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Screening the Antimicrobial and Antioxidant Potential of *Ventilago denticulata*, *Scolopia crenata* and *Rivea hypocrateriformis* from Maredumilli Forest, India

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ABSTRACT

Despite the numerous advances in medicine, the prevalence of infectious diseases continues to rise due to the emergence of antibiotic-resistant pathogens, which is attributed to the widespread use of antibiotics. Antioxidants help to deal with oxidative stress, which is caused by free radical damage. Thus, the search for new antibacterial and antioxidant agents from plants has gained increasing importance. The methanolic extracts of *Ventilago denticulata* bark, *Scolopia crenata* bark and *Rivea hypocrateriformis* roots were screened for their antimicrobial potential against seven bacterial species (*Bacillus cereus*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Salmonella* sp., *Staphylococcus aureus*) using minimum inhibitory concentration (MIC) and against three fungal species (*Aspergillus niger*, *Penicillium* sp. and *Trichoderma viride*) using the disc diffusion method. The extracts were also evaluated for their *in vitro* antioxidant activity using the FRAP method. *R. hypocrateriformis* showed the highest zone of inhibition (8-11 mm) followed by *V. denticulata* (7-9 mm) and *S. crenata* (6-10 mm). Phytochemical analysis showed the presence of saponins, alkaloids, steroids, cardiac glycosides, tannins and phenolics. This is the first report on the antioxidant property of *S. crenata*, which has excellent antioxidant potential (73.56 ± 1.34 mg GAE/g) among the three extracts tested. Results obtained in this study show that these plants have a broad spectrum antibacterial activity and constitute a potential source of new classes of antibiotics and antioxidants.

Keywords: methanolic extracts, antimicrobial activity, antioxidant activity, minimum inhibitory concentration, phytochemicals

INTRODUCTION

Infectious diseases are the leading cause of death worldwide. Antibiotic resistance has become a global concern. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens. Many infectious diseases are treated with herbal remedies, an age-old tradition. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Ujjwal *et al*. 2008). There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases (Parekh *et al*. 2007). The fact that microorganisms nowadays tend to develop resistance towards drugs, coupled with the undesirable side effects of certain antibiotics, offer considerable potentials for the development of new effective antimicrobial and antioxidant agents; medicinal plants are a prolific source (Teke *et al*. 2011).

In recent years, there has been a gradual revival of interest in the use of medicinal and aromatic plants in developed as well as in developing countries, because plant-derived drugs are reported to be safe and without any side-effects, especially when compared with synthetic drugs (Pandey *et al*. 2003; Iniaghe *et al*. 2009).

Various plant extracts possess bacteriostatic and bactericidal effects (Lee 2000) and most of these plants contain many active compounds. The most important of these plant-derived bioactive constituents are alkaloids, tannins, flavonoids and phenolic compounds (Hill 1952). Most of these secondary metabolites, in addition to possessing antimicrobial potential, can also act as potent antioxidants. Antioxidants are closely related to the prevention of degenerative illness such as cardiovascular, neurological diseases, cancer and oxidative stress dysfunctions (Diplock 1995; Black 2000). Therefore, the exploitation of natural antimicrobial agents and antioxidants, especially of plant origin, has increased considerably in recent years.

Ventilago calyculata Tul. (syn. *V. denticulata* Willd.), *V. madraspatana* Roxb., or *V. macrantba silbetiana* belong to the Rhamnaceae. The plants have medicinal qualities, as defined in the Ayurvedic system of medicine (Kirtikar *et al.* 1933). Plants of the genus *Ventilago*, e.g. *Ventilago leiocarpa* Bunge, have been used in Taiwan for the treatment of cough, rheumatism, and contused wounds (Kan 1980; Hsu 1995). Only six *Ventilago* species have been investigated chemically, i.e., *V. bombaiensis*, *V. calyculata*, *V. goughii*, *V. viminalis*, and *V. vitiensis* (Ali *et al.* 1994).

