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Original Article

Invitro Evaluation of Antioxidant and Antimicrobial Potential of leaves of *Atalantia racemosa* Wight ex Hook.

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ABSTRACT

Objective: *Atalantia racemosa* belongs to the family Rutaceae, comprises of 11 species which are closely-related. The present study was mainly done to evaluate the antioxidant and antimicrobial potential of leaves of *Atalantia racemosa*. **Methods:** Free radical scavenging activity for the leaves of *A. racemosa* was studied by invitro assays such as DPPH$^{\cdot}$, hydroxyl (OH$^{\cdot}$) radical scavenging, ABTS$^{\cdot+}$ radical cation, phosphomolybdenum and ferric reducing power assay respectively. Also, the antimicrobial studies were carried out by Agar well diffusion method in order to study the inhibition effect of plant extract against the test pathogens. **Results:** The IC_{50} value (Inhibitory concentration) of methanolic extract for DPPH$^{\cdot}$ radical was 95.92µg/mL. For hydroxyl (OH$^{\cdot}$) radical scavenging theIC_{50} value was 40.60µg/mL and for ABTS$^{\cdot+}$ radical theIC_{50} value was 41.81µg/mL. The antimicrobial effect was studied against the test pathogens, having higher inhibition of 20mm against gram positive bacteria *Staphylococcus aureus*. **Conclusion:** The study revealed that *Atalantia racemosa* could be considered as a potent antioxidant source against many free radicals. By performing these antioxidant activities, the anti-proliferative nature on selected cell lines shall be determined.

Keywords: Free radicals, Antioxidant, DPPH$^{\cdot}$ assay, ABTS$^{\cdot+}$ radical cation, IC_{50}, Antimicrobial activity.

1. INTRODUCTION

Nature has blessed India with plenty of medicinal plants, so often referred as the Medicinal Garden of the world. In Indian Vedas, the medicinal plants including every part of the plant from roots to flowers were used for curing several diseases. Medicinal plants have been selected and are used traditionally as potential and effective drug candidates since

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they possess definitive drug-like mechanism when compared to chemically-synthesized derivatives [1-2].

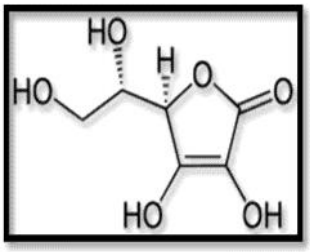
In 1820, isolation of Quinine was completed from Cinchona, which was considered to be an ancient herbal medicine which was changed into a chemical drug. This was an ethno-pharmacological process and scientists started using the above method for creating new drugs. Twenty-five thousand phytochemicals have been found to be prevailed in the plant flora. It's calculated that around 10,000 alkaloids and 4000 flavonoids are distributed in the plant source. The isolated phyto-compounds are gathered into many classes by number and types of atoms and the basic structure of the compound.

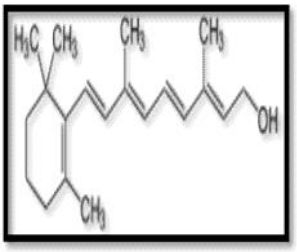
Phyto-medicine refers to making use of plants for effective medicinal purpose for treating, curing of diseases and improve human health system. The pharmacological property or the efficacy of medicinal plants is due to the presence of secondary metabolites such as Tannins, Alkaloids, Phenolic compounds, Steroids, etc. Each secondary metabolite has it's own pharmacologically active and valuable biological properties [1,3,4]. The major groups of phyto-constituents are Phenolic compounds (45%), Terpenoids and Steroids (27%), Alkaloids (18%) and others (10%). Due to the presence of phyto-compounds, the antioxidant potential against free radicals is highly determined, thereby protecting cardio-vascular diseases, aging factors, etc.[5, 6]

Antioxidants are compounds that are capable to inhibit or slow down the oxidation, which occurs due to environmental stress or Reactive Oxygen Species. Antioxidants are the first line of defense mechanism in neutralizing the free radicals. Fruits, vegetables, grains, nuts, pulses when consumed have a great effect in reducing many chronic diseases. The phytochemicals, derived from medicinal plants have significant role as antioxidant-rich molecules. Excess production of free radicals starts to form chain reactions, circulates in the human system thereby oxidize the low density lipoproteins (LDL) and slowly moving to lethal condition. The oxidation process gets initiated continuously due to specific substances, until a scavenging or neutralizing mechanism occurs [7-13].

