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PHYTOCHEMICAL, *IN VITRO* ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF *ARGYREIA PILOSA* WIGHT & ARN. (WHOLE PLANT)

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Keywords:

Argyreia pilosa, Antioxidant, Tannins, Flavonoids, Antibacterial and Phytochemical analysis

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ABSTRACT: Background: The whole plant of *Argyreia pilosa* Wight & Arn. (Convolvulaceae) continuously to be utilized in Indian indigenous systems as a remedy for cough, quinsy and applied externally in case of itch, eczema and other skin troubles, antidiabetic, antiphlogistic, rheumatism and reduce burning sensation. Still, there were constrained phytochemical or biological studies on the whole plant of *A. pilosa*, as well as no studies which align using its conventional medicinal uses. **Aim:** The aim of this study was to determine the total tannin and flavonoids contents, antimicrobial and antioxidant activity of two extracts of the whole plant of *A. pilosa* and compare them to find out the better extract for upcoming studies. **Materials and Methods:** The whole plant material was subjected to cold maceration with ethyl acetate and methanol to obtain ethyl acetate extract (APEE) and methanolic extract (APME). Phytochemical constituents of these extracts were determined as per standard procedure. Total Phenolics had been estimated by the Folin– Ciocalteu colorimetric method using tannic acid as standard. Total Flavonoids had been estimated by aluminum chloride assay using quercetin as standard and the antioxidant capacity was determined by the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay, hydrogen peroxide assay and reducing capacity. The antimicrobial activity was studied with the agar diffusion method and minimal inhibitory concentrations (MIC) of these extracts were determined. **Results:** APEE has better yield than APME. The phytochemical constituents present in both APEE and APME have revealed the presence of flavonoids, alkaloids, phenols, tannins, glycosides, amino acids and proteins except for steroids and acid compounds which are only present in APME. Total tannin content and total flavonoids content of APEE is more than in APME. Both REE and RME have antioxidant and antibacterial potency. Statistically there is no significant difference between the antioxidant potency of APEE and APME. But APEE and APME are statistically different from each other in terms of their antibacterial strength, APEE being better than APME in this case.

INTRODUCTION: Phytochemicals tend to be widely available at diverse ranges in several medicinal plants. Most of the plants utilized in conventional drugs are efficient for different disorders triggered by oxidative stress and microbial infections.

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Studies have revealed that medicinal plants show antioxidant along with antimicrobial activity [1]. Oxygen, the most crucial element for the endurance of living organisms is extremely reactive and may do severe harm to healthy cells of the body as free radical. Oxidation generates free radicals that begins sequence reaction within the cell that triggers harm to and also fatality of cell [2]. An antioxidant ends these type of radical responses by eliminating free radical intermediates, that can causes aging and disorders such as atherosclerosis, cancer, cardiovascular diseases and CNS disorders [3].

Therefore, play a important role in prohibiting or lagging the oxidation at the cellular level [4]. Man made antioxidants *i.e*., butylated hydroxytoluene, butylated hydroxyanisole and tert-butyl hydroquinone being examined all over the world for their possible health risks and have been found to promote hepatic damage and leading to cancer to laboratory animals [5]. In contrast, the herbs prove to have less or no side effects, [3] thus channelizing the interest towards the nature for antioxidant potency.

As a result of developing resistance to antibiotics of several microbes, plant extracts and plant derived compounds are of current interest as antiseptics as well as antimicrobial agents in medication. The effectiveness of the plants in treating various disorders is certainly well established along with a significant level of study has been carried out within this area by investigators in India as well as abroad. The worldwide breakthrough of multi-drug resistant bacterial strains is progressively restricting the potency of existing medicines and considerably leads to remedy failing of infections. For example include methicillin-resistant staphylococci, pneumococci resistant to penicillin and macrolides, vancomycin-resistant enterococci and multi-drug resistant Gram-negative organisms [1].

A. pilosa is an ornamental, in addition to a medicinal plant. All parts of this plant are widely used as a folklore medicine for the treatment of various ailments by the Indian traditional healer. Its root is utilized to cure a various illness like sexually transmitted diseases *viz*., gonorrhea and syphilis, blood diseases. Traditionally, the paste of the leaves is applied to the neck region for cough, quinsy and applied externally in case of itch, eczema and other skin troubles, antidiabetic, antiphlogistic, rheumatism, reduce burning sensation and antidiabetic [6]. Young wines are mixed together with rhizome of ginger are spread all around the body to relieve from fever [7]. The decoction of its root used to treat diarrhea and cathartic [8, 9].

