In vitro Assessment of Antibacterial and Antioxidant Activity of *Rhizophora apiculata* leaf extracts

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Abstract

In the present scenario, in the field of human disease management, there is a necessity to substitute the antibiotics with better alternatives so as to overcome the problem of multidrug resistance (MDR). Herbal drugs can best serve the purpose as they are being used as the traditional remedy for various ailments. Mangrove plants are being used in the modern medicine as they possess varied phytochemicals with therapeutic value. The phytochemicals in the leaves of Rhizophora apiculata were extracted into petroleum ether, ethyl acetate, methanol and double distilled water through Soxhlet extraction. The antibacterial and antioxidant activity of the leaf extracts was assessed by agar well diffusion, DPPH and FRAP assays respectively.

The leaf extracts of R. apiculata showed the range of inhibitory activity against P.aeruginosa 3.4±0.9 to 4.6±0.5; A.hydrophila 3.9±0.8 to 6.1±1.1; S. aureus 2.4±1.3 to 3.6±1.0, B.subtilis 1.8±0.7 to 3.1±0.7, Antioxidant activity; FRAP 123.6±0.9 to 148±1.1 and DPPH 72.3±0.9 to 83.1±1.4. The aqueous leaf extract of R.apiculata exhibited maximum potency for antibacterial, Pseudomonas aeruginosa (4.6±0.5mm), Aeromonas hydrophila (6.1±1.1mm), Staphylococcus aureus (3.6±1.0mm), Bacillus subtilis (3.1±0.7mm), antioxidant activity, DPPH 83.1±1.4 and FRAP 148±1.1AAEµg/ml.

Keywords: Mangroves, *Rhizophora apiculata*, Phytochemical screening, Anti-bacterial activity, FRAP, DPPH, TPC and TFC.

Introduction

Antibiotics are one of the commonly used therapeutic agents against various microbial infections. Continuous and indiscriminate usage of antibiotics has led to the development of resistance in pathogens and reduced antimicrobial efficiency thereby posing high health risks to the individuals[5].

Over the past three decades, multidrug resistance (MDR) has become a major challenge in treating infectious diseases. The long term usage of the antibiotics imposes negative effects on the host physiological processes by perturbation of microbiota that further results in inflammation, diarrhea and allergies etc.[11] As a result, development of the novel antimicrobial agents in the place of antibiotics is the need of the hour.

Herbal extracts are one of the best alternatives. The herbal extracts were used as holistic medicine from the ancient times in treating various human and animal diseases. According to WHO, nearly 80% of the world has been using phytotherapy[20]. Natural products constitute 50% of the modern panacea as they supply bioactive compounds that are potent to hinder the growth of multidrug resistant pathogens. The herbal drugs which are also known as ideal antibiotics, not only kill the pathogens but also affect their cellular pathways thereby impeding the development of anti-microbial resistance[28].

Secondary metabolites of the plants are a heterogeneous group of compounds that exhibit therapeutic properties. These compounds involve several ways to inhibit the microbial growth *viz.* disrupting the structure and function of cell membrane, interrupting quorum sensing and synthesis of DNA, RNA and interfering with the intermediary metabolism[21]. They also act as anti-inflammatory, anti-carcinogenic, anti-helminthic, anti-mutagenic, anti-microbial, anti-proliferative, antioxidant and anti-genotoxic agents[27,36].

Mangroves are one of the vast, diversified ecosystems in the world, escalate nearly one fourth of the world's coastline and are salt tolerant plants that were distributed in the tropical and subtropical regions. Mangrove plant extracts are ecofriendly, nontoxic and can efficiently inhibit the pathogenicity of animal and human pathogens[32]. The phytochemicals (alkaloids, tannins, saponins, phenols, terpenoids, steroids, glycosides, flavonols) of these plant species possess antibacterial, antifungal, antiviral, antiparasitic, anti-inflammatory, antioxidant, anti-tumor, antidiabetic, antifeedant, insecticidal, anti-diarrheal, cytotoxic properties. The extracts from the leaves, roots, flowers, stem, bark, fruits are capable of treating various ailments efficiently[22]. *Bruguiera, Avicennia, Acanthus, Ceriops, Excoecaria, Rhizophora, Lumnitzera, Suaeda* were reported to have antimicrobial properties[8,12,29].

