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Antimicrobial, Antioxidant and Phytochemical Potential of *Alternanthera pungens* HB&K

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Abstract:

Aim:

The present study was undertaken to explore the medicinal potential of *Alternanthera pungens* by investigating its antimicrobial, antioxidant activities and phytochemical properties.

Methodology:

Crude extracts of *A. pungens* were prepared in water, acetone, ethanol and petroleum ether by soxhlet apparatus. In vitro antimicrobial activity was assessed against six bacterial strains - *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Mycobacterium smegmatis, Bacillus subtilis* and *Chromobacterium violaceum* and against four fungal strains - *Aspergillus fumigatus, A. niger, Fusarium oxysporum* and *Penicillium expansum* using disc diffusion assay. Antioxidant potential of the plant extracts was evaluated by DPPH radical scavenging assay and correlated with total phenolic and flavonoid content.

Results:

The crude plant extracts showed a wide range of antibacterial activity against different bacterial strains with maximum zone of inhibition (18.6±1.50 mm) against *Mycobacterium smegmatis* with acetone extract (200 mg/ml conc.). Antifungal activity was observed only against *Aspergillus fumigatus* with acetone and aqueous plant extracts. Out of the four plant extracts, ethanolic extract exhibited highest antioxidant activity and minimum IC 50 value (100.79 µg/ml) followed by acetone (IC 50 value 203.56 µg/ml), aqueous (IC 50 value 324.43 µg/ml) and petroleum ether extracts (IC 50 value 931.63 µg/ml) and this correlated positively with total phenolic and flavonoid content of the extracts.

Conclusion:

Plant extracts showed a wide spectrum of inhibition against the bacterial strains. However, the antifungal activity was observed only against *Aspergillus fumigatus*. The plant extracts also revealed good antioxidant potential being rich source of bioactive compounds, thus justifying the use of plant in traditional medicine.

Keywords: DPPH assay, Disc diffusion method, Phenols, Flavonoids, Minimum inhibitory concentration, Phytochemicals

1. INTRODUCTION:

Plants were the first weapon used by man against different types of health ailments as they are the largest storehouse of the biochemical compounds. With the advancement of organic chemistry and modern technology man shifted from the traditional system towards synthetic drugs for healthcare needs. This was mainly because pure compounds were easily obtained and structurally modified to produce potentially more active and safer drugs. The synthetic drugs were preferred as there were many issues associated with the natural products like lack of authentication from legal authorities and there is also concern about the lack of validity of phytochemical products. Moreover, raw drugs from natural resources were mostly used by people from low income groups and poorly educated or due to superstition (1).

However, even with amazing advancement in science, technology and medicine, we have not been able to control the spread of infectious diseases. In fact, microbes are becoming more and more resistant to present day drugs as they possess extraordinary adaptive ability. Hence, again lot of interest has been generated in traditional and folk lore medicine as scientists consider the phytocompounds as effective therapeutic aid. Interestingly man is again moving towards nature and traditional ways from modernization to fulfill their healthcare needs. Due to this growing interest in herbal medicine, more and more plants have been explored and encoded as medicinal plants as they possess bioactive compounds. Nearly 6 % of the higher plant species available on the planet earth (approximately 2, 50,000) have been explored for their biological activity and a major fraction of plants still remains to be investigated for their beneficial properties (2).

As weeds are inexpensive sources of material, so to develop cost-effective products from weeds may be a method of choice to manage the weeds. This can also help to reduce the disturbance of weeds to other economical crops. Of special interest are the wild plants or weeds which are always looked down upon due to their nuisance value. It is believed that the losses due to the weeds to the cultivated crops are more than either diseases or insects (3). As weeds are resistant to microbial attack as compared to crops, it will be interesting to investigate weed extracts as antimicrobial agents [4,5]. Weeds have been used in traditional medicine in different parts of the world for curative and health care purposes. There are evidences to support that weeds contain bioactive phytochemicals with antioxidant and antimicrobial activities [6-9]. Hence, in the interest of the mankind, it is important that the fatalities by weeds should be remunerated by exploring their remedial efficacy especially in health care (10, 11). Keeping this in view, the present investigation was planned to study the medicinal potential of *Alternanthera pungens* HB&K commonly known as khaki weed which belongs to botanical family amranthaceae (Fig. 1). Though it is native of central and South America but is also reported from other tropical countries including India (12). It is a perennial herb and commonly noticed as a mat like structure in vacant lots, along roadside, railway tracks, lawns, etc. Its stem is hairy, 10-50 cm long, prostrate and

