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SCREENING OF SOLVENT EXTRACTS OF *RHEUM AUSTRALES* FOR ISOLATION OF ANTIMICROBIAL COMPOUND

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ABSTRACT

In the present study, the hydro-alcoholic and hexane extracts of the plant, *Rheum australes* (Family: Polygonaceae) were screened for antimicrobial activity against pathogenic and drug resistant strains. The non polar extracts did not showed any activity against any of the pathogenic strains. Amongst these extracts the hydro-alcoholic extracts of the plant, *Rheum australes*, showed potent antimicrobial activity against almost all the pathogens studied. These extracts also showed potent antibacterial activity against multidrug resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* in the ranges from 0.3 mg/ml to 0.7 mg/ml. The potent hydro-alcoholic extract was further subjected for isolation and characterization of potent antimicrobial compound via chromatographic and spectroscopic techniques. The pure antimicrobial compound isolated and characterized was Revandichinone. The compound was isolated as bright yellow needles, mp 214^{0}C. The IR spectrum showed absorption bands at 3441 cm^{-1} (–OH), 1625 cm^{-1} (chelated-C¼O) and 1749 cm^{-1} (ester–C¼O). The compound isolated was further subjected for antimicrobial screening against pathogens at varying concentrations.

Key words: Antimicrobial activity, *Rheum australes,* pathogenic and drug resistant strains, Revandichinone

INTRODUCTION

Plants are capable of synthesizing an overwhelming variety of low molecular weight organic compounds usually unique and complex in structure. Phytochemicals produced in plants are secondary compounds responsible metabolic activities and defense in purpose. Phytochemicals are produced by specific biochemical pathways, which occur inside the plant cells. At least 12,000 phytochemicals (secondary metabolites) have been isolated from plants, a number estimated to be less than 10 % of the total. Translation of ethno botanical information for isolation and identification of phytochemicals is required for revealing the pharmacological status of the important phytochemicals (Jaradat *et al*., 2004). One of the paramount reasons for pursuing natural products chemistry resides in the actual or potential pharmacological activity to be found in alkaloids, terpenoids, coumarins, flavanoids, lignans, glycosides etc. Since the advent of antibiotics in the 1950's, the use of plant derivatives as a source of antimicrobials has been virtually non-existent (Singh *et al*., 2009). Antimicrobial plant extracts have been recognized as a future source of new antimicrobials in the event of the current downturn in the pace at which these are being

derived from micro-organisms (Mathur *et al*., 2011). These antimicrobial compounds isolated from plants are found to be very effective in comparison to traditional medicines and produces no side effects. The biological activity of many plants has been known through scientific research and any literature search via the internet or elsewhere, would reveal that numerous new publications are added to the scientific literature every day. In the present investigation, *Rheum australes* extracts (hydro-alcoholic and hexane) were screened for antimicrobial activity against pathogenic and drug resistant microbial strains in lieu of isolation and identification of antimicrobial compound.

MATERIALS AND METHODS

Plant Materials Collection

The parts of the plant, *Rheum australes* were collected during September, 2010 from local gardens of North West Himalayan Garhwal regions of Uttarakhand and were further identified by Dr. Ajay Swami, Reader & HOD, Dept. of Botany, Chinmaya Degree College, Hardwar (U.K), India.

Preparation of Plant extracts

The plant parts were separated, washed with distilled water, dried under shade and pulverized. The method (Alade and Irobi, 1993) for preparation of extracts was slightly modified. Briefly 20 g portions of the powdered plant material was soaked in different solvents i.e. hydro-alcohol (50%v/v) and hexane for 72 h. Each mixture was stirred every 24 h using a sterile glass rod. At the end of extraction, each solvent was passed through Whatmann filter paper No. 1 (Whatmann, England) The filtrates obtained were concentrated in vacuo using water bath at 30^0C. The potent extracts of the plants were subjected for isolation and characterization of active principles.

Determination of Antimicrobial activity

Culture Media

The media used for antifungal test was Sabouraud's dextrose agar/broth of Hi media Pvt. Bombay, India while Soyabean Casein Digest agar/broth procured from Hi Media Pvt. Bombay, India was used for antibacterial assay.

Inoculum

The fungal strains were inoculated separately in Sabouraud's dextrose broth for 6 h and the suspensions were checked to provide approximately 10^5 CFU/ml. The bacterial strains were inoculated separately in Soyabean Casein Digest broth for 6 h and the suspensions were checked to provide approximately 10^5 CFU/ml.

Fungal strains used for the study

The clinical fungal test organisms used for study were *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404 were procured from National Chemical Laboratory (NCL), Pune, Maharashtra, India.

