (Luminous flux per unit area)
- mg (Milligram)
- MIC (Minimum inhibitory concentration)
- mm (Millimeter)
- MS (Murashige and Skoog’s medium)
- NAA (1-Napthalene acetic acid)
- NAM (Nutrient agar medium)
- OD (Optical density)
- *P. polyphylla* (*Paris polyphylla*)
- PGR’s (Plant growth regulators)
- ph (Potential of hydrogen or measure of acidity and alkalinity)
- Psi (Pound per square inch)

Introduction

Since time immemorial, use of plants has played significant role in the development of mankind and its culture (Petrovska, 2012; Hassan, 2012). Tribal healers used plants for medicinal purposes, either as a whole or extracts. Many plants (including food crops like mint, garlic, turmeric, etc.) are used directly and indirectly as medicines and long term practice leads to the progressive growth of modern medicines, though early uses of treatments are still widely practiced today (Tapsel et al., 2006). Nonetheless, it is no longer limited to indigenous or non-industrialized societies but intensified due to urbanization and globalization in recent few decades. Moreover, non-urbanized folk cultures gained knowledge about its usage and practical implications through steady interactions with the biotic environment.Throughout human history, plants play basic role in medical treatments and such folk medicines are still widely practiced today (Fabricant and Farnsworth, 2001). Indigenous practice has major advantage over modern drugs as it is cost effective in collection and plantations. Most of the world's population still depends on drugs derived from plants (Caufield, 1991).

Medicinal plants designed to produce new drugs are mostly due to the characteristic effects of secondary metabolites present in plants (Meskin et al., 2002). Isolation, purification, identification, and structure of bioactive compounds and chemicals found in plants, known as phytochemistry, specifically describes its secondary metabolites (Doughari, 2012). It includes terpenoids, alkaloids, saponins, polysterols, amines, glucossinolates, flavonoids, cyanogenic glycosides, phenolics, etc. (Sasidharan et al., 2011). Indefinite number of the pharmaceuticals currently available like aspirin, digoxin, quinine and opium are derived from plants (viz: *Filipendula ulmaria, Digitalis purpurea, Cinchona officinalis* and *Papaver somniferum*) that have millennia-long history drug information of use as folk herbalism (Swain, 1972).

Many ancient Indian literatures and Hindu scriptures like *Rigveda* (4500–1600 BC), *Charaka Samhita* (1000–800 BC), *Sushruta Samhita* (800–700 BC), etc. evidenced the use of herbal medicines from remote ages. In India approximately 75% population relies on traditional methods of herbal medicines. The Eastern Himalayas, Western Ghats and Andaman and Nicobar Islands are reported as the hot-spots of medicinal plant diversity (The Wealth of India 1988; Indian Herbal Pharmacopoeia 2002; Rana et al. 2014).

Paris polyphylla is a rhizomatous perennial herb native to China and the Indian Subcontinent. Diverse species of *Paris* is found extensively growing in Yunnan-Guizhou Plateau of China (Ji et al., 2006). The species is growing in China, Bhutan and Nepal. There are widely known subspecies and varieties of *P. polyphylla* distributed in Bhutan, Laos, Myanmar, Thailand and Vietnam as well (Liang and Soukup, 2000). In India, the species have been recorded from Arunachal Pradesh, Himachal Pradesh, Jammu and Kashmir, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Uttarakhand of Indian-Himalayan region (Paul et al., 2015). In Manipur, where the species is found in abundance, it is found growing in Hengbung, Maram, Purul and Makui regions of Senapati district and Puilong in Tamenglong district.

Fig. 1: Distribution of *Paris polyphylla*

Paris polyphylla plants are found in forest canopies and areas away from anthropogenic activities. It is a shade loving plant, prefers humic soil and high moisture content (Jamir et al., 2015). *Paris polyphylla* flourished well in soils with high amount of organic matter including nitrogen and phosphorus, whereas Potassium in soil plays adverse effects to its growth (Madhu et al., 2010).

Trade and marketing

All the harvested rhizomes of the *P. polyphylla* are traded to Myanmar and other South East Asian countries illegally routed through Assam and Manipur. Some middlemen are being involved in illegal trading either at local or directly to the regional level and then outside of the country (Paul et al., 2015). The rhizomes harvested from Dibang and Lower Dibang Valley district are traded to

Tinsukia/Dibrugarh via Roing. Upper Siang and East Siang district are traded to Dibrugarh/Tezpur via Pasighat. West Siang district is traded to Tezpur via Silapathar. While harvested rhizomes from Tawang and West Kameng district are traded to Tezpur via Bomdila. Similarly, the illegal exporting of rhizomes of *P. polyphylla* from Arunachal Pradesh to China via Myanmar has been reported (Basar, 2014). Illegal trade of the rhizomes to Myanmar through Indo-Myanmar border by the local traders have been reported (The Sangai Express, 2008). Trafficking of the rhizomes have been reported from Arunachal Pradesh, Manipur, Meghalaya and Nagaland to Myanmar (Mao et al., 2009).

Folk and traditional uses

Paris polyphylla commonly known as 'Rhizoma Paridis' in China was documented for the first time in the "Chinese pharmacopoeia" in 1985 (Man et al., 2009). The whole plant is primarily used to treat cancer and also it acts as a febrifuge. The dried rhizomes of *Paris polyphylla* have been used as a natural remedy for the treatment of microbial infection, snake bite, convulsions, fractures, throat swelling, diarrhoea and liver cancer. The rhizomes of Paris polyphylla possess rich bioactive compounds which can be utilized potentially as source of plant-derived drugs. Rajsekhar et al. (2016) reported large number of secondary metabolites from the rhizome of this plant including alkaloids, quinones, phenols and tannins.

Nearly 98 compounds were identified from the rhizome, which includes more than 30 steroidal saponins. Steroidal saponins are believed to be the main active ingredients in this species that showed antitumor, antibacterial and anti-viral, platelet agonist, and immune-stimulating properties, analgesic and sedative effects (Wang et al., 1990; Zhang et al., 2011). The steroidal saponins of stems and leaves of *Paris* possess antimicrobial and antioxidant activities (Shen et al., 2014).

Yunnan Baiyao', a very famous Yi ethno-medicine is prepared from the rhizome of various species of the genus *Paris.* This medicine is used against various diseases and injuries like back pain, bleeding, fractured bones, fungal diseases, poisonous snakes or insect bites, skin allergy, tumors and a variety of cancers (Long et al., 2003). Again various other Chinese traditional medicines, including analgesic, antiphlogistic, antipyretic, antitussive and depurative (Duke and Ayensu, 1984; Yeung, 1985) are prepared from the rhizome of *P. polyphylla*, whereas, the whole plant is used for the treatment of fever, liver and lung cancer and laryngeal carcinoma (Khanna et al., 1975; Ravikumar et al., 1979; Singh et al., 1980; Singh et al., 1982).

In Nepal, the rhizome is indigenously used against snake bites, insect bites, alleviate narcotic effects, internal wounds, external wounds, fever, food poisoning and are fed to cattle during diarrhea and dysentery (Baral and Kurmi, 2006; Dutta, 2007). It is also used to treat headache, vomiting and worms (Upretty et al., 2010). A drug called as Gong Xue Ning (GXN) capsule has been developed from the saponin extract of *P. polyphylla* var. *yunnanensis* in China for the treatment of abnormal uterine bleeding (AUB) (Zhao and Shi, 2005; Gou et al., 2008). Rhizome of the species is also used against uterine contractile effects (Tian et al., 1986; Zhou, 1991). *P. polyphylla* is a folk medicinal plant in the Indian Himalayan Region, traditionally used against analgesic, antibacterial, anti- phlogistic, antispasmodic, antitussive, any poisonous bites, burn, cut or injury, depurative, detoxification, diarrhea, dressing, dysentery, febrifuge, fever, gastric, gastritis, intestinal wounds, narcotic, poisoning, rashes or itching, scabies, skin diseases, sleeplessness, snake bite, stomach pain, typhoid, ulcer and wounds (Wu et al., 2004; Farooquee et al., 2004; Maity et al., 2004; Tiwari et al., 2010; Jamir et al., 2012; Lalsangluaii et al., 2013; Pfoze et al., 2013; Mir et al., 2014; Sharma and Samant, 2014). The name 'jack of all trades' has been given to *P.polyphylla* after reviewing its various medicinal properties (Shah et al., 2012).

Review of literature

Tissue culture

On studies conducted by Danu *et al.* 2015, the rhizome segments of *P. polyphylla* have been found to be responsive to vegetative propagation. Cutting rhizome subjected under different concentrations of plant growth regulators, the highest number of sprouting and rooting was observed together in applications of hormones GA3 and IBA.

Raomai *et. al.* (2014) were the first to report direct somatic embryogenesis (SE) from immature zygotic embryos (IZEs) in *P. polyphylla*. They used eight different basal media and studied the effect of each media on the rate of induction of somatic embryos from IZEs. The basal media used in producing somatic embryos includes B5, ½ B5, MS, ½ MS, N6, ½ N6, SH and ½ SH, out of which ½ MS media response significantly higher than the rest of the media used and the lowest being the MS media. Their result indicated that different concentrations or combinations of nutrients response differently on the formation of embryos from explant.

