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BIO-THERAPEUTIC AND NANO-BIOLOGICAL STUDIES FOR SOLVENT EXTRACT AND ENDOPHYTIC FUNGUS FROM LEAF SAMPLE OF BHUMI AMLA (*PHYLLANTHUS NIRURI*)

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ABSTRACT

Phyllanthus niruri (Bhumi amla) has been extensively used in medicinal and culinary activities in the Indian subcontinent. This plant has been little explored for their insight to the medicinal value applications. Studies need to be carried out in regards to its pharmacological importance. Present study is based on the qualitative analysis of different phytochemicals, quantitative estimation of Alkaloid, Flavonoids, Phenolics and Tannin that are mostly pharmacologically important plant-chemicals. In-vitro analysis such as antimicrobial, antioxidant, anti-inflammatory activity has been studied for their potential candidate as future drug. Analytical study for presence of different functional groups in extract was done by Fourier Transform-InfraRed (FT-IR) spectroscopy. Normal Phase High Performance Liquid Chromatography (HPLC) analysis using isocratic mobile phase revealed presence of 24 different compounds. Screening of potential for Green synthesis of silver nanoparticles from the extracts was studied. Purification of synthesized silver nano-particles (AgNP) was done and characterization of synthesized AgNP was done by Spectral analysis. Morphology study of synthesized AgNP was done by Transmission Electron microscopy (TEM) that shows aggregates that range between 25nm-70nm. In-vitro study (antimicrobial activity, anti-oxidant and anti-inflammatory) for extract + AgNP nanoparticle was studied along with the synthesized AgNP.

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1. INTRODUCTION

Phyllanthus are one of the important ethnobotanical utilized plants that have been used from old times. The extracts of *Phyllanthus amarus* used for antimicrobial, anti-oxidant and anti-inflammatory activity (Mao *et al.*, 2016; Devi *et al.*, 2016; Devi & Kumar 2017). Extensive reports on *Phyllanthus niruri* L. for their ethnobotanical prospect has been extensively reported in folk and traditional medicine systems to cure/treat various diseases including diabetes, joint pain, constipation, asthma, loss of appetite, conjunctivitis, gout disease, gonorrhoea in males and females, inflammation, skin ailments, liver disorders, kidney related diseases, female genital related diseases such as vaginitis, menorrhagia, irregularities in menstrual cycles, urogenital disorders, various bacterial and fungal diseases, viral infections and many more (Kaur *et al.*, 2016)

2. MATERIALS AND METHODS

2.1. Extract preparation

Leaf samples of Phyllanthus niruri (Bhumi amla) were washed properly, dried in shade and were milled to reduce size. Hydro-alcoholic and acetone plant extract was prepared by using Water:Ethyl alcohol (60:40) and 100% acetone respectively. After 48 hours, the mixture was filtered and the filtrate was collected as crude plant extract (Harborne, 1998).

2.2. Isolation of Endophytic fungus from leaf sample

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). The fermentation broth media after incubation was taken in a separation funnel and equal volume of ethyl acetate was added. The stop cock was fitted and shaken well for 10 minutes. Afterwards the separating funnel was kept in the stand until two distinctive layers of water and ethyl acetate were formed. The top organic layer (Ethyl acetate) layer was collected. The organic layer was reduced by using Rotatory/Vacuum evaporator and reconstituted by Dimethyl Sulphoxide (DMSO) and stored in amber colored bottle for further analysis (Shrestha *et al.*, 2001; Crozier *et al.*, 2006; Huang *et al.*, 2001).

2.3. Dessication of extract and Reconstitution

The extract was then dried using Vacuum evaporator (Xevgenos *et al.*, 2015). 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).

2.4. Qualitative screening of Phytochemicals

The presence of alkaloids, flavonoids, glycosides, carbohydrate, saponins, tannins and terpenoids can be tested qualitatively using the standard procedures to identify the presence or absence of the phyto-constituents. Every test is done in triplicates (Kokate *et al.*, 1995).

2.5. Quantitative estimation of Phytochemicals

2.5.1. Quantitative analysis

The quantitative analysis was carried out for Alkaloid content (Shamsa *et al.*, 2008), Flavonoid (Ramos *et al.*, 2017), Phenolic content (Amarowich *et al.*, 2009), and Tannin content (Polshettiwar *et al.*, 2007).

