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Studies on the Isolation, Identification and Antibacterial Activity of Bioactive Compound from *Andrographis paniculata* Leaf Extract

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ABSTRACT

All beings utilize plants to fulfil a wide range of requirements. Many plant-based products are being sold as herbal teas, nutritional supplements, health foods, and other goods. Traditional plant-based knowledge has become a useful resource in the hunt for new pharmacological and nutraceutical sources. According to research, Ayurveda is one of the oldest medicinal systems still in use today, both in India and across the world. Alkaloids, flavonoids, triterpenoids, and steroids are phytochemicals that are employed in the traditional Ayurvedic method. Such compounds are crucial for good health and can be used to treat a variety of conditions. Multiple chronic and infectious illnesses, including as sore throats, herpes, fever, and gastrointestinal and upper respiratory infections, have been treated with *Andrographis paniculata* in Asia from ancient times. The Indian Pharmacopoeia states that it is a key component in at least 26 Ayurvedic medicines. In this study, Andrographis paniculata leaves were extracted with ethanol using the Soxhlet method, and the resulting crude extract was then used for the qualitative identification of phytoconstituents. The findings revealed the existence of phenolic chemicals, alkaloids, flavanoids, tannins, and triterpenoids. Flavanoids were found in the ethanolic extract in places with Rf values of 0.92, 0.65, and 0.55, and an Rf value of 0.5 implies the presence of triterpenoids. The GC-MS chromatogram demonstrated the bioactive chemicals' substantial presence. Methyl salicylate, with a retention time of 3.628 (min), Diethyl Phthalate, with a retention time of 7.864, and tert-Butyl (5) isopropyl-2-methylphenoxy) dimethylsilane are the discovered chemicals (16.875 min). At 16.875 minutes, arsenic acid, tris(trimethylsilyl) ester, and tris(tert-butyldimethylsilyloxy) arsane were identified.

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Peaks for 2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene and 3-Benzo[g]quinoxalin-2-yl-propionic acid were observed at 17.244 minutes, whereas Diethyl Phthalate exhibits antibacterial, acetylcholinesterase-inhibiting, and neurotoxic characteristics. The column-purified phytocompound (diethyl phthalate) was found, and silver and gold metals were used to create nanoparticles of it. The ability of the synthesised metals (silver and gold) to prevent microbial growth was also assessed. Crude ethanolic leaf extract, *A. paniculata* leaf extract, diethyl phthalate, DPAgNPs, and DPAuNPs have demonstrated the highest anti-microbial activity against all of the target pathogenic strains among the study samples.

Keywords: *Andrographis paniculata*, Gas chromatography-Mass spectroscopy (GC-MS), Diethyl phthalate, Silver nanoparticles (AgNps) and Gold nanoparticles (AuNps).

Introduction

Plants are essential for all humans to serve a variety of desires (Phillips and Meilleur, 1998). Many plant-based products are offered for sale as herbal teas, nutritional supplements, health foods, and other goods. Distinct medical traditions are practised by various cultural and ethnic groupings (Leslie and Young., 1992). Traditional plant-based knowledge has become a useful resource in the hunt for new pharmacological and nutraceutical sources. Many individuals think that herbal treatments are safer than synthetic medications (Sharma and Mujundar, 2003). According to studies, Ayurveda is one of the earliest pharmacopoeias still in use today, both in India and across the world. Alkaloids, flavonoids, triterpenoids, and steroids are phytochemicals utilised in the traditional ayurvedic approach. These substances are important for maintaining good health and can be used to treat a variety of ailments and conditions. The Acanthaceae family includes Andrographis paniculata (Burm. F.) Wall. Ex. Nees (AP), sometimes known as Kalmegh or the "King of Bitters." It has been used in Asia for a very long time to treat a wide range of chronic and infectious illnesses, such as fever, herpes, sore throats, upper respiratory infections, and herpes. The Indian Pharmacopoeia states that it is a key component in at least 26 Ayurvedic medicines.

