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Phytochemical Screening, *in-vitro* Antioxidant and Anti-microbial evaluation of dried rhizomes extract of *Vetra*

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ABSTRACT

Medicinal plants are widely distributed throughout the world but most abundantly in tropical countries. *Vetra* plant extract contains numerous bioactive compounds and can be used as novel therapeutic agent. The objective of the current study to evaluate phytochemical screening, antioxidant and antimicrobial activity of hydroalcoholic extract of dried rhizomes of *Vetra*. The phytochemical screening reveals the presence of saponins, anthraquinones, tannins and flavanoids content. Antioxidant activity of extract was measured by DPPH Assay. Extract have antioxidant activity due to presence of phenolic and flavanoids and exhibits antibacterial activity and antifungal activity that may provide good efficacy against various infection and diseases. The antimicrobial activity was determined in the extracts using agar disc diffusion method. The antibacterial and antifungal activities of extracts (5, 25, 50, 100, 250 µg/ml) of hydroalcohol extract of rhizomes of *Calamus rotang* were tested against Staphylococcus aureus, Escherichia coli, human pathogenic bacteria; and fungal strains—Aspergillus niger, Candida albicans. Zone of inhibition of extracts were compared with that of different standards like Ampicillin, ciprofloxacin for antibacterial activity and griseofulvin and nystatin for antifungal activity. The results showed that the remarkable inhibition of the bacterial growth was shown against the tested organisms. The microbial activity of the Cassia fistula was due to the presence of various secondary metabolites. Hence these results demonstrating broad spectra of antibacterial and antifungal activity in hydroalchol extract of *calamus rotang* might be more helpful to discover a new compound that may be used as novel biochemical for various infectious diseases and may serve as leads in the development of new pharmaceuticals research activities.

Keyword:, *Vetra*, Calamus rotang, Phytochemical Screening, in-vitro, antioxidant evaluation, anti-microbial evaluation

1.Introduction

The on-going growing recognition of medicinal plants is due to several reasons, including escalating faith in herbal medicine. Medicinal plants are widely distributed throughout the world but most abundantly in tropical countries. It is estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants. In India, of the 17,000 species of higher plants, 7500 are known for medicinal uses. This proportion of medicinal plants is the highest proportion of plants known for their medical purposes in any country of the world for the existing flora of that respective country**.(1)**

1.1 Plant Profile of *Vetra* rhizomes

Vetra is the dried rhizomes of Calamus rotang L. (Family Arecaceae) a thorny climbing shrub occurring in central and southern india. It is restricted to the plains along the backwaters and coasts.

SYNONYMS- Vetraka, Tejana

1.1.1 Description

(a). Macroscopic: Rhizome horizontal and branched, Woody and stiff and rough in texture. Colour is light grey to brown. The size ranging from 1 to 5 cm long and 1 to 4 cm in cross section, Cut surface shows an inner creamy ring and an outer brownish narrow ring.brittle, and woody rhizomes are hard and fibrous. Fleshy rhizomes are quickly recognised in certain European species while woody rhizomes.

(b).Microscopy: TS of rhizomes are circular in outline, Epidermis is single in layer, Cortical cells are thin walled, Parenchyma polygonal towards the epidermis and gradually become circular with intercellular spaces. Cortex shows many resin canals which are red in colour. **(2)**

2. Material and Methods

2.1 Collection and extraction of plant material

Calamus rotang was selected for the study. Rhizomes of *Calamus rotang,* were collected and taxonomically identified and authenticated.