Bacterial strains used for the study

The multidrug resistant (MDR) bacterial strains (Super bugs) isolated from clinical samples of infected patients viz. *Staphylococcus aureus* and *Pseudomonas aeruginosa* procured from Dept. of Microbiology, Shoolini University, Solan (H.P) were used for the study. Besides MDRs other different pathogens viz. *Micrococcus luteus* ATCC 9341, *E.coli* ATCC 8739, *E.coli* mutans ATCC 11105, *Salmonella abony* ATCC 6017, *Lactobacillus plantarum* ATCC 8014 and *Staphylococcus epidermidis* ATCC 12228 were procured from National Chemical Laboratory (NCL), Pune, Maharashtra, India.

Determination of antifungal activity

The agar well diffusion method (Perez and Anesini, 1993) was modified. Sabouraud's dextrose agar (SDA) was used for fungal cultures. The culture medium was inoculated with the fungal strains separately suspended in Sabouraud's dextrose broth. A total of 8 mm diameter wells were punched into the agar and filled with plant extracts and solvent blanks (hydro alcohol, and hexane). Standard antibiotic (Fucanazole, concentration 1 mg/ml) was used as positive control and fungal plates were incubated at 37^oC for 72 h. The diameters of zone of inhibition observed were measured.

Determination of antibacterial activity

The same procedure was adopted for antibacterial activity. Soyabean Casein Digest Agar (SCDA) was used for bacterial cultures. The culture medium was inoculated with the bacterial strains separately suspended in Soyabean Casein Digest broth. A total of 8 mm diameter wells were punched into the agar and filled with plant extracts and solvent blanks. Standard antibiotic (Fucanazole, Chloramphenicol concentration 1 mg/ml) was used as positive control and culture plates were incubated at 37^{o}C for 72 h (for antifungal assay) and for 24 h (for antibacterial assay). The diameters of zone of inhibition observed were measured.

Determination of MIC and MBC

The antimicrobial plant extracts were then after evaluated to determine MIC and MLC values. The serial dilution method (Vollekova *et al*., 2001; Usman *et al*., 2007) was adopted by using N-saline for diluting the plant extract and was incubated for 48 h. The minimum dilution of the plant extract that kills the microbial growth was taken as MLC (Minimum lethal count) while the minimum dilution of plant extract that inhibits the growth of the organism was taken as MIC.

Phytochemical screening of the extract

The portion of the dry extract was subjected to the phytochemical screening using the method adopted (Trease and Evans, 1989; Harborne, 1993). Phytochemical screening was performed to test for alkaloids, saponin, tannins, flavanoids, steroids, sugars and cardiac glycosides (Mathur *et al*., 2011b)

Test for alkaloids

The 0.5 g of the plant extract was dissolved in 5 ml of 1% HCl and was kept in water bath for about 2 minutes. 1ml of the filtrate was treated with Dragendroff's reagent Turbidity or precipitation was taken as indicator for the presence of alkaloids.

Test for Tannins

About 0.5 g of the sample was dissolved in 10 ml of boiling water and was filtered. Few ml of 6% $FeCl_3$ was added to the filtrate. Deep green colour appeared confirmed the presence of Tannins.

Test for Flavanoids

About 0.2 gm of the extract was dissolved in methanol and heated for some time. A chip of mg metal was introduced followed by the addition of few drops of conc. HCl. Appearance of red or orange color was Indicator of the flavanoids.

Test for Saponin

About 0.5 g of the plant extract was stirred with water in the test tube. Frothing persists on warming was taken as a evidence for the presence of saponin.

Test for Steroids

Salkowaski method was adopted for the detection of steroids. About 0.5 g of extract was dissolved in 3 ml of chloroform and filtered. To the filtrate, conc. $H_2 SO_4$ was added to form a lower layer. Reddish brown color was taken as positive for the presence of steroids ring (Agarwal *et al*., 2011).

Test for Cardiac glycoside

About 0.5 g of the extract was dissolved in 2ml of glacial acetic acid containing 1 drop of 1% $Fecl_3$. This was under laid with conc. $H_2 SO_4$. A brown ring obtained at the interphase indicates the presence of deoxy sugar. A violet ring appeared below the ring while in the acetic acid layer a greenish ring appeared just above ring and gradually spread throughout this layer.

Test for reducing Sugars

1ml each of Fehling's solutions, I and II was added to 2 ml of the aqueous solution of the extract. The mixture was heated in a boiling water bath for about 2-5 minutes. The production of a brick red precipitate indicated the presence of reducing sugars.

