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Evaluation of the Free Radical Scavenging Activities and Antibacterial Activities of the Extracts of *Lindernia ruellioides* (Colsmann) Pennell.

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Abstract

Around the world, plants have been used as medicine to ameliorate a broad spectrum of ailments, leveraging their wealth of phytochemicals. Due to their availability and reasonably priced, plant-based medicines have occasionally supplanted synthetic pharmaceuticals. *Lindernia ruellioides,* locally called as "Thasuih" in Mizoram has been used by indigenous practitioners and traditional healers for treating various ailments. However, there is no scientific validation for this traditional use. Scientific validation at known doses may provide information about its safety and efficacy. Therefore, the present study endeavours to estimate the phytochemical contents and determine the free radical scavenging activity and antibacterial activity of various extracts of The results were evaluated statistically using SPSS (Online) and Graph pad prism (Online).The plant was washed and allowed to shade dried at room temperature and then powdered. It was then subjected to sequential cold maceration using different solvents such as petroleum ether, chloroform, ethanol and distilled water. Phytochemical analysis was carried out using standard procedures to identify the constituents. The ability of the extracts to inhibit the generation of various free radicals was determined by assessing the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH), 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and superoxide radicals. Antibacterial activity was determined using disc diffusion method and minimum inhibitory concentration. The results were evaluated statistically using SPSS (Online) and Graph pad prism (Online). The preliminary phytochemical screening disclosed that the plant incorporates an assortment of phytochemicals such as alkaloids, saponins, flavonoids, tannins, steroids, glycosides, phlobatannins and terpenoids. Among the various extracts of *L. ruellioides*, ethanol extract has the highest total phenolic (327.97 ± 1.77 mg GAE/g of dry extract) and flavonoid contents (264.95 ± 0.71 mg quercetin equivalent/g of dry extract). Different extracts of *L. ruellioides* when analysed for their scavenging activities showed significant inhibition of DPPH, ABTS and superoxide in a concentration dependent manner. The ethanolic extract showed the highest scavenging activities for DPPH and ABTS with IC_{50} 158.0 ± 4.82 µg/ml and 112.9 ± 6.47 µg/ml respectively. However, aqueous extract was found to possess the highest scavenging activity for superoxide with IC_{50} 135.5 ± 5.02 µg/ml. The different extracts were also found to be active against the test microorganisms, thus, justifying the folkloric use of the plant.

Keywords: *Lindernia ruellioides,* free radical scavenging, antibacterial, disc diffusion, minimum inhibitory concentration.

Introduction

During normal metabolic process, free radicals are normally generated in our body. Reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) are generally the by-products as a result of the cellular redox process. They play a dual role as both detrimental and beneficial effects (1). At high concentrations, they are highly reactive and toxic and cause damage to cell membrane, proteins, lipids and deoxyribonucleic acid (DNA) which leads to oxidative stress. This oxidative stress cause tissue damage resulting in several diseases such as neurodegenerative disease, cardiovascular disease, diabetes, cancer, aging, rheumatoid arthritis and cataract (2-4). Antioxidant play a key role in protecting our body against free radical damage. They balance free radical production and detoxify them when in excess (5-7).

Regardless of the extensive use as traditional medicine, scientific validation is still limited. *L. ruellioides,* belonging to the family Linderniaceae is an erect annual herb found commonly in semi-shaded areas (8). Traditionally, it has been used for dressing cuts, wounds, bruises, boils, jaundice, snakebite, dysentery, urinary trouble and quick healing of wounds when applied externally and the juices of the leaves are also used for massaging on strains (9,10). Regardless of the extensive use as traditional medicine, scientific validation is still limited.