2.1.1 Preparation of *Calamus rotang* Rhizomes extract : *Calamus rotang* Rhizomes (100 g) were cleaned, dried and crushed in a grinder to obtain a coarse powder. Dry powder of rhizomes was packed in a Soxhlet thimble and defatted with petroleum ether and defatted powder was again extracted with 80% methanol in water using Soxhlet apparatus and the solvent was evaporated under reduced pressure in rotator evaporator. The extract was stored in a Desiccator. **(3)**

2.2 Qualitative phytochemical screening of plant extract

Table2.1: Method of phytochemical investigation (4-6)

S.no.	Test	Procedure	Observation
1.	**Test of Alkaloids**	**a) Mayer's Test-** Test solution 1ml + few drops of Mayer's reagent (Potassium mercuric iodide solution)	The color change precipitate indicates the presence of alkaloid
		b) Dragendroff's Test- 1ml test solution few drops of Dragendroff's reagent (Potassium bismuth iodide solution)	The color change precipitate indicates the presence of alkaloid
		c) Tannic acid test- Test solution 1ml + few drops of 10% tannic acid solution.	The color change precipitate indicates the presence of alkaloid
2.	**Test for tannins**	**a) Ferric chloride ($FeCl_3$) test-** About 0.5 mg of dried powder of plant extract + 20 ml water in attest tube, boils and filters. A few drops of 0.1% ferric chloride solution will be added	The brownish-green or blue-black coloration will indicate the presence of Tannins.
3.	**Test for Steroids**	**a) Liebermann Buchard test-** Test solution 1 ml + few drops of acetic anhydride, boil and cool+ add concentrated Sulphuric acid	The brown ring at the junction of the two layers and the green layer in the upper layer will indicate the presence of the Steroids.
4.	**Test for Flavonoids**	**a) Alkaline reagent Test-** 1 ml test solution + few drops of sodium hydroxide solution	The yellow coloration which will disappear on the addition of dilute HCl indicates the presence of Flavonoids.
		b) Lead acetate Test- Test solution 1ml in a test tube + add few drops of lead acetate solution	The yellow color precipitate will indicate the presence of Flavonoids.
5.	**Test for terpenoids**	**a) Salkowski Test-** Test solution 1ml + 2ml chloroform + few drops of sulphuric acid. Shakewell and allow	The reddish-brown color will indicated the presence of Terpenoids.

		standing for some time.	
6.	**Test for Proteins**	**a) Ninhydrin Test-** Test solution boils with 0.2% solution of ninhydrin	The violet color will indicate the presence of proteins.
7.	**Test for Reducing sugars**	**a) Fehling Test-** Test sample 1ml + 0.5ml of Fehling A and Fehling B solutions and boil.	The brick-red coloration indicates the presence of reducing sugars.
8.	**Test for saponins**	**a) Frothy Test-** Test solution 1ml will place in a test tube containing water and shake well.	Note for a stable froth that persists for at least 2 min.

2.2 Thin layer Chromatography (TLC)

Hydroalcohol extract of Vetra extract was subjected to TLC studies. For the TLC analysis, the dimensional ascending method was used 20×20 cm. TLC plate coated with silica gel , was cut with a scissor in 14×3 cm shape. The plate was then marked with the pencil softly 1.5 cm far from the both bottom and top. Glass capillaries were used to spot the sample on the TLC plate on the pencil marked bottom line. Then Plate was placed in the fume hood to dry the plate and loaded the sample again until a dark spot is obtained. Then the solvent Hexane: Ethyl acetate: Acetic acid (4:4:2) about 20ml was taken in the chamber. The plate was placed in the chamber lining on the top. After the run, plates were dried in the fume hood and then used to detect the spots.

Detection of the spot

All the plates were dried and detected the spots with the help of UV light at 254nm and 366 nm . The movement of the active compound was expressed by the retention factor (Rf). **(7)**

2.3 Determination of total flavanoid content

Total flavonoid content was determined by Aluminium chloride method 14 using quercetin as a standard. 1ml of test sample and 4 ml of water was added to a volumetric flask (10 ml volume). Add 0.3 ml of 5 % Sodium nitrite, 0.3 ml of 10% Aluminium chloride was added after 5 minutes. After 6 minutes incubation at room temperature, 1ml of 1 M Sodium hydroxide was added to the reaction mixture and the final volume was make upto 10 ml with distilled water. Absorbance of sample was measured against the blank at 510 nm using a spectrophotometer. This Experiment was repeated three times for precision and values were expressed in mean ± standard deviation in terms flavonoid content (Quercetin equivalent, QE) per g of dry weight. **(8)**