The leaves and stem bark of Andrographis paniculata Linn were extracted using methanol, petroleum ether, acetone, and chloroform. A phytochemical analysis revealed that these extracts contained glycosides, phytosterols, saponins, tannins, flavonoids, and terpenoids (Pandey Jyoti *et al*., 2019). The Andrographis paniculata plant was tested for 14 phytochemicals using aqueous and ethanolic extracts. Alkaloids, phenols, tannins, phlobatannins, hydrolysable tannins, flavonoids, terpenoids, and saponins were all discovered to be present in the aqueous extract. Similar outcomes

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were also obtained using ethanolic extract, albeit cardiac glycosides rather than phlobatannins were found. Aqueous extract contained more tannins, terpenoids, and saponins whereas ethanolic extract had more alkaloids, total phenols, and flavonoids (S Nagajothi. *et al*., 2018). The features that may include anti-diabetic, anti-infective, anti-angiogenic, hepato-renal protective, sex hormone modulatory, liver enzymes modulatory, and insecticidal effects were assessed by Okhuarobo, A. *et al*,. (2014).

Materials and Methods

Sample Preparation

Fresh leaves of the plant *Andrographis paniculata* were harvested, dried after being rinsed with distilled water, and then stored for 4–5 days in a shed. In order to extract more compounds, the dried leaves were crushed into a fine powder and further subjected to solvent extraction.

Extraction of Phytochemical Compounds by Soxhlet Method

The Soxhlet apparatus was bagged with around 50 grams of powdered *Andrographis paniculata*, which was then put into new extract cloth. The device was set up on a heating mantle set to 40 °C, and a sample with a reflux condenser tube was snugly positioned above a solvent collector. 500 mL of ethanol was used as the extraction solvent to start the system and was used for 24 hours. The extract was filtered, kept at 4 °C for subsequent analysis, and the solvent was transferred to a conical flask after extraction.

Qualitative determination of Phytoconstituents

Test for Carbohydrates (Benedict's test)

About 0.5 mL of Benedict's reagent was added to 0.5 ml of ethanolic extract of *A. paniculata* The mixture was heated in a boiling water bath for 2 minutes. A characteristic coloured precipitate indicates the presence of sugar (Brain and Turner., 1975).

Test for Glycosides (Keller-Killiani Test)

About 2 mL of the *A. paniculata* leaf extract was added with glacial acetic acid and diluted the content with one drop of 5% $FeCl_3$ and concentrated H_2SO_4. A reddish brown colour appeared at the intersection of two liquid layers and the upper layer turned bluish green, indicating the presence of glycosides (Ansari, 2006).

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Test for Steroids

This was performed using the Salkowski test described by IP., (1996). 2 mL of extract was dissolved with 2 mL of chloroform and 2 mL of concentrated H_2SO_4. The reaction solution was shaken well. As a result, the chloroform layer turned red and the acid layer showed greenish yellow fluorescence.

Test for Alkaloids (Mayer's Test)

The detection of alkaloid content in study plant extract was examined using Ansari., (2006) methodology, and the ethanolic extract was evaporated in a test tube, afterward re-dissolved the residue with HCL, shaken well, and filtered. Mayer's reagent was added to the 2-3 mL of filtrate and observed the formation of a yellow precipitate, resulting in the presence of alkaloids.

Test for Flavanoids

Study extract was analysed for the presence of flavonoid content using the Shinoda Test described by Kokate., (1994). About 5 mL of 95% ethanol and a few drops of concentrated hydrochloric acid were added into 1 mL of *A.paniculata* extract. To this solution, 0.5 gm of magnesium turnings were added and observed. The pink coloration indicates the presence of flavanoids.

Test for Tannins

Lead acetate can be detected by adding the 1% lead acetate solution to the extract and developing a white precipitate, resulting in the presence of tannic acid (Mukherjee., 2002).

Test for Saponin

The Foam Test was a simple method reported in Ansari., 2006 by shaking the test extract of *A.paniculata* leaves vigorously with water. No persistent foam was formed and remained for more than 1 minute, indicating the presence of saponins.

Test for Protein (Ansari, 2006)

The Biuret test was employed in the olden days to detect the total protein content in samples. To 3 ml of A.paniculata leaf extract, a few drops of 4% NaOH and 1% CuSO4 solution were added. The tubes were observed for violet or pink colour formation.

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Test for Phenol

The total phenolic content was detected in the study extract using the ferric chloride test described by Mukherjee., (2002). The extract was diluted with 5 mL of distilled water and a few drops of a 5% ferric chloride solution were added. Exhibiting a green coloration indicates the presence of Phenols.