A vast range of phytochemical constituents has been separated from the genus Argyreia *i.e*., glycosides, alkaloids, amino acids, proteins, flavonoids, triterpene and steroids [10]. The genus Argyreia has been reported various biological activities including nootropic, aphrodisiac, antioxidant, antiulcer, immune-modulatory, hepatoprotective, anti-inflammatory, anti-hyperglycemic, antidiarrheal, antimicrobial, antiviral, nematicidal, anticonvulsant, analgesic, anti-inflammatory, wound healing and central nervous depressant activities [10-14]. Even though the drug has many uses, it's pharmacological and phytochemistry are very poorly explored [15].

Although the plant has been extensively used for its traditional value, but antibacterial and antioxidant activites remains unexplored. Therefore the current investigation had been carried out to study the antimicrobial and antioxidant potency of this medicinal plant.

MATERIALS AND METHODS:

Chemicals: All analytical grade chemicals were utilized in this study were procured form E. Merck, Germany. 2, 2-diphenyl-1picrylhydrazyl (DPPH), tannic acid, ascorbic acid, quercetin, Folin-Ciocalteu's reagent, aluminum chloride, hydrogen peroxide, ferric chloride, potassium ferricyanide, Dimethyl sulfoxide, agar powder, beef extract and peptone were obtained from Hi-Media, Mumbai; petroleum ether, toluene, acetone, ethyl acetate and methanol.

Plant Material: The plant material was obtained from Tirupati, chittoor district of Andhra Pradesh, India during the month of March 2016 and authenticated by Dr. K. Madhava chetty, Taxonomist, Sri venkateswara University tirupati, India. Voucher specimen No. 1922 was deposited at the herbarium for future reference. Then it was shade dried, powdered and sieved through 20 mesh and kept in an air tight container for future use [16, 17].

Extraction Method: The whole plant of *A. pilosa* were collected, washed in tap water to get rid of any contaminants and then dried to remove water from the surface of the plant. The plant was cut into small pieces and dried in shade in room temperature for 7 days. The dried plant material was grinded to coarse powder and defatted using petroleum ether. Equal quantity of defatted dried material of *Argyreia pilosa* was subjected to maceration with ethyl acetate and methanol for 72 hr each. It was then filtered and concentrated (rotary-evaporator) to obtain dried ethyl acetate

extract (APEE) and methanolic extract (APME). Percentage yield was calculated using the following Eqn. 1.

Percentage yield (%) = Weight of Extract (g)/ Weight of defatted dried plant material (g) X 100

Qualitative Phytochemical analysis: The extracts had been analyzed for the presence of alkaloid, glycoside, steroids, triterpenoids, flavonoids, proteins, amino acids, carbohydrates, reducing sugar, tannin and saponin using standard methods [15, 16, 17].

Quantitative Phytochemical analysis:

Determination of total flavonoids content: Total flavonoids contents of two extracts had been estimated by aluminum chloride colorimetric assay depending on the development of a complex flavonoid-aluminium, having a maximum absorbance at 510 nm [18]. Quercetin utilized to make the calibration curve. One milliliter of sample or Quercetin standard solution was added into a 10 mL volumetric flask, mixed with 4 mL of 60% ethanol and 0.3 mL of 5% $NaNO_2$ for 6 min, After that, 0.3 mL of 10% $AlCl_3$ was added to react for another 6 min. The reaction was stopped by 4 mL of 4% NaOH, and the total volume was topped up to 10 mL with 60% ethanol. The absorbance was measured after 15 min at 510 nm. Total flavonoids contents were expressed as mg Quercetin equivalents per gram dry weight of each extract [19]. All samples were analyzed in triplicate.