2.4 In-vitro antioxidant and antimicroial evaluation

2.4.1 *In- vitro* Anti-oxidant activity

A free radical may be an atom or molecules with one or more unpaired electrons like superoxide anion radical, hydroxyl radical, nitric oxide, peroxyl radical and alkoxy radical**.** Free radicals are capable of independent existence and cause oxidative tissue damage**.** The non- radical oxidants like hydrogen peroxide and hypo-chlorus acid, which do not possess unpaired electrons, are also capable of inciting oxidative tissue damage**.**

DPPH radical scavenging assay: DPPH [1,1 – Diphenyl – 2 picryl hydrazyl] is a stable free radical, which shows absorbance at 517nm. The antioxidant reacts with DPPH and converts it to 1, 1- Diphenyl-2-picryl hydrazine, which do not absorb at 517nm**.**

Procedure: The radical scavenging activities of plant extracts against 2, 2-Diphenyl-1-picryl hydrazyl radical (Sigma-Aldrich) were determined by UV spectrophotometry at 517 nm. The following concentrations of the extracts were prepared, 0.05, 0.1, 0.5, 1.0, 2.0 and 5mg/ml in DMSO. Vitamins C was used as the antioxidant standard at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, and 0.75 mg/ml. Extract 1ml was placed in a test tube, and 3 ml of DMSO was added followed by 0.5 ml of 1mM DPPH in a DMSO. A blank solution was prepared containing the same amount of DMSO and DPPH. The radical scavenging activity was calculated using the following formula:

$$\% \text{ inhibition} = \{[Ab - Aa]/Ab\} \times 100$$

Where Ab is the absorption of the blank sample and Aa is the absorption of the extract. **(9)**

2.4.2 Anti microbial evaluation

2.4.2.1 Assay of antibacterial activity

Antibacterial activity of plant extracts were carried out against bacterial pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* using agar well diffusion method. Initially, the stock cultures of bacteria were revived by inoculating in broth media and grown at 37ºC for 18 hrs. The Nutrient agar plates were prepared and wells were made in the plate. Each plate was inoculated with 18h old cultures (100 µl, 10^{-4}cfu) and spread evenly on the plate. After 20 min, the wells were filled with plant extracts (5,25,50,100,200 µg/ml). The control wells with Ampicillin and Ciprofloxacin were also prepared. All the plates were incubated at 37ºC for 24 h and the diameter of inhibition zone in mm were noted. **(10)**

2.4.2.2 Assay of antifungal activity

Antifungal activity of plant extracts were carried out against fungal pathogens such as *Aspergillus niger* and *Candida albicans* using agar well diffusion method. The stock cultures of fungal pathogens were revived by inoculating in broth media and grown at 27^0C for 48 hrs. The Potato Dextrose agar plates of the above media were prepared and wells were made in the plate. Each plate was inoculated with 18 h old cultures (100 µl 10^{-4}CFU) and spread evenly on the plate. After 20 min, the wells were filled with plant extracts (5 ,25,50,100,200

µg/ml) . The control plates with Griseofulvin and nystatin were also prepared. All the plates were incubated at 27^0C for 48h and the diameter of inhibition zone in mm were noted. **(11-12)**

3. Results

3.1 Preparation of extracts: The extraction of plant extract was carried out by the petroleum ether or 80% methanol as solvent. The nature, color and percentage yield of the extracts is given below in Table No 3.1

Table 3.1 : Percentage yield of the extracts

S. No.	Extract	Color	Consistency	%yield (w/w)
1.	Hydroalcohol extract of ***Calamus rotang*** rhizomes	Dark brown	Semi-solid	29.0 %

3.2 Qualitative phytochemical screening of plant extract:

The extract obtained from successive solvent extraction were subjected to qualitative phytochemical screening to detect the presence of various classes of chemical constituent in them. In Preliminary phytochemical screening It was found that hydroalcohol extracts of ***Calamus rotang*** rhizomes contained tannins, flavonoids, triterpenoids, steroids, glycosides, anthraquinones,sapoins, reducing sugars, carbohydrates, proteins, and amino acids. The results are given in Table No. 3.2