Rivea hypocrateriformis (Desr.) Choisy (Convolvulaceae) is a climbing shrub found throughout India. Ayurvedic physicians use this plant to prevent fertility in women (Shivalingappa *et al*. 2001, 2002). This plant has the ability of anti-implantation and is known to completely interrupt early pregnancy and influence the estrous cycle of albino rats (Shivalingappa *et al*. 2001). Leaves and young shoots are eaten as a vegetable and roots are given after parturition. Cooked leaves of this plant are used as a vegetable curry by the tribals of India (Nataraj *et al*. 2010). This plant has high vitamin A content (nearly 2.34 retinal equivalents) and has a high retention capacity (75 to 98%) of β-carotene upon pro-

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cessing (Rajyalakshmi *et al.* 2001, 2003). The ethanolic extract of *R. hypocrateriformis* leaf juice is used to treat rheumatic pain and hair scalp skin diseases (Saikat *et al.* 2010).

Scolopia crenata (Flacourtiaceae family) leaves are used for treating musco-skeletal pain (Kadavul *et al*. 2009) although the biological activity of *S. crenata* has not yet been reported. Therefore, our results are the first evidence demonstrating its antimicrobial and antioxidant activities along with its preliminary phytochemical screening.

The present study aims to evaluate the antibacterial potential of the methanolic extracts of the bark and/or roots of these three medicinal plants against bacteria and fungi. The minimum inhibitory concentration (MIC) for antibacterial activity was determined. Other objectives included the search for active phytochemicals present in the methanolic extracts of these plants and also to obtain a preliminary sketch about the chemical nature of the active ingredient(s) associated with their antimicrobial potential. Their antioxidant activity was also evaluated.

MATERIALS AND METHODS

Collection and identification of plant material

V. denticulata bark, *S. crenata* bark and *R. hypocrateriformis* roots were collected from Maredumilli forest located near Rampachodavaram (Mandal), East Godavari (district), Andhra Pradesh (State), South India with the help of a local Ayurvedic doctor. The plants for screening antimicrobial and antioxidant activity were selected based on their use in folk medicine for the treatment of infectious disease-causing agents and were correctly identified at the Department of Botany, Andhra University, Visakhapatnam. Only the most abundantly used part from each plant was selected in this study, based purely on its traditional use by the local tribe.

Extraction of plant material

Plant material was brought to the laboratory and washed under running tap water and blotted dry with filter paper and then shade dried on laboratory benches on top of newspaper. After having completely dried, the plant material was then ground into a powder with a hand mill. The powdered plant material (10 g) was placed into a Soxhlet apparatus and was exhaustively extracted using 100 ml of 90% methanol (60-80°C). The crude extracts were concentrated *in vacuo* at 40°C using a rotary evaporator (PBU 6D model; Superfit). The crude extracts were preserved in a freezer at -20°C until use. The successive extracts weights were 29.4, 23.2 and 31.6% (w/w) for *V. denticulata*, *S. crenata* and *R. hypocrateriformis*, respectively.

Test microorganisms and microbial culture

Bacterial cultures (*Bacillus cereus*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Salmonella* sp. and *Staphylococcus aureus*) fungal cultures (*Aspergillus niger*, *penicillium* sp. and *Trichoderma viride*) were obtained from the Department of Microbiology, Andhra Medical College, Visakhapatnam. Bacterial strains were cultivated at 37°C and maintained on a nutrient agar (Hi Media) slant at 4°C. Fungal strains were cultivated at 25°C and maintained on potato dextrose agar (PDA) slants at 4°C. Pure cultures were kept on agar slants at 4°C until needed. They were sub-cultured once a month. Each inoculum was prepared by inoculating the stock culture into freshly prepared media. All bacterial strains were incubated at 37°C for 24 h and fungi at 27°C for 48 h. The test organisms were grown overnight in the media defined above.

Screening of antimicrobial activity

The antimicrobial activity of organic extracts of the plant samples was evaluated by the paper disc diffusion method (Aida *et al*. 2001). To determine antibacterial activity, each overnight bacterial culture was adjusted to 0.5 using McFarland turbidity standards (Performance Standards for Antimicrobial Susceptibility Testing 2009).