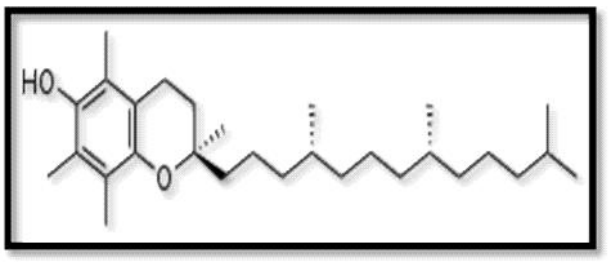
Molecules with an unpaired electron are said to be free radicals. These molecules are highly reactive species due to the presence of a free electron. They are highly important in natural mechanism involved in neurotransmission, vascular tone control and cytotoxic profile. Free radicals are formed when the weak bonds split each other, also they are unstable, react quickly with other compounds, seize the required electron to attain stability. Therefore, the desired/attacked molecule loses its electron and becomes a free radical and starts to produce chain reaction, thereby interfere with living cells. The human diseases that occur due to free radicals are Cancer, Alzheimer's disease, Fanconi syndrome, Fibrosis, Cardiomyopathy, Diabetes, Retinitis pigmentosa [14-16].

Example: Antioxidant rich foods – Vitamin A, Vitamin C, Vitamin E, tomato, apples, blueberries, dark chocolates, artichoke, kidney beans, etc..



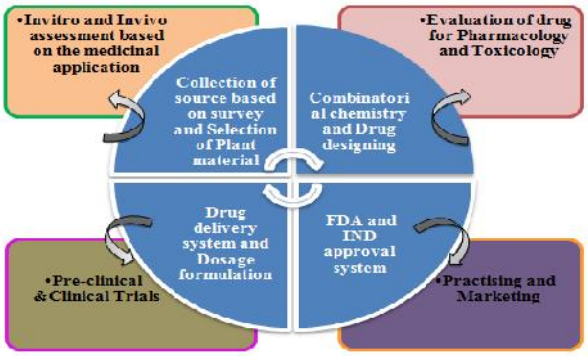


Vitamin C-Ascorbic acid ***Vitamin A-Retinol***



Vitamin E-Alpha Tocopherol

The genus Atalantia (Family: Rutaceae) comprises nearly 11 species which are very closely related one. *Atalantia racemosa* Wight. is covered in the evergreen forests of Peninsular India upto 1000m [17]. They are branched with long spines, branchlets terete and glabrous. The leaves arrangement is simple, alternate, spiral, petiole, plano-convex in cross section, articulate, elliptic ovate, apex emarginate, rounded, etc [18-20].



Therefore, it is necessary to enrich the dietary system with antioxidant molecules to protect the human system against various diseases [21,22]. Nowadays the research is focussed on food industry, natural products in order to derive phyto-molecules that could be turned as an active-drug. The antioxidant potential and also the effective compounds present in the selected medicinal plant have been studied.

2. MATERIALS AND METHODS

Collection of plant material

The leaves of *Atalantia racemosa* were collected from Damanur village, Villupuram, Tamilnadu and authenticated by Dr.P.Jayaraman, Department of Botany, Government Arts College for men (Autonomous), Nandanam, Chennai.

The leaves were separated and shade dried at room temperature for a period of 10 days. Then the leaves were ground into fine powder and sieved through fine mesh, finally stored in cool and dry place in a clean air-tight container.

Extraction process

Extraction of leaf powder with Hexane, Ethyl acetate and methanol was performed by direct method after the method [23].In this method, finely ground plant material was extracted with Hexane, Ethyl acetate and methanol in the ratio of 1:10 in conical flask in shaking condition at 120rpm for overnight. The extract was filtered through the Whatmann No.1 filter paper in a separate container. The above process was repeated 3 times with the same plant material but using fresh solvent.

Invitro Antioxidant Activity of crude extracts of *Atalantia racemosa*

The antioxidant activity of the *Atalantia racemosa* plant was analyzed by using the (solvents) hexane, ethyl acetate and methanol fraction.

(a) Free radical Scavenging Activity

The antioxidant activity was determined by DPPH$^{\cdot}$ scavenging assay accordingly [24].Various concentrations of the three crude extracts was been pipetted out in clean test tubes. Freshly prepared DPPH (1,1-Diphenyl-2-picryl hydrazyl) solution (1ml) was added to each tube and the samples were incubated in dark condition for 30 minutes at room temperature and read at 517 nm. The data were expressed as the percent decrease in the absorbance compared to the control. Ascorbic acid was used as reference compound.