Table 3.2 : Qualitative phytochemical screening of Hydroalcoholic extract of *Calamus rotang* rhizomes

S. No.	Chemical Test	Hydroalcoholic extract of *Calamus rotang* rhizomes
1.	Alkaloids	+ve
2.	Anthraquinone glycosides	+ve
3.	Saponin glycosides	+ve
4.	Cardiac glycosides	-ve
5.	Carbohydrates	+ve
6.	Flavanoids	+ve
7.	Tannins	+ve
8.	Steroids and Terpenoids	+ve
9.	Fats/lipids	-ve
10.	Proteins and Amino acids	+ve

3.3 TLC Identification:

TLC method was applied for identification of flavanoids in hydroalcoholic extract of *Calamus rotang* rhizomes. Fresh extracts showed brown or dark yellow zones, with these colors being specific for thiosulfinate compounds. After treatment with the detection system extracts of *Calamus rotang* rhizomes showed brown and yellow-brown zones with R_f values of 0.80, Moreover, the extract of *Calamus rotang* rhizome is distinguishable by the characteristic yellow-brown color and with R_f values of 0.2 to 0.88, respectively, indicating the presence of flavanoids was present in the hydroalcoholic extract of *Calamus rotang* rhizomes.

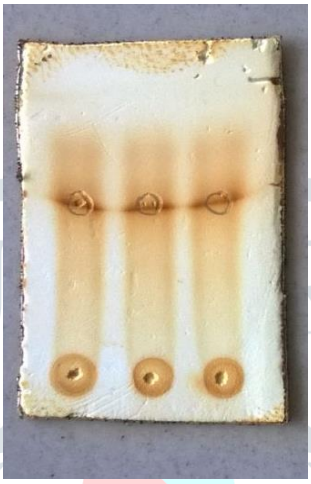

Figure 3.1 : TLC plate for the identification flavanoids

3.4 Determination of Flavanoid content

Flavonoids were reported in plant rhizomes of *Calamus rotang* extract. Total flavonoid content was found higher in ethanol extract > Petroleum ether > aqueous extract.

Table 3.3 : Flavanoid content in different solvents of Rhizomes of *Calamus rotang*

Plant Part	**Total Flavanoid content**		
	Aqueous	**Ethanol**	**Petroleum ether**
Rhizomes	22.50+0.80	57.12+0.35	53.7+0.22

3.5 *In- vitro* Antioxidant and Antimicrobial activity

3.5.1 *In- vitro* Antioxidant activity:

Table 3.4 : Result of antioxidant activity of Hydroalcoholic extract of *Calamus rotang* rhizomes

Sr No.	Conc. (µg/ml)	Control	Ascorbic acid % scavenging	Test %scavenging
1	10	0.812	62.80788	45.19704
2	20	0.812	75.61576	51.84729
3	40	0.812	85.83744	60.71429
4	80	0.812	89.65517	72.29064
5	100	0.812	94.45813	85.46798
7	IC_{50}		1 µg/ml	1.85 µg/ml

