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ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF ETHYL ACETATE EXTRACT FROM THE LEAVES OF *RHINACANTHUS NASUTUSIN* VITRO

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ABSTRACT

Orofacial Ethyl acetate extract from *Rhinacanthusnasutus*leaves has long been used to treat a variety of infectious disorders. The researchers wanted to see how much phenol and flavonoid was in the ethyl acetate extract from *Rhinacanthusnasutus* leaves, as well as how effective it was as an antioxidant and antibacterial. The leaves had increased phenolic contents, according to the test of flavonoid and phenolic level. The antioxidant properties of extracts from various plant sections were also assessed using the ABTS radical scavenging, lipid peroxidation, superoxide radical, and nitric oxide scavenging methods. The antioxidant capabilities of the radical scavenging were found to be higher than those of the other sections. The disc diffusion method were employed to check the antibacterial characteristics of an ethyl acetate extract from the *Rhinacanthusnasutus* leaves, and the floral extracts displayed better antibacterial activity against the four bacterial strains utilised in this work. The antioxidant and antibacterial characteristics of phenolics obtained from the ethyl acetate extract of *Rhinacanthus nasutus*are discussed in this study.

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INTRODUCTION

Plants used in traditional medicine and as food are very much likely to produce pharmacologically active chemicals. The medicinal characteristics of plants have been investigated in current scientific discoveries all over the world despite its high therapeutic efficacy and antioxidant activities, lack of adverse effects, and economic viability.Medicinal herbs are used as a origin of raw materials for pharmaceuticals that provide patients with effective and affordable health treatment. Phytochemicals, on the other hand, are produced by all plants and are helpful to our health because they cannot be created in the body (Kim, 2008). Plants are indeed abundant in biomolecules, vitamins, and minerals, which are all necessary for a healthy body.Many plants have theraupatic effects because of presence of metabolites. Plant metabolites are organic substances that are divided into two categories: primary and secondary metabolites. Primary metabolites are organic molecules such as glucose, starch, polysaccharide, protein, lipids, and nucleic acid, which are required for human functioning. Plants produce secondary metabolites such as alkaloids, flavonoids, saponins, terpenoids, steroids, glycosides, tannins, volatile oils, and others.

These secondary metabolites are essential for plants' medicinal efficacy in the treatment of a wide range of ailments (Prashant *et al*., 2008). Human infections, particularly those involving microbes such as bacteria, fungi, viruses, and nematodes, cause major damage in tropical and subtropical nations around the world.Multiple drug resistance in human pathogenic bacteria has emerged in recent years as a result of the indiscriminate use of commercial antimicrobial medications frequently used to treat infections. Intensive attempts have been done over the years to develop plants with antibacterial action for various ailments (Stankovic *et al*., 2016). The usage of phytomedicines is becoming increasingly scientific, with a greater emphasis on product safety and efficacy. Antimicrobial agents are abundant in medicinal plants. A wide variety of medicinal components are employed to produce phytochemicals with various therapeutic effects against various microorganisms. Many plant species are examined for antibacterial capabilities, however the majority was not sufficiently studied. The current study is based on a review of such plants, due to the great potentiality of plants as sources of antibacterial medicines. In Africa, bacterial infections are one of the most common causes of infectious diseases. Poverty and a shoddy health-care infrastructure continue to be roadblocks to

efficient health-care delivery (Kusuma *et al*., 2014). Infections produced by these pathogenic prokaryotic bacteria have become a great reason of mortality in immunocompromised people in underdeveloped countries on a global scale (Santharam *et al*., 2015). Despite the availability of a wide spectrum of antibiotics, bacteria are constantly evolving resistance to them, making coordinated efforts to tackle infectious diseases more challenging. Since the introduction of antimicrobials in the treatment of bacterial illnesses, bacteria have developed a variety of resistance mechanisms.As time passes, the amount and complexity of bacterial pathogen resistance mechanisms grows (Nandhakumar *et al*., 2016). Drug-resistant bacteria, such as *Staphylococcus aureus, Klebsiellapneumoniae, and Pseudomonas aeruginosa*, are becoming more widespread in healthcare settings and may be contributing to treatment failures.