Test for Glycosides

0.5 mg of extract was dissolved in 1 mL of water and then aqueous. The addition of NaOH solution to catalyse the reaction triggers the formation of a yellow color, which indicates the presence of glycosides. This methodology was adopted by Horbone., (1984).

Test for Triterepnoids

Triterepnoids were qualitatively detected using the protocol of Horbone., (1984). To the test solution, 2 mL of chloroform were added, followed by 3 mL of concentrated Sulphuric acid was pipetted out at the side of the test tube. An interface with a reddish brown coloration is formed due to the presence of Triterepnoids.

Detection of bioactive compound by Thin layer chromatography

The crude ethanolic extract of *Andrographis paniculata* was analyzed using the methodology of Biradar RS and Rachetti DB., (2013) and Kristanti *et al*., (2015) for the detection of Flavanoid and Terpenoid on the stationary phase of Silica gel G60 TLC (Merck) plate and the Mobile phase was prepared by dissolving the Ethyl acetate: Formic acid: Glacial acetic acid: Water at the of ratio of 10:1.1:1.1:2.6 for flavanoids and Toluene: ethyl acetate (93:7) for triterpenoids and add about 10µl of ethanolic extracts of respective samples (*A.Paniculata*) were dropped on TLC sheet at 2cm above from the bottom. Allow the chromatographic chamber for the separation of compounds as individual bands. Then chromatogram was developed by Sparing with following visualizing agents, 1% ethanolic aluminum chloride (Flavanoids) and Methanol + vanilin + Acetic acid + Conc. Sulfuric acid (95ml + 0.5g+ 5ml+ 3 drops) for triterpenoids; Then, visualized under UV at 325 nm. After chromatogram was developed, the Rf (Retention Factor) was calculated by using the formula,

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

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Column Purification of Bioactive compounds

Using silica gel column chromatography with ethyl acetate and hexane as the mobile phase at the ration of 1:1, the ethanolic extract of *A. paniculata* was subjected to further compound purification. Following solvent elution, the ethanolic extract was loaded onto the top of the column at a rate of around 4-5 ml. To collect the column fractions, the elution solvents were flown again. For subsequent examination, the collected fractions were kept in a freezer at 4 °C and later on analysed the presence of bioactive phytoconstituents using Gas chromatography-Mass spectroscopy analysis in Agilent Technologies: GC-MS (GC System-7820A) with the parameters of Over Temp -100 ^{o}C-270 ^{o} C (10 ^{o}C/min), Flow rate -1.2 and helium gas was used as mobile phase for the separation of phytocompounds.

Chemical synthesis of Diethyl phthalate Silver Nanoparticle and Gold Nanoparticles

Diethyl phthalate Silver Nanoparticle

The recovered fraction-4 was found to contain the compound diethyl phthalate, and silver nanoparticles were therefore synthesised. The collected fraction-4 was dried and then redissolved in 10 mL of aqueous. With the addition of 1 mL of 0.05M ascorbic acid (as a reducing agent) and 100 mL of 0.05M silver nitrate, the mixture was agitated using a magnetic stirrer until the colour of the solution changed. The mixture was centrifuged at 8,000 rpm for 20 minutes to obtain the synthesised diethyl phthalate silver nanoparticle (DP AgNps), and then the pellet was washed with deionized water. After three washes, the pellet was thoroughly re-dissolve distilled water and it was then placed onto a petri dish for overnight drying in the oven at 50 ^{o}C.

Gold Nanoparticles by Turkevich Method

By dissolving 0.25 mM gold chloride (HAuCl4) in 50 ml of solution and 5 mg of pure diethyl phathalate in a 250 mL conical flask containing 100 mL of distilled water, nanoparticles were created using the Turkevich Method. A 34.0 mM (1.0 wt%) solution of the reducing agent trisodium citrate (NaCt) was produced. The HAuCl4 solution was heated in a flask using a hotplate while being constantly and vigorously stirred. A certain volume of NaCt solution was swiftly introduced into the HAuCl4 solution after it had boiled under ambient pressure (Frens G., 1973). The color change was observed to determine the chemical synthesis of DP gold nanoparticles.