Rhizophora apiculata is a perennial mangrove plant, commonly known as Uppu ponna or kaaki ponna of family Rhizophoraceae and grow up to 2m with elliptical, smooth, leathery and dark green leaves. The extracts of the plant possess anti-bacterial, anti-fungal, anti-viral, anti-cancer, anti-malarial and anti-oxidant properties[25]. The present

study is confined to assess the antibacterial and antioxidant activity of *R. apiculata* leaf extracts.

Material and Methods

Chemical reagents: Petroleum ether, ethyl acetate, methanol, double distilled water, Mueller Hinton agar (MHA) media, ascorbic acid, oxytetracycline, ferric chloride, hydrochloric acid, 2,4,6-tri (2-pyridyl-s-triazine) (TPTZ), folin-ciocalteu reagent, aluminium chloride used in the present study were purchased from Merck.

Collection of plant material and extraction: The leaves of *R. apiculata* were collected from the Gilakaladindi mangrove forest (16.14° N, 81.16° E), 6km East to Machilipatnam, Andhra Pradesh, India. The plant species was confirmed by the taxonomist in the Department of Botany, Acharya Nagarjuna University, Guntur, India. The leaves were washed, dried under the shade, pulverized to fine powder and mixed with petroleum ether, ethyl acetate, methanol and double distilled water in Soxhlet apparatus to prepare extracts. The concentrated leaf extracts were used for the phytochemical screening, antibacterial, antioxidant activity, total phenol and flavonoid content.

Qualitative analysis of phytochemicals: The standard protocols were adopted for the qualitative screening of phytochemicals[35] *viz.* saponins (Foam test), tannins (Braymer's test), flavonoid (Alkaline reagent test), steroid (Liebermann-Buchard test), terpenoids (Salkowski's test), glycosides (Keller-Killiani's Test), carbohydrates (Fehling's test), proteins (Ninhydrin test), phytosterols (Liebermann-Buchard test), cardiac glycosides (Keller-Killiani's Test), alkaloids (Dragendroff's test), phenols (Ferric chloride test), reducing sugars (Fehling's test) and anthraquinones (Borntrager's test).

Antibacterial assay: The leaf extracts were assessed against *Pseudomonas aeruginosa* (MTCC1934), *Aeromonas hydrophila* (MTCC1739), *Staphylococcus aureus* (MTCC1430) and *Bacillus subtilis* (MTCC121) by agar well diffusion method[8]. 100 µl of bacterial strains were inoculated and spread on solidified Mueller Hinton agar (MHA) media. The wells of known diameter were loaded with 40µl of leaf extracts and antibiotic oxytetracycline (20µg/ml) was used as the positive control. The plates were incubated at 37^{0}C for 24 hr and zones of inhibition (mm) were measured.

Total phenol Content (TPC) and Total flavonoid content (TFC): For TPC, Folin-Ciocalteu protocol[3] was followed. 1ml of 0.5N folin-ciocalteu reagent was added to 250µl of the sample and undisturbed for 5 min. To this, 1.5ml of 20% (w/v) sodium carbonate solution was added, vortexed and incubated for 90min at 37^{0}C. The absorbance was noted at 760nm. TPC of the leaf extract was presented as mg GAE/g DW (mg gallic acid equivalent per gram dry weight). TFC was determined by aluminum chloride colorimetric method[18]. 250 µl of the extract was mixed with 2% methonolic aluminum chloride (250 µl) incubated for 45 minutes at room temperature and the absorbance was measured at 430nm in UV-vis spectrophotometer. TFC was expressed as mg rutin equivalent per gm of body weight (mgRE/g DW).

Antioxidant assay

FRAP assay: The antioxidant activity was assessed by the ferric reducing antioxidant power (FRAP) assay[6]. The FRAP reagent was prepared by mixing 300mM of acetate buffer, 10mM of 2,4,6-tri (2-pyridyl-s-triazine) (TPTZ) solution in HCl (40mM) and $FeCl_3$ (20mM) solution in 10:1:1 (v/v) respectively. To the FRAP reagent, 15.62, 31.25, 62.5, 125, 250, 500 µg/ml concentrations of leaf extracts were added, incubated for 15 minutes at 37^{0}C and the absorbance of the solution was measured using UV-Vis spectrophotometer at 593nm. Ascorbic acid was used as a standard and the results were noted as µg of ascorbic acid equivalents (AAE) per ml.