The affinities between somatic embryogenesis and basal media composition depicts the role and influence of nitrogen as the leading source among the several factors in inducing regeneration of somatic embryos in several plants (Hirai *et al*. 1997, Fisichella *et al*. 2000, Samson *et al.* 2006). It has been reported that ammonium nitrate as source of nitrogen is not required in high amount in SE of *P. polyphylla*. The explants (IZEs) were found to be more responsive in the absence of plant growth regulators in the nutrient media, which explicitly implies that the explant has potency of auxins to predestine its fate. Also, it results in direct formation of somatic embryos without an intervening callus phase (George 1993). Flow cytometry 33 indicates ploidy stability and the rapid accumulation of callose in the cell wall of somatic embryos after plasmolyzing pre-treatment. Hai-xia *et. al.* (2011) established the presence of diploid cells and morphology of regenerated plants similar to true breed in *P. polyphylla*.

A recent study on transverse thin cell layer culture technique for mini-rhizome formation in *P. polyphylla* by Raomai *et al.* 2015 reported that the phenolic compounds of explant (portion of rhizomes) are deleterious to regenerative potency and the growth of explants. The phenolic compounds present in different portions of rhizomes gave varied responses. The response of explant is effective to different range of cytokinin concentrations. Also, the types of cytokinin influence the induction of mini-rhizome. 6-Benzylaminopurine (BAP) and Thidiazuron (TDZ) were found to be more effective than Kinetin.

However, media containing BAP was found to induce higher responses than TDZ containing media. This result correlates with the findings of Han et al. (2005) on shoot formation from bulb scales. It has been reported that BAP are more efficiently biosynthesized than many other synthetic hormones. It can also be induced to produce natural growth regulators, eg. Zeatin (Zaerr and Mapes 1982). Cytokinins are known to promote cell division and implicate in the tuber induction (Fernie and Willmitzer 2001).

The induction of rhizome was more important as compared to root induction because rhizomes once formed could be easily acclimatized and undergo hardening. The rhizomes thus produced can be kept for long time, about six months, on the same medium without subsequent change in medium. With periodic subculture after every 60 days, it can be kept for 3 years or longer. Rhizomes in situ are generally more prone to pathogens while, mini-rhizomes produced in vitro are free from disease and response well in field conditions. Thus, mini-rhizomes could be potentially used for germplasm exchange, production of secondary metabolites and study the biosynthesis of phytomedicinal compounds including saponins (Raomai *et. al.* 2015)

Phenolic and flavonoid content

Antioxidants are those substances that prevent oxidation of other substances. Secondary metabolites in plants (eg. Phenolics, flavonoids, carotenoids) are natural or phytochemical antioxidants. Studies on phenolics and flavonoids have been confirmed to be more effective than other nather natural antioxidants like Vitamin C, E and carotenoids. Flavonoids are found to have medical importance as the compounds anticancer, antidiabetic and anti inflammatory properties and help to reduce coronary heart diseases (Ghasemzedah and Ghasemzedah, 2011; Saxena et al., 2012).

Various studies on different aspects and potentials of *Paris polyphylla* rhizomes further confirmed that steroidal saponins were the major bioactive constituents, which played an important role in the drug development for anti-tumour, immunity adjustment, anthelmintic and anti-inflammation activities (Man et al. 2009; Wang et al. 2010). The extracts of rhizome induce apoptosis, affect cell cycle distribution, inhibit angiogenesis and regulate immune function.

Antioxidant activity

There are various methods for determining the free radical scavenging activity of natural compounds. Techniques like DPPH assay, FRAP assay, Superoxide radical assay, hydroxyl radical assay were used to determine antioxidant activity of polysaccharides from the leaves and other aerial

parts of *Paris polyphylla* and the result showed exhibition of strong antioxidant activity *in vitro* (Shen et al., 2014). It has been reported that the underground rhizomes also possess significant antioxidant properties (Ravipati et al., 2012; Paonam and Sharma 2014).

Anti-bacterial activity

Volatile oils present in *Paris polyphylla* show strong inhibitory effects on *Micrococcus, Xanthomonas, Aerobacter, Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aureginosa*, *E. coli, Salmonella flexinerai* and *Brevibacterium* (Zhao et al., 2009; Chhetri et al., 2012; Liu et al., 2014). The roots have shown anti-bacterial action against *Bacillus* spp., *B. typhi, B. paratyphi, E. coli, Staphylacoccus aureas, Haemolytic streptococci, Meningococci,* etc. (Sharma et al., 2015). It has been reported that 25 compounds are present in stem and leaves of *P. polyphylla.* Out of these 25, 11 compoounds showed potent antibacterial activity against *Propionibacterium acnes* (Qin et al., 2012).

Anti-Fungal activity

A new steroidal saponin along with two other known compounds isolated from the rhizomes of *P. Polyphylla* showed antifungal activity against *Cladosporium cladosporioides, Magnaporthe oryzae* and various strains of *Candida albicans* (Deng et al., 2008; Zhang et al., 2011). The three pennogenin steroidal saponins isolated from the plant exhibit moderate antifungal activities against *Saccharomyces cerevisiae* and *Candida albicans* (Zhu et al., 2011).

Problem background

The rhizome is the main mode of regeneration though it regenerates from seeds, but it is meager. Uncontrolled and indiscriminate harvesting of whole plant without leaving any part of the rhizome, harvesting before reproductive/flowering or seed maturity period etc. are causing regeneration failure in its natural habitat. Conversely, regeneration of *P. polyphylla* from seed in wild, green house, laboratory is very poor because of long dormancy period and very slow growing nature (Madhu *et al.* 2010) which is affecting the growth and survival of the species. Over harvesting has caused the decrease in natural population of the species. The over exploitation, rampant illegal extraction and trade, wild populations of *P. polyphylla* are at risk of extinction from its natural habitat (Paul *et. al.* 2015).

IUCN and CAMP listed *Paris polyphylla* Sm. under vulnerable category (V) (Madhu *et. al.* 2010). The plant is declining continuously at an alarming rate by human interference such as unsustainable harvesting (over- and premature- collection), unscientific use, illegal/cross-border trade of rhizomes, habitat destruction, forest-fire, and soil-erosion. It was found that the existing threat to the population of *Paris polyphylla* is that people are heavily dependent on nature for the plant and there is no cultivation technology developed yet (Joshi and Bhatta 2014). Anthropogenic activities like shifting agriculture, forest resource collection, logging, etc. are affecting habitat and wild populations of the species. Heavy rainfall leading to the land erosion/landslides, etc. is also causing the habitat loss and population depletion of the species. Grazing is also one of the factors for loss of habitat/population of the species (Paul *et. al.* 2015).

The exploitation will certainly deteriorate the biodiversity which will lead to change and/or its extinction of the plant. Utilization should nevertheless be correlated to ensure sustainable use and conservation of the resources (Nwachukwu *et. al*. 2010).

Research objectives

- *In-vitro* regeneration from rhizome, seed, leaf and coleoptiles explants.
- To evaluate the free radical scavenging activity of methanol and water extract of rhizome of *Paris polyphylla* by DPPH assay.
- To estimate the total phenolic content.
- To estimate the total flavonoid content.
- To evaluate the antimicrobial (antibacterial and antifungal) activities and to determine relative percentage inhibition.

Materials and methods

Plant Material:

Rhizomes and seeds of *Paris polyphylla* were used for the present study. Different chemicals and instruments that are being used are as follows:

Table 1: List of chemicals and accessories used

Hi media	Product code
Ammonium nitrate (NH_4NO_3)	027202
Acetone	
Boric acid (H_3BO_3)	0249133
Calcium chloride ($CaCl_2.2H_2O$)	0278584
Cobalt chloride ($CoCl_2.6H_2O$)	027790
Copper sulphate ($CuSO_4.5H_2O$)	027849
Disodium EDTA (Na_2EDTA)	0373000500
Ferrous sulphate ($FeSO_4.7H_2O$)	028400
Inositol ($C_6H_{12}O_6$)	1036110
Manganese sulphate ($MnSO_4.H_2O$)	029147
Magnesium sulphate ($MgSO_4.7H_2O$	029117
Mercuric chloride	
Potassium dihydrogen phosphate (KH_2PO_4)	029605
Potassium iodide (KI)	0539000100
Potassium nitrate (KNO_3)	029638
Sodium molybdate ($Na_2MoO_4.2H_2O$)	030185
Sucrose ($C_{12}H_{22}O_{11}$)	0615500500
Zinc sulphate ($ZnSO_4.7H_2O$)	030621
Sigma	
Agar (C_{12} H_{18} O_9)	0077900500
6-benzyl aminopurine (BAP)	12750
Gibberellic acid (GA_3) ($C_{19}H_{22}O_6$)	0393000010
Indole-3-butyric acid (IBA) ($C_{12}H_{12}NO_2K$)	38559525
Indole-3-acetic acid (IAA) (C_{10} H_8NO_2Na	028400
Kinetin (KN) ($C_{10}H_9N_5O$)	
Merck	
Ethanol	20140110
Sodium hypochlorite	023029
Axygen	
Micro tubes (1.5 ml)	MCT-150-C

Micro tubes (2 ml)	MCT-200-C
Pipette tips (0.5–10µl)	T-300
Pipette tips (100–1000µl)	T-1000-B
Pipette tips (2–200 µl)	T-200-Y
Petri plates	460090-90 MM
Borosil	
Beaker (500 ml)	1000D24
Beaker(1000 ml)	1000D29
Conical flask (100 ml)	403010
Conical flask (500 ml)	441150
Conical flask (1000 ml)	441130
Culture tubes	10682424
Miscellaneous	**Description**
Aluminium foil	Scraft product Pvt.ltd,302, R.G Trade Tower , Netaji Subash Place , Pritampura, New Delhi-34
Absorbant cotton	Polylab industries Pvt.ltd Kundii Haryana
Autoclaved bag	Polylab industries Pvt.ltd Kundii Haryana
Brown paper	Polylab industries Pvt.ltd Kundii Haryana
Forceps	Polylab industries Pvt.ltd Kundii Haryana
Parafilm	Polylab industries Pvt.ltd Kundii Haryana
Scalpel blade	Polylab industries Pvt.ltd Kundii Haryana
Scalpel holder	Polylab industries Pvt.ltd Kundii Haryana
Scissors	Polylab industries Pvt.ltd Kundii Haryana