To determine antifungal activity, all fungal isolates were first adjusted to 10^6 colony-forming units (CFU)/ml. The bacterial and fungal broth cultures (100 µl each) were inoculated onto nutrient agar and PDA plates (90 mm diameter, Borosil), respectively by the spread plate method. At first, stock solutions (100 mg/ml) of each individual plant extract were prepared separately. Sterile filter paper (Whatman filter paper No. 1) discs 9 mm in diameter were prepared and 10 µl of each extract dilution were impregnated onto the discs and carefully placed at the centre of the previously seeded plates with 0.5 McFarland bacterial and 10^6 CFU/ml fungal cultures with sterile forceps. A disc with solvent alone served as the control. Streptomycin (10 µg/ml; Dr. Reddy's Laboratories Ltd.) for bacteria and Albendazole (10 µg/ml; Ranbaxy Laboratories Ltd.) for fungi were used as the standard antimicrobials for comparison. Bacterial culture plates were then incubated at 37°C for 24 h while fungal cultures were incubated at 25-27°C for 48 h. Antimicrobial activity was determined by measuring the zone of inhibition of growth around each paper disc (mm). For each extract (test) three replicate trials were conducted against each microorganism. Each zone of inhibition was measured with a ruler and compared with the control standard (Bauer *et al.* 1996).

Minimum inhibitory concentration

The MIC of the extracts was estimated against bacterial organisms by the NCCLS (2002) method. MIC was determined with different doses (10, 25, 50, 100 mg/ml) of extracts. To 0.5 ml of each concentration of the extracts, 10 ml of nutrient broth was added and then a loopful of the test organism previously diluted to 0.5 McFarland turbidity standard bacterial isolates was inoculated into a test tube. The procedure was repeated with the bacterial isolates using the standard antibiotic streptomycin. Tubes containing nutrient broth only seeded with the test organism served as the control. Tubes containing bacterial cultures were then incubated at 37°C for 24 h. The MIC was assessed from the tube with the least concentration of extract with no visible growth and turbidity.

Preliminary phytochemical screening

All extracts were analyzed for the presence of alkaloids, saponins, cardiac glycosides (Trease *et al*. 1989), tannins, phenols and flavanoids (Sofowora 1982; Adetuyi *et al*. 2001), and steroids (Odebiyi 1982).

In vitro antioxidant study

The ferric reducing antioxidant power (FRAP; Lim *et al*. 2007) property of the extract was determined by taking 1 ml of different dilutions of standard solutions of gallic acid (10-100 µg/ml) or methanolic extract that had been adjusted to come under the linearity range (500 µg/ml), placed in 10-ml volumetric flasks and mixed with 2.5 ml of potassium buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 20 min and 2.5 ml of 10% trichloroacetic acid was added to the mixture to stop the reaction. To the 2.5 ml of the above solution, 2.5 ml of distilled water was added and then 0.5 ml of 0.1% $FeCl_3$ was added and allowed to stand for 30 min before measuring the absorbance at 593 nm. The absorbance obtained was converted to gallic acid equivalents in mg/g of dry material (GAE/g) using a gallic acid standard curve (Avani *et al*. 2010) (**Fig. 3**).

RESULTS AND DISCUSSION

Antimicrobial activity

The present study was conducted to investigate the antibacterial properties of three selected plants from Maredumilli forest which have not received due scholarly attention, even though they are used in Indian folk medicine. Herbal remedies play a fundamental role in traditional medicine in rural areas of India where they serve as the therapeutic treatment of choice as an antiseptic, anti-inflammatory and in the treatment of infectious diseases, including diarrhea. In the present study, an attempt was made to link traditional herbal

Table 1 Results of antimicrobial activity of methanol crude plant extracts of three different plants against pathogenic bacteria and fungi (n = 3).

Microorganisms*	Zones of inhibition (mean ± SD) (mm)[a]			
	Crude plant extracts tested[b]			Antibiotics† positive standards
	Ventilago denticulata	*Scolopia crenata*	*Rivea hypocrateriformis*	
Bacterial strains				
Escherichia coli	7 ± 1.2	10 ± 1.2	11 ± 0.6	15 ± 0.9
Bacillus subtilis	7 ± 0.7	9 ± 1.4	10 ± 0.4	15 ± 0.4
Bacillus cereus	0	8 ± 0.9	10 ± 0.8	15 ± 0.6
Staphylococcus aureus	7 ± 0.9	0	8 ± 0.9	20 ± 0.8
Pseudomonas aeruginosa	7 ± 0.6	7 ± 0.9	10 ± 0.5	19 ± 0.6
Proteus vulgaris	6 ± 0.4	6 ± 0.8	9 ± 1.2	20 ± 0.3
Salmonella sp.	6 ± 0.9	9 ± 0.6	9 ± 1.5	18 ± 1.0
Fungal strains				
Trichoderma viride	9 ± 1.3	11 ± 0.4	6 ± 0.9	0
Penicillium sp.	9 ± 0.8	0	9 ± 0.7	20 ± 0.6
Aspergillus niger	7 ± 0.7	7 ± 0.7	0	10 ± 1.6

