Comparative in-vitrostudies for Leaf extract of Barleria prionitis (Procupine flower).

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ABSTRACT—Barleria prionitis(Procupine flower) has extensive medicinal properties and has long historyin the health care system of tropical countries. The plant is known in traditional health care systems. B.priontis is commonly known as “Vajradanti”. Little research has been carried out regarding its pharmacological importance. Present study is based on the Qualitative analysis of different phytochemical, Quantitative estimation of Flavonoids, Phenolics and Tannin, In-vitro analysis such as antimicrobial, antioxidant properties and anti-inflammatory activity, green synthesis of silver nanoparticles from the extracts and characterization and study of antimicrobial activity for extract and nanoparticle.

Keywords : Barleria prionitis(Procupine flower), Qualitative analysis, Quantitative estimation, Green synthesis, In-vitro analysis, AgNP.

I. INTRODUCTION

Plants are rich in variety of biochemical molecules that have various pharmacological aspects that helps in treatment of diseases (Balandrinet al., 1993).Barleria prionitisLinn. occupy a significant place in the Ayurvedic medicine in India. Detailed information on its traditional

uses, phytochemistry, pharmacology and toxicity of the extracts of different parts might be added value in the scientific evaluation of medicinal use of this plant. Barleria prionitis Linn. (Family: Acanthaceae) is a well-known perennial, Ayurvedic herb distributed in the tropical Asia, Africa and Yemen. Traditionally, the whole plant or its specific parts (leaf, stem, root, bark and flower) has been utilized for treatment of toothache, catarrhal affections, whooping cough, inflammations, glandular swellings, urinary infection, jaundice, fever, gastrointestinal disorders and as diuretic and tonic. Extensive literature survey revealed the promising pharmacological includes antimicrobial, anthelmintic, antifertility, antioxidant, antidiabetic, anti-inflammatory, anti-arthritic, cytoprotective, hepatoprotective, anti-diarrhoeal, enzyme inhibitory, diuretic and anti-nociceptive activities of the extract and isolated molecules of this plant without any toxic effects. In future study, the conversion of these pharmacological activities in to the modern drugs, proper scientific evaluation includes isolation of responsible phytochemicals, their mechanism of actions, toxicity and proper standardization need to be explored (Banerjee et al., 2012).

Endophytes are an endosymbiotic group of microorganisms that colonize in plants and microbes that can be readily isolated from any microbial or plant growth medium. They act as reservoirs of novel bioactive secondary metabolites, such as alkaloids, phenolic acids, quinones, steroids, saponins, tannins, and terpenoids that serve as a potential candidate for antimicrobial, anti-insect, anticancer and many more properties. While plant sources are being extensively explored for new chemical entities for therapeutic purposes, endophytic microbes also constitute an important source for drug discovery (Alvin et al., 2014; Andreote et al., 2014). Endophytes are a poorly investigated group of microorganisms capable of synthesizing bioactive compounds that can be used to combat numerous pathogens.

MATERIAL AND METHODOLOGY

A. Extract preparation:

Leaf sample of Barleria prionitis(Procupine flower)was washed properly, dried in shade and was milled to reduce size. Hydro-alcoholic and acetone plant extract was prepared by using water:ethyl alcohol (60:40) and 100% acetone respectively. After 48hours, the mixture was filtered and the filtrate was collected as crude plant extract.

B. Endophytic fungus isolation:

Endophytic Fungus was isolated from young disease free leaves by Water agar (16% Bacteriological grade Agar Agar) media and the metabolites were isolated by culturing the endophytic fungus in Potato dextrose broth (pH-5.9, 30°C ,70 r.p.m. / min) and after

incubation for about 10 days, the extract was extracted out using ethyl acetate (organic top layer), then dried using Vacumn evaporator(Schulz B. et al., 1993)

C. Dessication of extract and Reconstitution:

The hydro-alcoholic extract and acetone extract leaf and esterified extract of endophytic fungus were reconstituted to final concentration of 1mg/ml by dissolving dried extract with Dimethyl Sulphoxide (DMSO).

D. Qualitative screening of Phytochemicals:

The presence of alkaloids, flavonoids, glycosides, carbohydrate, saponins, tannins and terpenoids can tested qualitatively using the standard procedures to identifythe constituents.Every test is done in triplicates. (Kokateet. al.,1995)

E. Quantitative estimation of Phytochemicals:

1) Quantitative analysis:

The quantitative analysis was carried out for Alkaloid content (Fazel Shamsa et al., 2008), Flavonoid (Kumaran et.al.,2006), Phenolic content (Hagerman A. et.al.,2000), and Tannin content (Marinova et. al., 2005).