occasionally develop roots the nodes. Leaves are green and ovate to obovate in shape and are generally 0.5 to 4.5 cm long and 0.3 to 2 cm wide. Flowers are without stalk, sparsely velvety spikes with spiny bracts and bracteoles (13). In traditional medicine it was used as painkiller, for stomachache, swelling and nasopharyngeal infections and also reported for lactation stimulus in veterinary (14).

Fig. 1 - *A. pungens* grown at various places of Rohtak and surrounding areas

2. MATERIALS AND METHODS:

2.1. PREPARATION OF CRUDE EXTRACT:

Fresh aerial parts of *Alternanthera pungens* were collected from Rohtak district, Haryana, washed with tap water and again with distilled water. The plant material was then shade dried at room temperature for about 7 days and kept in an oven at 35°C for about 2-3 days for complete drying. The dried material was grinded with the help of a mixer grinder to make a fine powder which was stored in air tight containers at 4°C. The powdered plant material was extracted with the help of Soxhlet apparatus in four different solvents i.e. water, acetone, ethanol and petroleum ether (1:5 W/V) for 2-3 days till the solvent becomes colorless. The solvent was evaporated to dryness till the slurry was obtained. After this, the extract was weighed and stored at 4°C till further use.

2.2. IN VITRO ANTIMICROBIAL ACTIVITY:

Antimicrobial activity of crude plants extracts was evaluated by using disc diffusion method and minimum inhibitory concentration was determined by microbroth dilution assay. Three Gram negative bacterial strains namely *Klebsiella pneumoniae* (MTCC NO 109), *Pseudomonas aeruginosa* (MTCC NO 2453), *Chromobacterium violaceum* (MTCC NO 2656) and three Gram positive bacterial strains namely *Staphylococcus aureus* (MTCC NO 96), *Mycobacterium smegmatis* (MTCC NO 992) and *Bacillus subtilis* (MTCC NO 2057) and four fungal strains i.e. *Aspergillus fumigatus* (MTCC NO 3002), *A. niger* (MTCC NO 514), *Fusarium oxysporum* (MTCC NO 7392) and *Penicillium expansum* (MTCC NO 2818) were used in the investigation. Bacterial cultures were maintained in the culture tubes containing 10 ml of nutrient agar (NA) medium at 37°C. *Aspergillus fumigatus* and *A. niger* were maintained in the czapek dox agar and *Fusarium oxysporum* and *Penicillium expansum* were maintained in potato dextrose agar medium at 28°C. All the cultures were sub cultured monthly on a regular basis and stored at 4°C.

2.2.1. Disc diffusion assay:

Disc diffusion assay was performed for crude plant extracts as per standard method against all the six bacterial strains and four fungal strains. Four concentrations (200 mg/ml, 100 mg/ml, 50 mg/ml and 25 mg/ml) were prepared for each crude plant extract by reconstituting the plant material with the respective solvents. The bacterial strains (approximately 1.5×10^8 CFU/ml) were seeded separately into the nutrient agar (NA) medium in sterilized Petri Plates. Sterilized whatman filter paper discs (6 mm in diameter) soaked with the plant extract was placed on to the medium separately. Standard disc of ampicillin and solvent were used as positive and negative control, respectively. Petri Plates were then incubated at 37°C for 24 h and the diameter of the inhibition zone obtained was measured. The assay was carried out in triplicate for each extract and mean value was recorded. For antifungal activity, czapek dox agar was used for *Aspergillus fumigatus* and *A. niger* and potato dextrose agar was used for *Fusarium oxysporum* and *Penicillium expansum.* Miconazole was used as positive control and Petri plates were incubated at 28°C for three days.