Table 2: Various major and minor equipments used

S. No.	Equipment	Specifications	Use
1.	Autoclave	Ambient to150 °C, up to 30 psi	For sterilization of nutrient media glassware, decontamination of biological waste
2.	Electronic	Weighs from 0.02–310 g,	For precise weighing of

	balance	with Mono Bloc technology	compounds, media components, plant growth regulators etc.
3.	Hot air oven	Ambient to 80 °C; 0–10 rpm	For drying the glassware
4.	Laminar flow cabinet	HEPA and coarse filters	For providing contamination free work environment
5.	Magnetic stirrer	High and low speed	For dissolving the MS stocks and reagents etc.
6.	pH meter	pH range from 0–14 with temperature sensor	For adjusting the pH of MS media components, reagents etc.
7.	Refrigerator (4 ºC)	Temperature (4 ºC)	To preserve the growth hormones

Methods:

Step wise procedure for *in vitro* regeneration of *Paris polyphylla*:-

1. Sterilization of glassware

Glasswares used in this study were presterilized by

1. Washing with detergent and rinsed with distilled water.
2. Keeping in hot air oven for dry sterilization at 160ºC for 60-80 minutes, then wrap them with brown paper and kept in autoclavable bags.
3. The glasswares, media, distilled water, and others required equipments were autoclaved at 15 psi for 20 min at 121°C temperature.

2. Medium for plant tissue culture, growth and regeneration

For the tissue culture of *Paris polyphylla,* Murashige and Skoog salts (1962), vitamins of B5 (Gamborg *et al.* 1968) supplemented with different growth regulators, are used. The composition of the MS medium is shown in Table.

Table 3: Composition of Murashige and Skoog (MS) basal medium

Constituents	Working concentration (mg/L)	Stock solutions (mg/L)
Macronutrients		
NH_4NO_3	1650	16,500
KNO_3	1900	19,000
$CaCl_2.2H_2O$	440	4,400
$MgSO_4.7H_20$	370	3,700
$KH_2PO_4.H_2O$	170	1,700
Micronutrients		
KI	0.83	83.0
H_3BO_3	6.20	620.0
$MnSO_4.H_2O$	22.30	2,230
$ZnSO_4.7H_2O$	8.60	860
$Na_2MoO_4.2H_2O$	0.25	25.0
CuSO4.5H20	0.025	2.50
$CoCl_2.6H_2O$	0.025	2.50
Iron EDTA		
$FeSO_4.7H_2O$	27.80	2,780
$Na_2EDTA.2H_2O$	37.30	3,730
MS Vitamins		
Myo-inositol	100.0	10,000
Nicotinic acid	0.50	50
Pyridoxine HCl	0.50	50
Thiamine HCl	0.10	10
Carbon Source		
Sucrose	30,000	-
Gelling agents		
Agar	8,000	-

3. Plant growth regulators (PGR's)

Different growth regulators for regeneration purpose were sterilized and stored at recommended temperatures as shown in Table.

Table 4: Plant growth regulators stock solution preparation

Plant growth regulators	**Molecular weight**	**Solvent**	**Diluent**	**Storage condition**
IAA	175.2	95% Ethanol	H_2O	-0 ^oC
IBA	203.2	95% Ethanol	H_2O	-0 ^oC
NAA	186.21	95% Ethanol	H_2O	-0 ^oC
2,4-D	221.033	95% Ethanol	H_2O	-0 ^oC
BAP	225.3	1 M NaOH	H_2O	-0 ^oC
Kinetin	219.2	1 M NaOH	H_2O	-0 ^oC

4. Surface sterilization of Laminar Hood

The UV tube in the Laminar Air Flow was kept ON for 45 minutes before starting the work to remove the viral, fungal or bacterial contaminants and wiped the surface with 70% Ethanol. Then switched OFF the UV tube while working.

5. Media preparation for *In vitro* regeneration

1. MS basal medium for *In vitro* regeneration was prepared by dissolving MS salt in double distilled water in a conical flask.
2. The solidifying agent, agar (0.8%), was added, stirred steadily to dissolve completely and adjusted the pH to 5.7 using 1 N NaOH or 1 N HCl.
3. Then plug the conical flask with cotton cap and wrap it with brown paper.
4. Autoclave the media at 15 psi (1.06 kg/cm^2) for 20 min at 121^oC.
5. Thermo-labile plant growth regulators prepared in different solvents as per their nature were filter-sterilized and added to the autoclaved media when cooled to below 50^oC and mixed thoroughly in the laminar air flow hood.
6. The media were dispensed inside the Laminar Air Flow in autoclaved culture vessels or sterile Petri dishes as per requirement.

Media and their composition

Table 5: Different media compositions used in the present study

Media	**Composition**
Callus induction media	MS + Agar (0.8%) + BAP (0.5 mg l^{-1}) + NAA (0.5 mg l^{-1})
Callus induction media	MS + Agar (0.8%) + BAP (1 mg l^{-1}) + NAA (1.5 mg l^{-1})
Callus induction media	MS + Agar (0.8%) + BAP (1.5 mg l^{-1}) + NAA (2.5 mg l^{-1})
Bud induction media	MS+ Agar (0.8%) + BAP (2 mg l^{-1}) + IBA (1 mg l^{-1})
Bud induction media	MS+ Agar (0.8%) + BAP (1 mg l^{-1}) + NAA (0.2 mg l^{-1})

6. Surface sterilization of rhizome and leaf explants

1. The rhizome and leaf explants were washed thoroughly with tap water for 30 minutes to remove the contaminants, then cut into small pieces (0.5 cm× 0.5 cm).
2. The explants were treated with 4% Tween 20 for 10 minutes and again washed 4-5 times with double distilled water.
3. The explants were treated with 0.1% Mercuric Chloride ($HgCl_2$) for 10 minutes inside the Laminar Air Flow (LAF), followed by rinsing with double distilled water 4–5 times.
4. The explants were treated with 70% Ethanol for 30 seconds and finally washed 4-5 times with distilled water to remove all the chemical traces present on the explant surface.

Explant inoculation

1. The sterilized explants were dried on filter papers before inoculating into the culture vessels containing nutrient media with growth regulators.
2. The forceps are flame sterilized on gas burner inside the Laminar Air Flow.
3. The explants are inoculated near the flame to avoid any kind of Viral, Bacterial and Fungal contamination and sealed the culture vessels with parafilm.
4. The cultures were maintained at 25 ± 2ºC.

Preparation of plant rhizome extract

Soxhlet extraction

1. The rhizomes of *Paris polyphylla* were properly washed and dried at room temperature for 15 days.
2. Dried rhizomes were grind to fine powder.
3. 5g of the powder was extracted in Soxhlet apparatus with 300 ml solvent (methanol and distilled water) at 60˚C and 100˚C, respectively, for 8 hours.
4. The extract was evaporated using rotary evaporator.
5. The sample was collected and stored at 4˚C for later use.

Cold extraction

1. The dried rhizomes of the plant were crushed to fine powder using grinder.
2. 5g of powder was added in 200ml solvent (methanol and distilled water).
3. The solution was kept at room temperature for 72 hours with occasional stirring.
4. The solution was screened through Whatman filter paper.
5. The extract was evaporated in rotary evaporator.

DPPH Antioxidant Assay

The DPPH assay was carried out following the method of (Padmanadhan and Jangle, 2012).

Preparation of DPPH

1. 0.1mM DPPH solution was prepared by taking 3.94mg of DPPH in 100ml absolute methanol.
2. The solution was kept in dark for 30 minutes at room temperature.
3. Optical density was measured using spectrophotometer at 517nm.

Antioxidant activity

1. Different concentrations of sample were taken, ranging from 0.25-1.25 mg/ml. All the concentrations were prepared in triplicates.
2. 2 ml of DPPH methanol solution were added in each test tube.
3. The mixture was kept at room temperature covered with black paper or kept in dark for 5-10 minutes.
4. Absorbance was recorded at 517nm.
5. All the tests were done in triplicates.
6. DPPH radical scavenging activity was calculated using the following formula

$$\text{Scavenging effect (\%)} = \left(1 - \frac{\text{As}}{\text{Ac}}\right) \text{x } 100$$

Where, A_S = OD of sample with DPPH solution
A_C = OD of DPPH methanol solution.

Estimation of Total Phenolic Content

For the estimation of total phenolic content, Folin-Ciocalteu Colorimetric method was used following Ainsworth and Gillespie (2007).

Preparation of stock solutions

1. 5mg/ml Gallic acid solution was prepared by taking 100mg Gallic acid in 20ml distilled water.
2. 20% sodium carbonate was prepared by dissolving 20g in 100ml distilled water.
3. Plant extract was prepared by taking 1g rhizome powder in 60ml methanol and kept in water bath for 60 minutes at 40˚C followed by filtration.

Preparation of working solution

1. Different concentrations of Gallic acid was prepared in beakers as follows:

Table 6: Preparation of Gallic acid working solution

Gallic acid (ml)	Distilled water (ml)	Concentration of gallic acid (mg/100ml)
2	98	10
4	96	20
6	94	30
8	92	40
10	90	50

2. 20µl of gallic acid solution was taken from each beaker to test tubes.
3. For test sample, 20µl extract was taken.
4. 1.58ml of distilled water was added in each test tube followed by 100µl Folin-Ciocalteu and kept it for 5 minutes.
5. 300µl of sodium carbonate was taken and kept in water bath for 30 minutes at 40˚C.
6. OD was recorded at 765nm.
7. Distilled water was used as reference.
8. All the tests were done in triplicates.