Materials and Methods

Collection of plant material and preparation of extracts

L. ruellioides was collected from Reiek, Aizawl district, Mizoram, India (23.678ºN, 92.603ºE) during the months of June-August. Identification and authentication of the plant was done by Natural History Museum Mizoram, Mizoram University with accession no. NHMM-P/000160. *L. ruellioides* was washed and allowed to shade dried at room temperature and then powdered. It was then subjected to sequential cold maceration using different solvents such as petroleum ether, chloroform, ethanol and distilled water. The liquid extracts were then filtered and evaporated to dryness in an oven and was stored at 4°C for further use. Hereafter, the chloroform, ethanolic and aqueous extracts of *L. ruellioides* will be called as LRCE, LREE and LRAE respectively.

Phytochemical screening

Different extracts of *L. ruellioides* were analysed for the presence of alkaloids, saponins, flavonoids, tannins, steroids, glycosides, phlobatannins and terpenoids using standard protocol (11,12).

Quantitative analysis

Determination of total phenolic content (13)

5 ml of Folin-Ciocalteau's reagent (diluted ten-fold) was mixed with 1 ml of *L. ruellioides* extract, dissolved in their respective solvent, at the concentration ranging from 0.25-8.0 mg/ml. Sodium carbonate (4 ml, 0.115 mg/ml) was added to the mixture after 5 mins of incubation at room temperature. Then the mixture was incubated at room temperature for 2 hrs followed by measuring the absorbance at 765 nm using UV-Visible spectrophotometer. Calibration curve was also prepared by mixing methanolic solution of gallic acid (1 ml) with the reagents. All determinants were carried out in triplicate. The total phenolic content in each extract were expressed as gallic acid equivalents (GAE) mg/ml of the dry extract.

Determination of total flavonoids content (14)

0.25 ml of extract (0.25-8.0 mg/ml; dissolved in the appropriate solvent) and quercetin standard solution was mixed with 1.25 ml of distilled water followed by the addition of 75 µl of 5% (w/v) sodium nitrite solution. After few minutes, 150 µl of 10% (w/v) aluminium chloride solution was added and allowed to stand for further 5 mins before the addition of 0.5 ml of 1M sodium hydroxide. The mixture was then diluted with distilled water to a volume of 2.5 ml. At 510

nm, absorbance was immediately measured. Quercetin equivalents were used to represent the result (mg/g extract).

In vitro antioxidant assays

DPPH radical scavenging activity (15)

To different concentrations of various extracts of *L. ruellioides* (0.5 ml, 20-1000 µg/ml), 1 ml of methanolic solution of 0.1 M DPPH was added. The mixture was then allowed to stand in the dark for 30 mins and absorbance was measured at 523 nm. Methanol was utilized as the baseline correction. The results were compared with control prepared as above without sample. The antioxidant activity of the extract was expressed as IC_{50}, the concentration (µg/ml) of extract that inhibited 50% of DPPH radicals. Ascorbic acid was used as the standard and each study was performed in triplicate. The amount of scavenging was then calculated using the formula:

% scavenging = [(Ablank - Asample)/Ablank] × 100

where A_{blank} the is absorbance of the control reaction and A_{sample} is the absorbance of the solution containing the plant extract.

ABTS radical scavenging activity (16)

Equal volume of 2.45 mM potassium persulfate solution and a 7 mM ABTS solution were combined to create a stock solution. The solution was incubated at room temperature in the dark for 12 hrs to yield a dark-coloured solution containing $ABTS^{\cdot+}$ radicals. A working solution was prepared freshly before each assay by diluting the stock solution with 50% methanol for an initial absorbance of about 0.700 (± 0.02) at 745 nm. The scavenging activity was then evaluated by combining 150 µl of various extract fractions (20-1000 µg/ml dissolved in their respective solvents) with 1.5 ml of ABTS working standard. At 745 nm, the drop in absorbance was measured. Ascorbic acid was used as positive control. The scavenging activity was then estimated based on the formula:

% scavenging = [(Ablank - Asample)/Ablank] × 100where A_{blank} is the absorbance of the control reaction A_{sample} is the absorbance of the solution containing the plant extract.

