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Phytochemical Evaluation, Antibacterial and Antifungal Activity of Rauvolfia Tetraphylla L.

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Abstract
Rauvolfia tetraphylla L., like most other plants contain various secondary metabolites with great potentials. The present study aimed at evaluating the phytochemicals by using quantitative and qualitative analysis of ethanol, methanol and aqueous extracts with the help of standard techniques. The findings from quantification and phytochemical screening showed the presence of alkaloids, flavonoids, carbohydrates, phenols, saponins, tannins, quinones and terpenoids. Further, the quantitative analysis results revealed that ethanolic extract of roots was found to have more constituents when compared with other extracts. The antimicrobial activity of aqueous, methanol and ethanol extracts obtained from Rauvolfia tetraphylla roots were tested against bacterial and fungal species by agar well diffusion method. Better antimicrobial activity was observed when the methanol extracts showed maximum activity against Staphylococcus aureus followed by Bacillus subtilis. It was observed that methanol extract showed the highest antimicrobial activity against multi drug resistance S. aureus at 100 mg/ml concentration, while E. coli and K. pneumoniae were most susceptible to all extracts. In particular, the results obtained in the present study revealed that Rauvolfia tetraphylla root extract can be recommended for the control of infectious Gram positive bacteria. Among different fungi tested A. flavus and C. capsici were found to be more sensitive to the ethanol extract when compared to others.
Keywords: *Rauvolfia tetraphylla, phytochemicals, antimicrobial activity.*

I. Introduction

The plant *Rauvolfia tetraphylla* L. (syn. R. tomentosa Jacq.) belongs to the family Apocynaceae, and is native to Mexico, Central America, West Indies and northern South America and distributed throughout the tropics including India [1, 2]. In most of the moist and hotter regions of India, the species is seen frequently seen under cultivation and often observed as an escaped plant.

Family Apocynaceae is one of the frequently used angiosperm members as medicine in different Indian systems of medicine. At present, the genus *Rauvolfia* is represented by 77 species distributed in the world and known to contain various unique active phytochemicals in the form of flavonoids, phytosterols, oleoresins, steroids, tannins and alkaloids [3, 4, 5]. The plant is well known for its rich bioactive phyto-chemicals, especially alkaloids. *R. serpentina* (also known as Indian snakeroot or sarpagandha) and *R. tetraphylla* (also known as devil root or Bara Chandrika) are two very important distinctive medicinal shrubs growing in India having wide application in traditional system of medicine for curing human disease and various herbal formulations available in market using these plants [6]. The *Rauvolfia tetraphylla* plant is very similar to *Rauvolfia serpentina*, but the branches are harder. (**Figure 1**).

Figure 1: *Rauvolfia tetraphylla*

Historically, it is well known that Mahatma Gandhi, the father of Nation used *Rauvolfia* root to make an herbal tea to relax. Evidence proves that the roots contain an alkaloid called reserpine, which was first reported from Indian *R. serpentina*. Reserpine, a drug derived from the roots, is used in the treatment of high blood pressure and as a tranquilizer and has been used to cure snake bites, insomnia, and insanity [7, 8]. Reserpine, isolated in 1952, was the first active alkaloids found in the crude drug. This alkaloid is occasionally also used in treating hypertension. The roots of *R. tetraphylla* are often used as substitute or adulterant for *R. serpentina* [9].

Other regularly reported alkaloids are reserpine, ajmalicine, deserpidine, sarpagine, rescinnamine and yohimbine [10]. Reserpine is a potent alkaloid that depresses the lower blood pressure and central nervous system. The leaf extract of *R. tetraphylla* is intended for the treatment of cholera, fever and eye disease. It is also used as antihypertensive, also in intestinal disorders, diarrhoea and dysentery [11]. The leaves are crushed and applied over snakebite site [12]. Fruits of this plant are used to cure spleen disorders. Roots are supposed to stimulate uterine contraction in case of difficult delivery [13]. *R. tetraphylla* is becoming critically endangered due to its wide indiscriminate collection from wild, poor seed germination and lack of sufficient commercial plantation [14]. Literature reviews confirm that flavonoids, tri terpenoids, steroids, steroidal glycosides, and alkaloids have been reported which shows antioxidant activity [15, 16]. Flavonoids have been reported to be connected with anti-oxidative action in biological systems, performing as scavengers of singlet oxygen and free radicals [17, 18, 19].