Determination of total tannin content: Total tannin content (TTC) was estimated using Folin-Ciocalteu reagent (FCR) taking tannic acid as standard [20]. FCR being sensitive to reducing compounds including polyphenols produce blue colour upon reaction. The extracts utilized in this analysis should be in the concentration of 1 mg/ml. The reaction mixture was made by combining 0.5 ml of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent mixed with water, in addition to add 2.5 ml of 7.5% Na_2CO_3. Blank was prepared by same procedure without sample. The samples had been incubated in a thermostat at 45 C for 45 min. The absorbance was determined by making use of spectrophotometer at λ_{max} = 765 nm. The samples have been prepared in triplicate for each analysis and the mean value of absorbance had been acquired. The same method was repeated for the standard solution of tannic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was calculated (mg/ml) from the calibration line. All the determinations were done in triplicate. The total phenolic content is expressed in mg tannic acid equivalent (TAE) per gram.

Antioxidant Assays:

DPPH free radical scavenging assay: DPPH scavenging capacity of each extract was determined according to the method of Md. Nur Alam *et. al*. A stock solution made up of 40 µg/mL of DPPH (in anhydrous ethanol, w/v) had been prepared. 1 ml of sample was mixed with 4 ml of ethanol DPPH solution. The mixture had been shaken vigorously as well as incubated at room temperature for 30 min in the dark. The absorbance was determined at 517 nm. A control was prepared without sample. Ascorbic acid was used as positive controls. The DPPH radical scavenging ability was calculated according to the following equation:

$$\text{DPPH scavenging rate (\%)} = [(A_0-A_1)/A_0] \text{ X } 100$$

Where A_0 is the absorbance of control, and A1 is the absorbance of sample. Each sample was analyzed in triplicate [21].

Hydrogen peroxide scavenging activity assay: A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (50 mM pH 7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer. Extract (20–60 lg/mL) in distilled water is added to hydrogen peroxide and absorbance at 230 nm is determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging is calculated as follows:

$$\text{\% scavenged } (H_2O_2) = [(Ai - At) / Ai] \text{ X } 100$$

Where Ai is the absorbance of control and At is the absorbance of test [21, 22].

Reducing power method: In the method, 2.5 ml of 0.2 M phosphate buffer with pH 6.6 and 2.5 ml of $K_3Fe(CN)_6$ (1% w/v) are combined to 1.0 ml of sample mixed in distilled water. The resulting mixture is incubated at 50 °C for 20 min, followed

by the addition of 2.5 mL of Trichloro acetic acid (10% w/v). The mixture is centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 mL), mixed with distilled water (2.5 mL) and 0.5 mL of $FeCl_3$ (0.1%, w/v). The absorbance is then measured at 700 nm against blank sample [21, 23].

Antibacterial activity:

Test microorganisms: The antibacterial activity of the plant extracts had been examined against some Gram positive and Gram negative bacteria *i.e*., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *streptococcus epidermidis* and *Escherichia coli*, which are obtained from Laboratory of microbiology, NICU, SUM Hospital, Bhubaneswar.

Well diffusion method for determination of zone of inhibition: The antibacterial study of APCE, APEE and APME had been carried out through well diffusion method [24, 25]. The inoculation of the microorganism had been prepared from bacterial culture. 15 ml of nutrient agar medium was put in clean sterilized petri-plates and allowed to cool and solidify. 100µl of broth of bacterial stain was pipetted out and spread over the medium evenly by a spreading rod till it is dried properly. Wells of 6 mm in diameter are bored using sterile cork borers. Solutions of both the extracts (10 mg/ml) in DMSO (dimethyl sulphoxide) were prepared. 100 µl of plant extract solutions was added to the wells. The petri-plates were incubated at 37 °C for 24 h. Ciprofloxacin (1 mg/ml) was used as positive control and DMSO was taken as negative control. Antibacterial activity was evaluated by measuring the zone of inhibition diameters. All the determinations were performed in triplicate.

RESULTS AND DISCUSSION: Extraction of defatted plant material of *A. pilosa* had been performed utilizing chloroform, ethyl acetate and methanol as liquid extractor to yield dried APCE (Buff powder), APEE (Blackish green powder) and APME (Dark Green Powder). Defatting of plant material is a pre-extraction process that eliminates unwanted fatty and waxy substances from the plant material. On determining percentage yield (%) using Eqn.1, % yield of APME (5.38%) and APEE (2.25%) are shown in **Fig. 1**.

