The Pharma Innovation Journal 2018; 7(9): 149-153

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(9): 149-153

www.thepharmajournal.com
Received: 13-07-2018
Accepted: 15-08-2018

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Investigations on antifungal activity of *Rheum emodi* and *Podophyllum hexandrum*

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Abstract
The present study was carried out to investigate antifungal activity of aqueous and methanol extracts of rhizomes of *Rheum emodi* and *Podophyllum hexandrum* against two fungal strains *Fusarium solani* MTCC3871 and *aspergillus flavus* MTCC1037. The aqueous and methanol extract of *Rheum emodi* exhibited significantly higher (p<0.05) antifungal activity against the test organisms at 10mg/ml in comparison to *Podophyllum hexandrum*. The aqueous extract of *Rheum emodi* showed 19.7 ± 0.11 and 17.2 ± 0.09 antifungal activity against 16.9 ± 0.13 and 16.3 ± 0.14 when compared to *Podophyllum hexandrum* similarly methanolic extract of *Rheum emodi* also demonstrated higher antifungal activity 26.4 ± 0.17 and 24.2 ± 0.18 as against 24.3 ± 0.14 and 16.7 ± 0.13 of *Podophyllum hexandrum*. The extracts from *Rheum emodi* also showed higher zones of inhibition against fungal strains when compared to *Podophyllum hexandrum* extracts.

Keywords: *Rheum emodi*, *Podophyllum hexandrum*, extract, zone of inhibition, antifungal

Introduction
Many plant species belonging to different families have been reported to have a traditional medicinal use for a number of ailments by different communities of India. However these days we have new and different medicines for these diseases, which unfortunately are accompanied by various side effects. Subsequently, there is a need to have the active principals of natural origin which can be used for the treatment and/or prevention of diseases/infections with no side effects. Thus, there has been a growing interest in natural plant products as these are more compatible to the human body with little or no toxic side effects. Further, the pharmacological actions, if any, of many plants having medicinal potential remain unassessed by rigorous scientific research to define efficacy and safety [1]. *Rheum emodi* (Polygonaceae), commonly known as revand-chini, is the Himalayan species of Indian rhubarb found wild at an altitude of 4000–12 000 feet in Kashmir, Nepal, Sikkim and Bhutan. Rhubarb has been successfully grown in certain parts of Assam [2]. There have already been many reports about antibacterial and antifungal activities of the anthraquinones, the napthoquinones isolated from natural sources [3-5]. In addition several other biological activities such as laxative, diuretic, and *in vivo* inhibitory effect towards P388 leukemia in mice are also reported [6-7]. The anthraquinone derivatives from *Rheum emodi* is reported to possess anticancer activity [8]. The perennial herb *Podophyllum hexandrum* bearing the common names Himalayan May apple or Indian May apple, is native to the lower elevations of Himalayan countries like Afghanistan, Pakistan, India, Nepal, Bhutan, and in South West China rhizome. It is tolerant to cold temperatures, as would be expected of a Himalayan plant, but is not tolerant to dry conditions [9-11]. In India *Podophyllum hexandrum* is mostly found in Alpine Himalayas (3000-4000 m) of Jammu and Kashmir, Himachal Pradesh, Sikkim, Uttaranchal and Arunachal Pradesh [12]. In Kashmir it has been used in traditional system of medicine from time immemorial and is locally known as Banwangun, since its red colour fruit (berry) is of the size of a small brinjal.

Materials and methods
Plant material
The rhizomes of *Rheum emodi* and *Podophyllum hexandrum* were collected in the month of May - June from Sonamarg and Gulmarg area of Kashmir Valley and got identified at the centre of Plant Taxonomy, Department of Botany, University of Kasmir. The plant material (rhizome) was dried in the shade and ground into a powder using mortar and pestle and passed through a sieve of 0.3 mm mesh size. The powder obtained was extracted with water and methanol using a Soxhlet extractor (60-80° C). These extracts were concentrated using the rotary vacuum evaporator and then stored at 4^0 C for future use.