MATERIALS AND METHODS

Collection of Plant: Herbal garden of Government Siddha Medical College, Arumbakkam, Chennai-600106 provided the leaves of *Rhinacanthusnasutus.* Dr. S. Sankaranarayanan, Assistantprofessor, Department of Botany at Government Siddha Medical College, Arumbakkam, Chennai-600 106, Tamil Nadu, India, verified the plants' authenticity.

Phytochemical Analysis: Phytochemical screening of the aqueous extract from *Rhinacanthusnasutus* leaves was performed using established methods to evaluate the composition of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, glycosides, saponins, and polyphenols (Gul *et al*., 2017).

TLC Profile: The ethyl acetate extract from *Rhinacanthusnasutus* leaves was put onto pre-coated TLC plates (60 F_2 54). For formation of exudates onto silica gel plates, a solvent system containing Petroleum ether, Chloroform, and methanol (1:0.5:0.1, V/V/V) was utilised (10x20 cm, 0.2mmlayer). They are fluorescent with Ultra Violet light at 360nm and 240nm and is visible and non-visible.

ABTS assay: ABTS is diluted in water to a concentration of 7 mM. ABTS radical cation ($ABTS^{\bullet+}$) was produced by combining ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and leaving the mixture to sit in the dark at room temperature for 12-16 hours before use. The solution is dissolved in ethanol (1:89 v/v) and equilibrated at 30 °C to provide an absorbance of0.700 ± 0.020 at 734 nm in a 1 cm cuvette prior to the test. The concentration of extract sample that inhibited the blank absorbance by 20 percent to 80 percent was calculated and adapted. At each dilution of the standard, triplicate measurements were taken, and the % inhibition of the blank absorbance at 734 nm was shown with relation to Trolox concentration (Re *et al*. 1999). The concentration of Trolox with equal antioxidant activity reported as μmol/g sample extracts on a dry matter basis was designated as the measure of total antioxidant activity (TAA).

Inhibition of Lipid Peroxidation Activity: The method of Ohkawa *et al*. was used to examine lipid peroxidation generated by the Fe^{2+}ascarbate system in egg yolk as thiobarbituric acid reactive substances (TBARS) (1979). In final volume of 0.5 ml, 0.1 mL of egg yolk was included in the testing mixture(25 percent w/v) in Tris-HCl buffer (20 mM at pH 7.0) and various concentrations of ethyl acetate extract from *Rhinacanthusnasutus* leaves. For 1 hour, the experimental combination was kept at 37°C. After the incubation period, 0.4 ml of the sample was taken and treated with 0.2 ml sodium dodecyl sulphate (1.1%), 1.5 ml thiobarbituric acid (0.8%), and 1.5 ml acetic acid (20 percent , pH 3.5). With distilled water, the final amount was increased to 4.0 ml, which was then held in a water bath at 95 to 100 °C for 1 hour. To measure TBARS, the absorbance of the butanol pyridine layer was measured at 532 nm in a Deep Vision (1371) UV–Vis Spectrophotometer. The optical density (OD) of the test sample was compared to that of the control sample to assess whether lipid peroxidation was inhibited. As a control, ascorbic acid has been employed. The percent inhibition of lipid peroxidation by each extract has been determined using 1-(E/C) × 100, where C represents the absorbance of the totally oxidised control and E represents the absorbance of the test sample.