DPPH assay: To carry out the DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical scavenging assay[9], reaction mixture constituting 5ml of DPPH solution and leaf extract at varied concentrations (31.25, 62.5, 125,250, 500 µg/ml) was prepared and incubated in dark for 30min following which the absorbance of the coloured solution was measured at 517nm. Methanol and ascorbic acid were used as the blank and standard respectively. The scavenging activity was measured by:

$$\%\ \text{DPPH radical scavenging activity} = \left[\frac{(A0 - A1)}{A0}\right] \times 100$$

where *A*0 is the absorbance of the control and A1 is the absorbance of the sample of the tested extracts.

Statistical analysis: All results were presented in mean±standard deviation and n=6. One way ANOVA was used for the statistical comparisons. $P < 0.05$ was considered as significant difference. The relationship between TPC, TFC and antioxidant activity (FRAP and DPPH) was calculated by Pearson's correlation coefficient. SPSS software was used for the statistical analysis.

Results and Discussion

The qualitative phytochemical screening was carried out in the petroleum ether, ethyl acetate, methanol and aqueous extracts of *R.apiculata* leaves. Highest number of phytochemicals was recorded in the aqueous extract (Table1), unveiling that the bioactive compounds in the leaves might be highly polar[14].

Saponins, flavonoids, carbohydrates and phenols were present in all the four extracts. Phytochemicals were absent in solvents *viz.* tannins (petroleum ether), steroids (ethyl acetate and aqueous), terpenoids (petroleum ether, ethyl acetate), glycosides, proteins, phytosterols, anthraquinones (petroleum ether, ethyl acetate and methanol), cardiac

glycosides (ethyl acetate, methanol), alkaloids (petroleum ether) and reducing sugars (ethyl acetate and methanol).

The secondary metabolites naturally play a vital role in the plant's innate immunity, defense mechanism and are also proved to be capable enough to act positively against human and animal pathogens[15]. Alkaloids have antimicrobial, anti-inflammatory, antispasmodic, antimalarial properties. Tannins possess antitumor, antibacterial and antiviral activity; steroids have insecticidal and anti-bacterial activity and saponins act as anti-feedant, anti-inflammatory, antimicrobial agents[16]. Earlier, alkaloids, saponins, tannins, flavonoids, steroids, tannins and terpenoids were reported in *R. apiculata*[33].

The leaf extracts of *R. apiculata* in the present study have exhibited significant zones of inhibition (mm) against the bacterial strains *P.aeruginosa, A.hydrophila, S. aureus* and *B. subtilis* (Table 2). The highest zone of inhibition was exhibited by the aqueous extract against all the strains *P. aeruginosa* (4.6±0.5 mm), *A. hydrophila* (6.1±1.1 mm), *S. aureus* (3.6±1.0 mm) and *B. subtilis* (3.1±0.7 mm) and the lowest by petroleum ether extract against *P.aeruginosa* (3.4±0.9 mm), *A. hydrophila* (3.9±0.8 mm), *S. aureus* (2.4±1.3 mm) and *B. subtilis* (1.8±0.7 mm). The intermediary values were exhibited by ethyl acetate; *P. aeruginosa* (4.1±0.9 mm), *A. hydrophila* (5.4±1.4 mm) *S. aureus* (3.3±1.7 mm) and *B. subtilis* (2.6±1.2 mm) and methanol; *P.aeruginosa* (3.5±1.2 mm), *A. hydrophila* (4.3±0.7 mm), *S. aureus* (2.9±1.1 mm) and *B. subtilis* (2.4±0.6 mm). When the inhibitory zones of the leaf extracts against strains were compared with standard antibiotic oxytetracycline, a close resemblance was observed with that of aqueous extract. It divulges that the phytocompounds in the aqueous are highly potent to act against the bacterial strains.

The variation in the zones of inhibition among the different bacterial strains might be due to the difference in the resistance mechanisms adopted (alterations in the target sites, reduction of the intracellular inhibitory accumulation and structure of the cell wall (gram- positive and gram-negative)[1]. The Gram negative bacteria (*A.hydrophila* and *P.aeruginosa*) were more affected by the extracts than the gram positive (*S. aureus* and *B.* subtilis) which can be attributed by the active penetration of bioactive components across the cell membrane of gram negative strains and this was in accordance with the studies of Syawal et al[33].