Isolation of compounds from plant extracts by Gravity Gradient Column Chromatography

Sintered glass column fitted with stopper (i.d., 1.5 and length 100 cm) was filled with silica gel G (for column chromatography) with procedure described below. The elutions of the compounds

were carried out at the room temperature and pressure.

Loading of fraction

The potent extract of the plant having the highest antimicrobial activity were subjected to liquid-liquid fractionation. The highest antimicrobial fractions were chromatographed on 2x30 cm silica gel open column using a stepwise gradient of n-hexane and increasing amount of ethyl acetate (20 % at each step); ethyl acetate with increasing amount of methanol (10 % at each step); and finally at 40 % methanol. Collected fractions were evaporated under vacuum and examined by TLC. Homogeneous fractions were pooled to give large number of different fractions. These fractions were examined using Silica gel coated TLC plates to confirm the pure compound by changing the ratios of the solvent system components.

Elution of compounds

Once the column was filled with eluent, the column was allowed to run. A quick flow rate helps to give good separation. By adjusting the air pressure to give a swift flow rate, different coloured bands were appeared in the column. After collecting these fractions the column's progress were monitored by TLC.

Conventional preparative TLC

Silica gel G used for thin layer chromatography (TLC) was activated in hot air oven at 110°C for one hour.

Preparation of thin layer plates and loading of sample

The plates were developed in the solvent, Hexane: ethyl acetate: glacial acetic acid (65:35:0.5) to separate the compound. Chromatograms were detected with UV at 254 nm. The active fractions/pure compounds was scraped from the Silica gel plate and eluted from the silica gel with ethanol. The active compounds were filtered through Millipore filters (0.45 µm and 0.22 µm) to remove the silica gel and this yielded more of compound(s) fraction.

Structure elucidation of isolated compounds by combination of different techniques

Identification of compound was done by using a combination of different techniques including HPLC and FT-IR Besides these characterization techniques, R_f values and melting point of the active compounds were also determined.

High-performance liquid chromatography (HPLC)

HPLC analysis was performed in Roorkee Research and Analytical Laboratory Pvt. Ltd., Roorkee (Uttarakhand), India using a Shimadzo LC- 2010 HPLC system (Kyoto, Japan), equipped with a Shimadzo LC 2010 UV-VIS detector with a thermostated flow cell and a selectable two wavelengths of 190 - 370 nm or 371–600nm. The detector signal was recorded on a Shimadzo LC2010 integrator. The column used was a C-18 block heating-type Shim-pack VP-ODS (4.6 mm interior diameter × 150 mm long) with a particle size of 5 µm. Mobile phase was designed as per the nature of the compound, containing 50 % acetonitrile along with 50 % Phosphate buffer was used at a flow rate of 3.0 ml/min, column temperature 25°C. Injection volume was 40 µl and detection was carried out at specific wavelength having maximum absorbance as calculated by UV absorption spectra at maximum wavelength.

Fourier Transform Infrared (FTIR) studies

The IR spectrum of isolated compound was recorded in Roorkee Research and Analytical Laboratory Pvt. Ltd., Roorkee (Uttarakhand), India using a computerized FTIR spectrometer (Perkin Co., Germany) in the range of 4400-400 cm^{-1} by the KBr pellet technique.

RESULTS AND DISCUSSION

Antimicrobial activity

In this study, the hydro-alcoholic and hexane extracts of *Rheum australes* were screened for their antimicrobial activity against multi-drug resistant strains (isolated from Clinical samples, pus and blood of patients) and other pathogenic strains. Hydro-alcoholic extract of the plants showed significant antimicrobial activity in comparison to hexane extract against all the

Figure-1(a): Antimicrobial activity of Rheum australes against pathogenic and drug resistant strains.