(b) Phosphomolybdenum assay

Total antioxidant capacity can be calculated by the method described [25].Various concentrations of the crude extract from the prepared sample was been pipetted out and 1ml of the reagent solution was added, followed by incubation in boiling water bath at 95°C for 90mins. After cooling the sample to room temperature, the absorbance of the solution was measured at 695 nm in UV spectrophotometer. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions. Ascorbic acid was used as standard.

(c) Ferric (Fe^{3+}) reducing power assay

The crude extract was taken in various concentrations accordingly and was mixed with 2.5ml of phosphate buffer (0.2 M, pH 6.6) and 2.5ml of potassium ferricyanide (1%), and incubated at 50°C for 30minutes. Then, 2.5ml of trichloroacetic acid (10% v/v) was added to the mixture and then centrifuged at 3000 rpm for 10 min. Finally, 2.5ml of upper layer solution was mixed with 2.5ml of distilled water and 0.5ml $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm[26,27]. EDTA was used as standard.

(d) Hydroxyl radical ($OH^{\cdot}$) scavenging activity

The scavenging activity of methanolic extract of *Atalantia racemosa* on hydroxyl radical was measured according to the method [28]. Various concentrations of crude extract were added with 1.0ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5ml of EDTA solution (0.018%), and 1.0mL of dimethyl sulphoxide (DMSO) (0.85% v/v in 0.1M phosphate buffer, pH 7.4).The reaction was initiated by adding 0.5ml of ascorbic acid (0.22%) and incubated at 80–90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of1.0mL of ice-cold TCA (17.5% w/v). 3ml of Nash reagent was added and left at room temperature for 15min. The reaction mixture without sample was used as control. The intensity of the color formed was measured at 412 nm. The percentage of hydroxyl radical scavenging activity is calculated by the following formula:

%Hydroxyl radical scavenging potential = [(Control OD-Sample OD)/Control OD] X100.

(e) $ABTS^{\cdot+}$radical cation (2,2 – azinobis (3-ethylbenzo thiazoline-6-sulfonic acid)assay

This assay was performed according to the method[29]. The stock solution includes 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 hours at room temperature in the dark. Fresh ABTS solution was prepared for each assay. Plant extract of varying concentrations were allowed to react with 500µL of the ABTS solution for 15minutes in dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer.

Screening of crude extract for antibacterial activity

Agar Well diffusion assay

Nutrient agar medium was prepared, 24 hours grown bacterial pathogens were swabbed on the solidified nutrient agar plates [30]. Then, the stock crude extract individually (10mg/ml) was prepared. Varying concentration (250µg, 500µg, 750µg and 1000µg) of plant extract was loaded in the wells made using sterile Cork borer. Tetracycline was used as standard. The plates were then incubated at 37ºC for 24hours. After incubation the inhibition diameter was measured.

3. RESULTS AND DISCUSSION

Collection of plant material and Extraction process

The extracts (hexane, ethyl acetate and methanol)of *Atalantia racemosa* was condensed separately in rotary evaporator to obtain semi-crude consistency and gummy paste form. The extracted residues were weighed and re-dissolved in different solvents to yield 1mg/ml concentration for further analysis.

Fig 1: Dried leaves of *Atalantia racemosa*

Invitro Antioxidant Activity of crude extracts of *Atalantia racemosa*

Based on the presence of different phyto-constituents in the plant extract, the antioxidant effect was evaluated by different methods.

(a) Free radical Scavenging Activity

The antioxidant activity was carried out by DPPH assay according to the method of khalaf (2008) [24]. Antioxidant molecules can quench DPPH free radicals (i.e by providing hydrogen atoms or by electron donation, via a free radical attack on the DPPH molecule) and convert them to colorless. The percentage of DPPH scavenging activity was 70% (Table 3) in methanol fraction of *Atalantia racemosa* when compared to other two fractions.