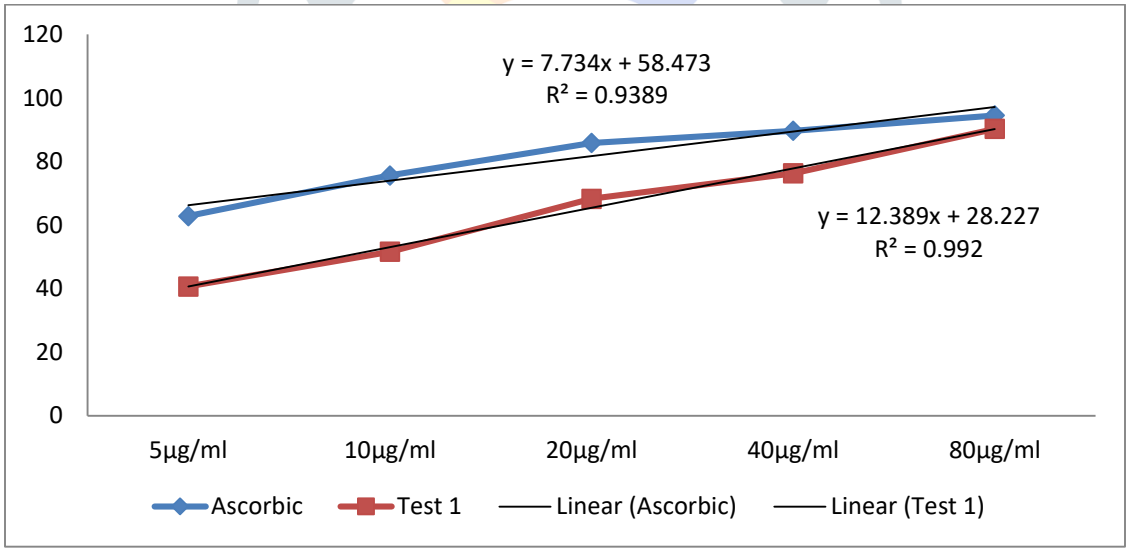

Figure 3.1 : Calibration curve of standard (Ascorbic acid) and Hydroalcoholic extract of *Calamus rotang* rhizomes

The DPPH radical scavenging activity of Hydroalcoholic extract of *Calamus rotang* rhizomes

is given in Table no 3.4. The results indicated that combination of plant extract had potent antioxidant activity.

3.5.2 Antimicrobial activity

3.5.2.1 Antibacterial activity of hydroalcoholic extracts of rhizomes of *Calamus rotang*

Table 3.5: Antibacterial activity of hydroalcoholic extracts of rhizomes of *Calamus rotang* against bacterial test organism

Antibacterial activity (Zone of inhibition)					
Microorganism	**Hydroalcohol extracts of rhizomes of *Calamus rotang* (µg/ml) Zone of inhibition in mm**				
	5 (µg/ml)	25 (µg/ml)	50 (µg/ml)	100 (µg/ml)	200 (µg/ml)
Staphylococcus aureus	_	14	16	17	18
Escherichia coli	_	16	17	18	20

Table 3.6 : Antibacterial activity of standard drugs against bacterial test organism

Antibacterial activity (Zone of inhibition)			
Drug	**Concentration (µg/ml)**	**Zone of inhibition in mm**	
		Staphylococcus *aureus*	**Escherichia *coli***
Ampicillin	5 (µg/ml)	10	14
	25 (µg/ml)	12	16
	50 (µg/ml)	14	18
	100 (µg/ml)	17	20
	200 (µg/ml)	19	21
Ciprofloxacin	5 (µg/ml)	17	20
	25 (µg/ml)	19	25
	50 (µg/ml)	20	27
	100 (µg/ml)	21	27
	200 (µg/ml)	22	28