2.2.2 Minimal inhibitory concentration (MIC):

MIC was determined by micro broth dilution technique using serially diluted (2 fold) plant extract as per the method of Sarkar et al., (2007) with slight modifications [15]. 0.1 ml of each plant extract and nutrient broth were added to the wells of a micro titer plate 0.01 ml of standardized inoculums and resazurin sodium salt indicator were also added into each well and incubated at 37°C for 24 h. Minimum concentration (highest dilution) of the extract that showed no color change (purple to pink) was considered as MIC for that particular bacterial strain. For antifungal activity, czapek dox broth was used for *Aspergillus fumigatus* and *A. niger* and potato dextrose broth was used for *Fusarium oxysporum* and *Penicillium expansum.* Plates were incubated at 28°C for three days and results were observed by visual identification of fungal growth.

2.3. ANTIOXIDANT ACTIVITY:

Antioxidant activity of the crude extracts was calculated by DPPH (1, 1-diphenyl-2 picrylhydrazyl) radical scavenging assay [16]. 2.5 ml of DPPH solution (0.5 mM) was added to 1 ml of plant extract (100 µg/ml to 1000 µg/ml) and incubated in dark. The absorbance was measured at 517 nm after 30 minutes. Ascorbic acid was used as standard. Experiments were carried out in triplicates for each assay.

Percentage scavenging activity for each extract was calculated as:

% Inhibition = (A control - A sample)/ A control× 100

2.4. PHYTOCHEMICAL SCREENING:

Phytochemical screening of the crude extracts was carried out for the presence of phenols, flavonoids, alkaloids, saponins, glycosides, steroids and tannins as per the standard methods [17]. Total phenolic content (TPC) and

total flavonoid content (TFC) was determined as per the method of Aiyegroro and Okoh, (2010) with some modifications [18].

2.5. STATISTICAL ANALYSIS:

Pearson correlation coefficient test was performed between IC 50 values and TPC and between TPC and TFC by using PAST3 software. Standard deviation was also determined for inhibition zone and MIC values of plant extracts.

3. RESULTS AND DISCUSSION:

Alternanthera is a large genus of family amranthaceae with nearly 137 species [19]. Though, many species of this genus have been investigated for their medicinal properties [21-24], but scanty information is available in literature about species *pungens.* Hence efforts were made in the present investigation to understand its antimicrobial, antioxidant and phytochemical properties. The results obtained are described and discussed below.

3.1 ANTIMICROBIAL ACTIVITY:

All the four crude extracts prepared from the aerial parts of *A. pungens* possess promising antibacterial activity. Among the four concentrations of plant extracts, significant observations were made with 200 mg/ml concentration; therefore, results obtained with this concentration are compared among different solvents.

The zone of inhibition obtained with the different crude extract against bacterial strains used is given in Table 1. The crude extracts were specific in action against test bacterial strains, signifying the presence of antimicrobial compounds in this plant. On contrary to our results, Zongo et al., (2011) observed weak antimicrobial and antioxidant activity of this plant [20].

Mycobacterium smegmatis (18.60±1.5 mm), *Staphylococcus aureus* (9.60±0.57 mm) and *Bacillus subtilis* (7.60±0.50 mm) revealed susceptibility with acetone extract. All the three Gram negative strains were found to be resistant to this crude extract as no activity was obtained with these strains in disc diffusion assay.

Aqueous extract revealed maximum inhibition zone 15.66±2.08 mm against *Bacillus subtilis* trailed by *M. smegmatis* (8.60±0.57 mm). Least activity was observed against *K. pneumoniae* and *P. aeruginosa* (7.0±0 mm) with this extract. Although the extract showed activity against both Gram positive and Gram negative bacteria, the extract was found to be more effective against Gram positive strains. Similarly, Kumari and Krishnan (2016) reported antibacterial activity of the aqueous extracts of two species of *Alternanthera* i.e. *sessilis* and *philoxeroides* against both Gram positive and Gram negative organisms [21].