[a]Values are mean of three replicates ± SD.
† Antibiotics used as positive standards: Streptomycin (for bacterial strains); Albendazole (for fungal strains)

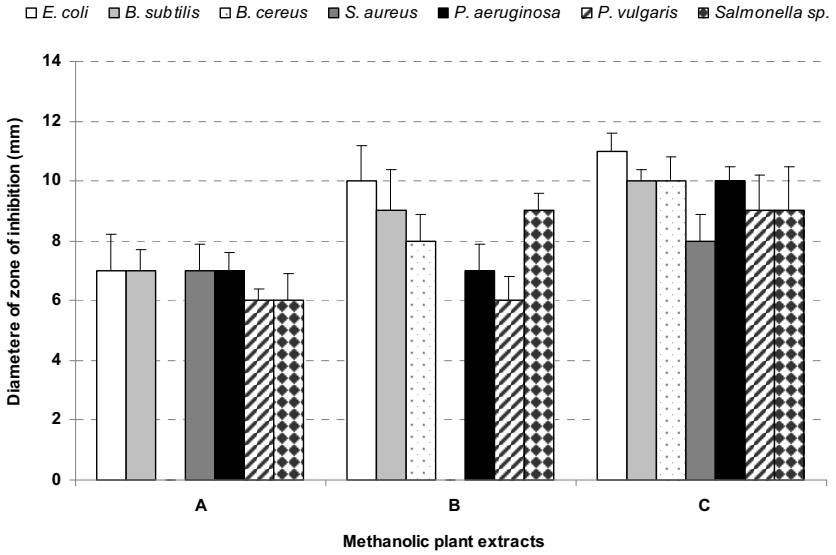

Fig. 1 Bar chart showing results of antibacterial susceptibility of test organisms to methanol extracts of plants. *Ventilago denticulata* (**A**), *Scolopia crenata* (**B**), *Rivea hypocrateriformis* (**C**).

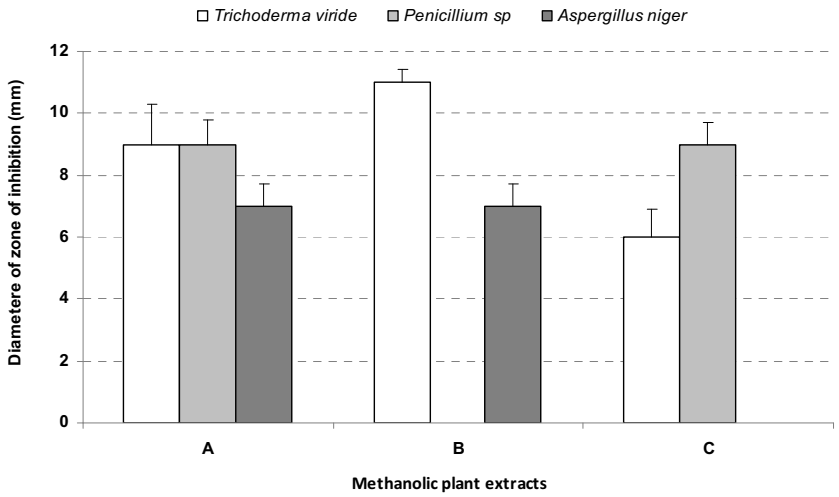

Fig. 2 Bar chart showing results of antifungal susceptibility of test organisms to methanol extracts of plants. *Ventilago denticulata* (**A**), *Scolopia crenata* (**B**), *Rivea hypocrateriformis* (**C**).

medicinal knowledge held by the Indian native people with modern scientific laboratory-based assays.

Antimicrobial screening of the methanolic extracts of *V. denticulata*, *S.crenata* and *R.hypocrateriformis* are presented in **Table 1** and **Figs. 1** and **2**. The present study reveals that the extracts of all three plants showed potent antibacterial and antifungal activity against all reference microbial (bacterial and fungal) strains.