Nitric Oxide Radical Scavenging Activity: The potentiality of goat urine to scavenge nitric oxide was ascertained using the method reported by Olabinri *et al* (2010). 0.1 ml sodium nitroprusside (10 mM) in phosphate buffer (0.2 M at pH 7.8) was combined with various concentrations of ethyl acetate extract from *Rhinacanthusnasutus* leaves and kept for 150 min maintained at room temperature. 0.2 ml Griess reagent (1 percent Sulfanilamide, 2 percent Phosphoric acid, and 0.1 percent N-(1-Naphthyl) ethylene diamine dihydrochloride was added to it following the treatment period. At 546 nm, the absorbance of the experimental sample was measured against a blank. Ascorbic acid was employed as a standard and entire measurements were taken in triplicate. The following equation was used to compute the percentage of inhibition:

% Nitric oxide radical scavenging capacity= $[(A_0-A_1)/A_0] \times 100$
Where A_0 was the absorbance of control and A_1 was theabsorbance of flavonoid rich fraction.

Superoxide Radical Scavenging Assay: The potentiality of goat urine to block the photochemical reduction of Nitroblue tetrazolium (NBT) in the presence of the riboflavin light-NBT combination was used to develop this assay (Tripati, Pandy, Sharma, 1999). Every 3 milli litre reaction solution comprised 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, 75 μM NBT, and various concentrations of ethyl acetate extract from *Rhinacanthusnasutus*leaves. It was kept visible in fluorescent light for 6 minutes, and absorbance was estimated at 560 nm with a Deep Vision: UV–Vis 1371 Spectrophotometer after that. As blanks, identical tubes with reaction mixture have been kept in the dark. By comparing the absorbance of the control and test sample solutions, the percentage suppression of superoxide radical activity has been calculated:

% Super oxide radical scavenging capacity= $[(A_0-A_1)/A_0] \times 100$
Where A_0 was the absorbance of control and A_1 was theabsorbance of both plant extracts fraction.

Culture Collection and Maintenance: Gram positive bacteria, such as *Staphylococcus aureus* MTCC 2833 and *Enterococcus faecalis* MTCC 1651, and Gram negative bacteria, such as *Klebsiella pneumoniae* MTCC 1589 and *Escherichia coli* MTCC 1246, were employed to test antibacterial activity. Microbial Type Culture Collection & Gene Bank, Institute of Microbial Technology Sector 39-A, Chandigarh-160 036, India provided these bacterial strains. To preserve stock culture, these bacterial strains were subcultured in nutrient agar medium, incubated at 37°C for 24 hours, and refrigerated at 4°C.

Antibacterial Property: The disc-diffusion method was employed to test the anti-microbial property of an ethyl acetate extract from *Rhinacanthus nasutus* leaf (NCCLS, 2002). Bacteria was cultivated overnight in Mueller Hinton agar plates, and 5 colonies have been suspended in 5 ml of sterile saline (0.9 percent) with a bacterial population of $3x10^8$ CFU/ml. To remove the excess fluid, a sterile cotton swab was immersed onto a solution and spun several times on the inside wall of the tube with

light pressure. The swab was then used to inoculate the dried surface of the MH agar plate 4 times, rotating the plate roughly 90 degrees between inoculations to ensure uniform dispersion.Prior to adding a sterile disc with a diameter of 9 mm, the medium is allowed to dry for around 3 minutes. To ensure uniform contact with the bacteria, every disc were firmly placed on the agar. Weighing and dissolving the bioactive ingredient (50 µg) in 1 ml of 7 percent ethanol. Each disc received a varying concentration of bioactive chemical, with the control disc receiving only 7% ethanol. The inhibition zone was measured and estimated after 24 hours of incubation at 37°C. The experiments were repeated three times in total. By estimatingthe zones of growth inhibition around the discs, the results (mean value, n=3) were recorded.

Minimum Inhibitory Concentrations (MICS): The isolated compounds' minimal inhibitory concentrations have been established by the dilution method (Sein *et al*.,2008). Strains are raised to exponential phase in Mueller Hinton broth with A*560* of 0.8, corresponding to 3.2×10^8Colony Forming Units/ml. The ethyl acetate extract from the leaves of *Rhinacanthusnasutus*was produced in various dilutions to yield solutions of 5, 10, 15, and 20 µg/ml. In individual test tubes containing 4ml of MHbroth injected with 0.5 ml bacterial suspension at a final concentration of 10^6CFU/ml, 0.5 ml of every concentration was added. Five independent tests were conducted in duplicate to ascertain each MIC.The bacterial control tubes included 4.5 ml of bacterial inoculates and 0.5 milli litre of 7 percent acetone, while the blank tubes had 4.5 ml of uninoculated MH broth and 0.5 ml PBS. The tubes were incubated at 37°C for 18 hours, and the absorbance at 560 nm was used to measure bacterial growth inhibition.