Estimation of Total Flavonoid Content

Aluminum Chloride Colorimetric method (Chang et al., 2002) was used for total flavonoid content estimation.

Preparation of stock solutions

1. 0.1mg/ml Quercetin solution was prepared in methanol.
2. 10% aluminum chloride solution was prepared in distilled water.
3. 1M potassium acetate solution was prepared by taking 9.82g potassium acetate in 100ml distilled water.
4. Plant extract was prepared by Soxhlet extraction method.
5. 10g rhizome powder of the plant was extracted in 300ml methanol for 10hrs
6. The extract was evaporated in rotary evaporator at 65˚C followed by preparation of 20mg/ml test sample in methanol.

Preparation of working solution

1. Different concentrations of Quercetin was prepared in beakers as follows:

Table 7: Preparation of Quercetin working solution

Quercetin (ml)	Methanol (ml)	Concentration of quercetin (µg/ml)
2	8	20
4	6	40
6	4	60
8	2	80
10	0	100

2. 500µl of quercetin was taken from each beaker to test tubes.
3. For test sample, 500µl extract was taken.
4. 1.5ml of methanol was added in each test tube.
5. 100µl Aluminum chloride was added and kept it for 5 minutes.
6. 100µl of potassium acetate was added followed by addition of 2.8ml distilled water.
7. OD was recorded at 415nm.
8. Aluminum chloride was replaced by distilled water for reference.
9. All the tests were done in triplicates.

Antimicrobial activity of *Paris polyphylla* rhizomes

1. **Antibacterial activity**

Preparation of media for bacterial culture

7. Nutrient agar was used for culture of two bacterial strains, *Escherichia coli* and *Staphylococcus aureus*.
8. pH was adjusted to 7.2 using 1 N NaOH or 1 N HCl.
9. Then plug the conical flask with cotton cap and wrap it with brown paper.
10. Autoclave the media at 15 psi for 15 to 45 min at 121°C.
11. The media were dispensed inside the Laminar Air Flow in autoclaved Petri plates as per requirement.

Determination of antibacterial activity:

Disk diffusion technique was used to determine the antibacterial activity of the methanolic plant extract following Balouiri et al., (2016).

1. The agar plates were inoculated with standardized inoculums of the test microorganism.
2. Then, filter paper discs (about 5mm in diameter), containing the test compound at a different concentrations, were placed on the agar surface.
3. Petri plate containing test organism and Nutrient agar media was kept as negative control and petri plate containing media with test organism along with streptomycin sulfate (antibiotic) disc was kept as positive control.
4. All the Petri plates were then left on the bench for 15 minutes for adequate diffusion of the extract and incubated at 37°C for 2 days.
5. After incubation, the diameter of the zones of inhibition around each well was measured to the nearest millimeters.

2. **Antifungal activity**

Preparation of media for fungal culture

Czepak Dox agar media was used for the culture of fungus, *Aspergillus niger* and *Trichoderma reeseii.*

1. 49.01g of Czepak Dox agar was added to distilled water and finally the volume was brought to 1 liter with constant mixing.
2. pH was adjusted to 7.3 using 1 N NaOH or 1 N HCl.
3. The media was autoclaved at 121°C for 15 minutes at 15 lbs pressure.
4. The media were dispensed inside the Laminar Air Flow in autoclaved Petri plates as per requirement.

Determination of Antifungal activity

Disk diffusion technique was used to determine the antibacterial activity of the methanolic plant extract following Balouiri et al., 2016.

1. The agar plates were inoculated with standardized inoculums of the test microorganism.
2. Then, filter paper discs (about 5mm in diameter), containing the test compound at a different concentrations, were placed on the agar surface.
3. Petri plate containing test organism and Czepak Dox media was kept as negative control and plate containing media with test organism and antibiotic disc was kept as positive control.
4. All the Petri plates were then left on the bench for 15 minutes for adequate diffusion of the extract and incubated at 27°C for 2 days.
5. After incubation, the diameter of the zones of inhibition around each well was measured to the nearest millimeters.

The complete antifungal analysis was carried out under strict aseptic conditions and repeated two times in order to get the accuracy.

Determination of relative percentage inhibition

The relative percentage inhibition of the test extract with respect to positive control was calculated by using the following formula (Ajay et al., 2003; Kumar et al., 2010)

Relative percentage inhibition of the test extract $= \frac{(X-Y)}{(Z-Y)} \times 100$

Where,

x: total area of inhibition of the test extract

y: total area of inhibition of the solvent

z: total area of inhibition of the standard drug

The total area of the inhibition was calculated by using area = πr^2, where, r = radius of zone of inhibition.

Results and discussion

Tissue culture

The composition of the media with hormone concentrations, which produced callus are given as:

Table 8: Media composition for callus production

Callus induction media	MS + Agar (0.8%) + BAP (0.5 mg l^{-1}) + NAA (0.5 mg l^{-1})
Callus induction media	MS + Agar (0.8%) + BAP (1 mg l^{-1}) + NAA (1.5 mg l^{-1})
Callus induction media	MS + Agar (0.8%) + BAP (1.5 mg l^{-1}) + NAA (2.5 mg l^{-1})
Callus induction media	MS + Agar (0.8%) + BAP (2 mg l^{-1}) + NAA (3.5 mg l^{-1})

Out of the above three compositions of MS media, the best results were found to be produced from MS + Agar (0.8%) + BAP (1.5 mg l^{-1}) + NAA (2.5 mg l^{-1}). The coleoptiles explants produced callus after 48 days of incubation in growth room.

There was no production of shoot or bud from any of rhizome explants used for the shoot or bud induction. This is due to the arbuscular mycorrhizal fungi (*Acaulospora, Glomus, Gigaspora, Scutellospora*, etc) infect the roots of *P. polyphylla* and form arbuscular mycorrhiza which are potent biological resources that stimulate the growth of the plant (Zhou et al., 2009). The broad diversity and taxonomic spectrum were exhibited by the endophytic fungi from *Paris polyphylla* that have been received much attention in recent years as they were believed to be an excellent source of biologically active compounds (Li et al., 2008). A rich fungal community and more than 180 OTUs were identified for each *P. polyphylla* rhizome. Further it has also been reported a rich diversity of bacteria in the rhizome of the species and concluded that this microbial diversity in the rhizome of *P. polyphylla* enhances the saponin content of the species (Yang et al., 2015).

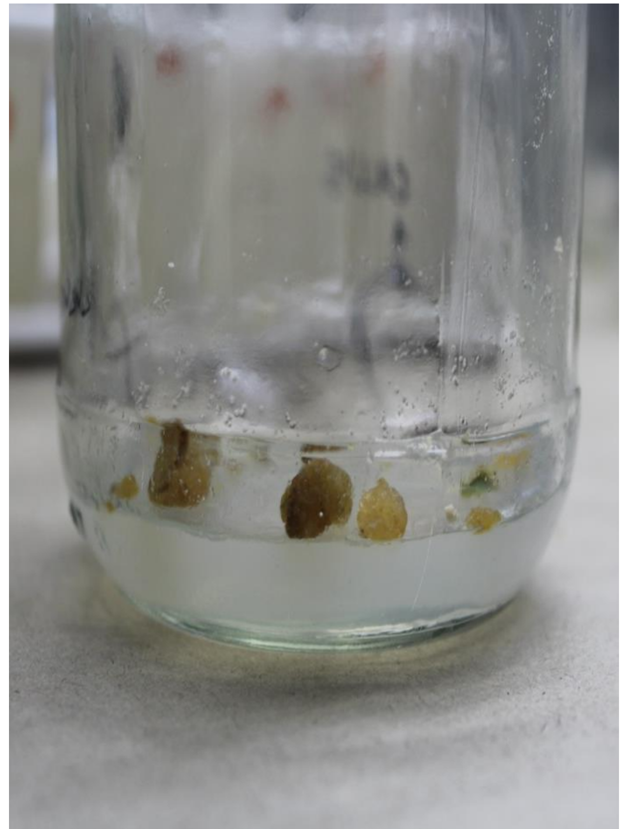
PLATE I

(A) Cutting of rhizome as explant

(B) Leaf explant on the MS media

(C) and (D) Rhizome as explant

PLATE II



Callus induction on MS + Agar (0.8%) + BAP (1.5 mg l^{-1}) + NAA (0.5 mg l^{-1}) + 2,4-D (1.0 mg l^{-1})

DPPH Antioxidant assay

DPPH radical is commonly used for the evaluation of the antioxidant activities of natural compounds. Alcohol solution of DPPH has strong absorption band at 517 nm. The reduced DPPH methanol solution changes its colour from purple to light yellow and absorbance reduced. The transfer of either an electron or a hydrogen atom to DPPH causes the scavenging mechanism of natural compounds (Naik et al., 2003).

The antioxidant activity of *P. polyphylla* rhizomes through the scavenging of DPPH radicals was studied. The scavenging activities of methanol and water extract were expressed as IC values. Lower IC value indicates higher scavenging ability.

It was observed that the methanol rhizome extract showed higher antioxidant activity than water extract. The results for rhizome extract IC50 values are in contrast to the results obtained by Shen et al. (2014). Further, our study showed that IC50 value for the aqueous extract was quite high than methanolic extract. The reason may be ascertained to the solubility of steroidal saponins in methanol and water. Steroidal saponins are more soluble in methanol than water.