Test microorganisms

The test organisms were obtained from Department of Microbiology, Government Medical College Srinagar. The fungal strains used in the study were *Fusarium solani* MTCC3871 and *aspergillus flavus* MTCC1037.

Preparation of inoculum

Stock cultures were maintained at 4°C on slopes of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Sabouraud dextrose broth (SDB) that were incubated without agitation for 24 h at 37°C and 25°C respectively. To 5ml of SDB, 0.2 ml of culture was inoculated and incubated till it reached the turbidity equal to that of the standard 0.5 McFarland solution (McFarland 1907) at 600 nm which is equivalent to 10^6– 10^8 CFU/ml.

Antifungal Assay

Extracts of *Podophyllum hexandrum* and *Rheum emodi* prepared with methanol (MERCK) were used to test their antifungal activity. Antifungal activity was demonstrated using a modification of the method originally developed by Bauer *et al*. which is widely used for the antimicrobial susceptibility testing. Liquid nutrient potato dextrose agar media and the petri plates were sterilized by autoclaving at 120^0C for 30 minutes. Under septic conditions in the laminar airflow chamber, about 20 ml of the agar medium was dispensed into each petriplate to yield a uniform depth of 4mm. After solidification of the media, the fungal strains were swabbed on the surface of the plates. Whatmann no.1 filter paper was cut into small discs of diameter 0.4cm and autoclaved. The discs were dipped into the different plant extracts of each four concentrations namely 2.5mg/ml, 5.0mg/ml, 7.5mg/ml and 10.0mg/ml. The dipped discs were placed on the appropriate swabbed petriplates such as that each petriplate have the four concentrations of each plant extract. Amphotericin B was used as the standard drug. It was then kept in incubator maintaining the temperature at about 25^0C for 48 hours and then the zones of inhibition were measured in mm.

Minimum Inhibitory Concentration (MIC) Assay

The MIC method was applied on extracts that proved their high efficacy against microorganisms by the disk diffusion method [13].The highest dilution of a plant extract that still retains an inhibitory effect against the growth of a microorganism is known as MIC [14]. Selected plant extracts were subjected to a serial dilution (25 mg/ml to 0.37 mg/ml) using sterile nutrient broth medium as a diluent. In a 96-well titre plate 20 µl of an individual microorganism and 20 µl of selected plant extract were loaded and inoculated at 37^0C for 24 h. The highest dilution of the plant extract that retained its inhibitory effect resulting in no growth (absence of turbidity) of a microorganism was recorded as the MIC value of the extract. A control experiment was run in parallel to study the impact of the solvent alone (without plant extracts) on growth of the test organisms. Methanol was diluted in a similar pattern with sterile nutrient broth followed by inoculation and incubation.

Statistical analysis

All the determinations were carried out in triplicates and results were expressed as mean ± SE and mean values were plotted in all figures. The level of significance was expressed using Students't-Test. All the analysis was carried out using Graph Pad Prism 5 software.

Results and discussions

The rhizomes of plants were extracted using methanol and water by the earlier mentioned method. In the extractive values were found 40.94%, 32.73%, 37.43% and 29.96% of methanolic and aqueous extract of *Rheum emodi* and *Podophyllum hexandrum* respectively (Table 1).

Table 1. Percentage yield of crude extract of *R. emodi* and *P. hexandrum*

Solvent	Wt. of powderd plant material *R. emodi*	Wt. of powderd plant material *P. hexandrum*	Vol. of solvent	Wt. of extract of *R. emodi*	Wt. of *P. hexandrum*	% yield of extract of *R. emodi*	% yield of extract of *P. hexandrum*
Aqueous	25 gm	25 gm	150ml	9 gm	9.25 gm	40.94	37.43
Methanol	25 gm	25gm	150ml	6 gm	6.34 gm	32.73	29. 96

Antifungal activity of *Rhum emodi* and *Podophyllum hexandrum*

The antifungal activity of aqueous and methnolic extract of *R. emodi* and *P. hexandrum* was assessed against two fungal strains *Fusarium solani* MTCC3871 and *aspergillus flavus* both the extract exhibited antifungal activity against the tested strains. Four concentrations 2.5 mg/ml, 5 mg/ml, 7.5 mg/ml and 10 mg/ml of both the extractrs were used to study the zone of inhibition against the tested fungal strains. Both the extracts exhibited antifungal activity against the tested organisms at 10 mg/ml by inhibiting the growth of test organisms (Table 2) and figure 1, 2, 3 and 4.