Table 1: DPPH· Radical scavenging activity of Hexane extract of leaves of *Atalantia racemosa*

S. No	Concentration µg/mL	% of inhibition (RSA)
1	100	19.21
2	200	23.38
3	300	27.81
4	400	33.07
5	500	36.76
6	600	39.53

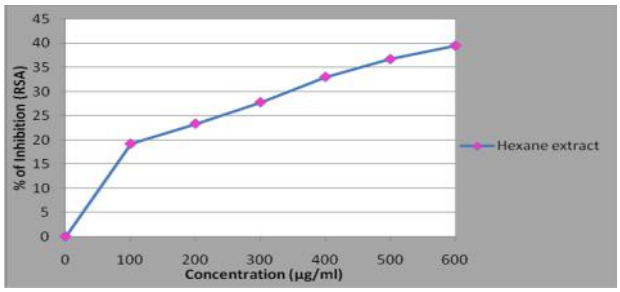


Fig 2: DPPH· Radical Scavenging Activity of Hexane extract of leaves of *Atalantia racemosa*

Table 2: DPPH· Radical scavenging activity of Ethyl acetate extract of leaves of *Atalantia racemosa*

S. No	Concentration µg/mL	% of inhibition (RSA)
1	200	22.77
2	250	35.16
3	300	43.44
4	350	49.75
5	400	54.67
6	450	60

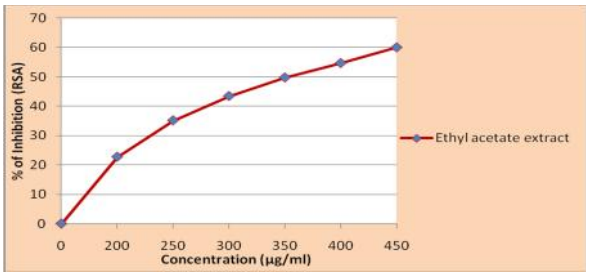


Fig 3: DPPH· Radical Scavenging Activity of Ethyl acetate extract of leaves of *Atalantia racemosa*

Table 3: DPPH· Radical scavenging activity of methanol extract of leaves *of Atalantia racemosa*

S. No	Concentration µg/mL	% of inhibition (RSA)
1	20	16.75
2	40	27
3	60	35.25
4	80	41.7
5	100	52.75
6	120	70

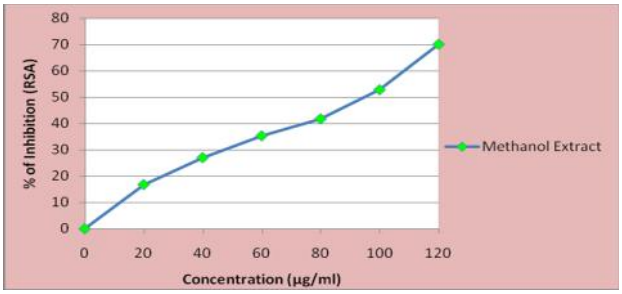


Fig 4: DPPH· Radical scavenging activity of methanol extract of leaves of *Atalantia racemosa*

DPPH method is one of the best screening method for evaluating the antioxidant capacity for herbal based drugs. The DPPH· (1,1-diphenyl-2-picryl hydrazyl) radical, which is purple in colour gets reduced by the antioxidant molecules present in the extract to 1,1-diphenyl-2-picryl hydrazine (yellow colour). The amount of scavenging potential by the plant extract can be determined spectrophotometrically at 517nm. The methanol extract of *A.racemosa* exhibited 41.7% radical scavenging activity at 80µg/mL concentration. The IC_{50} (Inhibitory Concentration) at 50% level can be calculated from the percentage values. From the current study, the ethyl acetate extract of *A.racemosa* possess 351.75µg/mL (Table 2) as IC_{50}value. Comparitively, the methanolic extract of *A.racemosa* possess 95.92µg/mL as IC_{50} value concentration, indicating the maximum effect.

(b) Phosphomolybdenum assay

The antioxidant activity was carried out by phosphomolybdenum assay according to the method of (Prieto *et al.*,1999) [25]. Increase in Absorbance was observed in the methanolic extract of *Atalantia racemosa* after the completion of incubation period.

Table 4: Phosphomolybdenum Reducing Potential of methanol extract of leaves of *Atalantia racemosa*

S. No	Concentration µg/mL	Phosphomolybdenum Reducing Potential
1	20	0.019
2	40	0.051
3	60	0.065
4	80	0.119
5	100	0.328
6	120	0.343

The Total antioxidant activity (or) phosphomolybdenum assay mainly detects ascorbic acid, phenolic compounds, carotenoids, etc.. (Harini *et al.*, 2012) [30]. The assay is based on the reduction of Mo (VI) to subsequent formation of green phosphate Mo (V) complex at acidic pH by the methanolic extract of *Atalantia racemosa.* The maximum absorbance was 0.343 at

120 µg/mL concentration (Table 4) showing increase in absorbance value at 695nm.