Table 9: Antioxidant activity of methanolic rhizome extract

Sample (µl)	Methanol (µl)	DPPH (ml)	Conc. of sample (mg/ml)	Absorbance at 517 nm			Mean±SD	% Scavenging Activity
				1st	2nd	3rd		
100	400	2	0.25	0.528	0.534	0.522	0.528±0.006	37.36
200	300	2	0.5	0.504	0.501	0.495	0.500±0.004	40.68
300	200	2	0.75	0.483	0.462	0.465	0.470±0.011	44.24
400	100	2	1.0	0.460	0.438	0.439	0.445±0.012	47.21
500	0	2	1.25	0.388	0.398	0.389	0.391±0.005	53.61

Concentration of stock solution= 5mg/ml

OD of DPPH at 517nm= 0.843

IC_{50}= 1.09 mg/ml

Table 10: Antioxidant activity of aqueous rhizome extract

Sample (µl)	Water (µl)	DPPH (ml)	Conc. of sample (mg/ml)	Absorbance at 517 nm			Mean±SD	% Scavenging Activity
				1st	2nd	3rd		
100	400	2	0.25	0.624	0.644	0.635	0.634±0.010	17.66
200	300	2	0.5	0.551	0.566	0.555	0.557±0.007	27.62
300	200	2	0.75	0.484	0.479	0.480	0.481±0.002	37.50
400	100	2	1.0	0.438	0.437	0.448	0.441±0.006	42.72
500	0	2	1.25	0.427	0.424	0.430	0.427±0.003	44.54

Concentration of stock solution= 5mg/ml

OD of DPPH at 517nm= 0.770

IC_{50}=1.33mg/ml

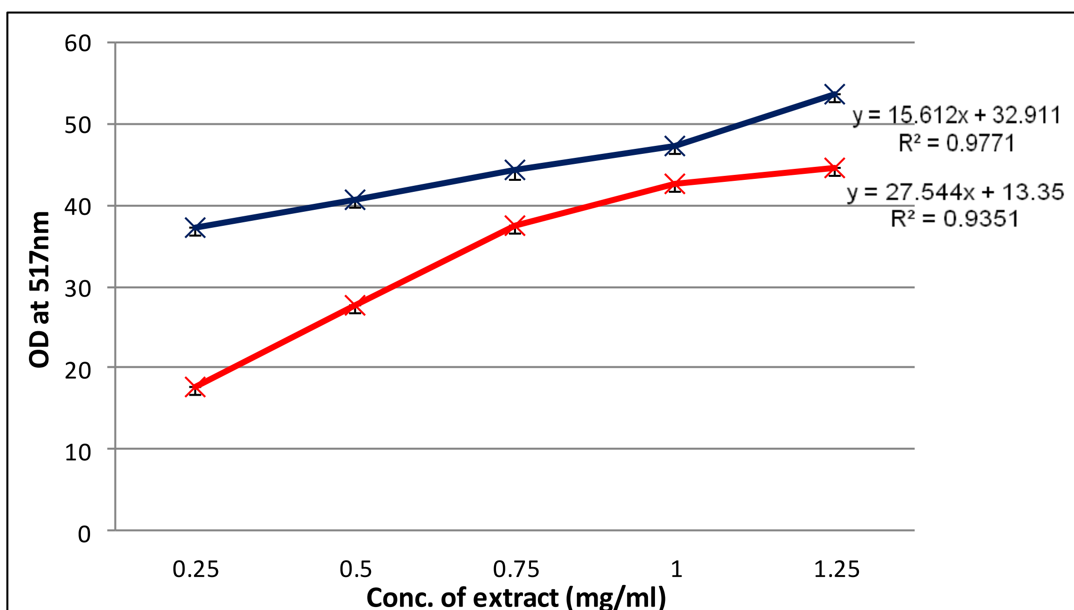


Fig 2: DPPH radical scavenging activity of *Paris polyphylla* rhizomes.

Estimation of total phenolic content

Phenols are important class of chemical compounds having various bioactivities like antioxidant, anticancer, anti-inflammatory properties. Natural phenolics from medicinal plants are novel anticarcinogen (Huang et al., 2010).

The methanol rhizome extract of *P. polyphylla* was found to contain significant amount of phenolics. The estimated total phenolic contents using the Folin-Ciocalteu's reagent is expressed as 43.01mg gallic acid equivalent/g dry weight of extract.

Table 11: Absorbance at 765 nm for phenolic content

Standard (Gallic acid)	Concentration of gallic acid (mg/ml)	Absorbance at 765 nm			Mean±SD
		1st	2nd	3rd	
1	0.002	0.114	0.115	0.117	0.115±0.001
2	0.004	0.232	0.229	0.217	0.226±0.007
3	0.006	0.368	0.372	0.373	0.371±0.002
4	0.008	0.499	0.480	0.481	0.487 ±0.010
5	0.010	0.601	0.598	0.599	0.599±0.001
Test sample		0.526	0.532	0.517	0.525±0.007

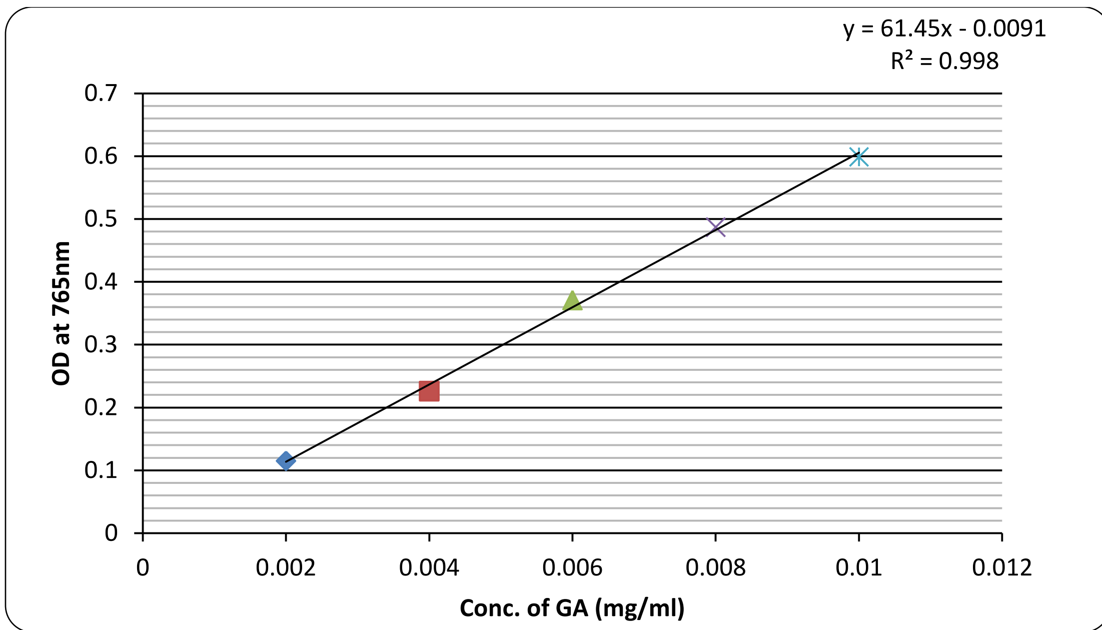


Fig 3: Standard curve of Gallic acid

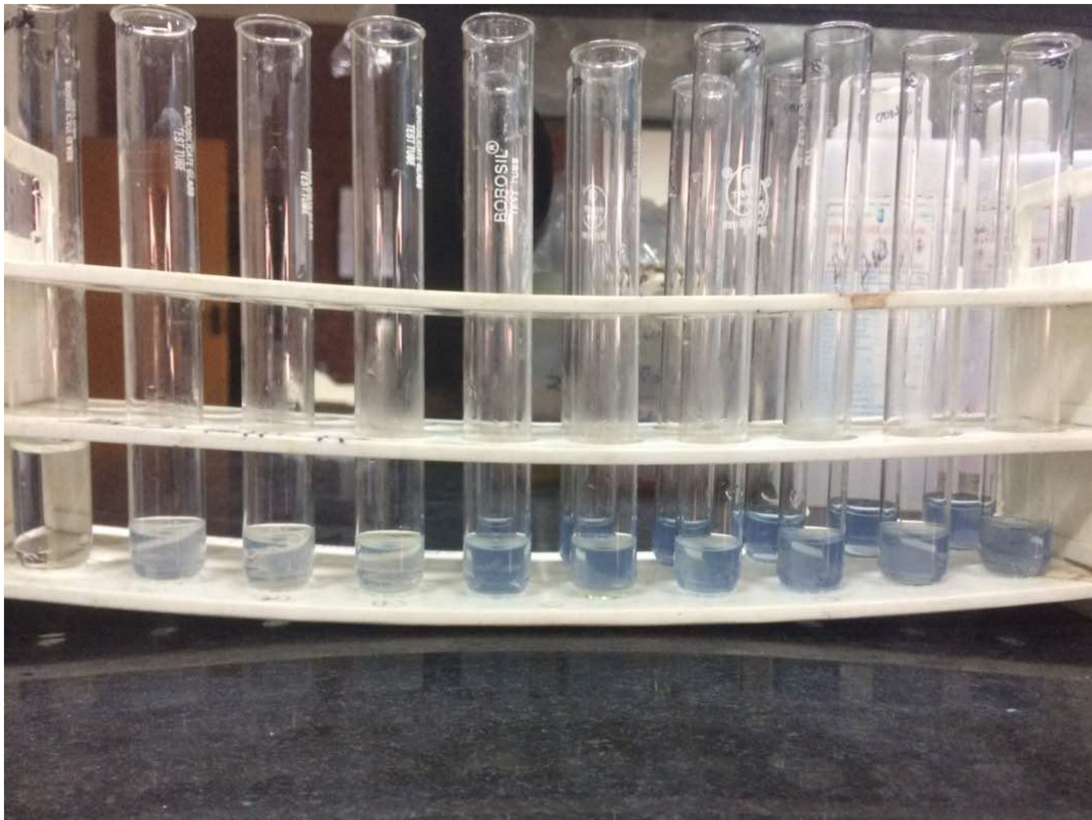


PLATE III: Test tubes containing Gallic acid solution

Estimation of total flavonoid content

Flavonoids are secondary metabolites widely distributed in plants. They are potential cytotoxic compounds. It possesses antioxidant properties (Maria et al., 2012).