Percentage (%) yield= Weight of extract (g) / Weight of plant material (g) X 100

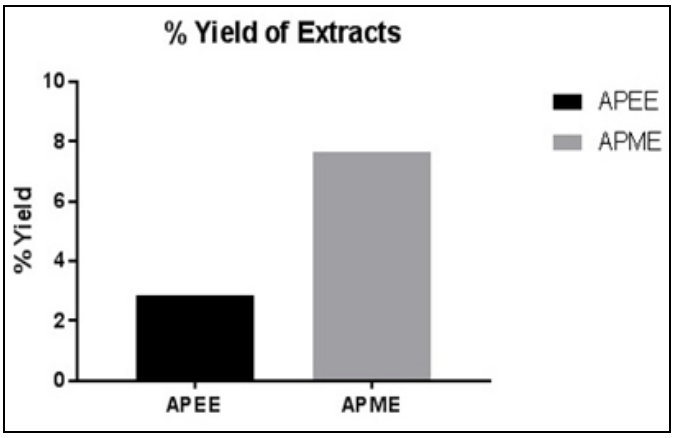

FIG. 1: PERCENTAGE (%) YIELD OF EXTRACTS

The results of qualitative phytochemical analysis of crude powder of *A. pilosa* are tabulated in **Table 1**.

TABLE 1: PHYTOCHEMICAL ANALYSIS OF VARIOUS EXTRACTS OF WHOLE PLANT OF *ARGYREIA PILOSA* WIGHT & ARN.

Phytoconstituents	Method	Ethylacetate Extract	Methanolic Extract
Flavonoids	Shinoda Test	+	+
	Zn. Hydrocholride test	+	+
	Lead acetate Test	+	+
Volatile oil	Stain test	-	-
Alkaloids	Wagner Test	+	+
	Hager's Test	+	+
Tannins & Phenols	$Fecl_3$ Test	+	+
	Potassium dichromate test	+	+
Saponins	Foaming Test	-	-
Steroids	Salkowski test	-	+
Carbohydrates	Molish test	-	-
Acid compounds	Litmus test	-	+
Glycoside	Keller-Killani Test	+	+
Amino acids	Ninhydrin test	+	+
Proteins	Biuret	+	+

The outcomes of quantitative estimation of total tannin content (TTC) and total flavonoid content (TFC) are being represented in **Fig. 2 and 3** respectively. It is definitely observable through the results that TTC and TFC in REE> RME.

