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Phytochemical analysis and antimicrobial efficacy of *Rhinacanthus nasutus* (l) Linn

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Abstract
Plant based natural constituents can be derived from any part of the plant like bark, leaves, fruits, flowers, roots, seeds etc. The therapeutic use of medicinal plant is becoming popular because of its inability to cause side effects and combat antibiotic resistant microorganisms. The present study provides information on phytochemical, antibacterial, antifungal and proximate analysis of petroleum ether, ethyl acetate, chloroform and methanol extracts of *Rhinacanthus nasutus* (Linn) leaf. This has been used in folk medicine for treating liver disorders, several skin diseases and other pharmacological effects. The leaf extracts contain secondary metabolites such as alkaloids, anthraquinones, carbohydrates, flavonoids, saponins, phytosterols, triterpenoids and poly-phenols. Cardiac-glycosides were absent in all the extracts. The proximate analysis value of the present data indicated that methanol (5.60%) extract showed higher extractives values when compared to other solvents. The four crude extracts of *Rhinacanthus nasutus* where investigated for their potential anti-bacterial and anti-fungal activities. Chloroform and Ethyl acetate extracts showed anti-bacterial and anti-fungal activities against all the organisms tested except *Pseudomonas aeruginosa* and *Proteus vulgaris.* Methanolic extract indicated significant activity only against *Staphylococcus aureus* and *Klebsiella pneumonia*. The present study on *Rhinacanthus nasutus* showed high antimicrobial activities that prove their use in traditional medicine.

Keywords: *Rhinacanthus nasutus*, Phytochemical, Antibacterial, Antifungal, Leaf Extracts.

1. Introduction
Medicinal plants are rich sources of antimicrobial agents. Plants are used medicinally in different countries and are the source of potential and powerful drugs [1]. According to World health organization (WHO) more than 80% of the world population relies on traditional medicine for their primary health care Needs [2]. The use of medicinal plants as a source of relief from illness can be traced back over five millennia to written documents of the early civilization in China, India and the north east, but it is thoughtless as art as old as mankind [3]. The potential of higher plants as a source for new drugs is still largely unexplored.
The genus *Rhinacanthus* comprises of about 25 species confined to the Old World tropics and subtropics. Within the *Acanthaceae* family, it placed in the Justiciinae subtype [4]. *R. nasutus* is widely distributed in some parts of sub-continent, in the region of Southeast Asia and China [5]. The *R. nasutus* (Nagamalle) is cultivated particularly as a medicinal plant has been used in treatments and preventions of diverse diseases as folklore medicines. Different parts of *R. nasutus* have used in traditional medicine for the treatment in diseases such as eczema, pulmonary tuberculosis, herpes, hepatitis, diabetes, hypertension and several skin diseases [6]. In the some experiments, it has potential effects for treatment of some diseases like to treat cancer, liver disorders, skin diseases, peptic ulcers, helminthiasis, scurvy, inflammation and obesity [7]. It is also used in the treatment of obesity, leprosy, scurvy and dhobi's itch. Leaves, roots and seeds act as an antidote for snake bites. In Madagascar, the juice of leaves and the root bark were used in the treatment of Herpes Circinatus [8-10]. *R. nasutus* species have demonstrated flavonoids, steroids, terpenoids, anthraquinones, lignans and especially naphthoquinone analogues as major constituents Naphthoquinones viz., *rhinacanthins* A, B, C, D, G, H, I, J, K, L, M, N, O, P, and Q were isolated and characterized from the leaves and roots of *R. nasutus* plant [11-13]. The rhinacanthone from leaves and stems [14, 15] and dehydro α-lapachone from roots were also isolated [11, 12]. The lignans *rhinacanthin*–E and -F were isolated from aerial parts [16]. The Benzenoids compounds *p*-hydroxy-benzaldehde, vanillic acid, syringic acid, 2 methoxy propiophenone, methyl valinate and syringaldehyde were isolated from leaves, roots and stems [17].
The antibacterial activity of extracts of *R. nasutus* was evaluated against clinically isolated bacteria from Thai cancer patients *viz*., *coagulase* positive *Staphylococci*, *coagulase* negative