Superoxide ($O_2^{\cdot-}$) radical scavenging activity (17)

To the reaction mixture containing 0.2 ml of nitroblue tetrazolium (1 mg/ml in dimethyl sulfoxide) and 0.6 ml of extract (20-1000 µg/ml), 2 ml of alkaline dimethyl sulfoxide (DMSO) (1 ml DMSO in 5 mM sodium hydroxide) was included to provide a complete volume of 2.8 ml. The absorbance was recorded at 560 nm. The blank consisted of pure DMSO instead of alkaline DMSO. Ascorbic acid was used as the standard and the ability of *L. ruellioides* extracts to scavenge the superoxide radical was calculated using the formula:

% scavenging = $(A_e - A_o / A_e) \times 100$

where Ablank is the absorbance of the control reaction and Asample is the absorbance of the solution containing the plant extract.Antibacterial activity

The antibacterial activity was assessed against three bacteria species: *Escherichia coli* (MTCC-40), *Bacillus subtilis* (MTCC-121) and *Klebsiella pneumoniae* (MTCC-39).

Disc diffusion method (18)

Agar plates were prepared which were inoculated with the test microorganisms. Then the paper disc containing two different concentrations (20 mg and 10 mg) of plant extract, 25 µg of streptomycin disc (standard) and paper disc (control) that contain 5% DMSO for LRCE and distilled water for LREE and LRAE were kept carefully on the surface of the prepared agar plate. Then, the plates were incubated for 24 hrs at 37°C in inverted position. After incubation, the extract possessing those activity was taken for measuring the zone of inhibition and compared with the standard antibiotic.

Minimum inhibitory concentration (MIC) (19)

Agar plates were prepared which were inoculated with the test microorganisms. Following that, the paper discs with six different concentrations (10 mg, 5 mg, 2.5 mg, 1.25 mg, 0.625 mg and 0.3125 mg) of plant extract and 25 µg streptomycin disc were carefully placed on the agar plate's surface. Negative control was not kept as 5% DMSO and distilled water does not show any antimicrobial activity in the previous disc diffusion experiment. The plates were then incubated for 24 hrs inverted at 37°C. The lowest concentration inhibiting the growth of microorganism was noted and considered as MIC for each test microorganisms.

Statistical Analysis

Data are expressed as mean ± standard error of mean. One-way analysis of variance (ANOVA) was performed to test the significant variations followed by Tukey multiple comparison of means. *P* value of less than 0.05 was considered statistically significant. SPSS (Online) and Graph pad prism (Online) were used for statistical and graphical evaluations.

Results and Discussion

Qualitative phytochemical analysis

Qualitative phytochemical screening showed the presence of various naturally occurring compounds like alkaloids, saponins, flavonoids, tannins, phlobatannins and terpenoids in LREE. Alkaloids, flavonoids, tannins and steroids were found in LRAE. Alkaloids, saponins and tannins were also found to be present in LRCE (Table 1). These phytochemicals are secondary metabolites that contribute to flavour and colour (20) and have been reported to possess several pharmacological potentials which includes antioxidants (21), antimalarial (22), antimicrobial activities (23) and reduce the risk of many diseases (24).

Table 1: Qualitative phytochemical evaluation of different *L. ruellioides* extracts.

Phytochemicals	Reagent	Colour indication	LRCE	LREE	LRAE
Alkaloids	Dragendroff's reagent	Reddish brown precipitate	+	+	+
Saponins	Olive oil	Whitish emulsion	+	+	-
Flavonoids	Sulphuric acid, Magnesium turnings	Pink red color	-	+	+
Tannins	Ferric chloride	Brownish green or blue-black color	+	+	+
Steroids	Sulphuric acid	Red colour	-	-	+
Glycosides	Glacial acetic acid, Ferric chloride, Sulphuric acid	Brown ring	-	-	-
Phlobatannins	Hydrochloric acid	Red precipitate	-	+	-
Terpenoids	Sulphuric acid	Reddish brown	-	+	-

'+' sign denotes the presence of phytochemicals while '-' sign denotes the absence of phytochemicals. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanolic extract; LRAE- *L. ruellioides* aqueous extract.