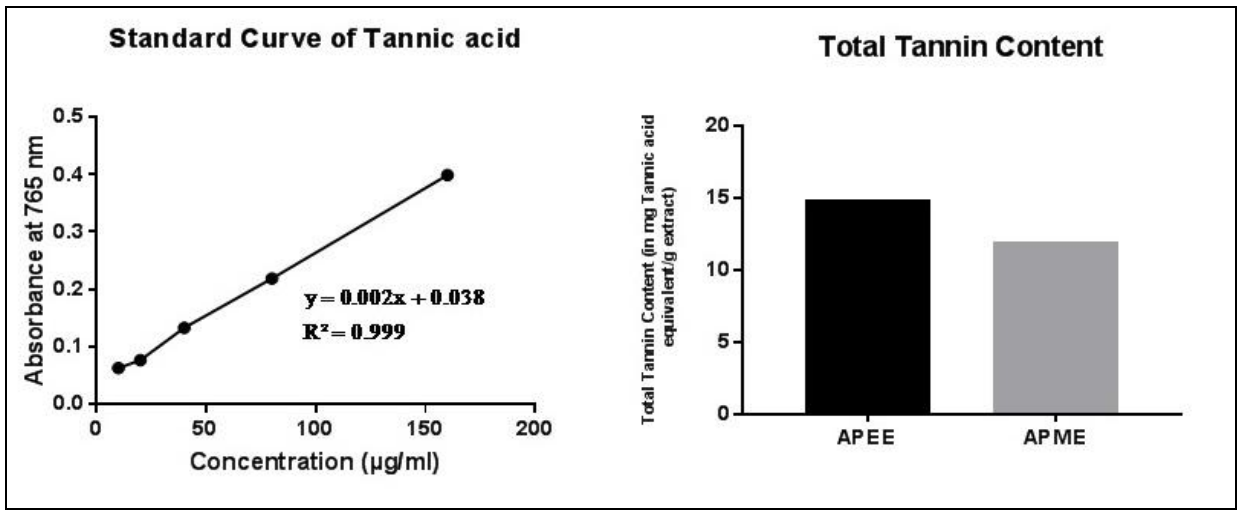

FIG. 2: STANDARD CURVE OF TANNIC ACID AND COMPARISON BY GRAPHICAL REPRESENTATION OF TOTAL TANNIN CONTENT

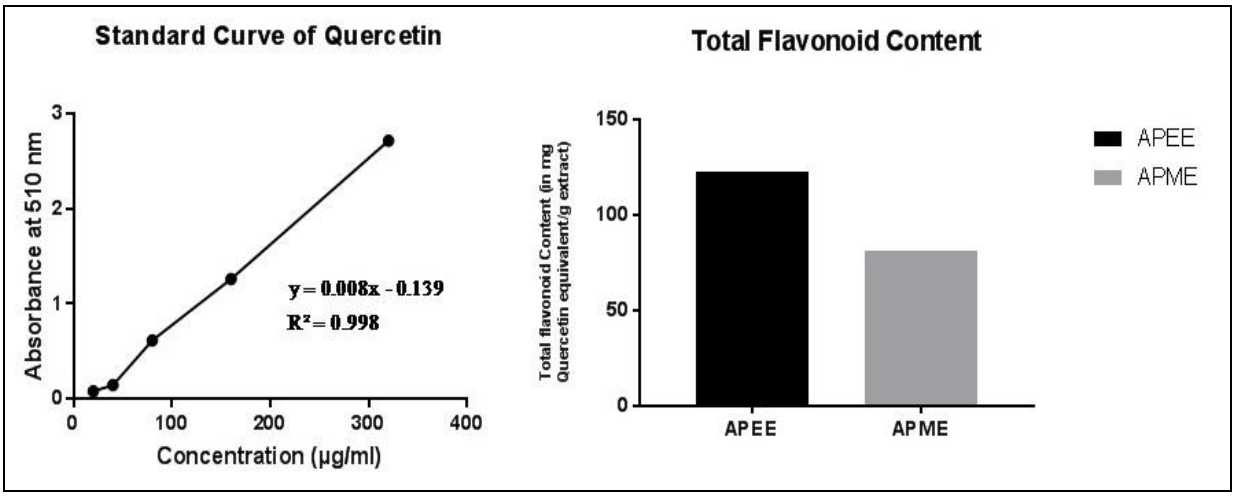

FIG. 3: STANDARD CURVE OF QUERCETIN AND COMPARISON BY GRAPHICAL REPRESENTATION OF TOTAL FLAVONOIDS CONTENT

Antioxidant activity of any crude extract might be because of several mechanisms. Binding of transition metal ion, radical scavenging, decomposition of peroxide, reducing capacity, prevention of continued hydrogen abstraction and prevention of chain initiation are some of these mechanisms. DPPH is a stable free radical having absorption at 515 nm. When reduced by an antioxidant to form DPPH, the natural deep violet colour of DPPH changes to pale yellow [26]. The change in the colour is proportional to the strength of the antioxidants and a significant decrease in the absorbance of the reaction mixture indicates significant free radical activity of the test material. Hydrogen peroxide (H_2O_2) scavenging activity of the plant extracts are assessed spectrophotometrically with the disappearance of H_2O_2 at 230 nm [27]. H_2O_2 generates hydroxyl radical within the tissues and is harmful to them. Therefore, removal of H_2O_2 is extremely important [28]. Results of Hydrogen peroxide (H_2O_2) scavenging activity is presented in **Fig. 4**.

Based on the reducing power of the screening substance, the yellow colour of the Fe(III) becomes to Fe(II), Perl's Prussian blue which may be measured spectrophotometrically at 700 nm [21]. The reductive capacity of APEE and APME is compared to that of the Ascorbic acid in **Fig. 5.**

It is noticed that there exists positive correlation between the antioxidant activity and reducing power of APEE and APME. Like the antioxidant activity study, reducing power of REE and RME also increases with the increase in concentration. Reductones, which exhibit the antioxidant activity by breaking the free radical chain and donating a hydrogen atom, are also responsible for the reducing properties [29].