Staphylococci, Hemolytic Streptococci, *Enterococci, E.coli*, *Klebsiella spp, Enterobacter sp, Pseudomonas aeruginosa.* The study on antimicrobial activity of the *Rhinacanthins-rich R. nasutus* extract as well as *Rhinacanthin-C* against *Streptococcus mutans*, *Propionibacterium acnes, Helicobacter pylori*, *Staphylococcus aureus Staphylococcus epidermidis* [18]. The ethanolic extract of *R. nasutus* leaves was also exhibited in vitro antibacterial activity against human pathogens [19]. The present investigation attempts to bring out the hitherto unearthed antibacterial, phytochemical and antifungal potentials of the leaf extracts of *Rhinacanthus nasutus* against some selected micro-organisms.

2. Materials and Methods

2.1 Plant Collection and Authentication

Fig 1: The fresh leaves of *Rhinacanthus nasutus* (L) Linn.

The fresh leaves of *Rhinacanthus nasutus* (L) Linn. of *Acanthaceae* family were collected from Tirumala Hills and Tirupati, Chittoor district of Andhra Pradesh, India and authenticated by professor P. Jayaraman, Botanist, Director, Plant anatomy research centre, Tambaram, Chennai, India in the month of May 2014 and registered Number of the Specimen is PARC/2014/2075.

2.2 Preparation of Extracts

Five hundred grams of coarse powder of shade dried leaves of *Rhinacanthus nastus* was extracted successively with petroleum ether (60-80 °C), chloroform, ethyl acetate and methanol in soxhlet extractor for 48 h. dark green residues were obtained after concentrating the extract under reduced pressure (yield 5.4%, 2.6%, 1.2% and 5.6% respectively). The obtained extracts were stored in a dessicator for further phytochemical and antimicrobial investigations. The dried material was tested for its constituents by standard methods [20-22]. The plant extracts were diluted with respective solvents to the final concentration of 20 mg/ml. Microorganisms like *Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgaris* and *Candida albicans* were used for testing.

2.3 Preliminary Phytochemical Screening

2.3.1 Test for alkaloids

To 2 ml of plant extract, 2 ml of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates the presence of alkaloids.

2.3.2 Test for Anthraquinones

To 1ml of plant extract few drops of 10% ammonia solution was added, appearance pink color precipitate indicates the presence of anthraquinones.

2.3.3 Test for carbohydrates

To 2ml of plant extract, 1ml of Molisch's reagent and few drops of concentrated sulphuric acid were added. Presence of purple or reddish color indicates the presence of carbohydrates.

2.3.4 Test for cardiac glycosides

To 0.5ml of extract, 2ml of glacial acetic acid and few drops of 5% ferric chloride were added. This was under layered with 1 ml of concentrated sulphuric acid. Formation of brown ring at the interface indicates presence of cardiac glycosides.

2.3.5 Test for flavonoids

To 2ml of plant extract, 1ml of 2ml sodium hydroxide was added. Presence of yellow color indicates the presence of flavonoids.

2.3.6 Test for saponins

To 2ml of plant extract, 2ml of distilled water was added and shaken in a graduated cylinder for 15minutes lengthwise. Formation of 1cm layer of foam indicates the presence of saponins.

2.3.7 Test for phytosterols

To 1ml of plant extract equal volume of chloroform is added and subjected with few drops of concentrated sulphuric acid appearance of brown ring indicates the presence of steroids.

2.3.8 Test for Triterpenoids

10mg of the extract was dissolved in 1 ml of chloroform; 1 ml of acetic anhydride was added following the addition of 2 ml of Conc.H2SO4. Formation of reddish violet colour indicates the presence of triterpenoids.

2.3.9 Test for Polyphenols

To 1ml of the extract, 2ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of blue or green color indicates presence of phenols or polyphenols.