2.5.2. Standard Calibration Curve

Total Alkaloid, Total Flavonoids, Total Phenolics and Total Tannin content were estimated from Atropine standard calibration curve, Quercetin

standard calibration curve, Gallic acid standard calibration curve, Tannic acid standard calibration curve respectively. The quantitative estimations were expressed as µg/mg extract equivalent of Atropine, µg/mg extract equivalent of Quercetin, µg/mg extract equivalent of Gallic acid, µg/mg extract equivalent of Tannic acid respectively.

2.6. *In Vitro* studies

2.6.1. Anti-microbial Study- Well diffusion method

The antibacterial activity of the different extracts was determined in accordance with agar-well diffusion method (Balouiri *et al.*, 2016; Khond *et al.*, 2009).

2.6.2. Antioxidant-DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) Radical Scavenging Assay

The antioxidant activity was measured in terms of radical scavenging (stable radical DPPH) ability of plant leaf extracts. The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radical scavenging ability of leaf extracts was carried out according to the method described by Blois *et al.*, 1958 with a slight modification (Brand-Williams *et al.*, 1995).

2.6.3. Antioxidant-Phophomolybdate Assay

The total antioxidant capacity of leaf extracts was determined by Phosphomolybdate assay (Bursal *et al.*, 2011; Kumaran *et al.*, 2006).

2.6.4. Anti-inflammatory assay- Albumin Denaturation assay

I*n vitro* anti-inflammatory activity of *Phyllanthus niruri* (Bhumi amla) leaf extracts was determined by using inhibition of albumin denaturation assay, carried out according to the method described by Mizushima *et al.*, 1968 and Sakat *et al.*, 2010.

2.7. FT-IR analysis for determination of functional group

FT-IR analysis was carried out to determine functional groups of phyto-compounds present in the leaf extracts of the plant. FTIR analysis was measured at room temperature using the KBr pellet technique in the range of 400-4000 cm-1 (Berthomieu & Hienerwadel, 2009).

2.8. HPLC analysis-Qualitative

The qualitative compound profiling was carried out by Normal High Performance Chromatographic (Normal HPLC) procedure to find out different compounds resolved into constituent fractions having different retention time and peak area (Malviya *et al.*, 2010).

2.9. Green Synthesis of Silver Nano Particles

Aqueous Silver nitrate solution was prepared. Aqueous leaf extract (1 part) mixed with 1mM aqueous silver nitrate solution (9 part). The mixture was heated in a hot water bath for 30-45minutes at 70˚C-80˚C (Veisi *et al.*, 2018, Saratale *et al.*, 2018, Gnanadesigan *et al.*, 2011).

2.9.1. Analysis of Nanoparticle formation

Change in coloration of the mixture into yellowish brown or reddish brown confirms production of silver nanoparticles (Saratale *et al.*, 2018; Gnanadesigan *et al.*, 2011).

2.9.2. 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 (Marco *et al.*, 2019; Hurst, 2020).

2.9.3. 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 (Falcone et

al., 2019; Menges, 2017).

2.10. *In-vitro* studies for (Hydroalcoholic Extract + Ag-NP)

In-vitro studies (Hydroalcoholic Extract + Ag-NP): Antimicrobial activity study (well diffusion method) (Balouiri *et al.*, 2016; Khond *et al.*, 2009), Anti-oxidant activity study by DPPH assay (Brand-Williams *et al.*, 1995) and Phosphomolybdate assay (Bursal *et al.*, 2011; Kumaran *et al.*, 2006), Anti-inflammatory activity (albumin denaturation assay) (Mizushima *et al.*, 1968; Sakat *et al.*, 2010).

3. RESULTS

3.1. Qualitative analysis for Phyto-constituents in leaf extract and extract from endophytic fungus

Overall Qualitative analysis of phyto-constituents in Leaf extracts revealed presence of Alkaloids, Flavonoids, Phenols, Tannins, Terpenoids, Saponins, Steroids, Glycosides, Carbohydrates with respect to the hydro-alcoholic and acetone extract. The esterified extract (dissolved in DMSO) of endophytic fungus isolated from leaf contain Flavonoids, Phenols, Tannins, Saponins, Steroids, Carbohydrates, Glycosides and Amino acids.

3.2. Quantitative Estimation of phyto-chemicals

Quantitative estimation for Alkaloid (µg Atropine A/mg extract), 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 Atropine (A) Quercetin(QE), Gallic acid(GA) and Tannic acid(TA) respectively (Table 1).

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

FTIR analysis of Hydro-alcoholic leaf extract of Phyllanthus niruri shows 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 2).

3.4. HPLC Analysis-Qualitative study

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

Table 1. Quantitative estimation of total alkaloid, total flavonoid, total phenolic and total tannin content in different Leaf extract and esterified extract of Endophytic Fungus.