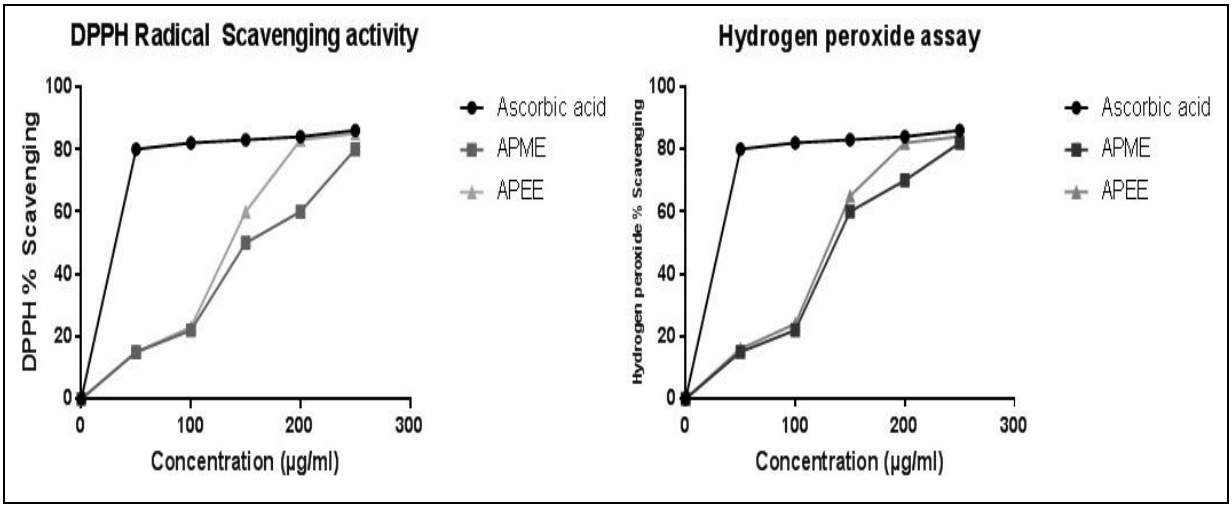

FIG. 4: ANTIOXIDANT ACTIVITY STUDY OF ASCORBIC ACID, APME AND APEE

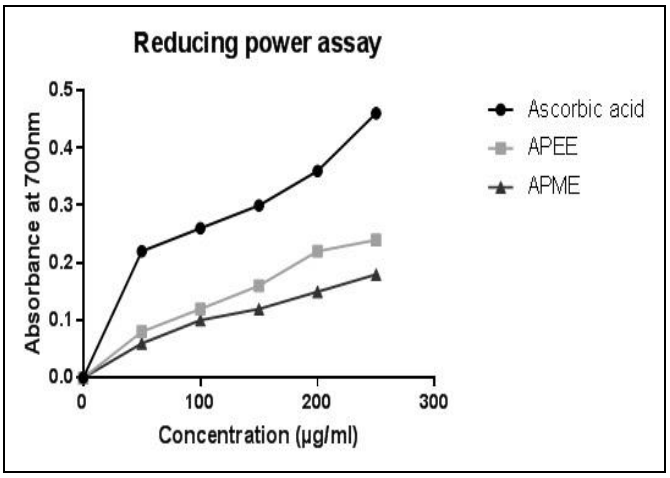

FIG. 5: REDUCING POWER ASSAY OF ASCORBIC ACID, APEE AND APME

The IC_{50} values of APEE and APME are portrayed in **Table 2**. The IC_{50} value of APEE is less than APME, which states the fact that APEE shows better antioxidant property than APME. The antioxidant activity has positive correlation with total phenolic and flavonoids content [1], which act as scavengers to various oxidising species [30]. For that reason, it was quite sensible to determine the TPC and TFC in the plant extract. TPC and TFC of REE is more than that of RME as evident from **Fig. 3B** and **Fig.** 3F, respectively reasoning the result obtained that IC_{50} of REE is less than that of RME.