2) Calibration Curve:

Total flavonoids, Total Alkaloid, Total phenolics, Total Tannin were estimated from Quercetin standard calibration curve, Atropine standard calibration curve, gallic acid standard calibration curve, gallic acid standard calibration curve respectively. The quantitative estimation were expressed as µg/mgextract equivalent of Quercetin, µg/mg extract equivalent of Atropine, µg/mg extract equivalent of gallic acid, µg/mg extract equivalent of gallic acid respectively.

F. Invitro studies

1) Anti-microbial Study

The antibacterial activity of the different extracts was determined in accordance with agar-well diffusion method described by Rious et. al.,1988).

2) Anti-oxidant-DPPH Radical Scavenging Assay

The antioxidant activity was measured in terms of radical scavenging (stable radical DPPH) ability of plant leaf extracts. The DPPH radical scavenging ability of leaf extracts was carried out according to the method described by Blois et al., 1958 with a slight modification (Gardeli et. al., 2008).

3) Anti-oxidant-Phophomolybdate Assay

The total antioxidant capacity of leaf extracts was determined by phosphomolybdate assay (Umamaheswari et. al., 2008).

G. FT-IR analysis

FTIR analysis was carried out to determine functional groups of phytocompounds present in the leafextracts of the plant. FTIR analysis was measured at room temperature using the KBr pellet technique in the range of 400-4000 cm^{-1}.

H. HPLC analysis-Qualitative

The qualitative compound profiling was carried out by Normal High Performance Chromatographic (Normal HPLC) procedure to find out different compounds resolute into constituent fraction having different retention time and peak area.

I. Green Synthesis of Silver Nano Particles

Aqueous solution of Silver nitrate solution was prepared. Aqueous leaf extract (1 part) mixed with of 1mMaqueous silver nitrate solution (9 part). The mixture was heated in hot water bath for 30minutes-45minutes at 70°C-80°C.

1) Analysis of Nanoparticle formation:

Change in coloration of the mixture into yellowish brown or reddish brown confirms production of silver nanoparticles.

2) Purification of Silver nanoparticle

The mixture was then centrifuged at 6000rpm for 1hour at 4°C.After centrifugation the supernatant was discarded. The pellet was again washed with distilled water to remove any contaminants or plant material. Again it is centrifuged at 6000 rpm for 30 minutes.The pellet was collected and the supernatant was discarded.

3) Confirmation of Silver nanoparticle formation by Spectral analysis

Absorbance Peaks were observed in the spectral analysis from 420nm-450nm that corresponds to the formation of Silver nanoparticles.

J. In-vitro studies

In-vitro studies (Hydroalcoholic Extract + Ag-NP): Antimicrobial activity study (well diffusion method), Anti-oxidant activity study (DPPH assay, phosphomolybdate assay), Anti-inflammatory activity (albumin denaturation assay).

II. RESULTS

A. Qualitative analysis

i. Qualitative analysis of phyto-constituents revealed presence of Alkaloids, Flavonoids, Phenols, Tannins, Terpenoids, Saponins, Steroids, Glycosides, Carbohydrates with respect to the hydro-alcoholic and acetone extract.

ii. The esterified extract of endophytic fungus isolated from leaf contain Flavonoids, Phenols, Tannins, Saponins, Steroids, Carbohydrates, Glycosides and Amino acids.

B. Quantitative Estimation of phytochemicals

Quantitative estimation for Flavonoid (µg Quercetin QE/mg extract) (Aluminium chloride method), Phenol (µg Gallic acid GA/mg extract) (Folin-Ciocalteu method) and Tannin (modified Folin-Ciocalteu method) was analysed by colorimetric method using standard calibration curve of Quercetin(QE), Gallic acid(GA) and Tannic acid(TA) respectively.

The Total Flavonoid content, Total Phenolic content and Total Tannin content for Hydroalcoholic, Acetone leaf extract and Esterified extract from endophytic fungus were 18.18±0.05µgQE/mg extract, 13.36±0.08µgQE/mg extract, 5.89±0.60µgQE/mg extract respectively; 13.48±0.01 µgGA/mg extract, 08.29±0.01µgGA/mg extractand 01.98±0.01µgGA/mg extract respectively; 2.87±0.03µgTA/mg extract, 1.19±0.69µgTA/mg extract and 0.68±0.33µgTA/mg extract respectively.

C. FT-IR Spectral analysis- Identification of functional group

FTIR analysis of Hydro-alcoholic leaf extract of Barleria prionitisshows characteristic absorption bands at 3748 cm-1 for hydroxyl (-OH) group, 3447 cm-1 for an amine (-NH) group, the band at 2924 cm-1 is due to C-H stretching. Absorption band at 1738 cm-1 confirms the presence of carbonyl group (C=O) (Figure 1), (Table 1).