R. hypocrateriformis exhibited the highest antibacterial activity against all reference strains (8-11 mm), 6 and 9 mm against *Trichoderma viride* and *Penicillium* sp. respectively and no activity against *Aspergillus niger.* Desmethylbergenin hemihydrate is a naturally occurring isocoumarin isolated from this plant (Zammarud *et al*. 2006). The phytochemical screening of *R. hypocrateriformis* tested positive for steroids, alkaloids, glycosides, saponins, fixed oils/fats,

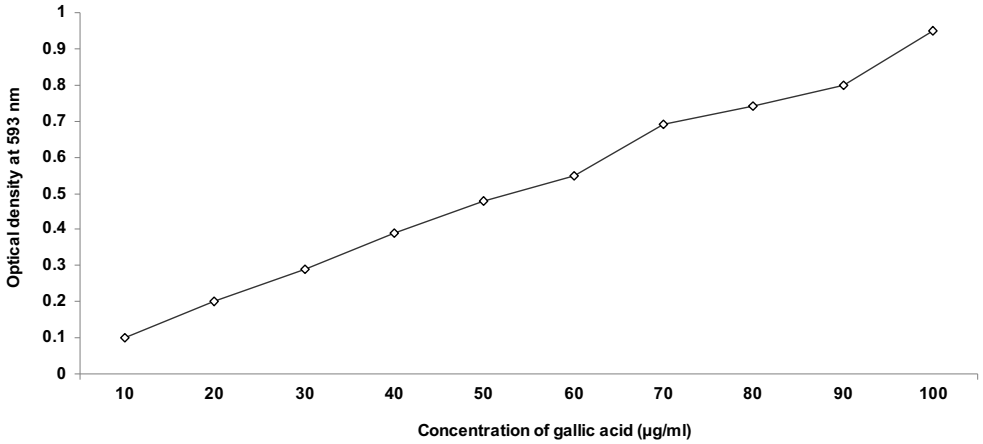

Fig. 3 Standard curve of ferric reducing antioxidant power (FRAP) method. R^2 values represented mean data set of n=3.

Table 2 Minimum inhibitory concentration (MIC) of methanol extracts (n = 3).

Plant extract	Test organism	I	II	III	IV
Ventilago denticulata	*Escherichia coli*	++	+	*	-
	Bacillus subtilis	++	+	+	*
	Staphylococcus aureus	++	+	+	*
	Pseudomonas aeruginosa	++	+	+	*
	Salmonella sp.	++	+	+	*
Scolopia crenata	*Escherichia coli*	++	+	*	-
	Bacillus subtilis	++	+	*	-
	Bacillus cereus	+	+	+	*
	Salmonella sp.	+	+	*	-
Rivea hypocrateriformis	*Escherichia coli*	+	*	-	-
	Bacillus subtilis	+	*	-	-
	Bacillus cereus	++	*	-	-
	Staphylococcus aureus	*	-	-	-
	Pseudomonas aeruginosa	++	+	*	-
	Salmonella sp.	+	*	-	-
	Proteus vulgaris	++	+	*	-

I = 10 mg/ml; II = 25 mg/ml; III = 50 mg/ml; IV = 100 mg/ml ;* = MIC; - = no growth; + = little growth; ++ = dense growth

Table 3 Results of preliminary phytochemical screening of plant extracts (n = 3).

Phytochemical tests	A	B	C
Tannins and phenols	+	-	+
Saponins	-	-	+
Alkaloids	-	-	+
Steroids	-	+	+
Cardiac glycosides	-	+	+
Flavonoids	-	-	-

+ = presence; - = absence

tannins, and phenolic compounds (Shivalingappa *et al*. 2002).

The next highest activity was observed in *S. crenata* with (6-10 mm) against bacterial strains, except for *S. aureus*, and antifungal activity of 7 and 11 mm against *A. niger* and *T. viride*, respectively and no activity against *Penicillium* sp.

V. denticulata showed activity against all bacterial strains (6-7 mm), except for *B. cereus*, and 7-9 mm activity against all fungal strains. Tambekar *et al*. (2009) also demonstrated antibacterial properties of *V. madraspatana* in which the acetone extract showed antibacterial activity against *Klebsiella pneumoniae*, *Enterobacter aerogenes* (8 mg/disc) and *Shigella flexneri* (10 mg/disc). The ethanolic extract was effective against *Staphylococcus aureus*, *Shigella flexneri* and *Enterobacter aerogenes* (10 mg/disc) while the aqueous extract only showed antibacterial effect against *Enter. aerogenes*. Basu *et al*. (2005) also observed similar antibacterial activity in the chloroform and ethanol extracts of *V. madraspatana*, from which emodin and physcion were isolated for the first time from the stem bark. *V. denticulata* also exhibited therapeutic anti-herpes simplex virus type 1 efficacy *in vivo* (Vimolmas *et al*. 2003).