TABLE 2: IC_{50} VALUES OF APEE AND APME

Testing sample	IC_{50} (µg/ml)	
	DPPH radical scavenging activity	Hydrogen peroxide assay
Ascorbic acid	34.14	32.67
APEE	140.23	132.32
APME	147.34	137.53

For the current study, the potency of APEE and APME is screened against gram positive and gram negative bacteria. The well diffusion method of screening antibacterial activity is considered to provide better results and is associated with the carrier solvent, DMSO which is likely to diffuse highly across the medium [31].

In this study, DMSO is employed as a solvent for solubilizing APEE and APME for antibacterial study. DMSO is a polar aprotic solvent which has the capability to dissolve both polar and nonpolar compounds. DMSO can be used as cryoprotectant for long term low temperature conservation of various biomaterials [32] and thus considered to have no impact on the growth or inhibition of growth on microorganisms. Although the literature facilitates the use of DMSO as solvent with regard to antibacterial studies, a negative control (*i.e.*, only DMSO) is also studied in each plate to be extra sure.

In the present circumstances of increasing resistance of microbes towards the worldwide used Antibacterial [33], it is crucial that the actual ingredients having antibacterial potential needs to be extracted. The current study is useful to find the extract that exhibits better antibacterial activity. In this study, the plant material was extracted with ethyl acetate and methanol by maceration. This study also compares the antibacterial activity of ethyl acetate and methanolic extracts of *A. pilosa*, APEE and APME, respectively. Results depicted in **Fig. 6** and **7** shows that both REE and RME at different concentrations 1, 10, 100, 250, 500 and 1000 µg/ml (100 µl incorporated in each well) possess antibacterial activity towards both gram-positive and gram-negative bacteria. DMSO shows no zone of inhibition (ZI).

All the results are mean±SD (n=3). The antibacterial activity of APEE and APME may be attributed to TTC and TFC in APEE and RME. Flavonoids and phenolic compounds are recognized for their role in protecting plants against microbial attack [30]. Tannins also act as astringents and have the ability to bind and precipitate and shrink proteins [34]. This ability of tannins directly damages the bacterial cell wall and precipitate proteins killing the bacteria [35, 36]. From the results of ZI depicted in **Table 3** and from the **Fig. 5** which compares the ZI formed by REE and RME against different bacteria, it is deemed fit to state that REE has better antibacterial activity than RME.

TABLE 3: ZONE OF INHIBITION (ZI) OF THE EXTRACTS (APEE AND APME) AGAINST DIFFERENT GRAM NEGATIVE AND GRAM POSITIVE BACTERIA

Test Organisms	APEE(µg/ml) ZI±S.D (mm)						APME(µg/ml) ZI±S.D (mm)					
	Concentration (µg/ml)											
	Standard (5µg/ml)	10	100	250	500	1000	Standard (5µg/ml)	10	100	250	500	1000
E. coli	18±0.577	5±0.232	8±0.179	12±0.545	15±0.318	17±0.253	18±0.225	4±0.179	6±0.325	12±0.291	14±0.381	16±0.485
P.aeruginosa	19±0.623	4±1.332	6±0.265	8±0.21	9±0.229	12±0.789	19±0.623	4±0.21	5±0.212	6±0.296	8±0.288	10±0.568
S. aureus	17±0.232	5±0.323	9±0.364	13±0.598	14±0.558	15±0.553	17±0.364	3±0.359	8±0.206	12±0.402	13±0.577	14±1.015
B. subtilis	16±1.23	4±0.623	6±0.485	7±0.307	8±0.615	10±0.458	16±0.485	4±0.489	5±0.223	7±0.525	8±0.750	10±0.5