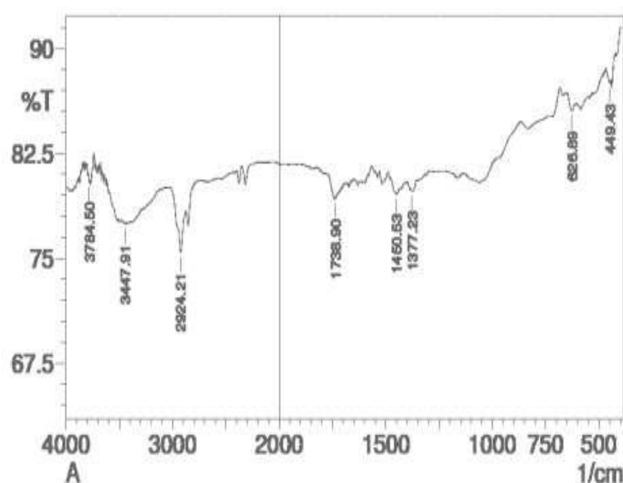

Figure 1: FT-IR spectrum

Table 1: FT-IR Spectrum functional group characterization

Wave number (cm-1)	**Functional group**
3784.50	-O-H group
3447.91	-N-H group
2924.21	C-H stretching
1738.90	C=O carbonyl group
1450.93	C-H bending
1377.23	C-H bending
626.89	C-O stretching
449.43	C-O stretching

D. HPLC Analysis-Qualitative study

Normal phase HPLC for the extract was done with isocratic mobile phase system where 24 different peaks at different Retention time (Rt) were determined from the chromatogram (Figure 2).

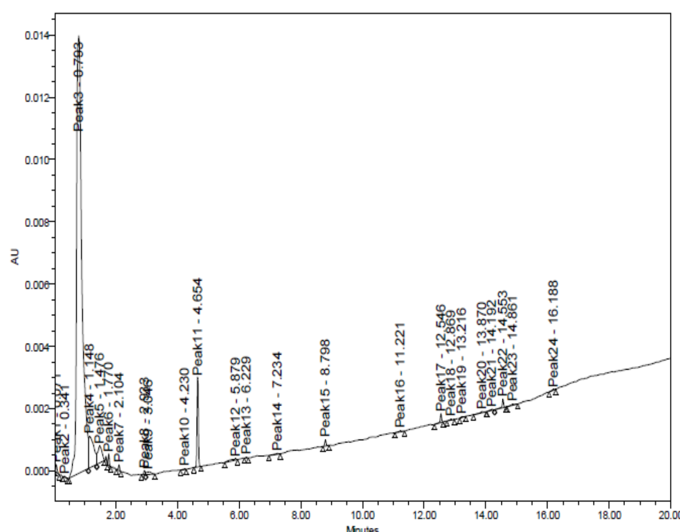

Figure 2: HPLC chromatogram

E. Green Synthesis of Silver Nano particles

The hydro-alcoholic plant extract had the capacity for green synthesis of nontoxic silver nanoparticles (Ag-NP) from Silver nitrate (toxic Ag+) which has been through the catalytic effect of the metabolites or enzymes present in the extract (Figure 3).

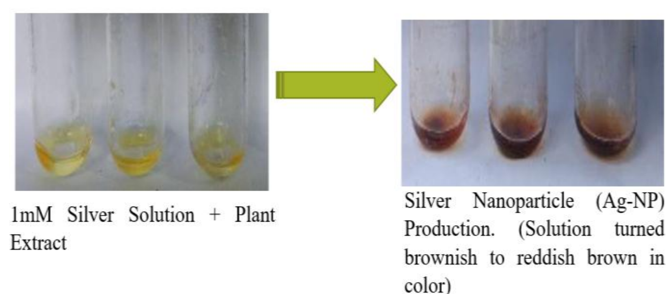

Figure 3: Screening for green synthesis of Silver nanoparticles. Hydro-alcoholic extract gave positive for potential to synthesize silver nano-particle

F. Characterization of Silver nano-particles (AgNP) Production

The Ag-NP was purified by continuous centrifugation, collecting and washing pellet that contained Ag-NP. The Absorbance Peaks were observed in the spectral analysis between 420nm-450nm that corresponds to the formation of Silver nanoparticles.