Ethanolic extract revealed maximum zone of inhibition (11.60±0.57 mm) against *M. smegmatis* followed by *C. violaceum* (10.60±0.57 mm) and *K. pneumoniae* (10.30±0.57 mm). The extract was almost ineffective against three bacterial strains i.e. *P. aeruginosa, S. aureus* and *B. subtilis* exhibited least inhibition zone (7.0±0 mm). Both Gram positive and Gram negative strains were found to be sensitive to ethanolic extract though to variable degrees as compared to aqueous extract. Similar to our results, ethanolic extract was reported to be ineffective against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* by Gupta et al., 2010 [22]. Studies carried out with *A. sessilis* also showed that *B. subtilis* and *K. pneumoniae* to be resistant to ethanolic and aqueous extracts [23]. Sivakumar and Sunmathi (2016) revealed that both Gram positive and Gram negative bacterial strains were susceptible to ethanolic extract of two species of *Alternanthera* i.e. *A. sessilis* and *A. philoxeroides* [24].

When the petroleum ether extract was evaluated for its antibacterial potential, maximum inhibition zone was observed against *Chromobacterium violaceum* (9.0±1.0 mm) followed by *Staphylococcus aureus* (8.30±0.57 mm). Minimum zone of inhibition was observed against *Pseudomonas aeruginosa* (7.0±0 mm) with this extract.

The positive control i.e. Ampicillin exhibited greater zone of inhibition when compared to all the plant extracts. This is probably due to the fact that crude plant extracts contains a number of impurities which may be inert and devoid of antibacterial activities. Ampicillin, is a pure chemical compound, and hence produces larger zone of inhibition even at a very low concentrations [25].

Table 1- Inhibition zone against six bacterial strains with extracts of *Alternanthera pungens* observed by disc diffusion assay.

Plant extracts (200 mg/ml) /Antibiotic (100 µg/ml)	Zone of inhibition diameter (mm)					
	Bacterial strains used					
	Klebsiella pneumoniae	***Pseudomonas aeruginosa***	***Staphylococcus aureus***	***Mycobacterium smegmatis***	***Bacillus subtilis***	***Chromobacterium violaceum***
Acetone extract	-	-	9.60±0.57	18.60±1.50	7.60±0.50	-
Aqueous extract	7.0±0	7.0±0	-	8.60±0.57	15.66±2.08	-
Ethanolic extract	10.30±0.57	7.0±0	7.0±0	11.60±0.57	7.0±0	10.60±0.57
Petroleum ether extract	-	7.0±0	8.30±0.57	-	-	9.0±1.0
Ampicillin	24.33±2.51	22.0±4.0	15.33±1.52	28.33±1.52	24.33±1.52	22.33±1.52

All values are mean±SD

MIC was performed by microbroth dilution assay only for those extracts that revealed inhibition zone with disc diffusion assay. Inhibition zone and MIC values obtained were contrarily related to each other which showed crude extracts with highest inhibition zone has least MIC value. The results are given below in table 2.

Table 2- MIC values against six bacterial strains with crude extracts of *A. pungens.*

Bacterial strains	Extract	MIC Value (mg)
Klebsiella pneumoniae	Aqueous	5.0±0
	Ethanol	1.60±0.72
Pseudomonas aeruginosa	Aqueous	5.0±0
	Ethanol	3.33±1.40
	Petroleum ether	5.0±0
Staphylococcus aureus	Acetone	0.51±0.17
	Ethanol	5.0±0
	Petroleum ether	1.66±0.72
Mycobacterium smegmatis	Acetone	0.41±0.17
	Aqueous	1.66±0.72
	Ethanol	0.51±0.17
Bacillus subtilis	Acetone	3.33±1.40
	Aqueous	1.04±0.36
	Ethanol	5.0±0
Chromobacterium violaceum	Ethanol	0.83±0.36
	Petroleum Ether	1.04±0.36