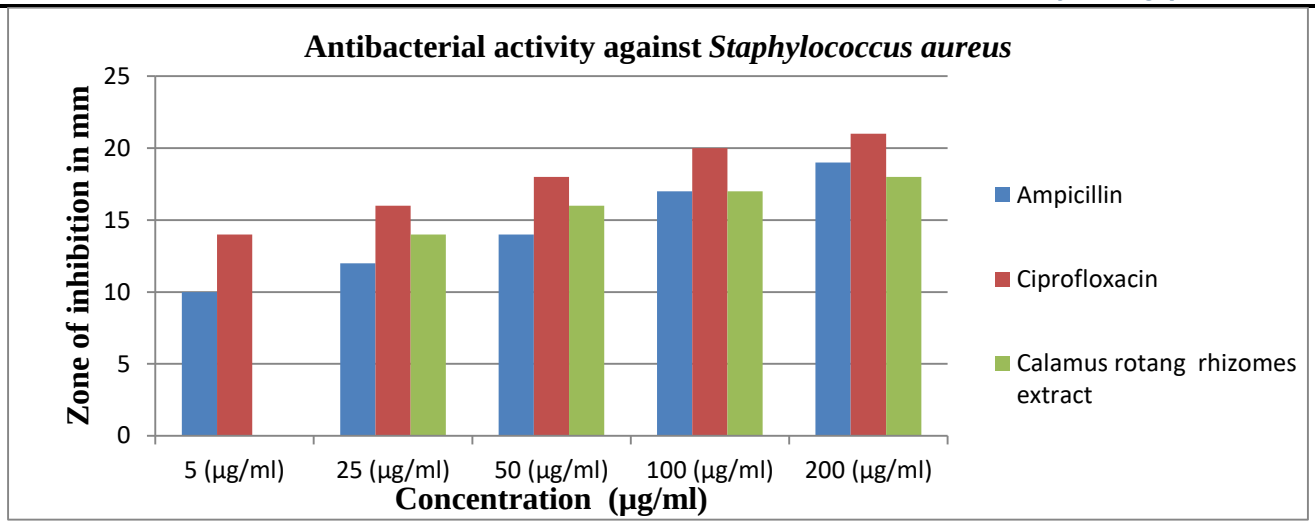

Figure 3.2 : Antibacterial activity against *Staphylococcus aures*

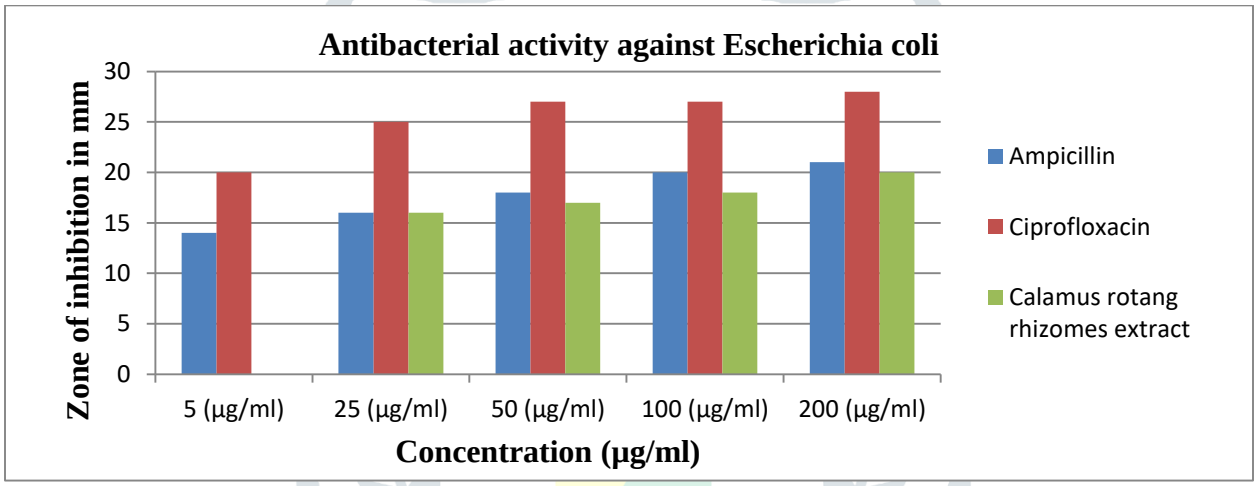

Figure 3.3: Antibacterial activity against *Escherichia coli*

Antibacterial potential of extracts were assessed in terms of zone of inhibition of bacterial growth. The results of the antibacterial activity are presented in Table No 3.5 and 3.6. The antibacterial activity of the extract increased linearly with increase in concentration of extracts (µg/ml). As compared with standard drugs, the results shows that in the extracts for bacterial activity, *Staphylococcus aureus* was more sensitive as compared with *Escherichia coli*. The growth inhibition zone measured ranged from 11 to 20 mm for all the sensitive bacteria. The above results show that the activity of hydroalcohol extracts of *Calamus rotang* rhizomes shows significant antibacterial activity.