The total flavonoid content of methanol rhizome extract was found to be 28mg quercetin equivalent/g dry weight of test sample.

Table 12: Absorbance at 415 nm for flavonoid content

Standard (quercetin)	Concentration of quercetin (mg/ml)	Absorbance at 415 nm			Mean±SD
		1st	2nd	3rd	
1	0.002	0.215	0.216	0.220	0.217±0.00
2	0.004	0.444	0.475	0.482	0.467±0.020
3	0.006	0.672	0.668	0.682	0.674±0.007
4	0.008	0.845	0.896	0.895	0.879±0.029
5	0.010	1.120	1.130	1.125	1.125±0.005
Test sample		0.626	0.667	0.626	0.640±0.023

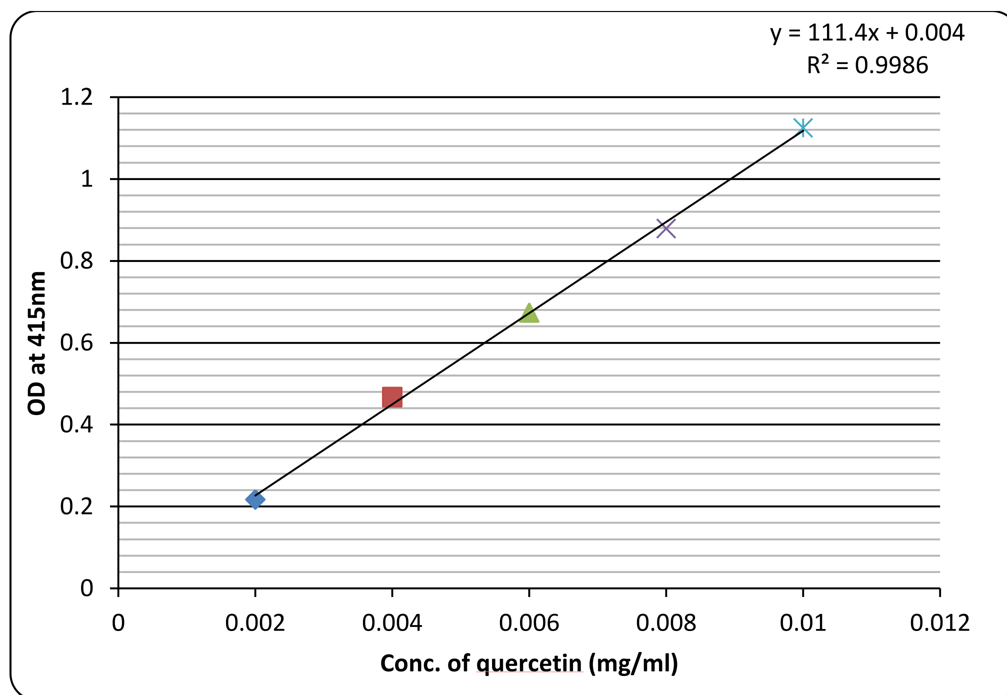


Fig 4: Standard curve of Quercetin

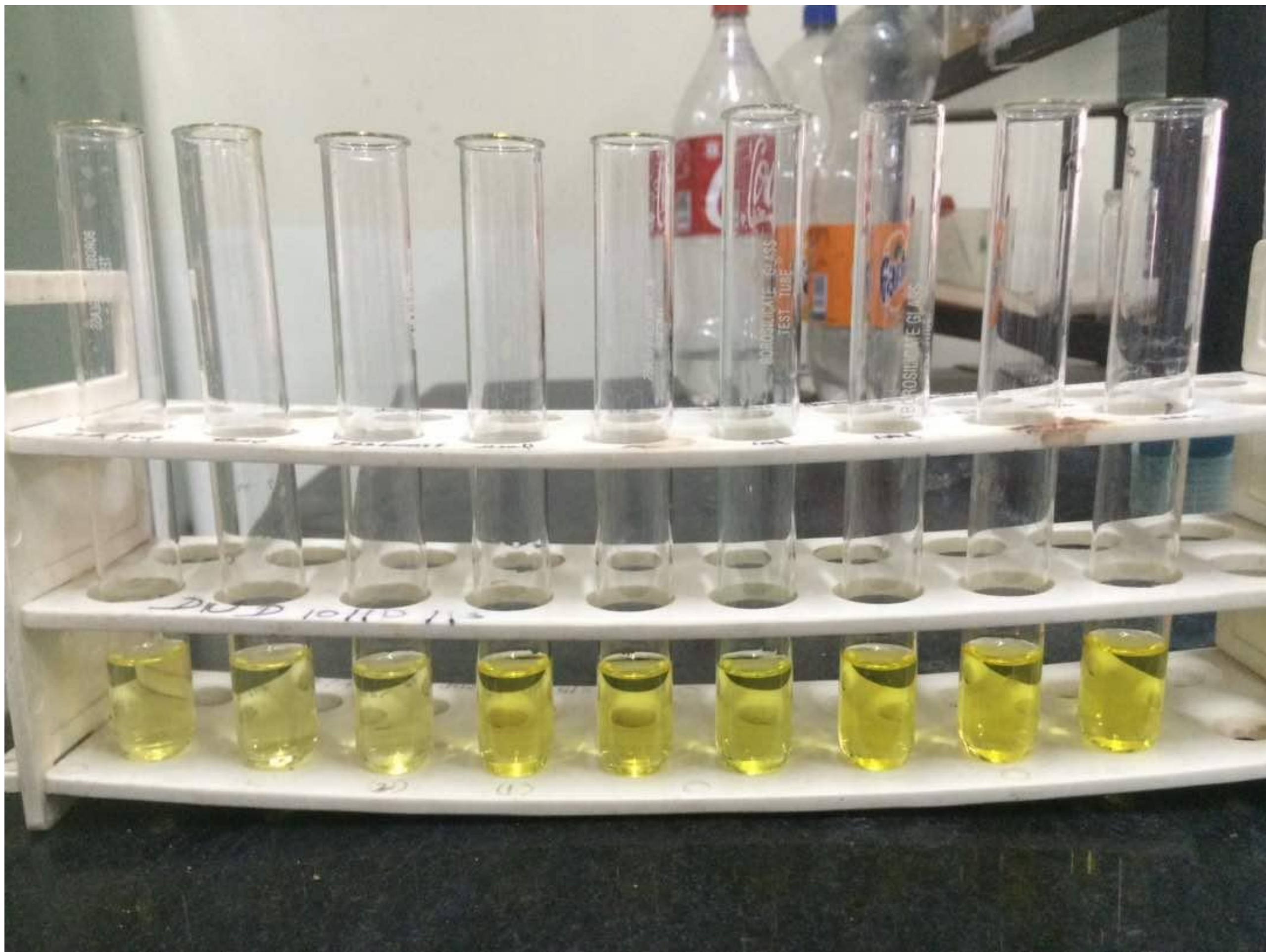


PLATE IV: Test tubes containing standard quercetin solution

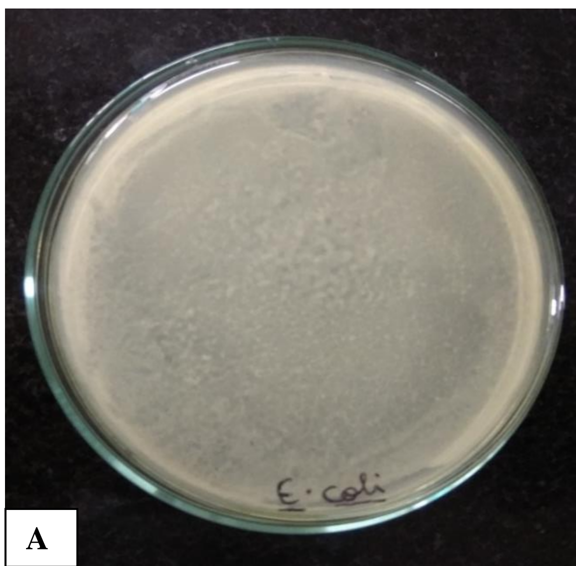
Antimicrobial activity

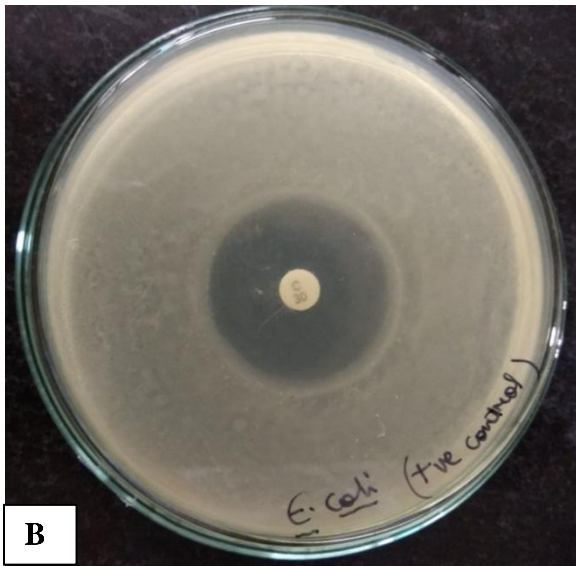
Paris polyphylla is a potent antimicrobial agent. The main compounds responsible for its antimicrobial activity are the steroidal saponins, which are concentrated mostly in its rhizomes (Qin et al., 2012). These compounds showed antimicrobial activity against a wide range of bacteria (*Agrobacterium tumifasciens, Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Helicobacter pylori, Xanthomonas vesicatoria, Staphylococcus haemolyticus, Pseudomonas sp.* Etc.) and fungi (*Aspergillus niger, Candida albicans, Fusarium graminearum, Phytophthora capsici, Botrytis cinerea, Magnaporthe oryzae*, etc.) (Zhao et al., 2010).