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Determination of Total phenolic and flavonoid content:

The total phenolic and total flavonoid content of *L. ruellioides* extracts increased in a concentration dependent manner (Figure 1 & 2). At 8 mg/ml, LREE has significantly higher (*P* 0.001) total phenolic content (327.97 ± 1.77 mg GAE /g of dry extract) than LRAE (209.63 ± 0.69 mg GAE /g of dry extract) and LRCE (196.51 ± 1.71 mg GAE /g of dry extract). For total flavonoid, LREE also has significantly higher (*P* 0.001) content (264.95 ± 0.71 mg quercetin equivalent/g of dry extract) than LRAE (217.44 ± 0.15 mg quercetin equivalent/g of dry extract) and LRCE (210.59 ± 0.41 mg quercetin equivalent/g of dry extract). Phenolic compounds have been reported to show antioxidant activity by scavenging or stabilizing free radicals due to their conjugated ring structures and presence of hydroxyl groups (25) and have been reported to exhibit antiallergenic, antimicrobial, anti-inflammatory and cardioprotective effects (26). Similarly, flavonoids are also reported to have antioxidative action and reduce their formation by chelating the metals (27). In addition, there have been reports of flavonoids to serve as health-promoting compound and protect against several diseases (28).

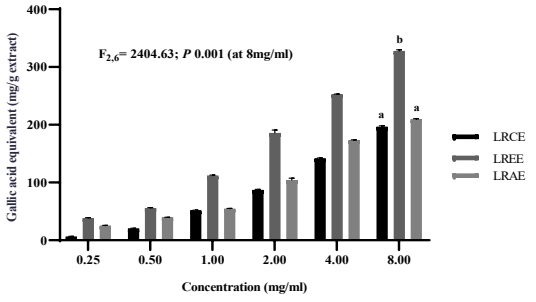

Fig. 1: Phenolic content of *L. ruellioides* extracts determined as Gallic acid equivalent. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

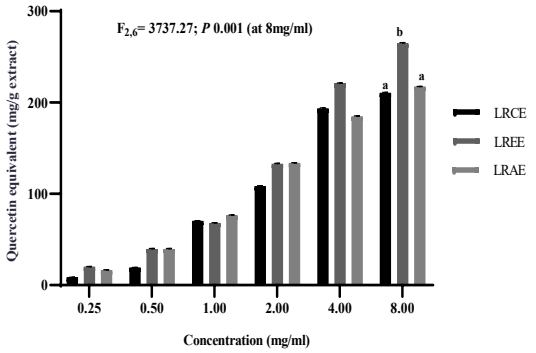

Fig. 2: Flavonoid content of various extracts of *L. ruellioides* determined as Quercetin equivalent. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

In vitro antioxidant assay

DPPH radical scavenging activity

L. ruellioides scavenged DPPH radicals in a concentration dependent fashion as indicated by the discolouration of DPPH. The maximum activity of LREE, LRCE and LRAE to scavenged DPPH was noted at 500 µg/ml, 600 µg/ml and 900 µg/ml respectively. LREE was most potent as it effectively inhibited DPPH radical formation and showed the highest scavenging activity (IC_{50} 158.0 ± 4.82 µg/ml) followed by LRAE (IC_{50} 276.4 ± 6.32 µg/ml) and the lowest scavenger was LRCE (IC_{50} 330.43 ± 5.34 µg/ml). IC_{50} of all the extracts are statistically significant when compared to the standard ascorbic acid (IC_{50} 12.47 ± 0.13 µg/ml). Different extracts of various plants have been shown to inhibit the generation of DPPH free radicals earlier (29-34). Compounds such as cysteine, glutathione, ascorbic acid, tocopherol, poly-hydroxyl aromatic compounds have been known to have the ability to reduce DPPH by hydrogenation (35,36).