Statistical Analysis

INTA Software was used to do the statistical analysis. Data was analysed using a one way analysis of variance (ANOVA) and Dunnet's test. Data was provided as a Mean SEM. *p<0.05 and **p<0.01 were considered statistically significant.

RESULT AND DISCCUSION

Phytochemical Screening: The composition of Terpenoids, alkaloids, flavonoids, saponin, glycosides, and phenolwas discovered in the aqueous extract from *Rhinacanthus nasutus* leaves investigated, but glycosides and tannin were absent (Table 1).

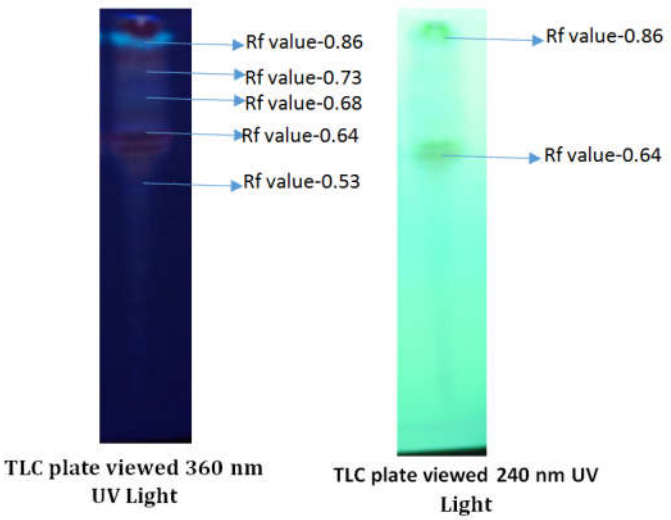
TLC partial characterization - ethyl acetate extract from rhinacanthus nasutus leaves: The leaves of *Rhinacanthus nasutus* were put onto pre-coated TLC plates (60 F_2 54 Merck) &established using a solvent system containing toluene, dioxan, and acetic acid in ratio of 9.5:2.5:0.4 was effective in extracting the antioxidant and antibacterial compound that will be employed for further research. The developed plate has been examined at Ultra violet light 240nm and 360nm (Fig-1).

Disc diffusion method for antibacterial activity: The ethyl acetate extract from the leaves of *Rhinacanthus nasutus* was evaluated against *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa*, and *Escherichia coli* at various concentrations (25, 50, 75, and 100 µl/ml).

Table 1. The Phyto-chemical screenings of aqueous extract from the leaves of *Rhinacanthusnasutus*

Sl. No.	Phytochemical Constituents	Observation	Aqueous of extract from the leaves of *Rhinacanthusnasutus*
1	Alkaloids -Dragendorff's test -Mayers test	Orange or red precipitate Cream pie ppt	+ +
2.	Flavonoids -Alkalai Reagent -Lead aceate test	Intense yellow colour Precipitate formed	+ +
3.	Glycosides -Keller-Killiani test	Pink colour (Ammonia layers)	+
4.	Tannin -$FeCl_3$ test	Blue-black colour	+
5.	Saponins -Frothing test	Foam	-
6.	Terpenoids -Salkowski test	Reddish brown colour ring formed in interface	-
7.	Polyphenols -Ferrozine test	Raddish blue	+
8.	Anthocyanin -Ammonia test	Pink color in ammonia layer	+

Positive result; - Negative result



At 100µl/ml concentration, the ethyl acetate extract from *Rhinacanthus nasutus*leaves displayed stronger bactericidal effect against B. subtilis (16.3mm) and S. aureus (15.3mm), with a larger inhibitory zone (Table-2). These findings corroborated previous studies that demonstrated methanol to be a suitable solvent than water and other organic solvents for more reliable extraction of antimicrobial chemicals from medical plants.Common phenolic compounds discovered in medicinal plant tissues include flavonoids, phenolic acids,tannins, coumarins, stilbenes, lignans, and lignins. These chemicals perform a wide range of biological functions, including antioxidant activity (Rubio *et al*., 2013).