1. Antibacterial activity

Table 13: Antibacterial activity against *Escherichia coli*

Serial no.	Conc. of the sample (mg/ml)	Zone of inhibition (mm)			Mean±SD
		1st	2nd	3rd	
1	1.25	25	19	20	21.33±3.21
2	2.5	27	26	20	24.33±3.78
3	3.75	27	30	29	28.67±1.53
4	5	28	31	29	29.33±1.53





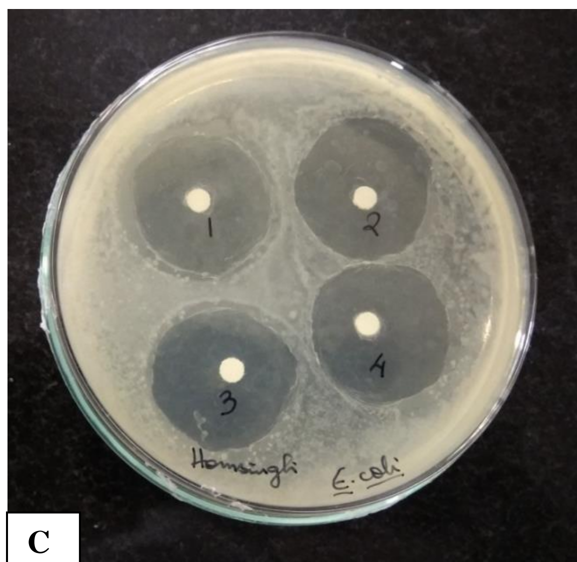
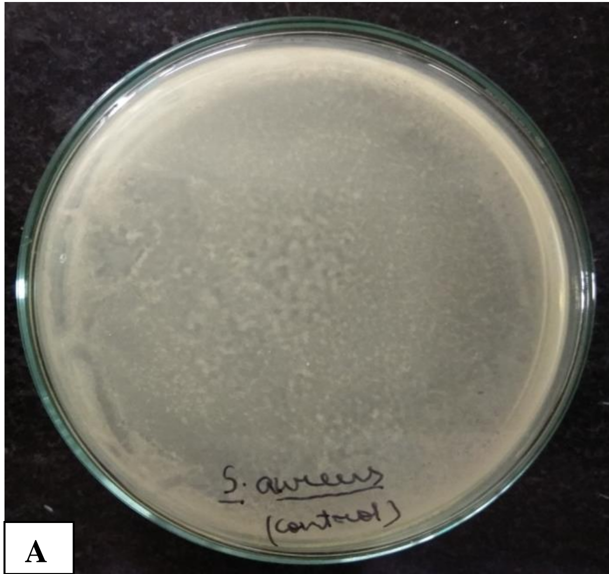
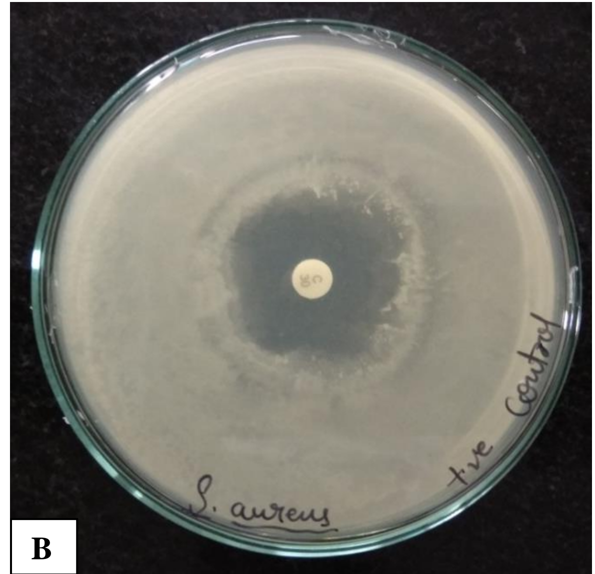


PLATE V: *E. coli* (A) negative control, (B) positive control and (C) bacterial plate treated with different concentrations of the plant rhizome extract.

Table 14: Antibacterial activity against *Staphylococcus aureus*

Serial no.	Conc. of the sample (mg/ml)	Zone of inhibition (mm)			Mean±SD
		1st	2nd	3rd	
1	1.25	25	19	25	23±3.46
2	2.5	27	19	27	24.33±3.62
3	3.75	29	29	27	28.33±1.15
4	5	30	30	28	29.33±1.15





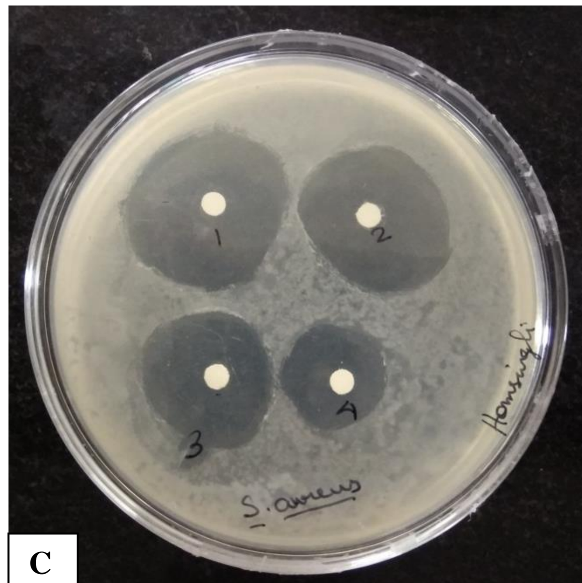
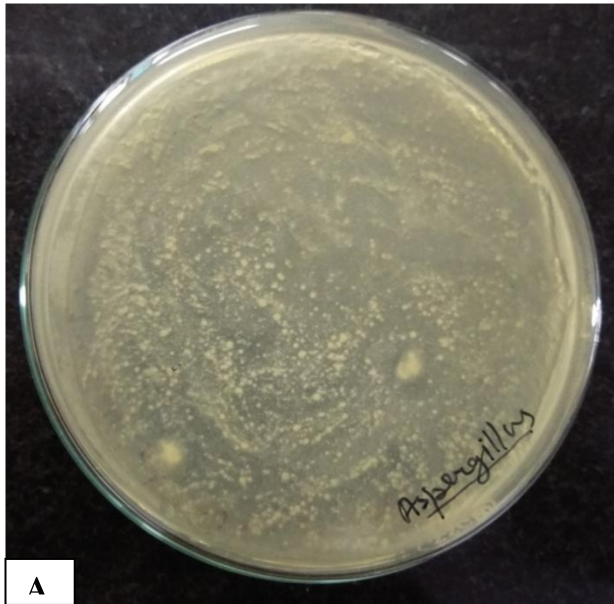


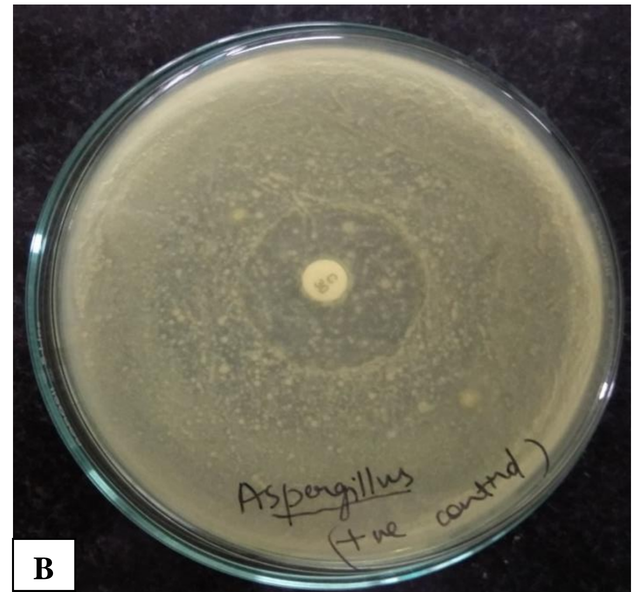
PLATE VI: *S. aureus* (A) negative control, (B) positive control and (C) bacterial plate treated with different concentrations of the plant rhizome extract.