(c)Ferric (Fe^{3+}) reducing power assay

The antioxidant activity of *Atalantia racemosa* extract was calculated according to the Makari *et al*., 2008, Hennebelle *et al*., 2008 [26, 27]. The ferric reducing power assay denotes the yellow color of the test solution changes to various shades of green to Pearl's Prussian blue depends upon reducing power of each compound.

Table 5: Fe^{3+} Reducing Potential (FRP) of methanol extract of leaves of *Atalantia racemosa*

S. No	Concentration µg/mL	Ferric Reducing Potential
1	20	0.029
2	40	0.053
3	60	0.097
4	80	0.151
5	100	0.203
6	120	0.396

The ferric reducing activity of methanolic extract of *Atalantia racemosa* was found to be increase in the reducing capability with increase in their respective concentrations. The reducing property was 0.396 at 120µg/mL concentration (Table 5). Reduction process from Fe^{3+} to Fe^{2+} was well-observed for the methanolic extract indicating higher activity.

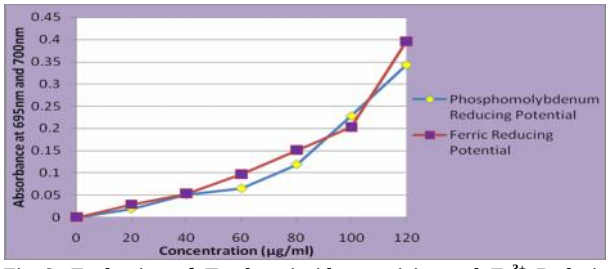


Fig 6: Evaluation of Total antioxidant activity and Fe^{3+} Reducing Potential (FRAP) of methanol extract of leaves of *Atalantia racemosa*

(d) Hydroxyl radical ($OH^{\cdot}$) scavenging activity

Hydroxyl radical is the most reactive oxygen centered species and causes severe damage to adjacent biomolecule. Hydroxyl radical scavenging activity was estimated by generating the hydroxyl radicals using ascorbic acid–iron EDTA. The hydroxyl radicals were formed bythe oxidation reaction with the Dimethyl Sulphoxide (DMSO) to yieldformaldehyde, which provides a convenient method to detect hydroxylradicals by treatment with Nash reagent.

The hydroxyl radical is formed by the combination of Fe (II) and hydrogen peroxide, which is a Fenton reaction. (Sanches 2002)[31]. This assay is used to quantify or measure the capacity of putative antioxidants to neutralize or stop the formation of precursor molecules (Magalh *et al.*,2008) [32]. These scavenging compounds would behave as antioxidant molecules in a preventive manner.

Table 6: Hydroxyl radical ($OH^{\cdot}$)scavenging activity of methanol extract of leaves of *Atalantia racemosa*

S. No	Concentration µg/mL	Hydroxyl radical ($OH^{\cdot}$) scavenging activity (%)
1	10	15.93
2	20	27.47
3	30	35,12
4	40	49.26
5	50	61.46
6	60	63.57

This particular scavenging activity is considered to be important since, the OH radical is highly reactive. The hydroxyl radical is a reactive free radical generated in biological/living systems. The maximum activity of hydroxyl radical scavenging potential was found to be 49.26% (Table 6). The IC_{50} of hydroxyl radical scavenging activity was found to be 40.60µg/mL concentration.

(e) $ABTS^{\cdot+}$radical cation (2,2 – azinobis (3-ethylbenzo thiazoline-6-sulfonic acid)assay

ABTS (2,2 – azinobis (3-ethylbenzo thiazoline-6-sulfonic acid) assay measures the relative ability of antioxidant to scavenge the ABTS generated in aqueous phase. ABTS is generated by reacting with a strong oxidizing agent (Potassium per sulfate) with ABTS salt. Reduction of blue green ABTS-radical coloured reaction by hydrogen-donating antioxidant is measured at 734nm.