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Anti-Microbial Potential of Crude *A. paniculata* extract, Diethyl phthalate (DP), DP AgNPs and AuNPs

The medium was prepared by dissolving 38 g of Muller Hinton Agar Medium (Hi Media) and for anti-fungal activity, 65.0 g Sabouraud Dextrose Agar in 1000 ml of distilled water. The dissolved medium was autoclaved at 15 Lbs pressure at 121^0C for 15 min (pH 7.3). The autoclaved medium was tend to be cooled, assorted well and poured petriplates (25 ml/plate) the plates were swabbed with Pathogenic Bacteria culture viz. analysis *Staphylococcus aureus, Enterococcus feacalis, Escherichia coli, Proteus sps ,Streptococcus epidermis, Pseudomonas sps, Aspergillus niger, Asergillus flavus, Candida sps and Rhizhopus sps* Finally, About 20 µL of samples (ethanolic extract of *A.Paniculata,* Diethyl phathalate and synthesized metal (Ag & Au) nanoparticles were loaded onto the disc then placed on the surface of Mullar-Hinton medium and the plates were kept for incubation at 37°C for 24 hours. At the end of incubation, inhibition zones were examined around the disc and measured with transparent ruler in millimetres. The size of the zone of inhibition (including disc) was measured in millimeters. The negative activity was determined by absence of zone inhibition (Kohner *et al*., 1994; Mathabe *et al*., 2006). The activities are expressed as zone of clearance, if the zone of inhibition was less than 7 mm, intermediate (8-10 mm) and sensitive if more than 11 mm (Assam *et al*., 2010).

Results and Discussion

Qualitative detection of Phytocompounds

Followed by Soxhlet extraction, the crude filtered ethanolic extract of *A. paniculata* was analysed for the presence of phytocompounds. The results of the biochemical tests revealed the presence of alkaloids, flavanoids, tannins, triterpenoids, and phenolic compounds in ethanolic extract **(Table.1 and Figure.1)** This showed the promising prevalence of bioactive compounds and their biological potential across a wide range of diseases and disorders.

Table: 1. Phytochemicals assessment of Leaf Ethanolic Extract of *Andrographis paniculata*

Phytoconstituents	**Results**
Carbohydrate	Negative
Protein	Negative
Glycosides	Negative
Steroids	Negative

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Alkaloids	Positive
Flavanoids	Positive
Tanins	Positive
Saponins	Negative
Phenols	Positive
Terpenoids	Positive

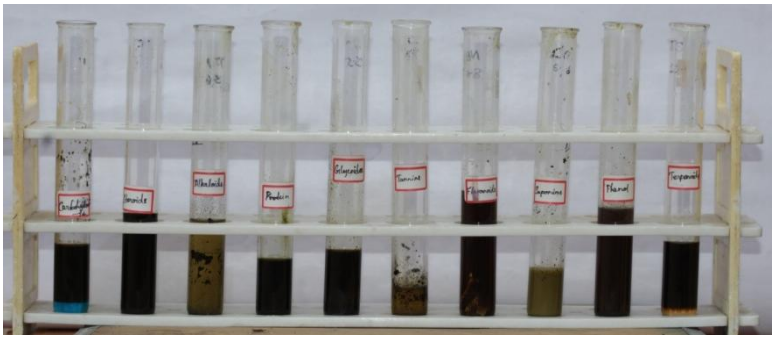

Figure: 1. Phytochemicals assessment of Leaf Ethanolic Extract of *Andrographis paniculata*

Detection of bioactive compound by Thin layer chromatography

The chromatogram was developed using respective visualising agents for flavonoids and triterpenoids (1% ethanolic aluminium chloride and Methanol + vanilin + Acetic acid + Conc. Sulfuric acid). The developed chromatogram was observed for the presence of bands. One spot for flavanoid and four spots for triterpene detection were revealed. The Rf value of 0.6 with an orange spot in UV (325nm) exhibited the presence of flavonoids, and Rf values of 0.92, 0.65 (pink), 0.55 (light purple) and 0.5 (light yellow) showed the presence of triterpenoids in ethanolic extract of *A. paniculata* leaves **(Table.2 and Figure.2)**.