G. Morphology study by Transmission Electron Microscope

i. The Morphology of the Silver nano-particles are highly variable.

ii. The assemblies were found to be aggregated of Silver nano-particle (Ag-NP) in the range 25nm-70nm. (Figure 4)

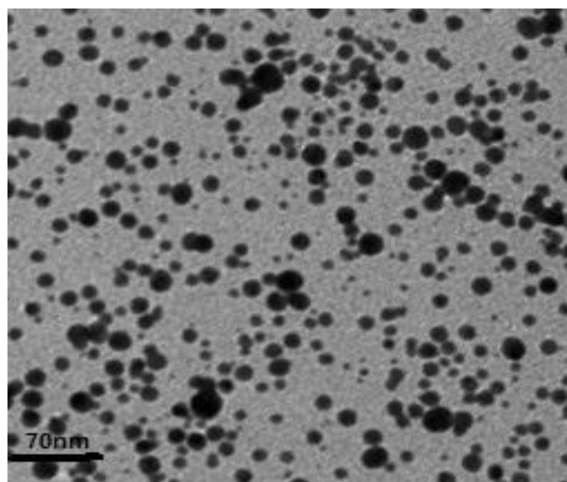

Figure 4: Assemblies for aggregated of Synthesized Silver nano-particle (Ag-NP).

H. In-vitro assay:

1) Antimicrobial assay:

i. The Hydro-alcoholic extract and acetone extract have antimicrobial activity against pathogens such as Escherichia coli, Staphylococcus, Pseudomonas, A.niger, A. Flavus, Candida sp.
ii. No significant antimicrobial activity was found in esterified extract from endophytic fungus.
iii. Studies for Hydroalcoholic Extract + Ag-NPalso revealed increase in antimicrobial activity (increase in zone of inhibition) for E.coli, Pseudomonas, Salmonella, A.niger and A.Flavus and gain of antimicrobial activity against Staphylococcus sp. and Candida albicans.

2) **Anti-oxidant assay-DPPH assay**

i. The percentage of inhibition by DPPH assay for standard ascorbic acid was 87.56±0.17%.
ii. The percentage of inhibition for hydro-alcoholic extract and Acetone extract of leaf sample and esterified extract of endophytic fungus found 44.33%±0.18%, 57.49%±0.06% and 14.03±0.03% respectively.
iii. The antioxidant property of purified Silver nanoparticles was 04.63%±0.58% whereas for Hydroalcoholic Extract + Ag-NPwas found 64.89%±0.26%.
iv. There is 12.5% (approx.) increase in activity of Hydroalcoholic Extract + Ag-NPas compared to Hydroalcoholic Extract alone.

3) **Anti-oxidant assay-Phosphomolybdate assay**

i. The percentage of inhibition by Phosphomolybdate assay for standard ascorbic acid was 49.26%±0.85%.
ii. The percentage of inhibition for hydro-alcoholic extract and Acetone extract of leaf sample and esterified extract of endophytic fungus found 34.47%±0.20%, 23.06%±0.87%, and 00.00±0.00% (didn't determined) respectively.
iii. The antioxidant property of purified Silver nanoparticles was 03.93%±0.33% whereas for Hydroalcoholic Extract + AgNP was found 38.25%±0.23%.
iv. There is 11.0% (approx.) increase in activity of Hydroalcoholic Extract + AgNP as compared to Hydroalcoholic Extract alone.

4) **Anti-inflammatory- Albumin denaturation assay**

i. Anti-inflammatory activity was studied using Indomethacin as standard. The anti-inflammatory activity for standard was 78.34%±0.95%.

ii. The anti-inflammatory activity for hydro-alcoholic extract and Acetone extract of Leaf sample and esterified extract of endophytic fungus were found to be 61.65%±0.20%, 42.85%±0.89%, and 28.65±0.45% respectively.
iii. The anti-inflammatory property of purified Silver nanoparticles was 18.63%±0.58% whereas for Hydroalcoholic Extract + Ag-NP was found 66.99%±0.75%.
iv. There is 8.5% (approx.) increase in activity of Hydroalcoholic Extract + Ag-NP as compared to Hydroalcoholic Extract alone.

III. FUTURE STUDIES:

Further studies will enable identification of compounds from hydro-alcoholic extracts) by Mass Spectroscopic method.

These identified compounds along with commercially available API (standards) are to be studied and determined for their anti-inflammatory activity (Docking studies) against molecular targets such as COX-2 (PDB ID: 4COX) and IL1β (PDBID: 1T4Q), IL6 (PDBID: 19PM), TNFα (PDBID: 2AZ5), that plays the crucial role in inflammations whose 3D structures were retrieved from RCSB-PDB database (Berman et al 2000).

Ligand generation (Ligand sketch) will be carried out using some standalone software or online ligand generation tools available.

Flexible docking of ligands will be carried out by using AutoDock Vina tool (Trott and Olson, 2010). The comparative docking interactions shall be studied using visualization software available.

IV. CONCLUSION:

Exploring new prospect in extraction, purification, analysis and down-stream process can give new insight to new drug chemistry and pharmacological aspects.

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