2.4 Physicochemical Screening

2.4.1 Total Ash Content

Accurately weigh a quantity of the Test Sample, representing 2 to 4 g of the air-dried material, in a tarred crucible, and incinerate, gently at first, and gradually increase the temperature to 675 ± 25 ºC, until free from carbon, and determine the weight of the ash. If a carbon-free ash cannot be obtained in this way, extract the charred mass with hot water, collect the insoluble residue on an ash less filter paper, and incinerate the residue and filter paper until the ash is white or nearly so, then add the filtrate, evaporate it to dryness, and heat the whole to a temperature of 675 ± 25 ºC. If a carbon-free ash cannot be obtained in this way, cool the crucible, add 15ml of alcohol, break up the ash with a glass rod, burn off the alcohol, and again heat the whole to a temperature of 675 ± 25 ºC. Cool in desiccators, weigh the ash, and calculate the percentage of total ash from the weight of the drug taken.

2.4.2 Acid-Insoluble Ash
Boil the ash obtained as directed under Total Ash, above, with 25 ml of 3 N hydrochloric acid for 5 minutes, collect the insoluble matter on a tarred filtering crucible or ash-less filter, wash with hot water, ignite, and weigh. Determine the percentage of acid-insoluble ash calculated from the weight of drug taken.

2.4.3 Water-Soluble Ash
Boil the ash obtained as directed for Total Ash with 25 ml of water for 5 minutes. Collect the insoluble matter in a sintered-glass crucible or on an ash-less filter paper. Wash with hot water, and ignite for 15 minutes at a temperature not exceeding 450 ºC. Subtract the weight of this residue, in mg, obtained under Total Ash, and calculate the percentage of water-soluble ash with reference to the weight of sample as determined under Total Ash.

2.4.4 Foreign Matter
Spread the sample out in a thin layer, and separate the foreign organic matter by hand as completely as possible. Weigh it, and determine the percentage of foreign organic matter in the weight of drug taken.

2.4.5 Moisture Content
Hot extraction method under alcohol soluble extractives, except to use water in place of alcohol and Cold extraction method under alcohol soluble extractives, except to use water in place of alcohol.

2.5 Anti-bacterial Screening
Disc diffusion method was adopted for the antibacterial study [23, 24]. Ciprofloxacin at conc of 10mcg/disc was used as a standard. The filter paper impregnated with extracts (separately in each extracts at a concentration of 20 mgml-1) and ciprofloxacin disc were placed aseptically on the seeded agar medium (Bio-zone Research Technologies Pvt. Ltd., Chennai) which was already swabbed with the test organisms and incubated at 37 °C for 24h. The zone of inhibition in mm was measured.

2.6 Anti-fungal Screening
The antifungal activity of the crude extracts was determined against *Candida albicans* by disc diffusion method [23-24]. Ketoconazole (10 mcgdisc-1) was used as standard. The filter paper disc impregnated with various extracts (20 mgml-1) individually and ketoconazole disc were placed aseptically on the seeded sabouraud dextrose agar medium (Bio-zone Research Technologies Pvt. Ltd., Chennai) which was already swabbed with the test organism and incubated at 37 °C for 48 h. The zone of inhibition (in mm) was measured and recorded.