Table 1
Qualitative phytochemical of *R. apiculata* leaf extracts

Phytochemicals	Petroleum ether	Ethyl acetate	Methanol	Aqueous
Saponin	+	+	+	+
Tannin	-	+	+	+
Flavonoid	+	+	+	+
Steroid	+	-	+	-
Terpenoid	-	-	+	+
Glycosides	-	-	-	+
Carbohydrates	+	+	+	+
Proteins	-	-	-	+
Phytosterols	-	-	-	+
Cardiac glycosides	+	-	-	+
Alkaloid	-	+	+	+
Phenols	+	+	+	+
Reducing sugars	+	-	-	+
Anthraquinones	-	-	-	+

+ represents presence, - represents absence

Table 2
Antibacterial activity of *R. apiculata* leaf extracts

Test bacteria	Zone of inhibition (mm)				
	Oxytetracycline	Petroleum ether	Ethyl acetate	Methanol	Aqueous
P. aeruginosa	5.1±0.9	3.4±0.9	4.1±0.9	3.5±1.2	4.6±0.5
A.hydrophila	6.4±0.8	3.9±0.8	5.4±1.4	4.3±0.7	6.1±1.1
S.aureus	4.1±1.2	2.4±1.3	3.3±1.7	2.9±1.1	3.6±1.0
B. subtilis	3.8±0.6	1.8±0.7	2.6±1.2	2.4±0.6	3.1±0.7

Results were expressed in mean ± SD, n=6

Phytochemicals in the leaf extracts of *R. apiculata* are potent antibacterial agents against *Mycobacterium* sp., *Corynebacterium* sp., *Vibrio cholera* and *Staphylococcus aureus* and the antibacterial activity could be due to the presence of phytochemicals alkaloids, terpenes, phenols, saponins, steroids, tannins and flavonoids[30].

The aqueous and petroleum ether extracts of *Acanthus ilicifolius* leaves acted effectively against *Staphylococcus aureus*, *Bacillus thuringiensis* and *E.coli*[7], diethyl ether and acetone leaf extracts of *A. marina* against *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *S.aureus*[12] with inhibitory zones of 0.2-1.9mm and 0.2-1.8mm respectively. Acetone, petroleum ether, aqueous and diethyl ether extracts of *E.agallocha*, aqueous and ethanol extracts of *S.maritima* inhibited the bacterial growth of *P.aeruginosa*, *S.aureus*, *B.subtilis*, *K.pneumonia* with zone ranging from 0.5 to 3.1mm[13] and 1.1 to 4.9mm[8] respectively. The antibacterial potency of *Rhizophora* leaf extracts was previously reported by Rahayu et al[23] in the methanol extract of *R. apiculata* against *S. aureus* (14mm) and *E. coli* (21.9mm), in n-hexane (8±0.5mm), ethyl acetate (10±0.3mm), methanol (11±0.3mm) extracts of *R. apiculata* against *S. aureus*[26].

The estimated phenol and flavonoid contents of the leaf extracts showed that these two phytochemicals were highest in aqueous extract followed by ethylacetate, methanol and petroleum ether (Figure 1). Ferric reducing antioxidant power assay (FRAP) evaluates the antioxidant activity of a compound based on its capability to reduce ferric (Fe^{+3}) ions to ferrous (Fe^{+2}) ion. The antioxidant reacts with the ferric tripyridyltriazine complex (Fe^{+3}- TPTZ) and produces a coloured complex ferrous tripyridyltriazine complex (Fe^{+2}-TPTZ)[6].