Table: 2 Retention factor values of Leaf Ethanolic Extract of *Andrographis paniculata*

S. No	**Sample Code**	**Obtained Rf Value**	**Band Intensity and Colour**		**Detected Compound**
			Visible	**UV**	
1.	Ethanolic extract of ***A.Paniculata***	0.6	Light green	Orange	Flavanoid
2.	Ethanolic extract of ***A.Paniculata***	0.92 (Spot-1)	Light yellow	Pink	Triterpenoids
3.		0.65 (Spot-2)	Nil	Pink	

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4.		0.55 (Spot-3)	Nil	Light purple	
5.		0.5 (Spot-4)	Nil	Light yellow	

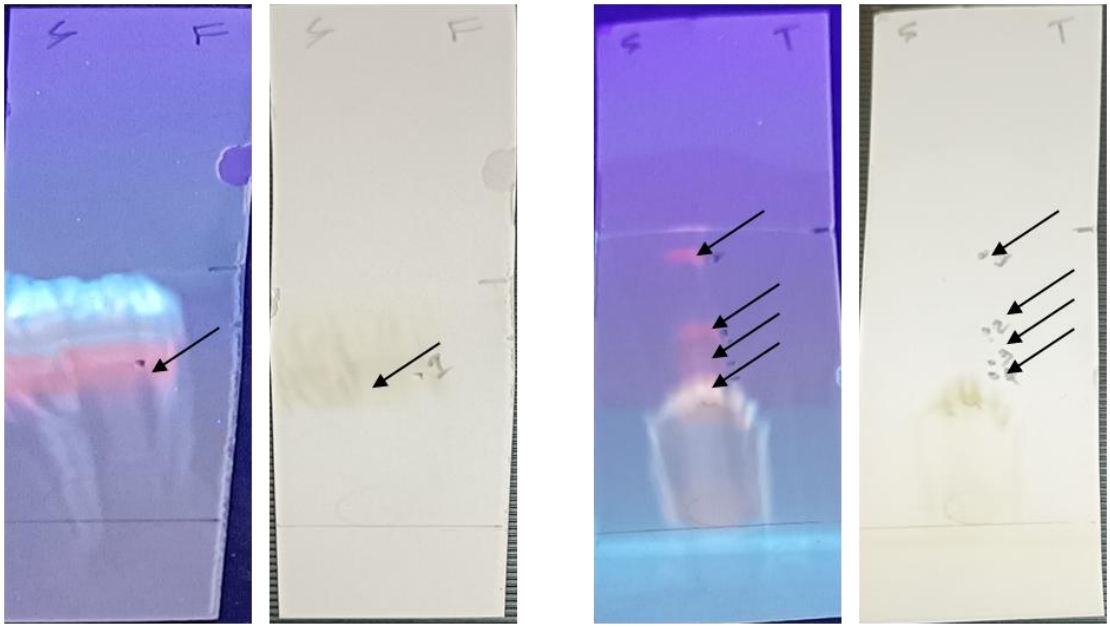

Figure: 2 Retention factor values of Leaf Ethanolic Extract of *Andrographis paniculata*

Column Purification GC-MS Identification of Bioactive compounds

The crude ethanolic extract was subjected to column purification using a suitable solvent mobile phase and elution was collected as eight fractions **(Figure.3)**. The collected column fractions were further identified with the presence of bioactive compounds using a gas chromatography-mass spectrometry approach. The GC-MS chromatogram showed the significant presence of bioactive compounds. The identified compounds are Methyl salicylate at a retention time of 3.628 (min), Diethyl Phthalate at 7.864 and tert-Butyl (5) isopropyl-2-methylphenoxy) dimethylsilane (16.875 min). At 16.875 minutes, arsenic acid, tris(trimethylsilyl) ester, and tris(tert-butyldimethylsilyloxy) arsane were detected. Peaks were observed for the compounds 2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene and 3-Benzo[g]quinoxalin-2-yl-propionic acid at 17.244 min. Methyl salicylate, for example, has antimicrobial, anti-oxidant, and anti-cancer properties, while Diethyl Phthalate has antimicrobial, acetylcholinesterase, and neurotoxic properties. The antireflective coating polymer properties of 2-(Acetoxymethyl)-3-(methoxycarbonyl) biphenylene

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compounds isolated from the ethanolic extract of *A. paniculata* were investigated **(Figure. 3.a)**.