2. **Antifungal activity**

Table 15: Antifungal activity against *Aspergillus niger*

Serial no.	Conc. of the sample (mg/ml)	Zone of inhibition (mm)			Mean±SD
		1st	2nd	3rd	
1	1.25	25	20	22	22.33±2.52
2	2.5	30	25	25	26.67±2.89
3	3.75	32	25	28	28.33±3.51
4	5	30	26	30	28.67±2.31





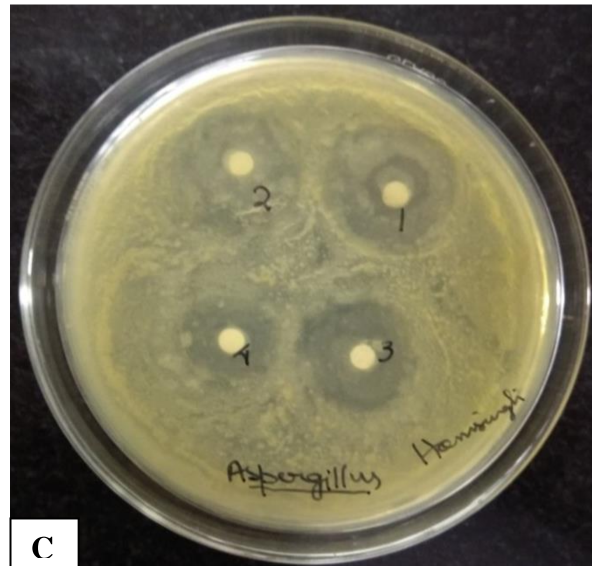
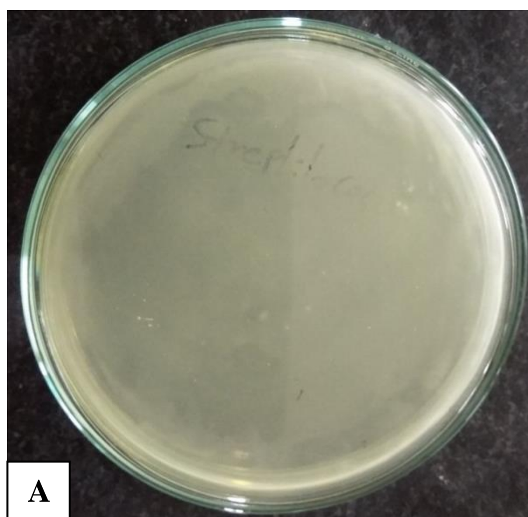
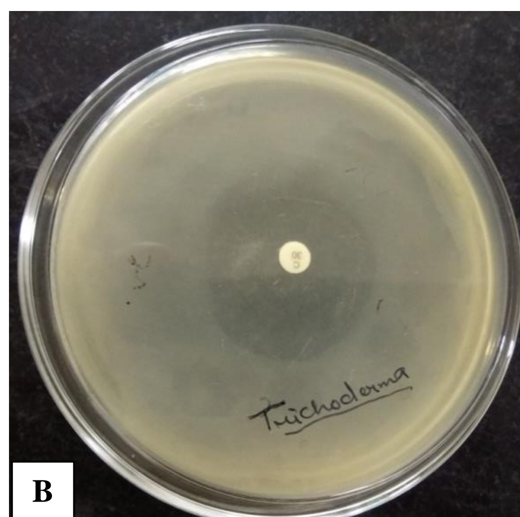


PLATE VII: *A. niger* (A) negative control, (B) positive control and (C) fungal plate treated with different concentrations of the plant rhizome extract.

Table 16: Antifungal activity against *Trichoderma reesei*

Serial no.	Conc. of the sample (mg/ml)	Zone of inhibition (mm)			Mean±SD
		1st	2nd	3rd	
1	1.25	20	28	25	24.33±4.04
2	2.5	24	29	27	26.67±2.52
3	3.75	27	29	29	28.33±1.15
4	5	28	30	30	29.33±1.15





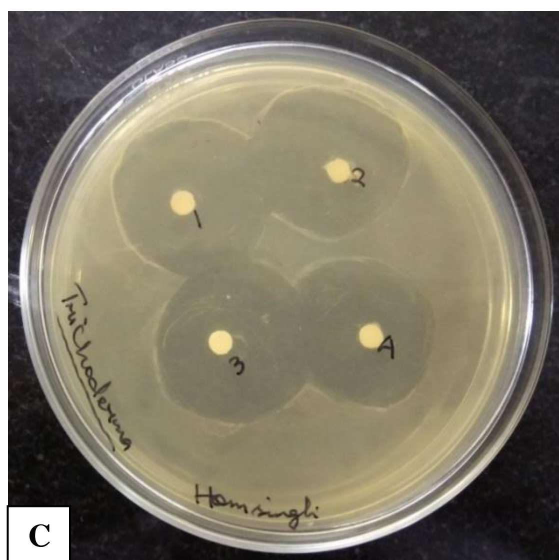


PLATE VIII: *T. reesei* (A) negative control, (B) positive control and (C) fungal plate treated with different concentrations of the plant rhizome extract.

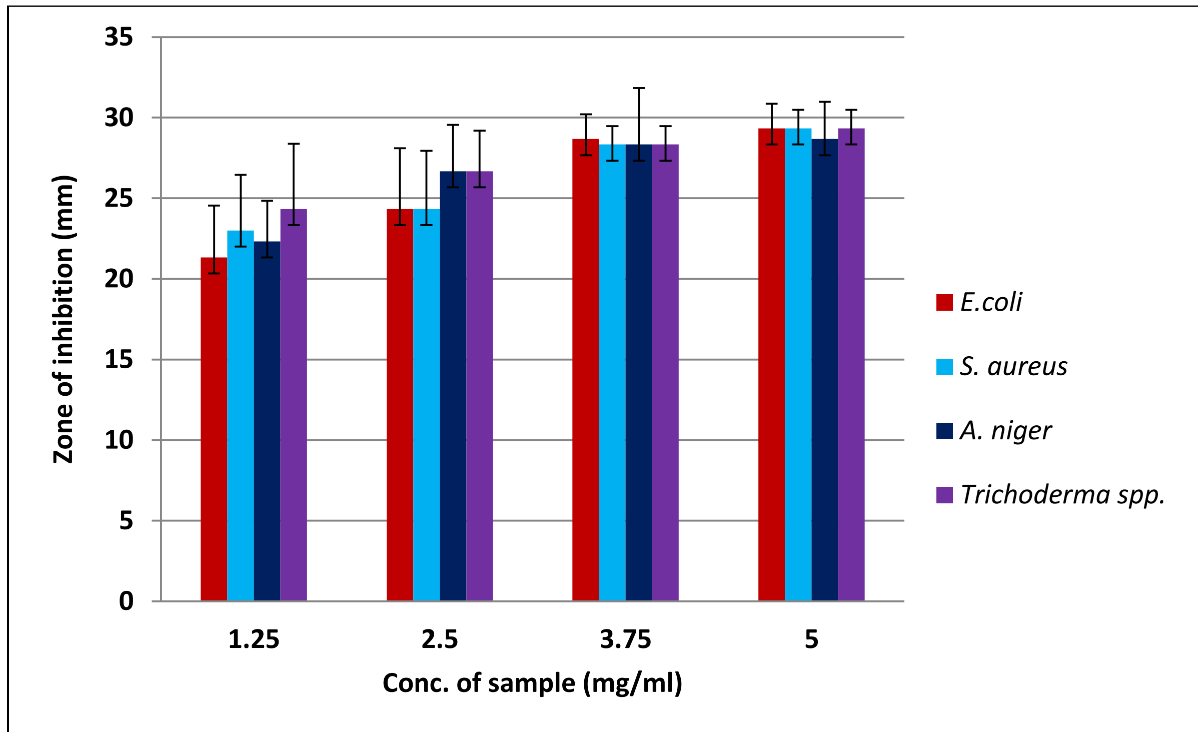


Fig 5: Antimicrobial activity of test sample on different bacterial and fungal strains

Determination of relative percentage inhibition

Methanol extract of rhizome showed the maximum relative percentage inhibition against *A. niger* (97.74%) followed by *S. aureus* (95.58%), *E. coli* (95.58%) and *T. reesei* (74.41%), respectively.

Table 17: Relative percentage inhibition of the test organisms

Conc. of sample (mg/ml)	Relative percentage inhibition (%)			
	E. coli	*S. aureus*	*A. niger*	*T. reesei*
1.25	50.55	58.78	59.29	51.21
2.5	65.77	65.77	84.58	61.53
3.75	91.33	89.18	95.43	69.42
5	95.58	95.58	97.74	74.41

The present results reveal that the relative percentage inhibitions of all the test organisms are dependent on the concentration of rhizome extract.

Summary and conclusion

Paris polyphylla Sm. is one of the medicinal plants listed as vulnerable by the IUCN and CAMP (Anonymous, 2001). Seeds have reduced viability in situ and in vitro (Madhu et al., 2010). People unaware of this condition results in random collection of plants, uprooting the rhizomes which causes tremendous loss of *P. polyphylla* (Jamir et al., 2015). Unsustainable extraction of the species owing to its high commercial demand has led to decline in populations and becoming endangered in its natural habitat. The species is facing tremendous pressure because of over exploitation due to its high market demand. Anthropogenic activities like road construction, shifting agriculture, forest resource collection, logging, etc. are affecting habitat and wild populations of the species. Heavy rainfall leading to the land erosion/landslides, etc. is also causing the habitat loss and population depletion of the species. Grazing is also one of the factors for loss of habitat/population of the species (Paul et al., 2015). The plant is declining continuously at an alarming rate by human interference such as unsustainable harvesting (over- and premature-collection), unscientific use, illegal/cross-border trade of rhizomes, habitat destruction, forest-fire, and soil-erosion. Recent reports have shown its cross border trade from NE Indian states to Burma and China. So, present plant need conservation strategies for protecting it undergo extinction.

Extensive studies and researches conducted on the different efficacies of this plant, yet much potential are yet to be exploited. Since the seed viability of the plant is very low, almost nil, the tissue culture studies should be taken for this medicinal herb. Tissue culture of this plant was done using different explants. This wonder herb requires further studies to evaluate its properties.

The plant rhizomes have good antioxidant properties and also show potent source for phenolics and flavonoids.

The rhizomes of the plant *Paris polyphylla* has shown very good antibacterial and antifungal activities. The study showed a potential to be carry out further for the analysis and assessment of capabilities present in *Paris polyphylla* for production of new antibiotics and metabolites.

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