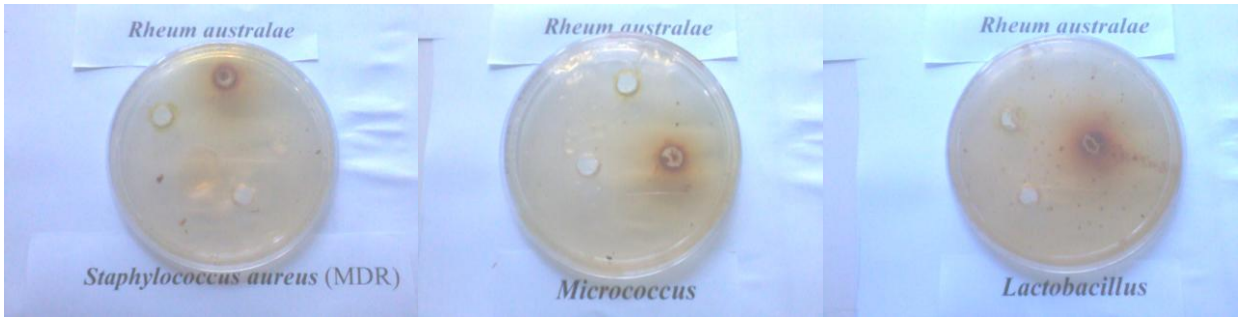


Table 1: Antifungal activity of the extracts of *Rheum australes*

Plants	Solvent Extract	Diameterof zone of inhibition (mm)		MIC(mg/ml)		MLC(mg/ml)	
		AN	CA	AN	CA	AN	CA
Rheum australes (Chirrchey)	HA (Hydro alcoholic)	13	NA	0.6	NA	0.8	NA
	HX (Hexane)	07	NA	0.8	NA	0.9	NA

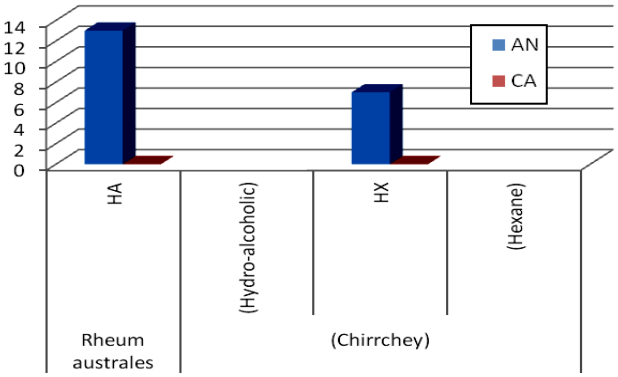
*AN, *Aspergillus niger*; CA, *Candida albicans;* NA, No activity

pathogens and against MDR *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Hydro-alcoholic extract of the plant showed potent antifungal activity against *Aspergillus niger* (zone of inhibition: 13mm) in comparison to hexane extract (zone of inhibition: 7mm). None of the extract showed any anti-fungal activity against *Candida albicans*. The hydro-alcoholic extracts of the plant was found to have significant antifungal activity at MIC value, 0.8 mg/ml in comparison to hexane extract which showed MIC value, 0.9 mg/ml. The hydro-alcoholic extracts showed significant antibacterial activity against *Salmonella abony* (zone of inhibition: 27 mm) followed by *Pseudomonas aeruginosa* (zone of inhibition: 26mm) and *Lactobacillus plantarum* (zone of inhibition: 20mm). The results are shown in Table 1 and 2; Figure 1 (a) & (b) and 2. Hydro-alcoholic extract showed potent antibacterial activity against multidrug resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* in the ranges from 0.3 mg/ml to 0.7 mg/ml.

Phytochemical Screening of plant extracts

The results showed that alkaloids, flavanoids, tannins, glycosides and reducing sugars are present specifically in hydro-alcoholic extract of the plant while steroids and saponin are found in hexane extract of the plant. The results are shown in Table 3.

Figure 1 (b): Antifungal activity of the extracts of *Rheum australes*



Isolation and Identification of the antimicrobial compound:

Chromatographic and spectroscopic techniques

Table 2: Antibacterial activity of the extracts of *Rheum australes*

Plants	Solvent Extract	Diameterof zone of inhibition (mm)							
		SA	PA	ML	ECO	ECO M	S. ABONY	LP	SE
Rheum australes (Chirrchey)	HA (Hydro-alcoholic)	27	26	19	18	19	19	20	16
	HX (Hexane)	NA	NA	14	12	12	15	17	13

*SA, *Staphylococcus aureus*; PA, *Pseudomonas aeruginosa*; ML, *Micrococcus luteus*; ECO, *E. Coli*; ECO M, *E.coli mutans*; S. ABONY, *Salmonella abony*; LP, *Lactobacillus plantarum*; SE, *Staphylococcus epidermidis;* NA, No activity

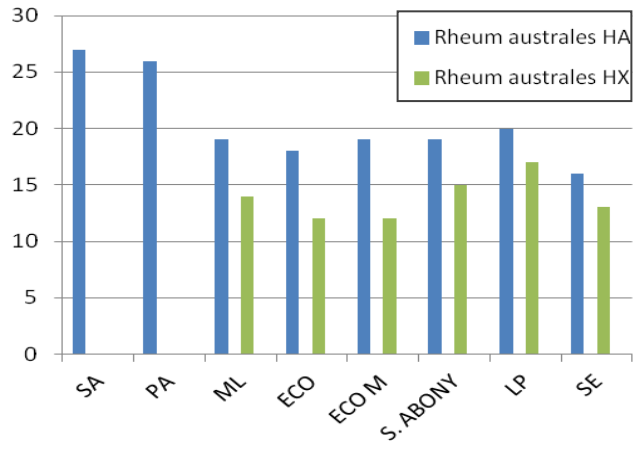
Table 3: Phytochemical screening of the active constituents

