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Quantitative estimation of phytochemicals and antimicrobial activity of *Podophyllum hexandrum*

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Abstract
The present study was performed to assess the presence of phytochemical constituents in aqueous and methanol extracts of rhizomes of *Podophyllum hexandrum* and to investigate their antibacterial activity. The estimation of phytochemical constituents in methanol proved to be effective solvent for the extraction of secondary metabolites from the rhizomes of *Podophyllum hexandrum*. Methanol extract exhibited significantly higher (p<0.05) amounts of secondary metabolites and antibacterial activity against the test organisms at 10mg/ml, 28.6 ±0.21 and 24.4±0.17 in comparison to standard antibiotics 19.8 ± 0.16 and 19.9 ± 0.21 and aqueous extract 17.9±0.22 and 18.3±0.18 respectively. The methanol extract also demonstrated higher zone of inhibition against bacteria from 8-11 mm while aqueous extract exhibited zone of inhibition from 8-10mm.

Keywords: *Podophyllum hexandrum*, phytochemical estimation, extract, antibacterial activity

Introduction
Plants are the rich source of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, phenols, steroids, glycosides, saponins and anthraquinones [1] which gives them a wide variety of medicinal properties. It is therefore essential to identify these constituents from medicinal plants responsible for the treatment and cure of various ailments. In addition studies into antimicrobial activities of medicinal plants will provide the alternate sources of therapeutic agents [2]. 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. In India *Podophyllum hexandrum* is mostly found in Alpine Himalayas (3000-4000 m) of Jammu and Kashmir, Himachal Pradesh, Sikkim, Uttaranchal and Arunachal Pradesh [3]. *Podophyllum hexandrum* or Himalayan Mayapple is used in the treatment of ulcers, hepatic disorders, wounds, cuts, tuberculosis, constipation, mental disorders and as anti-cancerous agents [4-5]. The major active constituents of *Podophyllum* are podophyllotoxin, quercetin, lignans and kampherol which show anticancerous, antirheumatic, radioprotective, antimicrobial and antihelminthic properties [6-9]. Although *Podophyllum hexandrum* has received significant attention for its tumour necrotizing properties, very few studies have been done on the antimicrobial activity of *Podophyllum hexandrum*. The rhizome extract has been reported to inhibit growth of *Candida albicans* and *Aspergillus niger* [10].

Materials and methods
Plant material
The rhizomes of *Podophyllum hexandrum* were collected in the month of May - June from Gulamarg 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 at 30 ± 2 °C 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.

Phytochemical Screening
Chemical tests were conducted on aqueous and methanolic extracts of *Podophyllum hexandrum* for qualitative analysis of various phytoconstituents using standard protocols as described by (Sofowora 1993, Trease and Evans 1989, Herborne 1973) [11-13].

Quantitative phytochemical estimation:
The alkaloids were estimated spectrophotometrically by the method of Singh and Sahu 2006 [14], flavonoids were estimated by the method of Zhishen *et al*., 2010 [15]. Saponins, tannins, phenolic content and sterols were estimated by the methods of Obadoni and Ochuko 2001, Graham 1992, Singleton and Rosi 1965) [16-18].

Test microorganisms
The test organisms were supplied by Department of Microbiology, Government Medical College Srinagar. The bacteria strains used in the study were *Bacillus megaterium* MTCC 1684 and *Psedomonas aeruginosa* MTCC 3541

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 Mueller-Hinton broth (MHB) and incubated without agitation for 24 h at 37 °C and 25 °C respectively. To 5ml of MHB 0.2 ml of culture was inoculated and incubated till it reached the turbidity equal to that of the standard 0.5 McFarland solution at 600 nm which is equivalent to 10^6– 10^8 CFU/ml.