QUANTITATIVE ESTIMATION	UNIT	HYDRO-ALCOHOLIC LEAF EXTRACT	ACETONE LEAF EXTRACT	ESTERIFIED EXTRACT ENDOPHYTIC FUNGUS
Total Alkaloid content	µgA/mg extract	02.18±0.15	01.12±0.28	00.91±0.69
Total Flavonoid content	µgQE/mg extract	16.18±0.05	12.36±0.08	05.29±0.60
Total Phenolic content	µgGA/mg extract	12.45±0.01	06.49±0.01	02.98±0.01
Total Tannin content	µgTA/mg extract	01.14±0.29	2.37±0.07	00.58±0.37

Acetonitrile:Water:Formic acid (55:43:2) and the stationary phase selected was C18 column with column temperature at 25°C. The flow rate was 1.5ml/min and Detector λmax at 230nm.

Figure 1. FT-IR spectrum

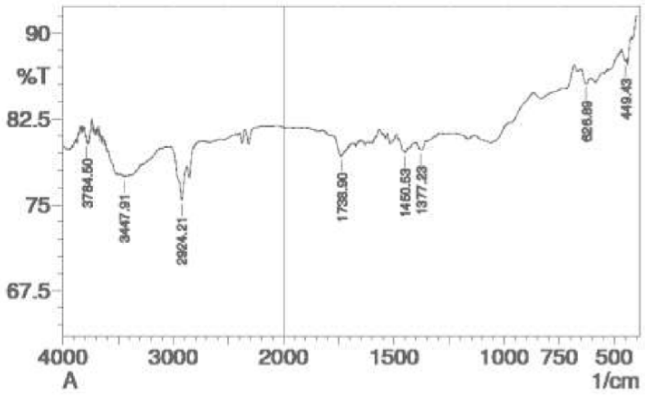


Table 2. FT-IR Spectrum functional group characterization

Wave number (1/cm)	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

3.5. 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).

3.6. Characterization of Silver nano-particles (Ag-NP) Production

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

3.7. Morphology study by Transmission Electron Microscope

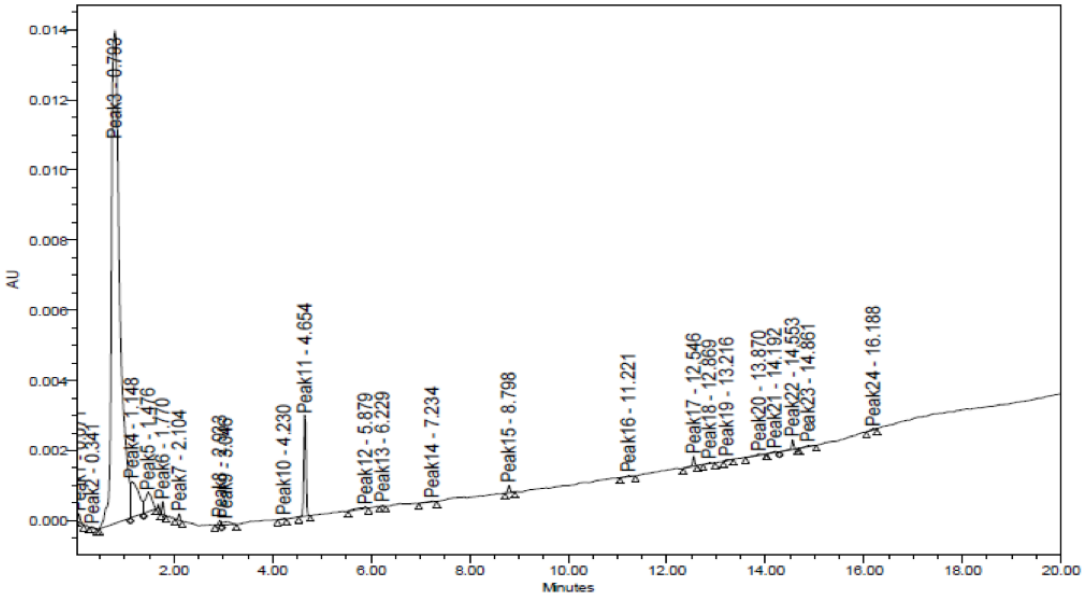
The Morphology of the Silver nano-particles are highly variable. The assemblies were found to be aggregated of Silver nano-particle (Ag-NP) in the range 25nm-70nm (Figure 4).