3.5.2.2 Antifungal activity of hydroalcoholic extracts of rhizomes of *Calamus rotang*Table 3.7 : Antifungal activity of hydroalcoholic extracts of rhizomes of *Calamus rotang* against bacterial test organism

Antifungal activity (Zone of inhibition)					
Microorganism	**Hydroalcohol extracts of rhizomes of *Calamus rotang* (µg/ml) Zone of inhibition in mm**				
	5 (µg/ml)	25 (µg/ml)	50 (µg/ml)	100 (µg/ml)	200 (µg/ml)
Candida albicans	_	14	15	18	18
Aspergillus niger	_	14	17	18	20

Table 3.8 : Antifungal activity of standard drugs against fungal test organism

Antifungal activity (Zone of inhibition)			
Drug	**Concentration (µg/ml)**	**Zone of inhibition in mm**	
		Candida albicans	***Aspergillus niger***
Griseofulvin	5 (µg/ml)	17	18
	25 (µg/ml)	20	22
	50 (µg/ml)	21	24
	100 (µg/ml)	22	25
	200 (µg/ml)	23	26
Nystatin	5 (µg/ml)	17	17
	25 (µg/ml)	20	18
	50 (µg/ml)	23	24
	100 (µg/ml)	24	27
	200 (µg/ml)	25	28