The MIC value for different extracts ranged between 0.41±0.17 mg to 5.0±0 mg. For *Klebsiella pneumoniae* minimum MIC value was observed with ethanolic extract (1.60±0.72 mg) followed by aqueous extract (5.0±0 mg). MIC observed for *Pseudomonas aeruginosa* with ethanol extract was minimum (3.33±1.40 mg). Similar MIC value was observed with aqueous and petroleum ether extract (5.0±0 mg). For *Staphylococcus aureus*, MIC value (0.51±0.17 mg) acetone extract was found to be lower than the petroleum ether extract (1.04±0.36 mg) and ethanolic extract (5.0±0 mg). Acetone extract also revealed least MIC value against *Mycobacterium smegmatis* (0.41±0.17 mg) as compared to the ethanol extract (0.51±0.17 mg) and aqueous (0.83±0.36 mg) extract. MIC values of aqueous extract, acetone extract and ethanolic extract with *Bacillus subtilis* were 1.04±0.36, 3.33±1.40 and 5.0±0 mg respectively. Minimum MIC value 0.20±0.09 mg was reported against *Chromobacterium violaceum* with acetone extract followed by ethanol extract (0.62±0 mg) and petroleum ether extract (1.25±0 mg). Ethanol extract exhibited MIC value of 0.83±0.36 mg for *Chromobacterium violaceum* followed by petroleum ether extract (1.04±0.36 mg).

All crude extracts prepared from the aerial parts of *A. pungens* possess weak antifungal activity and the results obtained are given in Table 3. *A. niger, F. oxysporum* and *P. expansum* were found to be resistant against all the four crude extracts. *A. fumigatus* was inhibited by acetone extract and aqueous extract with zone of inhibition 9.66±0.57 mm and 7.66±0.57 mm respectively. Ethanolic and petroleum ether extract were found to be ineffective against *A. fumigatus.* Sivakumar and Sunmathi in 2016 reported antifungal activity of two species of *Alternanthera* i.e. *sessilis* and *philoxeroides* against *Candida albicans* (24). Antifungal activity of *Alternanthera philoxeroides* were also found to be against *Aspergillus niger, Aspergillus flavus* and *Trichoderma viridae* (26). The MIC value obtained for *Aspergillus fumigatus* with acetone and aqueous extracts were 1.45±0.95 and 1.66±0.72 mg respectively. The results are shown in Table 4.

Table 3- Inhibition zone against four fungal strains with extracts of *Alternanthera pungens* observed by disc diffusion assay.

Plant extracts (200 mg/ml) /Antibiotic (100 µg/ml)	Zone of inhibition diameter (mm)			
	Fungal strains used			
	Aspergillus fumigatus	***Aspergillus niger***	***Fusarium oxysporum***	***Penicillium expansum***
Acetone extract	9.66±0.57	-	-	-
Aqueous extract	7.66±0.57	-	-	-
Ethanolic extract	-	-	-	-
Petroleum ether extract	-	-	-	-
Miconazole	18.33±1.52	15.0±4.58	8.33±0.57	24.33±2.51

All values are mean±SD

Table 4- MIC values against *Aspergillus fumigatus* with crude extracts of *A. pungens.*

Fungal strains	Extract	MIC Value (mg)
Aspergillus fumigatus	Acetone extract	1.45±0.95
Aspergillus fumigatus	Aqueous extract	1.66±0.72

3.2 ANTIOXIDANT ACTIVITY OF PLANT EXTRACTS

The crude extracts of *A. pungens* were investigated for their free radical scavenging activity by DPPH assay. The antioxidant potential of the extracts was found to be concentration dependent (Fig. 2). The results obtained are displayed as IC 50 values which ranged between 100.79 µg/ml to 931.63 µg/ml (Fig. 3). Minimum IC 50 value (100.79 µg/ml) was observed with ethanolic extract followed by acetone (IC 50 value 203.56 µg/ml), aqueous (IC 50 value 324.43 µg/ml) and petroleum ether extracts (IC 50 value 931.63 µg/ml). The study assured free radical scavenging efficiency of *A. pungens* to a firm level. Though, the free radical scavenging efficacy varied with solvents used for extraction. Based on the results obtained, we observed that polar solvents are more effective antioxidants as compared to the non polar petroleum ether crude extract. This is because secondary metabolites are more soluble in polar solvents.