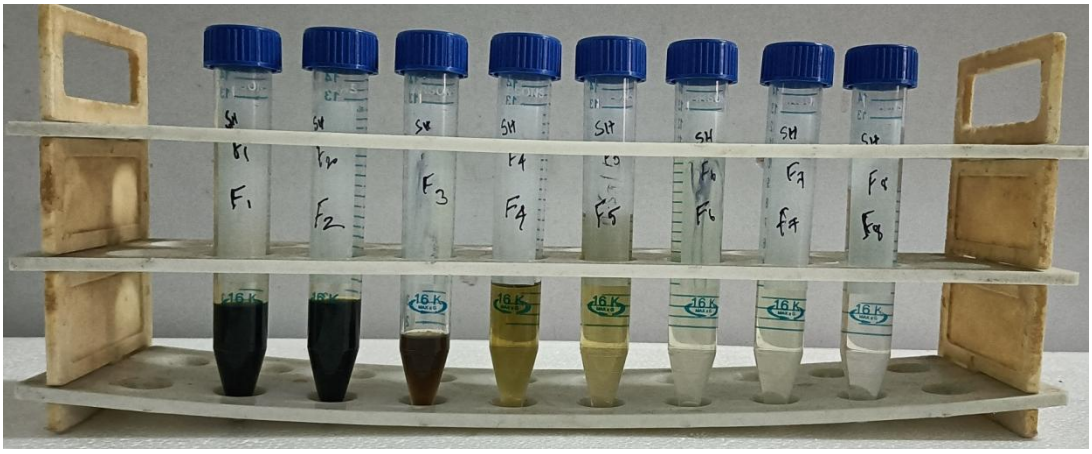

Figure: 3. Different Fractions of Leaf Ethanolic Extract of *Andrographis paniculata* Collected by Column Chromatography

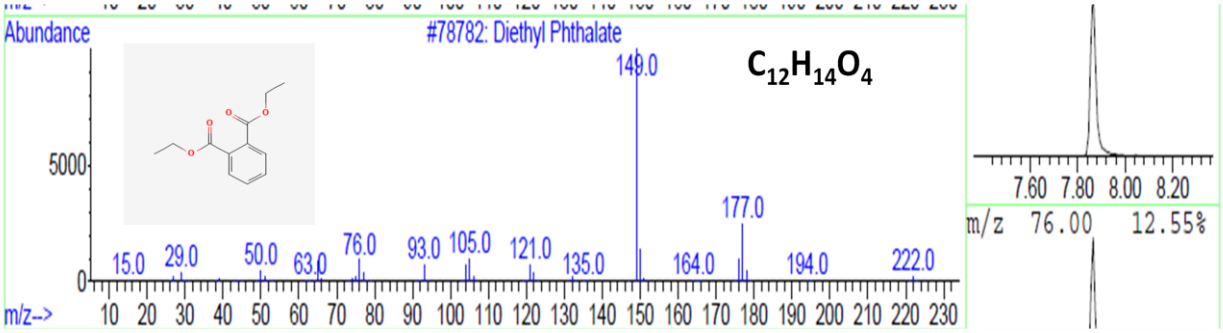

Figure: 3.a. Identification and Separation of Diethyl Phthalate of Sample *Andrographis paniculata* Ethanolic Extract by GC-MS

Synthesis of Silver and Gold Nanoparticles with Diethyl Phthalate

The identified Diethyl Phthalate from column fraction-4 was further concentrated and subjected to nanoparticle synthesis using silver (Silver nitrate) and gold metals (Gold chloride). The presence of 0.05M ascorbic acid (reducing agent) aided in the formation of nano-sized particles conjugated with Diethyl Phthalate (Fraction-4) and has been identified as the colour change of the silver nitrate solution into gray. On the other hand, gold chrolide was reduced with a 34.0 mM (1.0 wt%) solution of the reducing agent trisodium citrate (NaCt) in the mixture of Diethyl Phthalate (Fraction-4) and turned the yellowish gold solution into brick red, confirming the production of nano-sized gold particles. Later, the synthesised nanoparticles (Ag and Au) have been concentrated, filtered, and oven dried for further storage and study for biological potential.