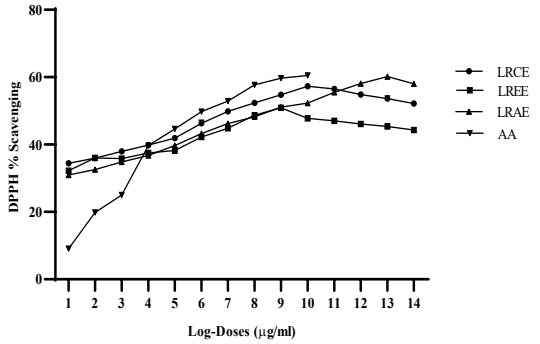

Fig. 3: DPPH radical scavenging activity of several extracts of *L. ruellioides* and standard ascorbic acid. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract; AA- Ascorbic acid. Values are expressed as mean ± SEM, n=3.

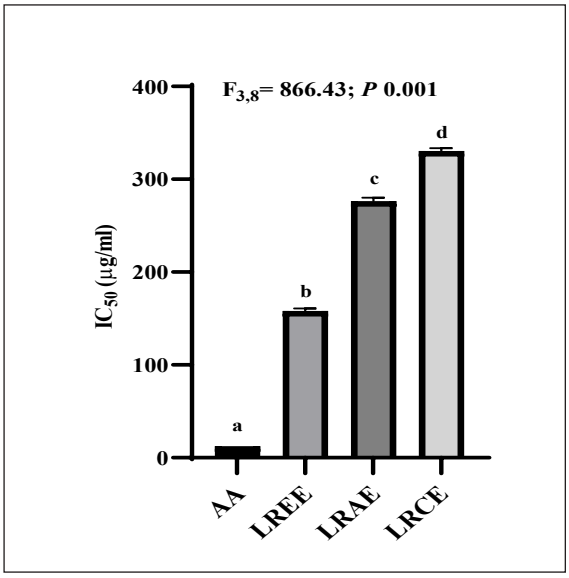

Fig. 4: IC_{50} (µg/ml) for DPPH. AA- Ascorbic acid. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

ABTS radical scavenging activity

L. ruellioides also showed a concentration dependent increase in the scavenging of ABTS radical. The maximum scavenging activity was observed at 200 µg/ml for both LREE and LRAE and 500 µg/ml for LRCE. LREE scavenged the ABTS radicals more efficiently as it showed the highest scavenging activity (IC_{50} 112.9 ± 6.47 µg/ml), followed by LRAE (IC_{50} 222.33 ± 3.18 µg/ml). LRCE was least effective in neutralizing the ABTS (IC_{50} 318 ± 12.19 µg/ml). IC_{50} of all the extracts were statistically significant when compared to the standard ascorbic acid (IC_{50} 13.52 ± 0.25 µg/ml). Some earlier studies had reported a resembling effect by using different plant extracts (37,38). Stable free radical ABTS is produced when a potent oxidizing agent reacts with another substance with the ABTS salt. A dark-coloured solution's reduction by an antioxidant that donates hydrogen is assessed by its distinctive long-wave (745 nm) absorption spectra (39). The level of decolourization indicates the effective inhibition of the $ABTS^{\cdot+}$ (40). The $ABTS^{\cdot+}$ scavenging activity could be due to high phenolic contents.

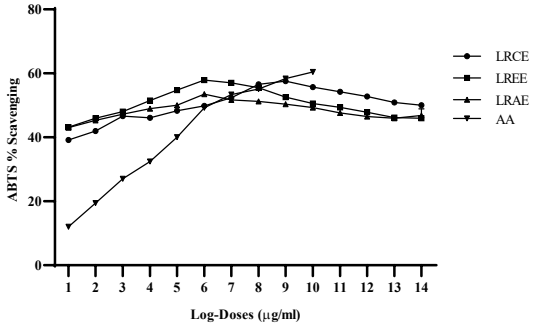

Fig. 5: ABTS radical scavenging activity of various extracts of *L. ruellioides* and standard ascorbic acid. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract; AA-Ascorbic acid. Values are expressed as mean ± SEM, n=3.