(Table-4). Lipid peroxidation can be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid) because it is the outcome of any free radical attack upon membrane as well as other lipid components of the system.Egg yolk was used as a non-enzymatic substrate for free radical-driven lipid peroxidation in the study.Usually, phenolic substances' antioxidant activity is mediated by neutralising lipid free radicals thereafter preventing hydroperoxide degradation into free radicals (Gabriela *et al*., 2015).

Assay of Superoxide Scavenging: Table-4 shows the findings of a photochemical reduction of nitro blue tetrazolium (NBT) in the

Table 2. Antibacterial property of the ethyl acetate extract from the leaves of *Rhinacanthusnasutus*by disc diffusion method

Pathogenic bacteria	Ethyl acetate extract from the *Rhinacanthusnasutus*leaves exhibited the Zone of inhibition (mm)[a]				
	Positive control 10µl Ampicillin	Different concentrations extract (µl/ml)			
		25 µl	**50** µl	**75** µl	**100** µl
Bacillus subtilis	13.7±0.2	10.3±0.8	12.4±1.2	14.3±0.5	16.3±1.4
Staphylococcus aureus	12.6±0.3	9.2±0.3	11.2±0.4	13.4±0.4	15.3±1.6
Pseudomonas aeruginosa	13.4±0.3	6.5±0.7	8.4±0.4	10.2±0.2	12.3±0.8
Escherichia coli	13.2±0.4	7.5±1.2	9.4±1.4	11.3±1.6	13.7±1.7

[a] Calipers were used to measure the inhibitory diameter. The assays were all replicated, and the mean results were recorded.

Table 3. The efficacy of an ethyl acetate extract of Rhinacanthus nasutus to scavenge free radicals was tested using the ABTS method

Different concentration of extract	Percentage of lipid peroxidation	
	Ethyl acetate extract from the leaves of *Rhinacanthusnasutus*	Standard Vitamin-C
25 µl/ml	19.64±1.89	16.34±2.37
50 µl/ml	41.32±2.34	38.64±1.73
75 µl/ml	56.34±1.46	52.34±2.64
100 µl/ml	70.49±2.78	65.32±1.64
EC_{50} value	61.23	64.21

[a]The results are expressed as a % of ABTS radical scavenging against control. Each value indicates the mean+standard deviation of three experiments.

Table 4. Inhibited lipid peroxidation activityof ethyl acetate extract of *Rhinacanthusnasutus*

Different concentration of extract	Percentage of lipid peroxidation	
	Ethyl acetate extract from the leaves of *R.nasutus*	Standard Vitamin-C
25 µl/ml	22.31±2.34	18.32±1.54
50 µl/ml	48.32±1.45	42.34±2.31
75 µl/ml	61.23±1.65	58.32±1.25
100 µl/ml	77.32±1.24	73.32±1.49
EC_{50} value	56.32	59.32

[a]The results are expressed as a percentage inhibition of lipid peroxidation in comparison to the control. Each value indicates the mean+standard deviation of three experiments.

Table 4. Superoxide scavenging assay activityof ethyl acetate extract from the leaves of *Rhinacanthusnasutus*

Different concentration of extract	Superoxide scavenging activity in percentage	
	Superoxide scavenging activity of ethyl acetate extract of *R. nasutus*	Standard Vitamin-C
25 µl/ml	16.37±2.34	14.32±2.34
50 µl/ml	31.24±1.49	27.34±1.78
75 µl/ml	53.35±1.69	48.35±2.34
100 µl/ml	67.32±1.24	65.34±1.56
EC_{50} value	63.21	69.32

[a]The results are expressed as a percentage of Superoxide scavenging activity vs control. Each value indicates the mean+standard deviation of three experiments.