Antimicrobial assay
Disc diffusion method
Bauer *et al.*, 1966 [19] was followed for disc diffusion assay. *In vitro* antimicrobial activity was screened by using Mueller Hinton Agar (MHA) obtained from Himedia. The MHA plates were prepared by pouring 15 ml of molten media into sterile petriplates. The plates were kept for 5 min to solidify and 0.1 % inoculum suspension was swabbed uniformly and was allowed to dry for 5 min. The different concentrations of extracts (2.5, 5, 7.5 and 10 mg/disc) were loaded on 5 mm sterile individual discs. The loaded discs were placed on the surface of medium and the compound was allowed to diffuse for 5 min and the plates were kept for incubation at 37 °C for 24 h. Negative control was prepared using respective solvent. Erythromycin 25 µg/disc and tetracycline 10 µg/disc were used as positive control. At the end of incubation, inhibition zones formed around the disc were measured with transparent ruler in millimeter. These studies were performed in triplicate.

Minimum Inhibitory Concentration (MIC) Asssay
The MIC method was applied on extracts that proved their high efficacy against microorganisms by the disk diffusion method [19]. The minimum concentration of a plant extract that still retains an inhibitory effect against the growth of a microorganism is known as MIC [20]. Selected plant extracts were subjected to a serial dilution 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 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. The 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 GraphPad Prism 5 software.

Results and discussions
The rhizomes of plants were extracted using methanol and water. In the extractive values were found 37.43% and 29.93%, of aqueous and methanol extract of *Podophyllum hexandrum* (Table 1). The extracts were subjected to qualitative phytochemical investigation for the presence of alkaloids, carbohydrates, glycosides, phenolic compounds, flavonoids, amino acids, terpenoids, tannins, saponins and anthraquinones. The Presence and absence of different phyto constituents are presented in (Table 2).

Table 1: Percentage yield of crude extract of *Podophyllum hexandrum*

Solvent	Wt. of powdered plant material *P. hexandrum*	Vol. of solvent	Wt. of extract of *P. hexandrum*	% yield of extract of *P. hexandrum*
Aqueous	25 gm	150ml	9.25 gm	37.43
Methanol	25 gm	150ml	6.34 gm	29.93

Table 2: Phytochemical Screening of *Podophyllum hexandrum*

	Rheum emodi	
Tests	**Methanolic**	**Aqueous**
Alkaloids	-	-
Carbohydrates	-	-
Tannins	++	+
Steroids	++	+
Flavonoids	++	+
Terpenes	+	-
Glycoside	+	+
Terpenoids	+	+
Anthraquinones	-	-
Saponins	+	-
Amino acids	++	+
Phenol	+	-
Anthocyanin	+	+

+ = trace amount, ++ = moderately present, +++ = highly present, - = absent

The result obtained from the quantitative estimation of alkaloids, flavonoids, saponins, tannins, phenolics and sterols from methanol and aqueous extracts are depicted in table 3. The quantitative estimation of crude extract of *Podophyllum hexandrum* in aqueous and methanol indicates that methanol was potent in the extraction of flavonoids and sterols while aqueous extract was found to be a significant solvent for the extraction of saponins and tannins. The results indicate that *Podophyllum hexandrum* are lacking in alkaloids and phenolics

Table 3. Quantitative estimation of phytochemical constituents in Aqueous and Methanol extract

Extract	Alkaloids mg/g	Flavonoids mg/g	Saponins mg/g	Tannins mg/g	Sterols mg/g	Phenolics mg/g
Aqueous Extract	-	93±0.13	87±0.15	122±0.17	69±0.09	-
Methanol extract	-	127±0.14*	83±0.20*	110±0.19*	98±0.24*	-

Values are mean of three determinations ± standard error (SE)

Antibacterial activity of *Podophyllum hexandrum*

The antibacterial activities of aqueous and methanol extracts of *Podophyllum hexandrum* against the bacterial strains *Bacillus megaterium* MTCC 1684 (Gram-positive) and *Pseudomonas aeruginosa* MTCC 3541 (Gram-negative) whose virulent strains can result in a number of ailments such as anthrax, meningitis, urinary tract infection, gastrointestinal infection, septic shock, pneumonia and many more were tested along with standard antibiotics *erythromycin* and *tetracycline* are shown in table 4. The four concentrations 2.5 mg/ml, 5mg/ml, 7.5 mg/ml and 10 mg/ml of the extract were used to study the zone of inhibition (Fig. 1 and 2). The methanolic extract of *Podophyllum hexandrum* showed significant antimicrobial activity at 10 mg/ml concentration against the tested microorganisms when compared to aqueous extract and standard antibiotics.