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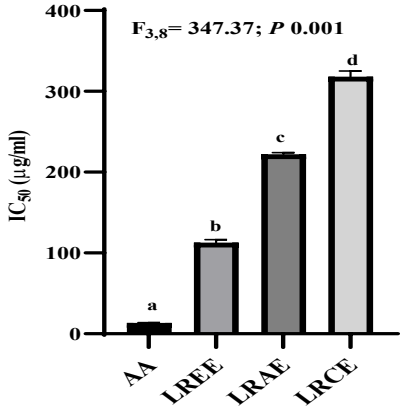

Fig. 6: IC_{50} (µg/ml) for ABTS. AA- Ascorbic acid; LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

Superoxide radical ($O_2^{\bullet-}$) scavenging activity

Various extract of *L. ruellioides* inhibit the generation of superoxide radicals in a concentration dependent manner. LRAE scavenged the $O_2^{\bullet-}$ most effectively with a peak scavenging activity at 600 µg/ml (IC_{50} 135.5 ± 5.02 µg/ml). For LREE and LRCE peak scavenging activity occurred at 700 µg/ml (IC_{50} 214.9 ± 3.59 µg/ml) and 1000 µg/ml (IC_{50} 366.43 ± 6.57 µg/ml) respectively. The standard ascorbic acid had an IC_{50} 15.18 ± 0.14 µg/ml for $O_2^{\bullet-}$. A number of plant extracts and some compound plant formulations were found to inactivate the formation of $O_2^{\bullet-}$ radicals in a dose dependent manner (31,33,34,38,41). Neutralization of superoxide radical is necessary to protect the cells from oxidative stress (42). It has also been reported that antioxidant properties of some flavonoids are effective mainly through scavenging superoxide radical (43). Thus, the presence of flavonoids in *L. ruellioides* might be responsible for their scavenging activity.

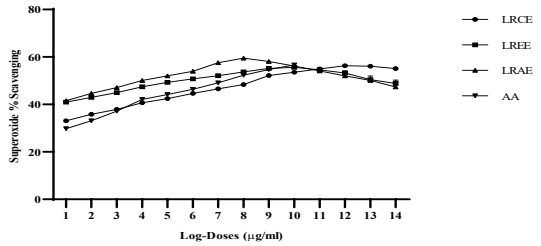

Fig. 7: Superoxide radical scavenging activity of various extracts of *L. ruellioides* and standard ascorbic acid. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract; AA- Ascorbic acid. Values are expressed as Mean ± SEM, n=3.

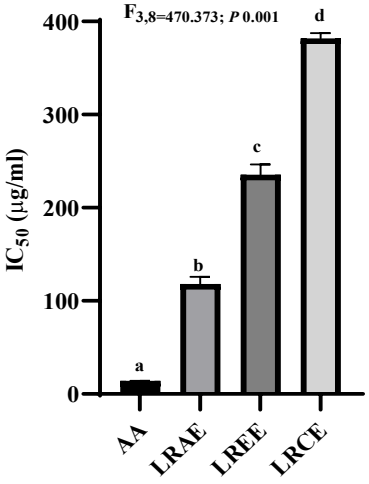

Fig. 8: IC_{50} (µg/ml) for superoxide radical. AA- Ascorbic acid; LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

Antimicrobial activity of plant extract

Disc diffusion method

The present study revealed that LRCE, LREE and LRAE were active against the test organisms as they show remarkable zone of

inhibition. At 10 mg/ml, LREE was most active against the three microorganisms with zone of inhibition of 7.6 ± 0.16 mm, 6.1 ± 0.16 mm and 7 ± 0.28 mm for *E. coli*, *B. subtilis* and *K. pneumoniae* respectively. However, at 20 mg/ml, LRCE was most effective with zone of inhibition of 9.3 ± 0.16 mm, 8.1 ± 0.16 mm and 9.1 ± 0.16 mm for *E. coli*, *B. subtilis* and *K. pneumoniae* respectively. Moreover, from the study, it is seen that 5% DMSO and distilled water, the solvents used for dissolving the extract does not show any antibacterial activity.