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Anti-microbial potential of Crude *A. paniculata* extract, Diethyl phthalate (DP), DP AgNPs and AuNPs

The synthesized silver and gold nanoparticles were evaluated the potential of inhibiting the microbial growth in growth medium comparatively. The data of anti-microbial study performed using disc diffusion method was showed the maximum zone of inhibition of *A. paniculata was 12 mm* against *Staphylococcus aureus* and an 11 mm zone of clearance were widely observed in *Enterococcus feacalis, Proteus sps ,Streptococcus epidermis, and Pseudomonas sps* except *Escherichia coli.* DPAgNPs showed quite fine out-put compared with diethyl phthalate and DPAuNPs against a wide range of pathogenic bacteria (Table and Figure). While exhibiting anti-fungal effects, DPAgNPs and DPAuNPs exhibited a considerably greater zone of inhibition against all target fungal strains than crude A. paniculata extract and diethyl phthalate (Fraction-4). The zone of inhibition was elaborated in **Table 3-3.a and Figure 4-4.a**.

Table: 3. Anti-bacterial potential of Study Samples

Bacteria	**Positive**	**Negative**	***A. paniculata***	**DP**	**DP AgNpps**	**DP AuNps**
S. aureus (G+)	18	-	12	8	9	10
E. feacalis (G+)	17	-	11	8	6	7
E.coli (G-)	14	-	-	6	12	11
Proteus sps (G-)	13	-	11	7	8	10
S. epidermis (G+)	18	-	11	9	10	10
Pseudomonas sps (G-)	16	-	11	-	10	11

Table: 3.a. Anti-bacterial potential of Study Samples

Fungal Strains	**Positive**	**Negative**	***A. paniculata***	**DP**	**DP AgNpps**	**DP AuNps**
A. niger	12	-	-	5	10	11
A. flavus	18	-	-	10	12	11
Candida sps	27	-	-	9	14	13
Rhizopus sps	11	-	-	11	-	-

Keywords: ***PC*** *Positive control (Streptomycin),* ***NC*** *Negative control, "-" No Zone,* ***mm***

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*(Millimetre), **G+** (Gram Positive Organism),**G-** (Gram Negative Organism),*

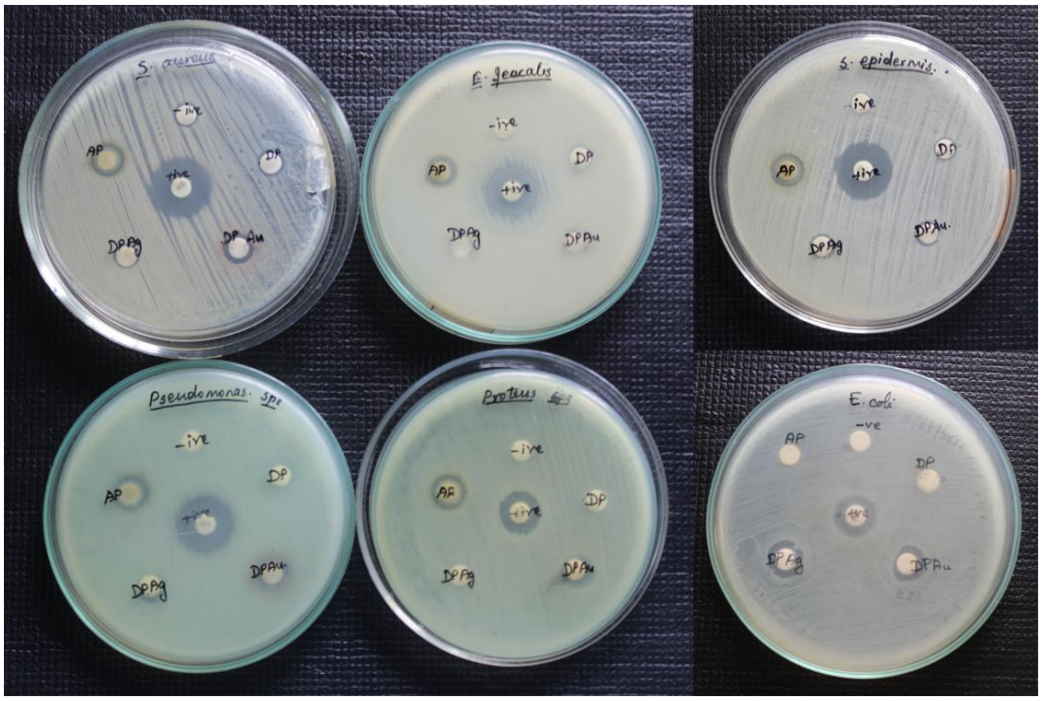

Figure: 4. Anti-bacterial potential of Ethanolic Extract of *Andrographis paniculata* Leaf