3.3 PHYTOCHEMICAL ANALYSIS

Plant acts as a natural repository of a wide spectrum of bioactive compounds which are responsible for their biological properties. Moreover these compounds are unique sources for food additives, flavors, and other industrial materials [27]. These metabolites have enormous therapeutic potential and are effective in treatment of various diseases also mitigate the side effects often associated with the synthetic drugs (28). The extracts of *A. pungens* prepared in four different solvents were screened for the presence of different phytochemical. The results obtained are given in Table 5. Crude extracts of *A. pungens* contains phenols, flavonoids, alkaloids, glycosides, steroids, saponins and tannins. Several studies have linked the biological activities of plants to phenolic compounds that they contain. There is now a strong consensus that phenolics and flavonoids are responsible for much of the antioxidant activity of plants [29-31].

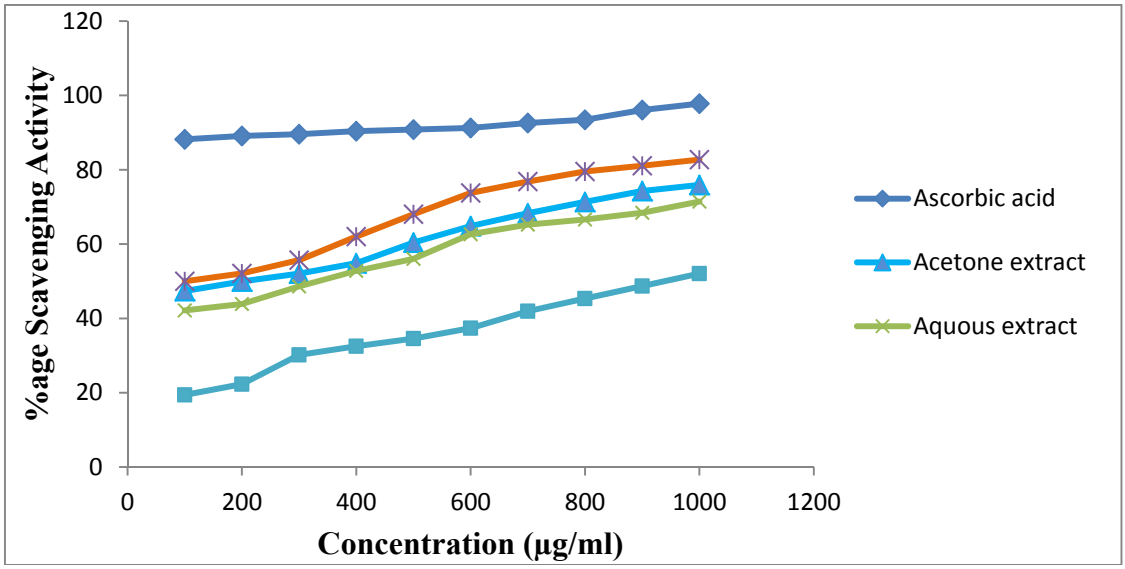

Fig. 2. %age scavenging activity obtained against different concentrations of *A. pungens* extracts prepared in different solvents and Ascorbic acid.