3.8. *In-vitro* assay

3.8.1. Antimicrobial assay:

The Hydro-alcoholic extract and acetone extract have antimicrobial activity against pathogens

Figure 2. HPLC chromatogram



such as *Escherichia coli*, *Staphylococcus*, *Pseudomonas*, *Aspergillus niger*, *Apergillus flavus*, *Candida* sp. No significant antimicrobial activity was found in esterified extract from endophytic fungus. Studies for Hydroalcoholic Extract + Ag-NP also revealed increase in antimicrobial activity (increase in zone of inhibition) for *Escherichia coli*, *Pseudomonas*, *Salmonella*, *Aspergillus niger*, *Apergillus flavus* and gain of antimicrobial activity against *Staphylococcus* sp. and *Candida albicans*.

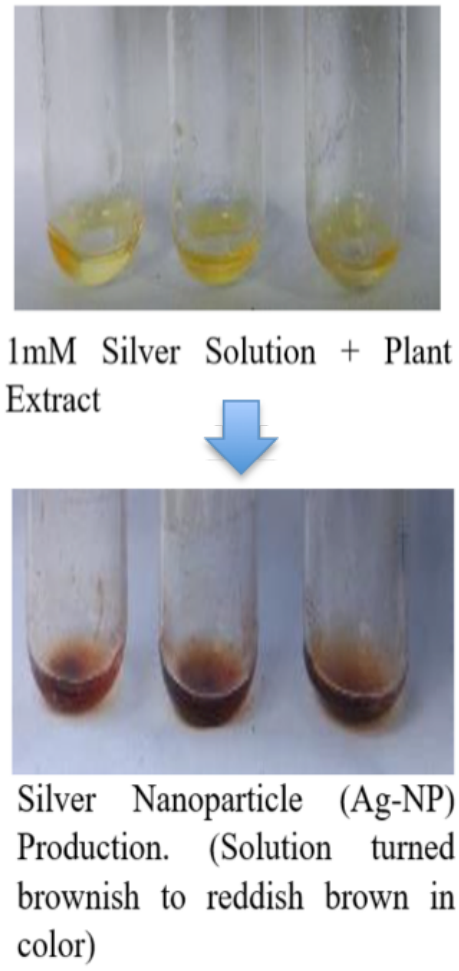


Figure 3. Screening for green synthesis of Silver nanoparticles. Hydro-alcoholic extract gave positive for potential to synthesize silver nano-particle

3.8.2. Anti-oxidant assay-DPPH assay

The percentage of inhibition by DPPH assay for standard ascorbic acid was 87.56%±0.17%. 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. The antioxidant property of purified Silver nanoparticles was 04.63%±0.58% whereas for Hydroalcoholic Extract + Ag-NP was found 64.89%±0.26% (Table 3). There is 12.5% (approx.) increase in activity of Hydroalcoholic Extract + Ag-NP as compared to Hydroalcoholic Extract alone.

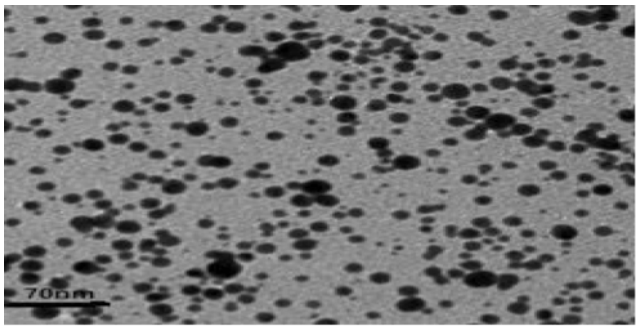


Figure 4. Assemblies for aggregates of Synthesized Silver nano-particles (Ag-NP).

Table 3. Anti-oxidant assay of Sample extracts, esterified extract (endophytic fungus), AgNP and Extract+AgNP

Sl. No.	Sample	Percentage of inhibition (DPPH assay)
1	Ascorbic acid (Standard)	87.56%±0.17%
2	Hydro alcoholic Leaf extract	44.33%±0.18%
3	Acetone Leaf extract	57.49%±0.06%
4	Esterified extract of endophytic fungus	14.03%±0.03%
5	Purified Silver nanoparticles (AgNP)	04.63%±0.58%
6	Hydro alcoholic Leaf Extract + Ag-NP	64.89%±0.26%

3.8.3. Anti-oxidant assay- Phosphomolybdate assay

The percentage of inhibition by Phosphomolybdate assay for standard ascorbic acid was 49.26%±0.85%. 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. The antioxidant property of purified Silver nanoparticles was 03.93%±0.33% whereas for Hydroalcoholic Extract + AgNP was found 38.25%±0.23% (Table 4). There is 11.0% (approx.) increase in activity of Hydroalcoholic Extract + AgNP as compared to Hydroalcoholic Extract alone.