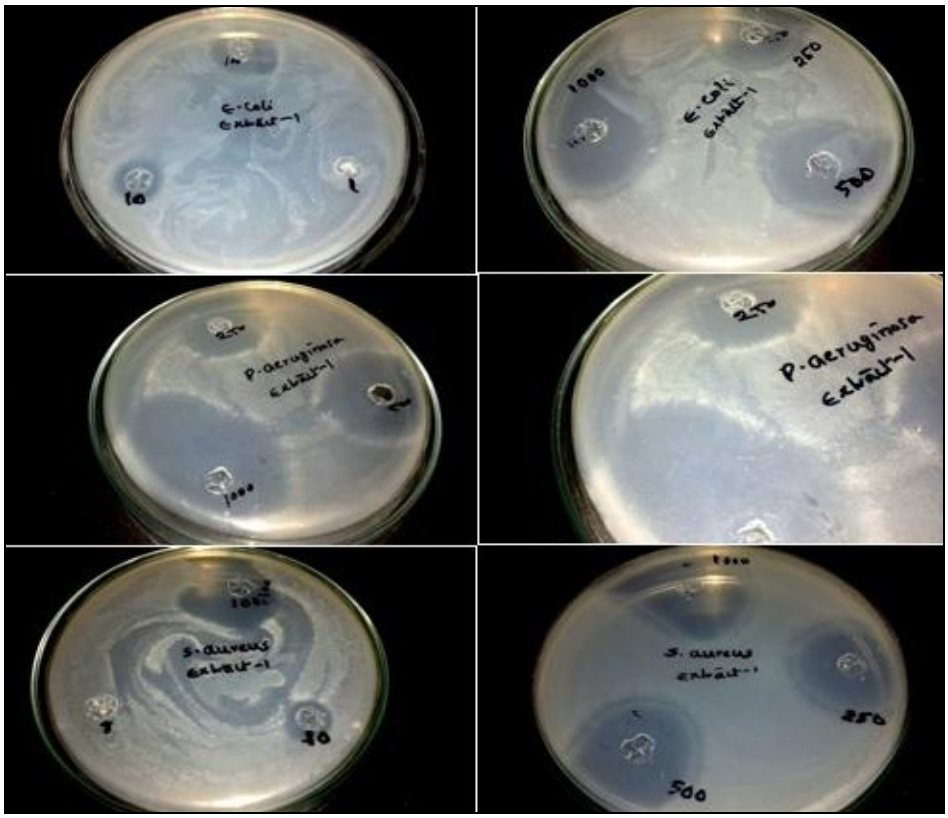

FIG. 6: ANTIBACTERIAL ACTIVITY OF APEE AND APME AGAINST E*SCHERICHIA COLI, PSEUDOMONAS AERUGINOSA, STREPTOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIDIS*

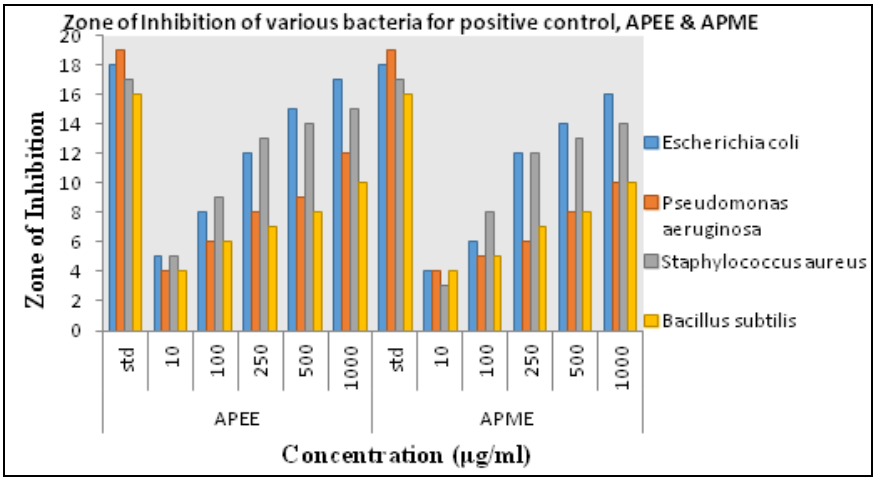

FIG. 7: ZONE OF INHIBITION OF VARIOUS BACTERIA FOR STANDARD, APEE AND APME

CONCLUSION: Whole plant of *A. pilosa* was extracted with ethyl acetate and methanol to obtain APEE and APME. Both APEE and APME was subjected to phytochemical screening and it was found that both contain flavonoids, alkaloids, tannins, glycosides, proteins and amino acids except for steroids and acid compounds which is only present in APME. TTC and TFC is more in APEE than APME. Both APEE and APME show antioxidant and antibacterial activity, APEE is claimed to have more antibacterial and antioxidant potency than APME.

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