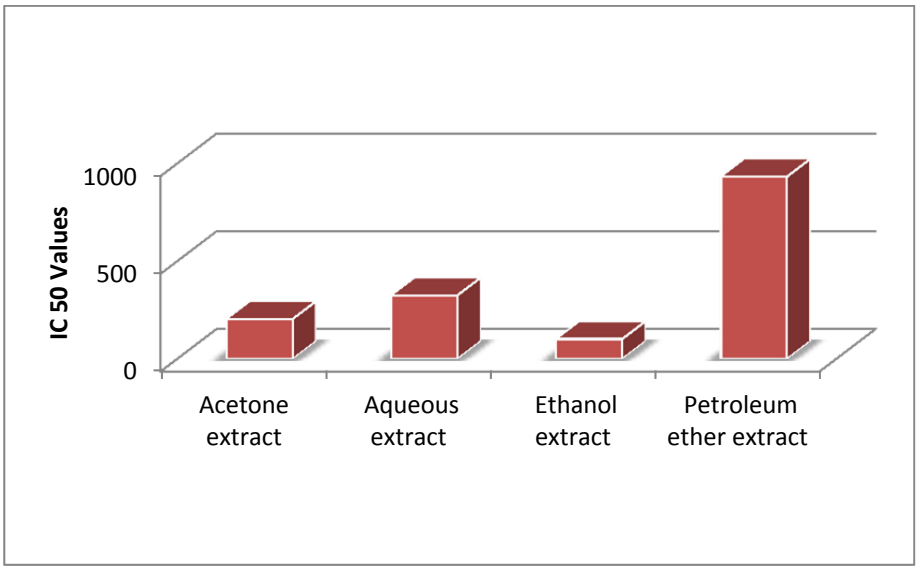

Fig. 3. IC 50 values obtained with different extracts of *A. pungens*

Table 5- Phytochemical analysis of different extracts of *A. pungens*

Phytochemical	Crude plant extracts			
	Acetone	Aqueous	Ethanol	Petroleum ether
Alkaloids	-	+	+	-
Phenols	++	+	+	+
Flavonoids	++	++	++	+
Glycosides	+	-	+	-
Tannins	+	+	-	+
Saponins	+	-	+	-
Steroids	+	+	+	-

Presence: +, absence: -

Total Phenolic Content reported as Gallic acid equivalents varied widely, ranging from 12.11 mgGAE/g obtained with ethanolic extract to 2.55 mgGAE/g obtained with petroleum ether extract (Fig. 4).

Similar to phenolic content, flavonoid content was also highest in ethanolic extract i.e. 9.23 mgQE/g and minimum in petroleum ether extract (2.07 mgQE/g) (Fig. 5). All values were Quercetin equivalents.

Correlation coefficient between IC 50 values and total phenolic content of different extracts was recorded as -0.844 which revealed a negative correlation between them. On the other hand, correlation coefficient between total phenolic content and total flavonoids content (0.967) showed that these two variables are positively co-related.

Similar to *pungens* other species of *Alternanthera* have been reported to possess antioxidant properties and this is mainly due to the presence of phenolic and flavonoids. Ethanolic extract was found to be exhibited highest percentage scavenging activity and total phenolic and flavonoid content of the ethanolic extract was significantly high and this is correlated with highest percentage scavenging activity. As not much information is available in literature with regards to the *A. pungens* results are discussed and compared with the other species of *Alternanthera*. Similarly, Borah et al (2011) investigated total phenolic and flavonoid content and correlated it with the antioxidant potential of *Alternanthera sessilis* and concluded that these secondary metabolites are liable for its antioxidant potential [32]. Murugan et al (2013) also reported antioxidant activity of *Alternanthera sessilis* [33]. *Alternanthera bettizicikiana* has also been shown to possess good antioxidant activity [34]. Hence other plant species of genus *Alternanthera, A. pungens* also attributed with various biological properties including antibacterial and antioxidant potential.