ABTS Assay Provides free Radical Scavenging Ability: The radical scavenging ability have been determined using the ABTS test, shown in Table-3. On average, ethyl acetate extract of *R.nasutus*EC_{50} value61.23µl/ml had stronger free radical-scavenging values compared to pure ascorbic acid 64.21µl/ml. Nevertheless, the concordance between these assays in the investigation suggests that these actions were most likely caused by phenolics. Even though these chemicals have not been examined, their implications to the antioxidant/antiradical activities of the *R.nasutus* ethyl acetate extract studied may be insignificant. The antioxidant assay relies on electron transfer, whereas the ABTS assay relies on both electron and H atom transfer (Prior *et al*., 2005).

Peroxidation Activity is Inhibited: In egg yolk homogenates, the ethyl acetate extract of *R. nasutus* leaves prevented lipid peroxidation induced by ferrous sulphate. The highest inhibition percentage was discovered in *R. nasutus* ethyl acetate extract (77.32 percent), and the less inhibition % of ascorbic acid has been observed in 73.32 percent

existence of a riboflavin-light-NBT system to investigate the influence of goat urine on superoxide radical.All of the investigated samples significantly scavenged superoxide radicals and standard chemicals (ascorbic acid) at concentrations ranging from 25 to 100 µl/ml. The ethyl acetate extract of *R. nasutus* has the maximum radical activity of 67.32 percent when compared to the positive control of 65.34 percent. Although superoxide radicals are a relatively poor oxidant, they can breakdown to create more reactive oxidative species such as hydroxyl radicals and singlet oxygen (Shanmugam *et al*., 2014).

Assay for Nitric Oxide Radical Scavenging: The radical quenching activity of goats' urine against nitric oxide determined and compared to that of ascorbic acid. Ethyl acetate extract of *R. nasutus* similarly inhibited nitric oxide in dose dependent way, with an EC_{50} of 57.34µg/ml (Table-5). Vitamin C was employed as a reference molecule, and 59.32µg/ml Vitamin C was required for 50%

inhibition. The extract's EC_{50} value was lower than the standard's. At 100µg/ml, the % inhibition of *R. nasutus* ethyl acetate extract was 74.38 percent, whereas Vitamin-C was 72.31 percent. The decrease in nitric oxide radical activity concentration in both plants was larger than in the control. This could be because antioxidant polyphenolic compounds are present (Alasalvar et al., 2006).

Table 5. Nitric oxide radical scavenging activity of an ethyl acetate preparation of *Rhinacanthus nasutus* leaves

Different concentration of extract	Nitric oxide radical scavenging activity in percentage	
	Nitric oxide scavenging activity of ethyl acetate extract of *R. nasutus*	Standard Vitamin-C
25 µl/ml	17.32±2.34	15.34±2.34
50 µl/ml	38.32±1.78	34.65±1.78
75 µl/ml	51.32±2.34	47.32±1.39
100 µl/ml	74.38±1.56	72.31±1.45
EC_{50} Value	57.34	59.32

[a]The results are expressed as a percentage of Nitric oxide radical activity in comparison to the control. Each value indicates the mean+standard deviation of three experiments.

CONCLUSION

Finally, the study shows that the anti-oxidant and anti-microbial activities of *Rhinacanthus nasutus* leaf ethyl acetate extract differ. The results show that the leaf extract has the strongest antioxidant and antibacterial activities. Based on the toxicity test, the leaf extract was shown to be non-toxic. However, more pharmacological and toxicological research is required to confirm this suggestion. Phytochemical research should be performed to describe the components in *Rhinacanthusnasutus* ethyl acetate extract which function as antioxidants and antibacterial agents. This study shows that *Rhinacanthusnasutus* can be used to improve human health by acting as anti-oxidant and anti-microbial agent.

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