In recent years, secondary plant metabolites (Phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents. Thus, it is anticipated that phytochemicals with adequate antibacterial efficacy will be used for the treatment of the bacterial infections like typhoid [20]. The treatment of infectious diseases with antimicrobial agents continues to present problems in modern-day medicine with many studies showing a significant increase in the incidence of bacterial resistance to several antibiotics [21, 22]. Multiple drug resistance in human pathogenic microorganisms has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. This situation have led scientists to search for new antimicrobial substances from various sources which are the good sources of novel antimicrobial chemotherapeutic agents [23, 24].

The principle aim of the present work was to evaluate the phytochemical components and study the antimicrobial activity of *Rauvolfia tetraphylla* (Aqueous, Methanolic and Ethanolic extracts) of roots against both human and plant pathogenic bacteria including *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and fungi like *Aspergillus flavus, Fusarium solani, Colletotrichum capsici.*

II. Materials and Methods

Chemicals:
All the Media were purchased from HiMedia (Mumbai, India). The chemicals and reagents used for this study were of analytical grade and purchased from SD fine chemicals, India.

Microbial strains:
The following strains were procured from microbial type culture and collection (MTCC), Chandigarh, India. Five bacterial strains namely *Bacillus subtilis* MTCC B2274, *Escherichia coli* MTCC B1560, *Klebsiella pneumoniae* MTCC B4030, *Pseudomonas aeruginosa* MTCC B2297, *Staphylococcus aureus* MTCC B3160, and three fungal strains such as *Aspergillus niger* MTCC F4325, *Fusarium solani* MTCC 9668, *Colletotrichum capsici* MTCC 3414.

Collection of Plant materials:
The roots of Rauvolfia tetraphylla were collected from Marathwada region namely districts Parbhani, Nanded, Latur, Maharashtra, India; they were identified with the help of Gamble's flora.

Preparation of powder:
The roots of plants were collected and dried under shade. These dried materials were mechanically powdered sieved using 80 meshes and stored in an airtight container. These powdered materials were used for further physiochemical, phytochemical and antimicrobial activity.

Extraction of plant material:
Various extracts of the study plant roots were prepared according to the methodology of Indian Pharmacopoeia [25]. The plant roots were dried in shade and then subjected to pulverization to get coarse powder. The coarse powder material was subjected to Soxhlet extraction separately and successively with Ethanol, Methanol and Distilled water. These extracts were concentrated to dryness in flash evaporator under reduced pressure and controlled temperature (40-50 ^{o}C). The extracts were stored in a refrigerator in air tight containers. The extracts were then analysed for phytochemical screening of compounds, antimicrobial and pharmacological activity [26-28].

Qualitative Phytochemical Screening:

Test for Carbohydrates: The presence of carbohydrates was confirmed when 2 ml of extract was treated with 1 ml of Molisch's reagent and few drops of concentrated sulphuric acid which resulted in the formation of purple or reddish color.
Test for Tannins: To 1 ml of extract, 2 ml of 5% ferric chloride was added. Formation of dark blue or greenish black indicates the presence of tannins.
Test for Saponins: 2 ml of extract, 2 ml of distilled water were added and shaken in a graduated cylinder for 15 min. It resulted in the formation of a 1 cm layer of foam that indicated the presence of saponins.
Test for Alkaloids: To 2 ml of extract, 2 ml of concentrated hydrochloric acid was added. Then a few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates the presence of alkaloids.
Test for Flavonoids: To 2 ml of extract, 1 ml of 2N sodium hydroxide was added. Presence of yellow color indicates the presence of flavonoids.
Test for Quinones: To 1 ml of extract, 1 ml of concentrated sulphuric acid was added. Formation of red color indicates the presence of quinones.
Test for Phenols: 2 ml of distilled water followed by a few drops of 10% ferric chloride was added to 1ml of the extract. Formation of blue or green color indicates presence of phenols.
Test for Terpenoids: 0.5 ml of the extract was treated with 2 ml of chloroform and conc. sulphuric acid. Formation of red brown colour at the interface indicates the presence of terpenoids.
Quantitative Determination of Secondary Metabolites:
Estimation of Alkaloids: Alkaloids were determined using Harborne method [29]. Five grams of the sample was weighed into a 250 ml beaker, 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.
Estimation of Flavonoids: The total flavonoid content in the sample was estimated by the method of Chang [30]. A volume of 0.25 ml of the sample was diluted to 1.25 ml with distilled water. 75 µl of 5% sodium nitrite was added and after six minutes 0.1 5 ml of aluminium chloride solution was added. 0.5 ml of 0.1M NaOH was added after 5 min and made up to 2.5 ml with distilled water. The solution was mixed well and the absorbance was read at 510 nm along with standard quercetin at 5 - 25 µg concentration. The results are expressed as mg of flavonoids as quercetin equivalent / gm of dried sample.
Total Phenolic Content (TPC): Total phenolic content of extract was determined according to the Folin-Ciocalteu method of Slinkard and Singleton [31] with some modifications. Briefly, 0.1 ml of extract (200, 600 and 1000 µg/ml), 1.9 ml distilled water and 1 ml of Folin-Ciocalteau's reagent were seeded in a tube, and then 1 ml of sodium carbonate was added. The reaction mixture was incubated at 25 °C for 2 h and the absorbance of the mixture was read at 765 nm. The sample was tested in triplicate and a calibration curve with six data points for catechol was obtained. The results were compared with catechol calibration curve and the total phenolic content of the sample was expressed as mg of catechol equivalents per gram of extract.
Total Tannins Content (TTC): Tannins - phenolics were determined by the method of Peri and Pompei [32]. 1 ml of the sample extracts of concentration 1mg/ml was taken in a test tube. The volume was made up to 1ml with distilled water and 1 ml of water serves as the blank. To this 0.5 ml of Folin's phenol reagent (1:2) followed by 5ml of 35% sodium carbonate was added and kept at room temperature for 5 min. Blue colour was formed and the colour intensity was read at 640 nm. A standard graph (gallic acid - 1 mg/ml) was plotted, from which the tannin content of the extract was determined. The total tannin content was expressed in mg/g of extract.
Total Saponins: The total Saponins content was determined by the method of [33]**.** The roots were ground and 20 g of powder was taken into a conical flask and 100 ml of 20% ethanol was added to the sample. The sample was heated over a hot water bath for 4 h with continuous stirring at about 55 ºC. The mixture was then filtered and the residue re-extracted with another 200 ml of 20% ethyl alcohol. The combined extracts were reduced to 40 ml over a water bath at about 90 ºC. The concentrate was then transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added to the extract and vigorously shaken. The aqueous layer was recovered while the diethyl ether layer was discarded and the purification steps were repeated. 60 ml of n- butanol was added and the combined n- butanol extracts were washed twice with 10 ml of 5% sodium chloride. The remaining solution was then heated in a water bath and after evaporation; the samples were dried in the oven to a constant weight and values are expressed as mg/g of extract.

Antimicrobial screening:

The lyophilized culture of each bacteria was sub-cultured and concentration of working stock culture was assessed as 10^{-6} CFU/ml. Specified quantity of Nutrient Agar was prepared and plated in aseptic conditions. The agar well diffusion technique was followed for antimicrobial susceptibility test for roots extracts of

*Rauvolfia tetraphylla (*Aqueous, Methanol and Ethanol) and (DMSO as negative control), the activity was compared with tetracycline disc (10 μg/disc). After 24 h of incubation at 37 °C the zone of inhibition was measured by using an antibiotic zone reader scale (Hi Antibiotic Zone Scale-c). For the antifungal activity, the same method as for bacteria was adopted, however Sabouraud Dextrose Agar was used. The inoculated medium was incubated at 25 °C for 3-5 days for the fungal strains and fluconazole (5μg) was used for comparison.

The zone of inhibition was calculated by measuring the diameter of the inhibition zone around the well (in mm) including the well diameter. The readings were taken in three different fixed directions in all three replicates and the average values were tabulated. Each experimental data from triplicates were subjected to one way ANOVA using Minitab version 15. A significant level of $p < 0.01$ was used for all statistical analyses.