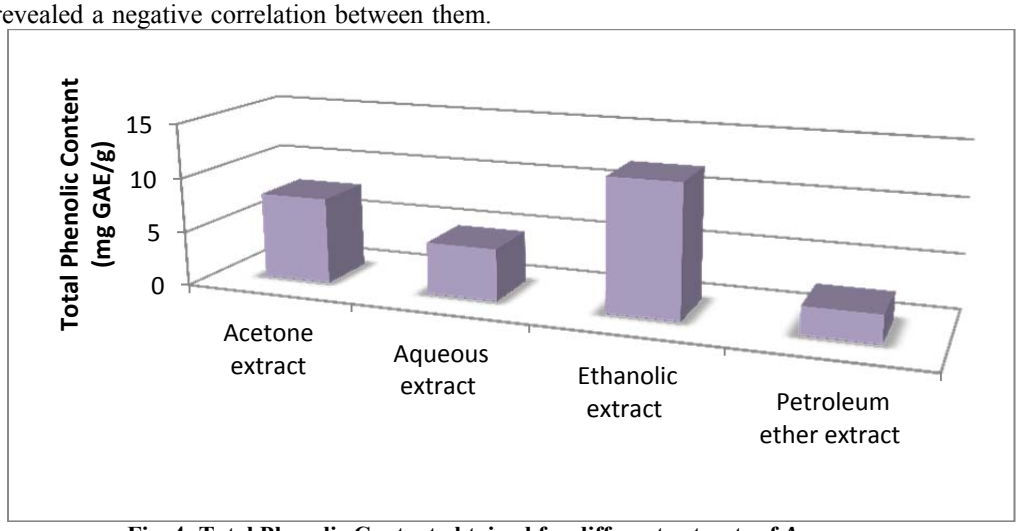

Fig. 4: Total Phenolic Content obtained for different extracts of *A. pungens*

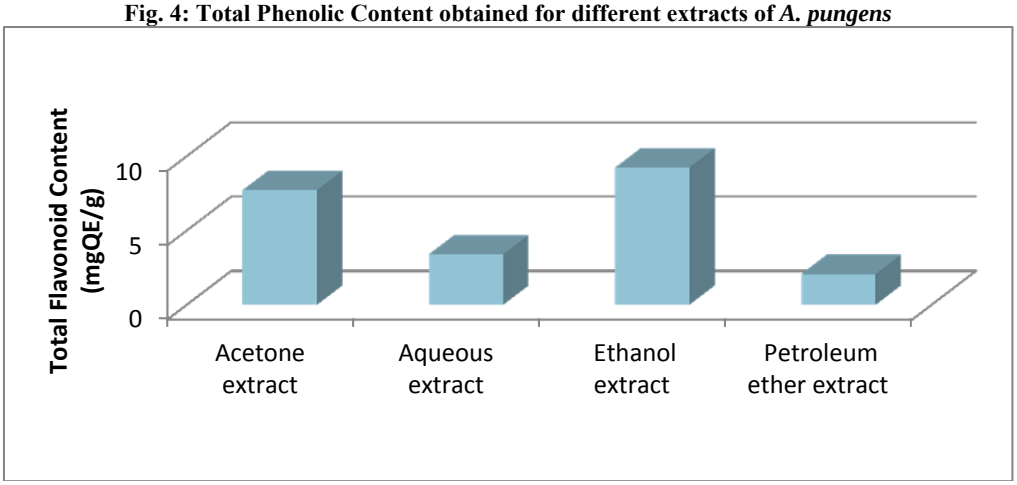

Fig. 5. Total Flavonoid Content obtained from different extracts of *A. pungens*

4. CONCLUSION

The present in vitro study revealed that crude extracts of *Alternanthera pungens* inhibits some particular bacterial strains effectively. The zone of inhibition with diameter of more than 10 mm was observed against *Klebsiella pneumoniae* (with ethanolic extract), *Mycobacterium smegmatis* (with acetone and ethanolic extract), *Bacillus subtilis* (with aqueous extract) and *Chromobacterium violaceum* (with acetone extract). Plant showed poor antifungal activity as reported only against *Aspergillus fumigatus* with acetone and aqueous extracts. Ethanolic extract was found to possess better antioxidant activity by DPPH radical scavenging activity followed by petroleum ether and aqueous extracts. Total phenolic content and total flavonoids content of different extracts, exhibits a significant relation with scavenging activity of respective extracts. The present study revealed the medicinal potential of plant and ascertains the value of this plant in development of new herbal drugs. However the plant needs more detailed investigation with ethno-pharmacological approach are required for its better use in therapeutics.

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