Table 2: Antimicrobial activity of LRCE, LREE, LRAE on different test microorganisms.

Extracts (10µg/disc)	Conc. (mg/ml)	Zone of inhibition (mm)*		
		E. coli	*B. subtilis*	*K. pneumoniae*
LRCE	10	7.1 ± 0.16	6.1 ± 0.16	6.5 ± 0.28
	20	9.3 ± 0.16	8.1 ± 0.16	9.1 ± 0.16
	Standard	17.5 ± 0.28	19.1 ± 0.16	17.3 ± 0.16
LREE	10	7.6 ± 0.16	6.1 ± 0.16	7 ± 0.28
	20	8.1 ± 0.16	7.1 ± 0.16	8.1 ± 0.16
	Standard	17.6 ± 0.16	19.6 ± 0.16	16.8 ± 0.16
LRAE	10	6.6 ± 0.33	5.3 ± 0.16	6.6 ± 0.16
	20	7.6 ± 0.16	6.1 ± 0.16	7.1 ± 0.16
	Standard	16.1 ± 0.16	16.1 ± 0.16	17.1 ± 0.16

* Indicate the zone of inhibition shown above is the mean of three readings and includes the diameter of the paper disc, i.e., 5 mm. LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanolic extract; LRAE- *L. ruellioides* aqueous extract.

MIC

The minimum concentration of the crude extract that inhibits the growth of the test microorganism was determined and recorded as MIC of the extract on that particular organism.

Table 3: Minimum inhibitory concentration (MIC) for different extracts of *L. ruellioides* on different test microorganisms.

Test organisms	MIC of LRCE	MIC of LREE	MIC of LRAE
Escherichia coli (MTCC- 40)	1.25 mg/ml	0.625mg/ml	2.5 mg/ml
Bacillus subtilis (MTCC- 121)	2.5 mg/ml	0.625mg/ml	5 mg/ml
Klebsiella pneumoniae (MTCC- 39)	0.625 mg/ml	1.25 mg/ml	5 mg/ml

MIC- Minimum inhibitory concentration; LRCE- *L. ruellioides* chloroform extract; LREE- *L. ruellioides* ethanol extract; LRAE- *L. ruellioides* aqueous extract.

Our result (Table 3) showed that LREE is quite active even at low concentrations with an MIC value of 0.625 mg/ml on *E. coli* and *B. subtilis* and 1.25 mg/ml on *K. pneumonia*. LRCE is also quite active on *E. coli* and *K. pneumonia* having MIC at 1.25 mg/ml and 2.5 mg/ml for *B. subtilis.* LRAE show MIC for *E. coli* at 2.5 mg/ml and for *B. subtilis* and *K. pneumonia* at 5 mg/ml. The presence of phytochemicals like flavonoids and tannins may be the cause of its antibacterial properties (44,45). These findings are in accordance with earlier studies (46-48). The above findings tell us that *L. ruellioides* has antibacterial activity even at low concentration,

however, isolation and purification of bioactive compounds responsible for the antibacterial activity need further investigation.

Conclusion

The results of phytochemical analysis validated the presence of various classes of bioactive chemical constituents. It also demonstrates that various extracts of *L. ruellioides* exhibit a concentration dependent inhibition against DPPH, ABTS and superoxide which might be due to presence of significant amounts of phenolic and flavonoid contents. Notably, its antibacterial activity is also quite remarkable against the test microorganisms. It may be concluded that the significant number of phytochemicals present in *L. ruellioides* attributed to their free radical scavenging activity and antibacterial property as well. Thus, the results of the present study substantiate the folkloric use of *L. ruellioides* urging in-depth exploration to unravel its mechanism of action.

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