Table 2: Antifungal activity of aqueous and methanol extract of *R. emodi* and *P. hexandrum*

Microorganism	*R. emodi* A. extract	*R. emodi* M. extract	*P. hexandrum* A. extract	*P. hexandrum* M. extract
F. solani MTCC3871	19.7±0.11	26.4 ±0.17	16.9±0.13	24.3 ±0.14
A. flavus MTCC1037	17.2±0.09	24.2 ±0.18	16.3±0.14	16.7±0.13

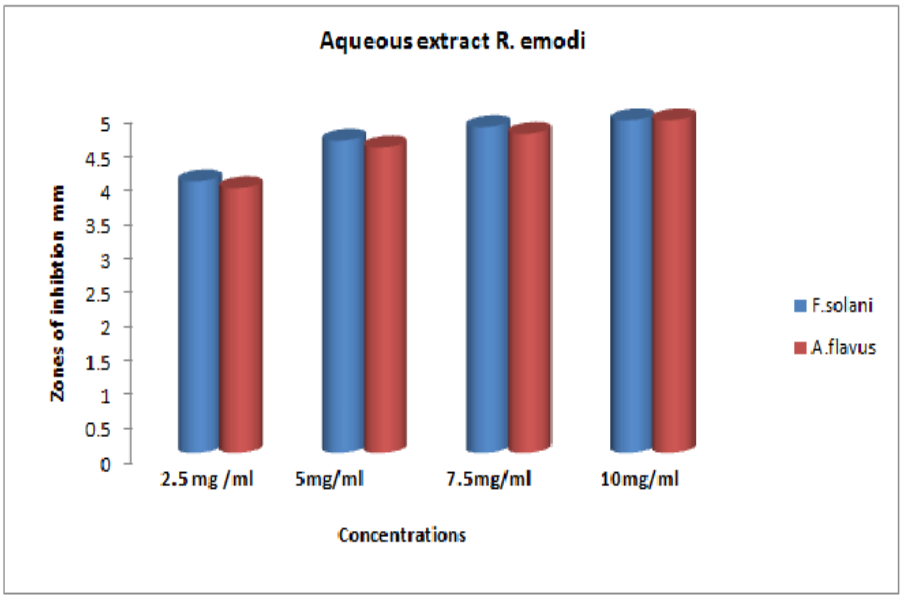

Fig 1: Zones of inhibition (mm) in *Rheum emodi* aqueous extract

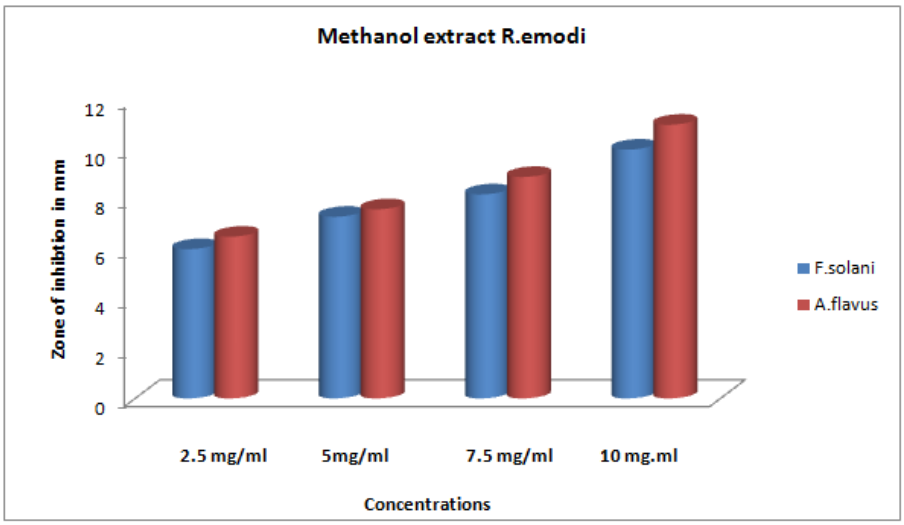

Fig 2: Zones of inhibition (mm) in *Rheum emodi* methanol extract

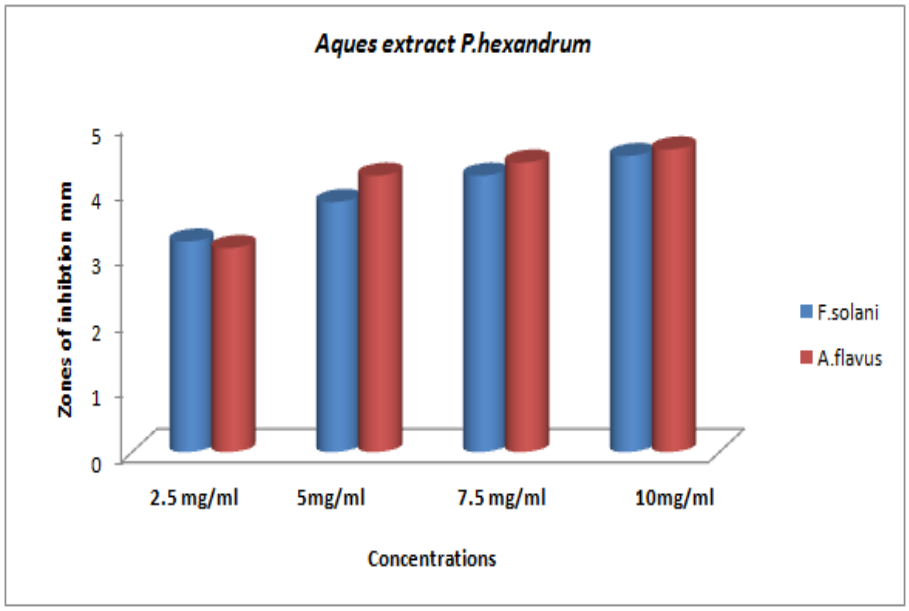

Fig 3: Zones of inhibition (mm) in *P. hexandrum* aqueous extract

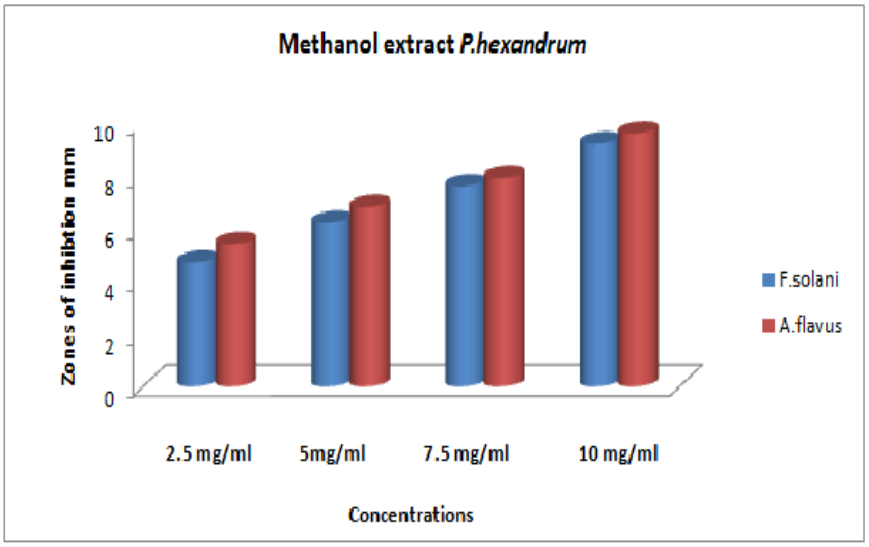

Fig 4: Zones of inhibition (mm) in *P. hexandrum* methanol extract

Minimum inhibitory concentration

Minimum inhibitory concentration is the concentration at which there is no visible turbidity observed. In this broth dilution technique was used where different concentrations of extract were inoculated with 0.2 ml of standard suspension of test organism after 24 h. of incubation at 37^0C. The test tubes were observed for turbidity. The minimum ihibtory concentration of test organism was observed by transferring 5 ml. of nutrient broth into various test tubes containing concentrations of 10 mg/ml to 2.5 mg/ml of extract against *Bacillus megaterium, Pseudomonas aerugionasa, Fusarium solani* and *Aspergillus flavus.* The lowest concentration of test tube that did not show any visible growth was considered as MIC (Table 3).