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Table 4: Antimicrobial activity of aqueous and methanol extract of *P.hexandrum*

Microorganism	*R. emodi* A. extract	*R. emodi* M. extract	*Erythromycin*	Tetracyclin
B. megaterium MTCC 1684	17.9±0.22	28.6 ±0.21*	22.9±0.14	19.8±0.16
P. aeruginosa MTCC 3541	18.3±0.18	24.4±0.17*	20.7±0.23	19.9±0.21

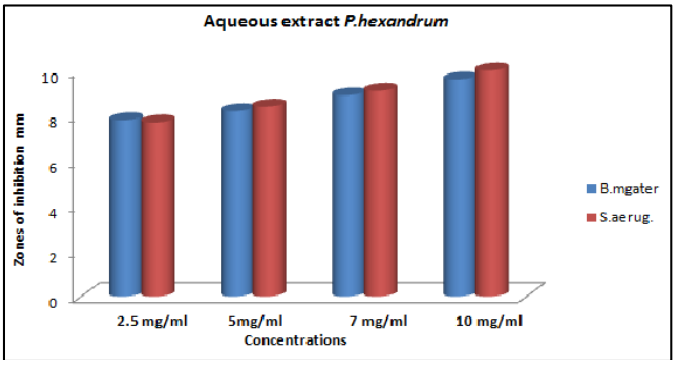

Fig 1: Zones of inhibition (mm) in *P. hexandrum* aqueous extract

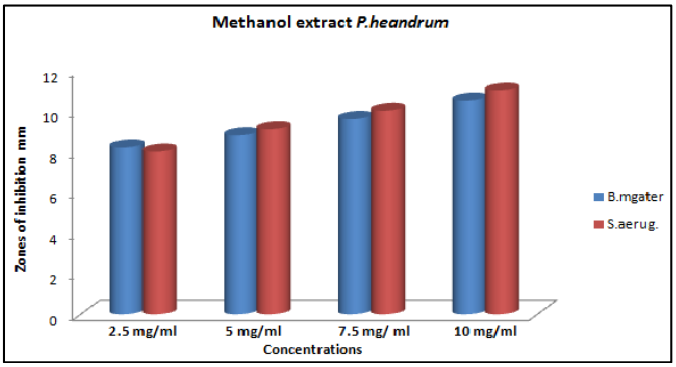

Fig 2: Zones of inhibition (mm) in *P. hexandrum* methanol extract

The preliminary phytochemical screening of *Podophyllum hexandrum* revealed the presence of terpenoids, steroids, flavonoids, saponins, tannis, glycosides, amino acids and the absence of alkaloids, carbohydrates and anthraquinones. Kumar and Dhillon 2015 [21] also have reported the presence of flavonins, terpenes, glycosides and saponins however they have also reported the presence of alkaloids and carbohydrates. In present study no carbohydrates and alkaloids were detected. Wani *et al*., 2012 [22] and Sultan *et al*., 2008 [23] also have reported the absence of carbohydrates, alkaloids and anthraquinones from *Podophyllum hexandrum* rhizome extract so present findings are in concurrence with the findings of many workers [22, 23].

Although Podophyllum hexandrum has received significant attention for its tumour necrotizing properties, only few studies have been done for its antimicrobial activity. The antimicrobial activities of aqueous and methanolic extract of *Podophyllum hexandrum* against the bacterial strains used were assessed by the presence of inhibition zones. Methano extract demonstrated higher zone of inhibition. Phani *et al*., 2010 [24] also reported that extracts of *Podophyllum hexandrum* at concentration between 8-250 µg/ml exhibited significant antibacterial effect against *Bacillus subtilis*, *Salvia sclarea* and *Pseudomonas aeruginosa* with MIC of 8 µg/ml. *Podophyllum hexandrum* leaf has been found to be highly effective against Bacillus subtilis [25]. So our results are in concurrence with previous findings. MICs of the aqueous extracts *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.

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