III. Results and Discussion

Phytochemical Screening: The phytochemical analysis of various extracts of roots of *R. tetraphylla* is shown in **Table 1**. From the qualitative analysis results, it is observed that the roots of *R. tetraphylla* of different extracts confirmed the presence of alkaloids, flavonoids, phenols, saponins, tannins, and terpenoids. However, alkaloids, flavonoids, phenols and terpenoids components are present in all the three extracts of aqueous, methanol and ethanol. While, carbohydrates, saponins and tannins was only found in two extracts namely ethanol and aqueous extract in more amount. The study revealed that ethanolic root extract was found to have more amounts of constituents when compared with other extracts.

The quantitative estimation of phytochemicals of various extracts of roots of *R. tetraphylla* is shown in **Table 2**. It is observed that alkaloids, flavonoids and phenols are in more quantities in the ethanolic extracts from all the three districts; showing 18 mg/g alkaloid from Latur, phenol content of 20.3 mg/g from Nanded region and 27.5mg/g of flavonoids from Parbhani and 24.0 mg/g from Latur. The Saponins and tannins are also determined however, they are produced in less amount compared to alkaloids and flavonoids. The higher amount of Flavonoids observed indicates good antioxidant activity. The amount of alkaloids, which are localized in the roots are significant and can be used in formulations of herbal drugs for the treatment of hypertension, cardiovascular diseases, and intestinal disorders and as a sedative agents [34, 35].

Table 1: Qualitative Phytochemical Analysis

Sr. No	Phytochemical Components	Aqueous Extract	Methanolic Extract	Ethanolic Extract
1	Carbohydrates	++	+	++
2	Alkaloids	++	++	+++
3	Flavonoids	++	+	+++
4	Phenols	++	+	++
5	Saponins	++	+	++
6	Tannins	++	+	++
7	Quinones	+	+	+
8	Terpenoids	+	+	+

Note: ++ present in moderate; +++ present in more amount

Table 2: Quantitative estimation of phytochemical of *Rauvolfia tetraphylla*

Sr.No.	Phytoconstituents of *Rauvolfia tetraphylla* (root)	*R. tetraphylla* Plant root extract from Parbhani (mg/gram)			*R. tetraphylla* Plant root extract from Nanded (mg/gram)			*R. tetraphylla* Plant root extract from Latur (mg/gram)		
		Aq.	Meth.	Eth.	Aq.	Meth.	Eth.	Aq.	Meth.	Eth.
1	Alkaloids	7.6	10.6	12.7	8.5	5.8	10.3	11.9	17.1	18.0
2	Flavonoids	20.0	27.3	27.5	14.0	17.0	18.2	12.0	16.0	24.0
3	Saponins	4.6	4.4	6.0	4.3	5.6	6.8	3.8	6.9	7.2
4	Tannins	4.5	9.5	10.4	3.4	5.9	6.8	4.6	7.9	9.4
5	phenols	7.2	19.0	19.4	19.3	13.8	20.3	10.0	18.0	19.3

Note: Aq.: Aqueous extract, Meth.: Methanol and Eth.: Ethanolic extract

Antimicrobial activity

The results are summarized in **Table 3**, demonstrating that the methanol and ethanol extracts exhibited high antimicrobial activity, while aqueous extracts showed low activity. Methanol extract exhibited the highest inhibition zone value against *S. aureus,* followed by *B. subtilis* at the concentration of 100 mg/ml when compared with the other organisms. The observed zone of inhibition was 24 ± 0.8 mm against *S. aureus* and 23 ± 0.9 mm for *B. subtilis*.

Among the bacterial species, *S. aureus* was highly sensitive to all the extracts, whereas *K. pneumonia* and *E. coli* were found to be highly resistant. Moreover, methanol extract did not exhibit antimicrobial action against *E. coli.* The antibacterial effect of aqueous extract was comparatively less, when compared with Methanol and ethanol extracts.

The negative control of DMSO had no effect on microbial growth. The absence of inhibition zones confirmed that DMSO could not act as antimicrobial agent.