Table 3: The MIC of aqueous and methanol extract of *R. emodi* and *P. hexandrum*

Sample	*F. solani*	*A. flavus*
R. emodi (aq. extract)	7.5	10
R. emodi (meth. extract)	5	5
P. hexandrum (aq. extract)	10	10
P. hexandrum (meth. extract)	7.5	7.5

The valley of Kashmir has been a hub for medicinal plants. The people there are using these medicinal plants for the cure and prevention of various diseases since ancient times. A total of 937 plant species belonging to 129 families have so far been reported to have a traditional medicinal use by indigenous communities of Jammu and Kashmir. The investigations into antimicrobial activities of local medicinal plants will expose the plants as potential sources of therapeutic agents. The extracts of *Rheum emodi* and its active ingredients have been reported to possess antioxidants, anticancer, antibacterial, antifungal and antiviral activities [15-17]. The extract of *Podophyllum hexandrum* and its constituents have been reported to possess anticancer, antiviral, antihelminthic, antifungal and in the treatment of rheumatoid arthritis [18- 21].

The aqueous extract of *Rheum emodi* inhibited the growth of fungi strains between the ranges of 4-5.2 mm while methanol extract inhibited the growth of fungi strains in the range of 6 mm to 11.3 mm. The antifungal properties of *Rheum emodi* has also been reported by many workers [22-23]. *Podophyllum hexandrum* have received significant attention for its tumour necrotizing properties [11], treatment of warty lesions [24] and radioprotective [25]. They are used as starting compound for the chemical synthesis of etoposide and teniposide [26]. Only few studies have been done for its antimicrobial activity. In the present study four concentrations 2.5 mg/ml, 5 mg/ml, 7.5 mg/ml and 10 mg/ml of aqueous and methanolic extracts were used to assess the antifungal of activity of *Podophyllum hexandrum.* The aqueous extract exhibited the zone of inhibition between 3.5 to 5 mm while methanol extract had zone of inhibition between 4.7 to 9.6 mm. Atta-ur- Rahman *et al*., 1995 [27] reported that *P. hexandrum* showed strong antifungal activity against *Epidermophyton floccosum, Curvularia lunata, Nigrospora oryzae, Microsporum canis, Allescheria boydii* and *Pleurotus ostreatus.* Wani *et al*., 2013[28] also reported that rhizome extracts of *Podophyllum hexandrum* have antifungal activity against pure cultures of clinical isolates of Aspergillus niger ATCC 1197 and Candida albicans ATCC 1023. So our results are in concurrence with previous findings. MICs of the aqueous extracts of *Rheum emodi* and *Podophyllum hexandrum* showed inhibtory values less than methanolic extracts. This may be due to the solubility of the antimicrobial compounds in the respective solvents used. In the present study the extracts of *R. emodi exhibited* better antibacterial and antifungal inhibition than *Podophyllum hexandrum*. This may be due to the presence of a number of phytoconstituents.

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