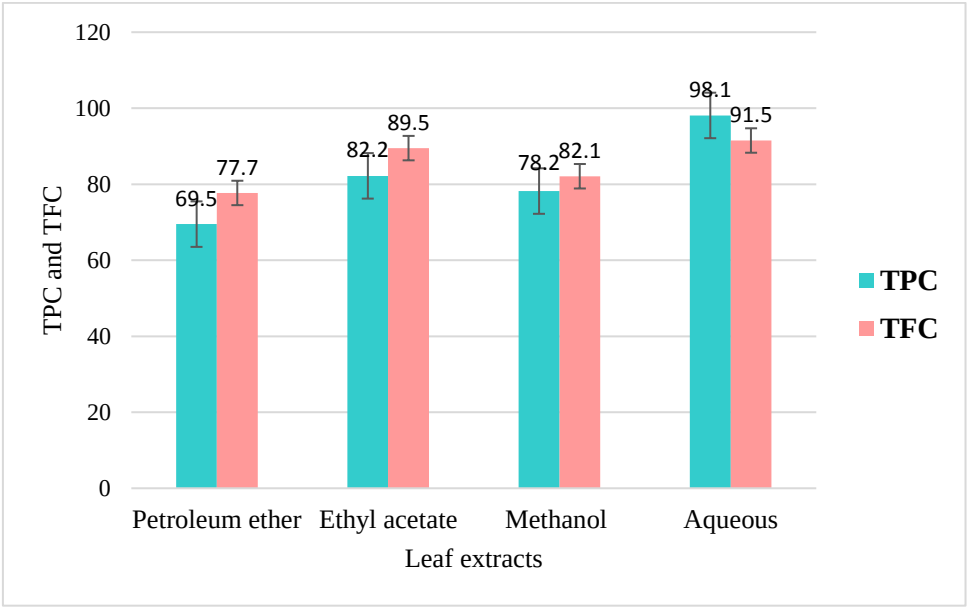

Figure 1: TPC and TFC of *R.apiculata* leaf extracts represented as mg GAE/g DW and mg RE/gDW respectively

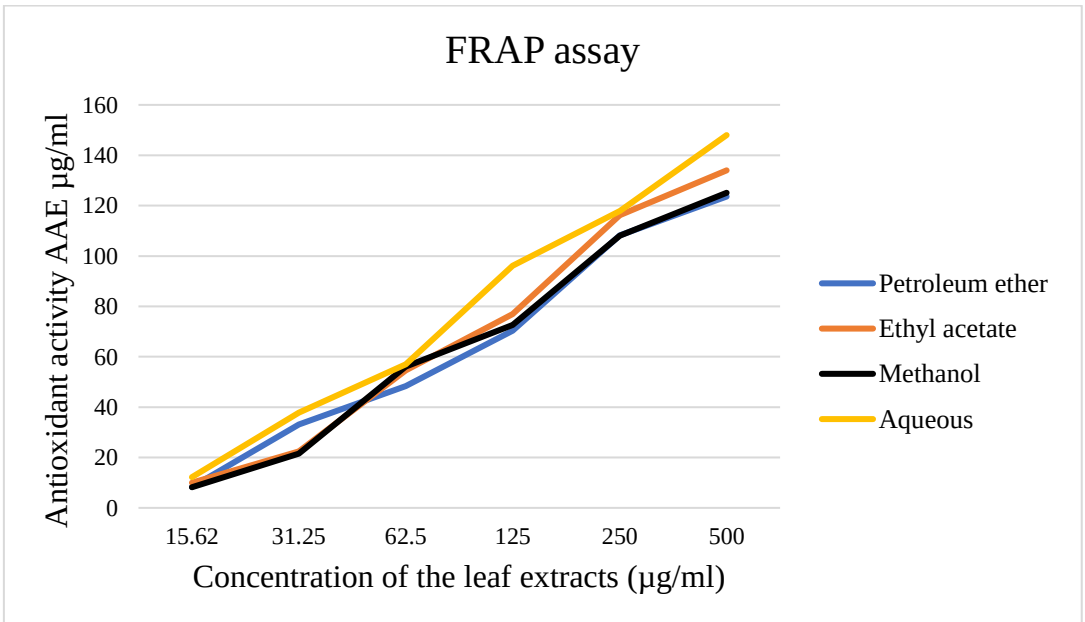

Figure 2: FRAP assay of *R.apiculata* leaf extracts represented as Ascorbic acid equivalents (µg/ml)

The results of the FRAP assay disclosed that the antioxidant activity of the extracts was increased with increase in the concentration[17]. At a concentration of 500 µg/ml, the highest antioxidant activity (AAE µg/ml) was exhibited by the aqueous extract (148±1.1) followed by ethyl acetate (134.4±1.6), methanol (125.1±1.0) and petroleum ether (123.6±0.9) (Figure 2).

The free electron of DPPH radical is responsible for its purple colour and it becomes colourless upon receiving an electron from an antioxidant[24]. DPPH radical scavenging activity of the leaf extracts increased with the concentration. The extracts exhibited maximum scavenging activity at 500 µg/ml; highest activity was recorded in aqueous extract (83.1±1.4) followed by ethyl acetate (82.8±1.3), methanol (78.2±0.5) and petroleum ether (72.3±0.9) (Figure 3).