The MIC results of the extracts are shown in **Table 2**. Among the three plant extracts, *R. hypocrateriformis* was more potent with least MIC values of 10 and 25 mg/ml against *S. aureus*, *E. coli*, *B. cereus*, *B. subtilis* and *Salmonella* sp. whereas *S. crenata* and *V. denticulata* had MIC values of 50 and 100 mg/ml against the tested strains.

Preliminary phytochemical analysis of the methanolic extracts of these plants showed the presence of saponins, alkaloids, steroids, cardiac glycosides, tannins and phenolics (**Table 3**).

Capacity of the FRAP method

At low pH, measuring the change in absorption at 593 nm can monitor the reduction of a ferric complex to the ferrous form, which has an intense bluish green color. The change in absorbance is directly related to the combined or "total" reducing power of the electron-donating antioxidants present in the reaction mixture (Avani *et al*. 2010).

The FRAP method showed the methanolic extracts of *V. denticulata* bark, *S. crenata* bark and *R. hypocrateriformis* roots to have 67.25 ± 0.28, 73.56 ± 1.34 and 47.46 ± 0.72 mg GAE/g of sample, respectively (**Table 4**). The antioxidant activity of other members of the Rhamnaceae is briefly reviewed. Kaempferol 3-*O*-β-isorhamninoside (K3O-ir) and rhamnocitrin 3-*O*-β-isorhamninoside (R3O-ir), isolated from the leaves of *Rhamnus alaternus* L., inhibited NBT photoreduction and scavenged the free radical ABTS (+). At 150 µg/assay the two compounds showed the most potent inhibitory activity against the superoxide anion by 80.4 and 85.6%, respectively (Bhouri *et al*. 2011). Free radical scavenging activity of the methanolic extract of *Ziziphus rugosa* Lam. fruit pericarp exhibited radical scavenging activity with an IC_{50} = 61.88 µg/ml by the DPPH method (Kekuda *et al*. 2010).

The total antioxidant activity of some Australian Flacourtiaceae species was assessed based on scavenging activity of stable ABTS free radicals, among them the leaf extract of *Casearia* sp. (RB 3051), the mature stem extract of *Casearia grayi* and the stem extract of *Scolopia braunii* had the highest antioxidant activity (IC_{50} = 2.9 µg/ml) (Mosaddik *et al*. 2004).

The antioxidant activity of the methanolic extracts of *Rivea hypocrateriformis* leaf, stem and flower was esti-

Table 4 Results of ferric reducing antioxidant power (FRAP) method on extracts added at 500 µg/ml (n = 3).

Plant extracts	mg GAE/g of extracts
Ventilago denticulata	67.25 ± 0.28
Scolopia crenata	73.56 ± 1.34
Rivea hypocrateriformis	47.46 ± 0.72

GAE = gallic acid equivalents based on standard curve (**Fig. 3**)

mated using the FRAP reagent (containing 2.5 mL of 20 mmol/L ferric 2,4,6-tripyridyl-*S*-triazine complex [Fe(III)-(TPTZ)$_2$]$^{2+}$ solution in 40 mmol/L HCl plus 2.5 mL of 20 mmol/L $FeCl_3 \cdot 6H_2O$ and 25 mL of 0.3 mol/L acetate buffer (pH 3.6) resulted in substantial activity ranging from 484.2 to 1279.5 µmol Fe(II)/g extract (Nataraj *et al*. 2010).

Another Convolvulaceae member, *Evolvulus alsinoides* L. was also tested for its antioxidant activity in the 2,2′-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid radical cation decolorization assay using ethanolic extracts and water infusion. The results from the ABTS assay showed that the ethanolic extract of *E. alsinoides* was IC_{50} = 33.39 µg/ml and the relative antioxidant capacity for the water infusions was IC_{50} = 172.25 µg/ml (Auddy *et al*. 2003).

CONCLUSION

Our results favor the use of these plants as potential additives or nutraceuticals to replace synthetic antioxidant compounds. However, further analyses of the antioxidant capacity of the beneficial compounds of these plants are needed to better understand how to implement them in functional medicine. The broad spectrum of antimicrobial activity may help to discover new chemical classes of antibiotic substances that could serve as selective agents for infectious diseases, chemotherapy and control.

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