Table 7: $ABTS^{\cdot+}$ assay of methanol extract of leaves of *talantia racemosa*

S. No	Concentration µg/mL	$ABTS^{+}$ radical cation activity (%)
1	10	24.03
2	20	34.12
3	30	42.41
4	40	47.83
5	50	52.08
6	60	58.64

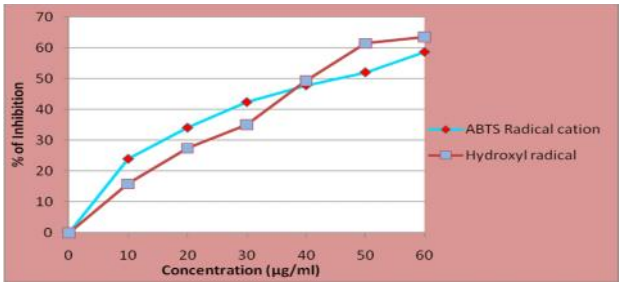


Fig 7: Hydroxyl radical ($OH^{\cdot}$) scavenging activity and$ABTS^{\cdot+}$ assay of methanol extract of leaves of *Atalantia racemosa*

Interaction between the antioxidants and $ABTS^{\cdot+}$ radical cation can be studied by this method. The $ABTS^{\cdot+}$ radical is stable and soluble in water and organic solvents, thereby determining the antioxidant capacity of both hydrophilic and lipophilic compounds. This assay is mostly used by the food industries in order to measure the antioxidant capability of foods. The methanolic extract of *Atalantia racemosa* was evaluated for $ABTS^{\cdot+}$ radical cation scavenging activity. The maximum percentage was 47.83% at 40 µg/mL concentration (Table 7) and also the IC_{50} of $ABTS^{\cdot+}$ radical cation was found to be 41.81 µg/mL.

4.6 Screening of crude extract for antibacterial activity by Agar Well diffusion assay

The inhibitory activity for the methanolic extract was observed after 24hours of incubation. The zone of inhibition was measured using zone scale and is represented in Table 8.

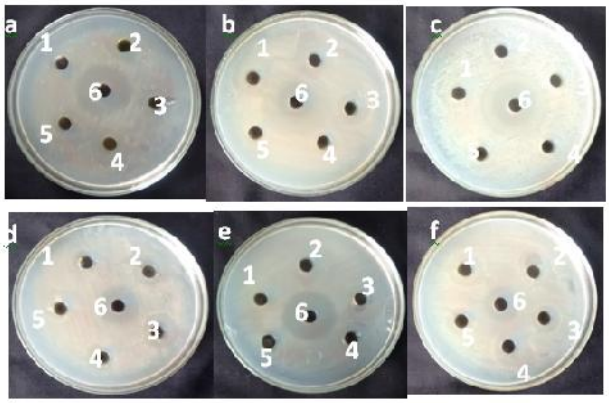


Fig 8: Antibacterial activity of methanolic extract of *Atalantia racemosa* 1,3,4,5 – Crude extract in varying volume; 2-Methanol (solvent) as control; 6-Standard – Tetracycline as positive control

Table 8: Zone of inhibition of Antibacterial activity of methanolic extract of *A. racemosa*

S.No	Bacterial pathogens	Zone of inhibition(mm)				Standard
		250µg	500µg	750µg	1000µg	
1	*(a) S. aureus*	-	16mm	18mm	20mm	22mm
2	*(b) K. pneumonia*	14mm	15mm	17mm	19mm	20mm
3	*(c) P. vulgaris*	10mm	10mm	11mm	11mm	34mm
4	*(d) E. coli*	14mm	15mm	17mm	19mm	20mm
5	*(e) B.subtilis*	13mm	17mm	18mm	19mm	20mm
6	*(f) M. luteus*	12mm	12mm	14mm	16mm	29mm

The antibacterial property of*Atalantia racemosa* was tested against set of bacterial pathogens. The zone of inhibition of LME (250µg/mL) was found to be 17mm and 12mm respectively against *S. aureus, K. pneumoniae.*(Jayaprakash priya *et al.*, 2013). The antibacterial activity results of *Atalantia racemosa* was comparatively found to be higher against *S. Aureus* inhibiting zone of 20mm whereas, the inhibitory effect was comparatively moderate for *K. pneumonia*(19mm), *E. coli*(19mm), *B.subtilis* (19mm), *Micrococcus luteus*(16mm), *P. vulgaris* (11mm).

4. CONCLUSION

From the present investigation, the leaves of *Atalantia racemosa* possessed significant antioxidant activity, thereby proving that the leaves contain rich-antioxidant molecules on natural basis. Also, new antimicrobial drugs can be developed for treating various diseases from the selected plant. The leaves also contain higher amount of phenolic compounds. Further, the individual active compounds can be isolated by chromatographic techniques and the fractions shall be evaluated separately for FTIR, NMR to identify the compound functional group, nature and structure for converting as a new active drug.

5. ACKNOWLEDGEMENT

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