Free radicals are the molecules with unpaired electrons in the outermost orbital that are produced due to endogenous (phagocytosis, respiration, fattyacid metabolism) or exogenous processes (environmental radiations) in humans whereas plants produced during the hostile environmental conditions or nutritional deficiencies that ultimately led to the development of oxidative stress. Phenols and flavonoids in specific are the strong antioxidants and can efficiently act against oxidative stress. The hydroxyl group of phenols and its derivatives terminates the cycle of new free radical generation by interacting with the free radicals (reactive oxygen and reactive nitrogen species)[10].

The ethanol extract (149 AAE µg/ml) and aqueous extract (132 AAE µg/ml) of *S. maritima*[8], petroleum ether (47.6 AAE µg/ml) and aqueous extract (44.8 AAE µg/ml) of *E. agallocha*[13] showed antioxidant activity. Suganthy et al[31] reported the antioxidant activity of hexane (87±0.2%) and chloroform (74.79±0.01%) extracts of *R. mucronata* leaves. The aqueous leaf extracts of *R.apiculata* exhibited an antioxidant activity of 76.74±0.76%[4].

However, in the present study, the antioxidant ability of the *R. apiculata* leaf extracts might be attributed to the presence of phenols and flavonoids. Earlier, positive correlation between phenol, flavonoid content and antioxidant activity was reported in *B.gymnorrhiza* [2], *B.cylindrica* and *C.tagal* [19]. In the present work, the aqueous leaf extracts of mangrove plant *R. apiculata* showed significant antibacterial and antioxidant activity.

Mangrove plants are being constantly exposed to the environmental fluctuations made them synthesize the secondary metabolites with antioxidant activity[34]. One way Anova was applied to the antibacterial activity of the selected extracts and it revealed a significant relation with *P* 0.001 to *P* 0.04. TPC and TFC of the all extracts have shown a linear correlation with the antioxidant activity (FRAP and DPPH) (Table 3) (Figure 4 represents correlation of aqueous extract).

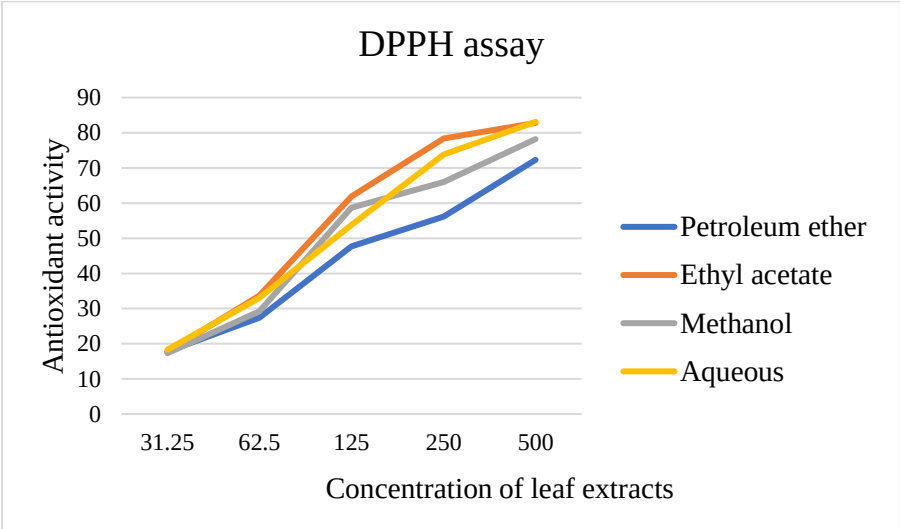

Figure 3: DPPH assay of *R.apiculata* leaf extracts represented as Ascorbic acid equivalents (µg/ml)

Table 3
Correlation between antioxidant activity and TPC, TFC

Extract	R (R^2)			
	Total Phenol Content (TPC)		Total Flavonoid Content (TFC)	
	FRAP	DPPH	FRAP	DPPH
Petroleum ether	0.903 (0.8147)	0.753 (0.5668)	0.922 (0.85)	0.936 (0.877)
Ethyl acetate	0.879 (0.773)	0.916 (0.8393)	0.934 (0.8726)	0.964 (0.9296)
Methanol	0.950 (0.9022)	0.368 (0.1358)	0.755 (0.5698)	0.634 (0.4024)
Aqueous	0.953 (0.9075)	0.968 (0.938)	0.723 (0.5222)	0.589 (0.3475)