Rheum australes	Phytochemical constituents						
	Alkaloids	Flavanoids	Tannins	Steroids	Saponin	Glycosides	Reducing sugars
Hydro-alcoholic extract	+	+	+	_	_	+	+
Hexane extract	_	_	_	+	+	_	_

*+, present; _, absent

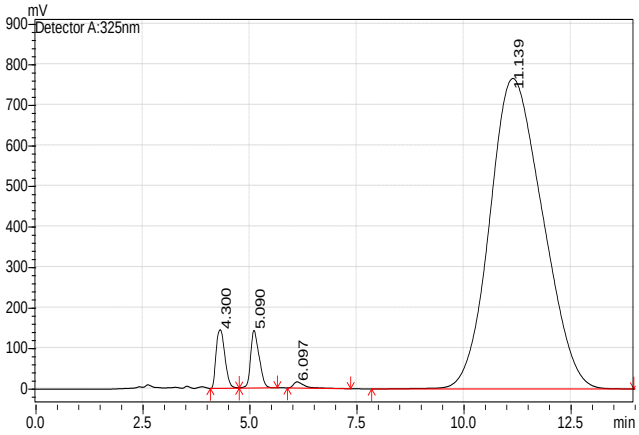
The pure antimicrobial compound isolated and characterized from this plant was revandichinone.

Figure 2: Antibacterial activity of the extracts of *Rheum australes*



The obtained compound was found to be in the form of bright yellow needles, mp 214^{0}C. The IR spectrum showed absorption bands at 3441 cm^{-1} (–OH), 1625 cm^{-1} (chelated-C¼O) and 1749 cm^{-1} (ester–C¼O). The compound showed R_f value 0.45 similar to that of the standard compound, Revandichinone.

Fig. 3: Compound determined through HPLC



The pure compound, revandichinone (as compared with that of standard) was further subjected for antimicrobial and antioxidant screening (data not shown). The pure compound fraction was further subjected to HPLC and

FTIR analysis. The results are shown in Figures 3 to 6.

Fig. 4: Standard compound chromatogram

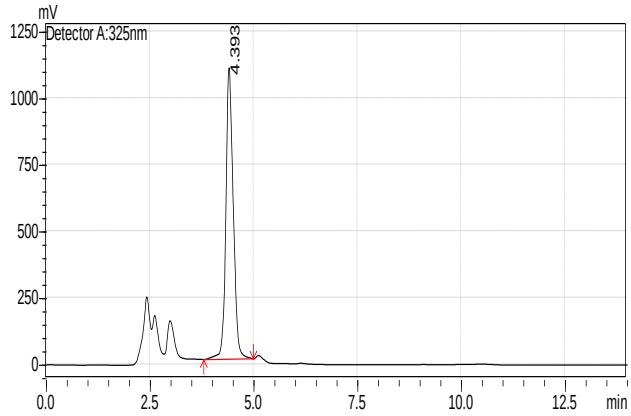
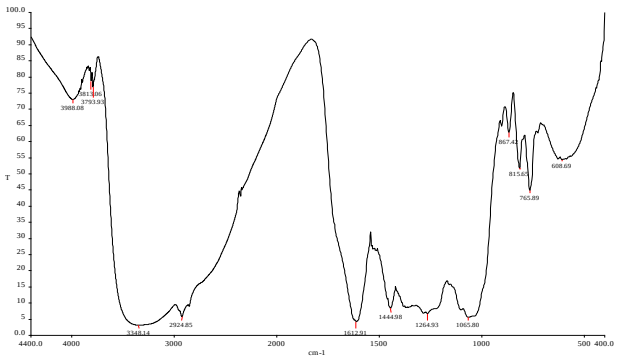


Figure-5: TLC Chromatogram of pure compound detected in UV light

Fig. 6: FT-IR spectra of isolated compound, Revandichinone-1 from *Rheum australes*



CONCLUSION

The use of the plant in the treatment of pathogenic diseases associated with the infection of these pathogens is validated, scientifically supported by the results obtained in this work. The present study thus suggests that compound isolated, Revandichinone-1 characterized is found to be most potent antimicrobial agent and can be further utilized in formulating a new antimicrobial drug. The results thus signify that the drug prepared from the compound isolated can be utilized in treatment of infections associated with such pathogens.

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