3. Results and Discussions
The Phytochemical analysis of leaves extract of *Rhinacanthus nasutus* revealed the presence of various components such as Alkaloids, Anthraquinones, Carbohydrates, Flavonoids, Saponins, Phytosterols, Triterpenoids and Poly-phenols (Table 1.0). Comparing the extraction solvents Triterpenoids and Steroids were present whereas, Cardiac glycosides were absent in all the extracts.
Ethyl acetate and methanol leaf extracts depicted the presence of all the phytochemicals when compared with other extracts.
Physicochemical parameters and extractive value of leaves were studied and the results were shown in Table 2.0 respectively. The moisture content was 5.15% (leaves) and the total ash content, acid insoluble ash, Water soluble ash and foreign matter values which were determined to be not more than 7.80%, 1.70%, 2.76%, 1.58% respectively. While study of extractive values can serve as a valuable source of information and provide suitable standards to determine the quality of plant material in future investigation. The proximate analysis value of the present data indicated that methanol (5.60%) extract showed higher extractives values when compared to other solvents (Table 3.0).
The four crude extracts of *Rhinacanthus nasutus* where investigated for their potential anti-bacterial and anti-fungal activities. Petroleum ether extract was infective for the organism tested. Standard antibiotics ciprofloxacin (10mcg/disc) and ketoconazole (10mcg/disc) showed good inhibitory action on the micro-organisms tested. Chloroform and Ethyl acetate extracts showed anti-bacterial and anti-fungal activities against all the organisms tested except *Pseudomonas aeruginosa* and *Proteus vulgaris.* Methanolic extract indicated significant activity only against *Staphylococcus aureus* and *Klebsiella pneumonia* (Table 4.0).
The GC-MS analysis of the leaf sample of *R. nasutus* was carried out to identify the nature of the components present. The GC-MS output also showed the presence of two major components at retention times 4.14 and 7.8. The mass spectrum of the compound with retention time 4.14 gave three major peaks (m/z) at 129.7826, 101.8992, and 76.0586. The element combination of the molecular ion peak at 129.7826 shows the probability for C8H19N, C7H5NO, C6H11NO2, C9H7N and C5H7NO3. The above molecular formula indicates the possibility of an aromatic Nitrogen containing compound that is an alkaloid. The mass spectrum of the compound with retention time 7.8 gave eight major peaks (m/z) at 221, 203, 165, 147, 119, 102, 91 and 75. Therefore, it may be concluded that the compound with retention time 7.8 may be a poly-phenolic compound [25].

4. Conclusion
The presence of the identified phytochemical components makes the leaves pharmacologically active. In the proximate analysis the leaf nutrients in the plants that are useful for many pharmacological activity. We are currently studying other possible mechanisms of action of these leaves.

5. Authors' contribution
Collected the plant material and prepared the extract carried out assays and drafted the manuscript. Conceived and designed the study, revised the manuscript. Both the authors read and approved the final manuscript.

6. Acknowledgement
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7. Tables

Table 1.0: Phytochemical screening of *Rhinacanthus nasutus* leaf

Test	PE	EE	CE	ME
Alkaloids	-	+	-	+
Anthraquinones	-	+	-	+
Carbohydrates	-	-	-	+
Cardiac glycosides	-	-	-	-
Flavonoids	-	+	-	+
Saponins	-	+	-	+
Phytosterols	+	+	+	+
Triterpenoids	+	+	+	+
Polyphenols	-	+	-	+

PE – Petroleum Ether Extract; EE - Ethyl Acetate extract; CE - Chloroform Extract; ME - Methanol extract;
+ve – Present; -ve – Absent;

Table 2.0: Physicochemical constants of *Rhinacanthus nasutus* leaf

S. No.	Parameters	Values
1	Total ash content	7.80%
2	Acid insoluble ash	1.70%
3	Water Soluble ash	2.76%
4	Foreign matter	1.58%
5	Moisture content	5.15%

Table 3.0: Proximate analysis of *R. nasutus* leaf powder

S. No.	Parameters	Values
1	Petroleum Ether	1.60%
2	Chloroform	2.60%
3	Ethyl acetate	1.20%
Solubility		
1	Methanol	5.60%

Table 4.0: Evaluation of antibacterial and antifungal activity of *Rhinacanthus nasutus* Linn

S. No.	Micro-organisms	Zone of inhibition (mm)				
		Std.	PE	CE	EE	ME
1.	*Escherichia coli*	21	9	9	9	NS
2.	*Klebsiella pneumonia*	25	05	04	20	16
3.	*Staphylococcus aureus*	31	16	15	20	20
4.	*Pseudomonas aeruginosa*	20	NS	NS	NS	NS
5.	*Proteus vulgaris*	30	NS	NS	NS	NS
6.	*Candida albicans*	22	9	20	18	NS

PE – Petroleum Ether Extract; EE - Ethyl Acetate extract; CE - Chloroform Extract; ME - Methanol extract; NS = Not Specified; Std. – Standard;

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