R- coefficient of correlation, R^2-Coefficient of determination

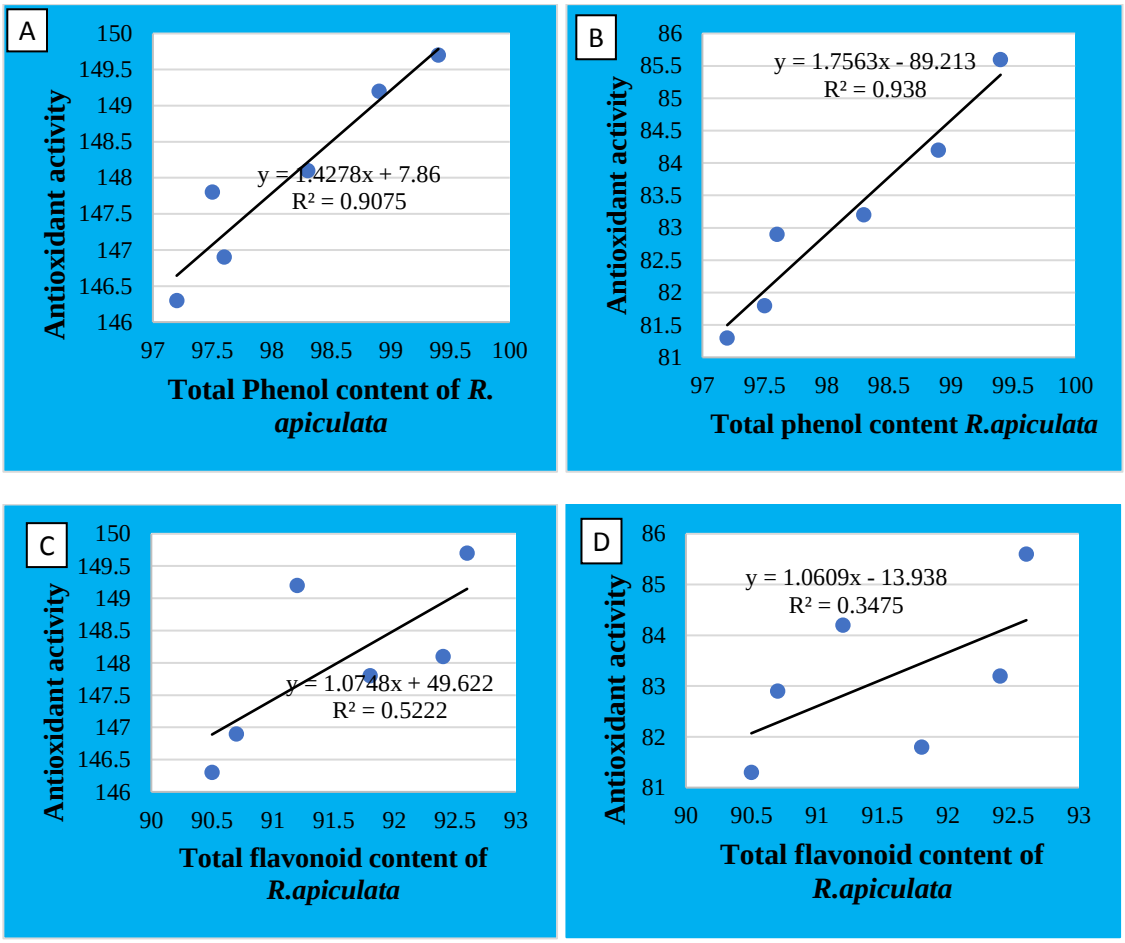

Figure 4: Correlation between TPC, TFC and antioxidant activity of Aqueous leaf extract (A) TPC vs FRAP, (B) TPC vs DPPH, (C) TFC vs FRAP and (D) TFC vs DPPH

Conclusion

In the present study, the aqueous extract has reported high yield of phytochemicals from the leaves of *R.apiculata* and has exhibited highest antibacterial and antioxidant activity showing that the extract owns bioactive phytocompounds. Increased MDR in the pathogens has become a leading cause for the reduced efficacy of antibiotics in treating the bacterial infections. It is evident from the results that the leaf extracts of *R.apiculata* act as potent bio-controllers against the bacterial strains and can be used as a suitable substitute in the place of antibiotics.

The present work can be further carried out for the characterization of phytocompounds in the leaf extracts that are responsible for the antimicrobial activity and can be used for the development of novel herbal antibacterial drugs.

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