The results of antifungal activity are given in **Table 4**. The fungal strains tested were most susceptible to Ethanol followed by Methanol extract. Ethanol extract were most effective against *Aspergillus flavus* and *Colletotrichum capsici*, weakly active against *Fusarium solani*. The aqueous extract expressed nil activity against the *Aspergillus flavus* and *Colletotrichum capsici*.

Table 3: Inhibition zone of Aqueous, Methanol and Ethanol extracts of *Rauvolfia tetraphylla* roots against bacterial pathogens

Sr. No	Test Organism	Zone of Inhibition in mm[a]									
		Aqueous			Methanol			Ethanol			Tet.
		50mg	75mg	100mg	50mg	75mg	100mg	50mg	75mg	100mg	
1	*S. aureus*	15±0.9	19±0.8	20±0.2	20±0.3	22±0.8	24±0.8	15±0.2	18±0.5	21±0.8	18
2	*B. subtilis*	-	-	-	17±0.2	19±0.9	23±0.9	15±0.5	18±0.8	20±0.9	18
3	*E. coli*	10±0.8	12±0.6	14±0.3	-	-	-	13±0.4	15±0.4	17±0.3	21
4	*K. pneumoniae*	11±0.2	13±0.5	14±0.9	-	-	-	13±0.7	14±0.2	15±0.3	21
5	*P. aeruginosa*	15±0.4	18±0.9	19±0.7	10±0.6	13±0.8	15±0.4	18±0.6	20±0.6	22±0.8	24

[a]: Each value is the mean of three replicates with standard deviation; P value is < 0.01 extremely significant when compared with standard, Tet: Tetracycline; –: No activity

Table 4: Inhibition zone of Aqueous, Methanol and Ethanol extracts of *Rauvolfia tetraphylla* roots against fungal pathogens

Sr. No	Test Organism	Zone of Inhibition in mm [a]									
		Aqueous			Methanol			Ethanol			Flu.
		50mg	75mg	100mg	50mg	75mg	100mg	50mg	75mg	100mg	
1	*A. flavus*	-	-	-	18±0.2	24±0.8	25±0.8	23±0.8	24±0.5	26±0.9	26
2	*F. solani*	15±0.2	18±0.6	19±0.8	18±0.2	21±0.9	23±0.9	18±0.9	19±0.8	21±0.8	24
3	*C. capsici*	-	-	-	19±0.8	20±0.7	23±0.7	21±0.7	24±0.2	25±0.3	25

[a]: Each value is the mean of three replicates with standard deviation; P value is < 0.01 extremely significant when compared with standard, Flu: Fluconazole, –: No activity

Many research articles on the antimicrobial activity of plant extracts of *R. tetraphylla* against human pathogenic bacteria were reported in the recent years [36]. Suresh *et al.* (2008) reported the best antimicrobial activity of ethanol extract obtained from *Rauvolfia tetraphylla*, which showed maximum activity against *E. coli* and *Enterobacter aerogenes*, and various tested fungi such as *A. niger* and *Penicillium* sp., were found to be more sensitive to crude extract when compared to others [37]. Several studies conducted on antifungal potential of *R. tetraphylla* revealed its effectiveness against human pathogens and phytopathogenic fungi including dermatophytes and seed-borne fungi. Aqueous leaf extract of *R. tetraphylla* was effective as antifungal agent against *Fusarium indicus* and *Aspergillus flavus* independently. Aqueous extract of *R. tetraphylla* leaves exhibited concentration dependent inhibition of test bacteria viz. *Escherichia coli* and *Klebsiella pneumoniae*

[38]. Ethanol extract of leaves were effective in causing concentration dependent inhibition of bacteria. *Salmonella typhi* and *Micrococcus luteus* inhibited to the highest and least extent, respectively [39].

IV. Conclusion

From the present study it is evident that *R. tetraphylla* have potential antimicrobial activity. The antibacterial activity of plant extracts was not likely to be due to any one of the main active constituent but because of the combined action of additional other phytochemical compounds. The purified components may have even more potency with respect to inhibition of microbes. Further this research will focus on the types of phytoconstituents and purification of individual groups of bioactive components and reveal the exact potential of the plant to inhibit several pathogenic microbes.

Conflict of Interest

Authors declare no conflict of interest for this research paper.

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