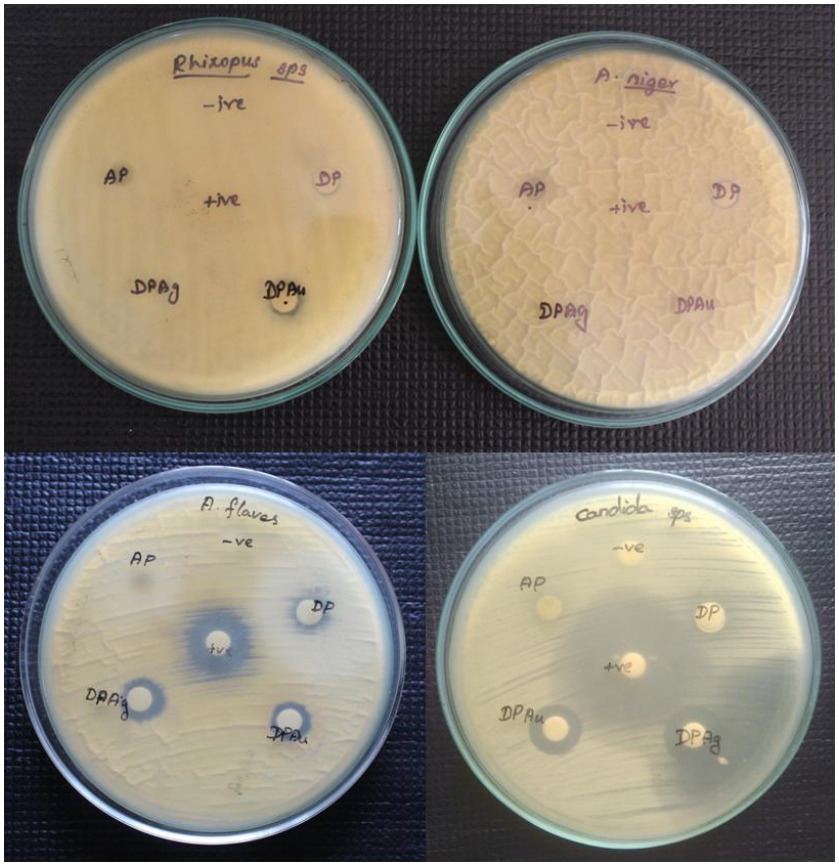

Figure: 4.a. Anti-Fungal potential of Ethanolic Extract of *Andrographis paniculata* Leaf

Conclusion

Andrographis paniculata has been used for their wide range of medicinal values traditionally. Many literatures showed the significant presence of bioactive phytocompounds i.e., plant secondary metabolites responsible for biological potentials such as anti-microbial, anti-oxidant, anti-inflammatory and anti-cancer property so on. According this investigation, these phytocompunds were examined in ethanolic extract of *Andrographis paniculata* leaves and identified the plants constituents using gas chromatography-mass spectroscopy method. Diethyl phthalate was picked and reviewed for their potential of antimicrobial, acetylcholinesterase, and neurotoxic effects. The identified and column purified phytocompound (Diethyl phthalate) was developed as nano particles using silver and gold metals. The synthesized metals (silver and gold) nanoparticles were further evaluated for their effect on microbial growth inhibition. Among the study samples such as *A. paniculata* leaves extract, diethyl phthalate, DPAgNPs and DPAuNPs, crude ethanolic leaf extract and DPAgNPs have showed finest anti-microbial effects against all of target pathogenic strains. The nanotechnology has promising strategies to develop nano scaled drug carrier system conjugated with potential bioactive compounds hence further molecular characterization helps to understand the interactions between drug and target molecules.

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