Table 4. Anti-oxidant assay of Sample extracts, esterified extract (endophytic fungus), AgNP and Extract+AgNP

Sl. No.	Sample	Percentage of inhibition (Phosphomolybdate assay)
1	Ascorbic acid (Standard)	49.26%±0.85%
2	Hydro alcoholic Leaf extract	34.47%±0.20%
3	Acetone Leaf extract	23.06%±0.87%
4	Esterified extract of endophytic fungus	00.00%±0.00%
5	Purified Silver nanoparticles (AgNP)	03.93%±0.33%
6	Hydro alcoholic Leaf Extract + Ag-NP	38.25%±0.23%

3.8.4. Anti-inflammatory- Albumin denaturation assay

Anti-inflammatory activity was studied using Indomethacin as standard. The anti-inflammatory activity for standard was 78.34% ±0.95%. 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. The anti-inflammatory property of purified Silver nono-particles was 18.63%±0.58% whereas for Hydroalcoholic Extract + Ag-NP was found 66.99%±0.75% (Table 5). There is 8.5% (approx.) increase in activity of Hydroalcoholic Extract + Ag-NP as compared to Hydroalcoholic Extract alone.

Table 5. Anti-inflammatory assay of Sample extracts, esterified extract (endophytic fungus), AgNP and Extract+AgNP

Sl. No.	Sample	Percentage of inhibition (Albumin denaturation assay)
1	Indomethacin (Standard)	78.34%±0.95%
2	Hydro alcoholic Leaf extract	61.65%±0.20%
3	Acetone Leaf extract	42.85%±0.89%
4	Esterified extract of endophytic fungus	28.65%±0.45%
5	Purified Silver nanoparticles (AgNP)	18.63%±0.58%
6	Hydro alcoholic Leaf Extract + Ag-NP	66.99%±0.75%

4. DISCUSSION

Advances in the field of nano-science, nanotechnology and nanomaterial have immense importance in the medical, biomedical and pharmaceutical sciences (Zhang *et al.*, 2016). The approach for the green synthesis of silver nano-particles using plant extracts can be a better alternative as these methods are simple, eco-friendly, non-toxic and bio-compactable methods (Sapsford *et al.*, 2011) as compared to physical and chemical methods that are expensive and hazardous (Gurunathan *et al.*, 2015). However irrespective of various biological methods available for the green synthesis, plant based methods for synthesis of nano-particles have proven to be a better alternative (Jadoun *et al.*, 2021) due to wide variability in the chemical compounds present in different plant extracts may be due to flavones, terpenoids, sugars,

ketones, aldehydes, carboxylic acids, and amides, along with different plant enzymes present in the extract (Prathna *et al.*, 2010). Many previous research findings have shown the potential of plant extracts and silver nano-particles in various biological and therapeutical aspects. Endophytic fungus also plays a major role in the finding potential bio-active compounds that have immense therapeutical potential (Subbulakshmi *et al.*, 2012; Turner *et al.*, 2013). Many secondary metabolites that are produced from the endophytic micro-biome are alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, saponins, tannins, terpenoids, tetralones, xanthones, and many others (Zhao *et al.*, 2011a; Zhao *et al.*, 2011b). The present study is based upon the assessment of different biotherapeutical agents present, their potential for green synthesis of silver nano-particles and also an experimental insight to the biological study for integration of extracts and synthesized silver-nano-particles.

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 & Olson, 2010). The comparative docking interactions shall be studied using visualization software available.

5. CONCLUSION

The experimental study revealed that the hydro-alcoholic leaf extract of Bhumi amla had better anti-microbial, anti-oxidant and anti-inflammatory activity than the acetone extract. The esterified extract of endophytic fungus didn't have significant anti-microbial, anti-oxidant and anti-inflammatory activity. This may be due to the presence of phytochemicals such as Alklaoids, Flavonoids, Phenolic and Tannin that have different pharmacological activities. In case of the potential of green synthesis of Silver nano-particles, the hydro-alcoholic extract showed positive for converting toxic elemental Silver (Ag) into Silver nano-particles (AgNP's). Present study also revealed that there is increase in the pharmacological activity of Extract+AgNP in comparison to the activity study of the extracts alone revealing strategies for drug efficacy and safety. Exploring new prospects in extraction, purification, analysis and down-stream process can give new insight to new drug chemistry and pharmacological aspects.

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7. FUNDING

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