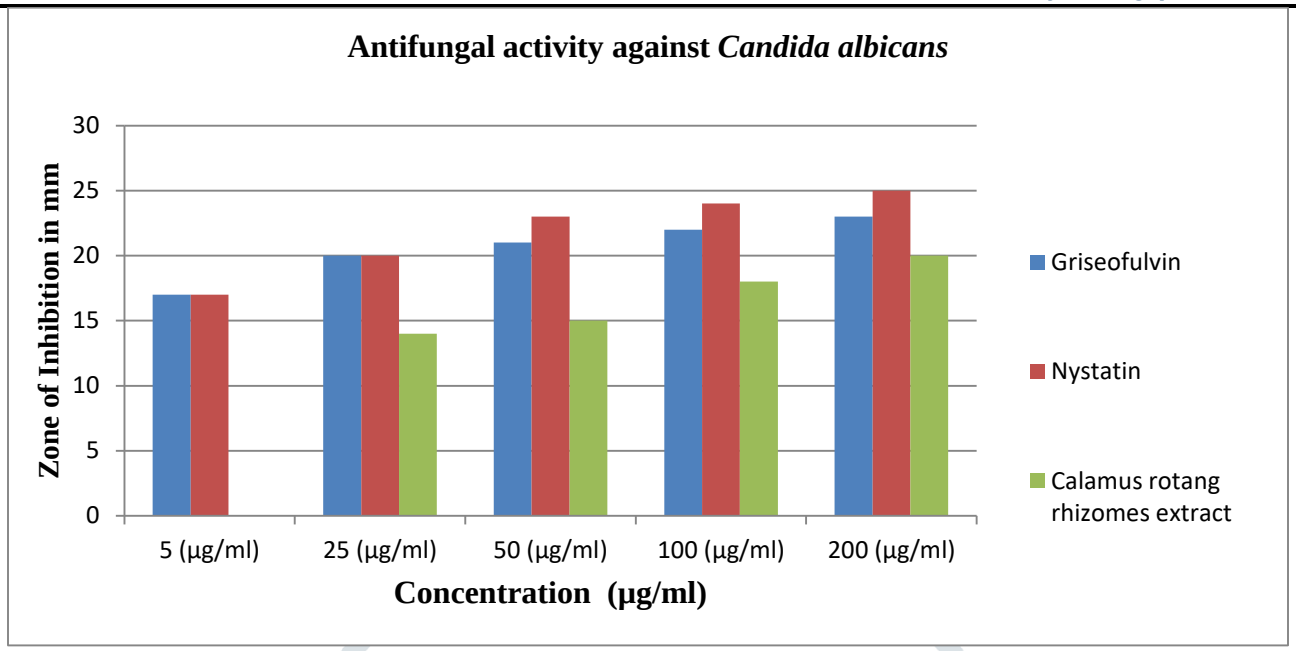

Figure 3.4 : Antifungal activity against *Candida albicans*

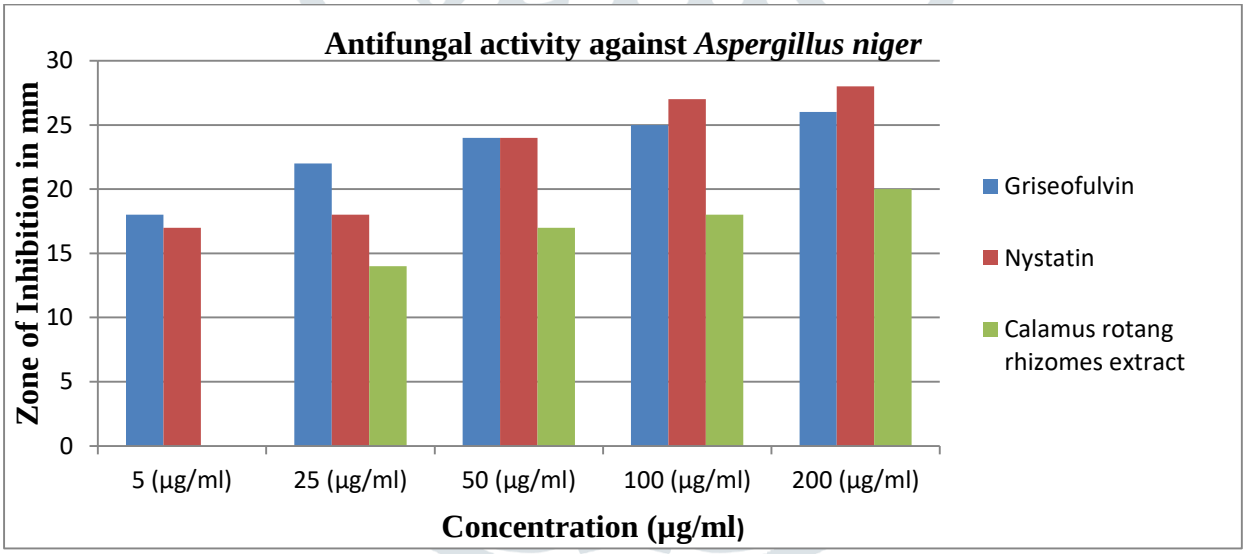

Figure 3.5 : Antifungal activity against *Aspergillus niger*

Antifungal activity of extracts were assessed in terms of zone of inhibition of bacterial growth. The results of the antifungal activity are presented in TableNo. 3.7 and 3.8. The antifungal activitity of the extract increased linearly with increase in concentration of extract (µg/ml). As compared with standard drugs(Griseofulvin and nystatin) the results shows that in the extract for fungal activity, *Candida albicans* was more sensitive as compared with, *Aspergillus niger* for fungal activity.. The growth inhibition zone measured ranged from 14 to 22 mm for fungal strains. The above results show that the activity of hydroalcohol extracts of *Calamus rotang* rhizomes shows significant Antifungal activity.

Conclusions

The current work was focused on the Phytochemical Screening, *in-vitro* Antioxidant and Anti-microbial evaluation of extract of dried rhizomes of *Vetra*. Initially the phytochemical screening reveals the presence of saponins, anthraquinones, tannins and flavanoids content and this data confirmed the presence of antioxidant activity due to presence of phenolic and flavanoids in rhizomes of *calamus rotang* extract. Secondly, microbiological screening exhibits antibacterial activity and antifungal activity that may provide good efficacy against various infection and diseases. In conclusion, these results demonstrating broad spectra of antibacterial and antifungal activity in hydroalchol extract of *calamus rotang* might be more helpful to discover a new compound that may be used as novel biochemical for various infectious diseases and other biological functions in the future.

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