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Anti-inflammatory Activity and Isolation of Luteolin from *Plagiochasma appendiculatum* Methanol Extract

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Abstract

Plagiochasma appendiculatum, a flavonoid rich liverwort, is reported to be used in the treatment of cuts, burns, outside wounds, bacteriostatic, pulmonary tuberculosis, neurasthenia, fractures, convulsions, scalds, uropathy, and inflammation by tribal communities. In the present study, flavonoid luteolin, reported to possess anti-inflammatory potential, is isolated from *P. appendiculatum* methanol extract and extract is evaluated for anti-inflammatory effect. Anti-inflammatory potential was studied by protein denaturation assay and carrageenan-induced rat paw edema method. Plant flavonoid is isolated from methanol extract by column chromatography, using petroleum ether and ethyl acetate in various proportion, as mobile phase. Further identified by spectroscopic analysis. *In vitro* studies, at a concentration of 800 µg/ml, *P. appendiculatum* methanolic extract (PAME) exhibited 74% inhibition while indomethacin showed 79% inhibition at the same concentration. The extract exhibits a decrease in inflammation in rat hind paw when tested with carrageenan-induced paw edema. PAME at a dose of 150 mg/kg displayed the most potent anti-inflammatory activity compared to the other groups during the course of the observation period. The methanol extract yielded a yellow color crystalline compound which is characterized as 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-chromenone, commonly known as luteolin. Isolation of luteolin from *P. appendiculatum* is first time reported, which supports the ethnobotanical claims and provides scientific explanation for anti-inflammatory use of mentioned liverwort.

Keywords: Anti-inflammatory, Carrageenan, Luteolin, *Plagiochasma appendiculatum*

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Introduction

In both traditional systems of medicine and modern medicine, medicinal plants have a significant role as a source of raw material and represent a substantial proportion of the global drug market. In the developing world, mostly rural population depends on medicinal plants as their leading source of health care.[1] For the medicinal values, higher plants have been investigated so far, while bryophytes are still waiting to be explored for their medicinal value for a long time. Bryophytes are used in traditional medicine to cure cuts, burns, outside wounds, pulmonary tuberculosis, bacteriosis, neurasthenia, fractures, convulsions, scalds, uropathy, inflammation, and pneumonia worldwide.[2] *Plagiochasma appendiculatum* is an important Indian liverwort and belongs to the family Aytoniaceae. It is a thalloid liverwort that is characterized by 30 species but in India, only 10 species are described.[3] *P. appendiculatum* generally grows to an altitude up to 3000–8000 feet from water level and geographically distributed in the Central India, Eastern, Western Himalayas, and South India.[4,5] In folk medicine, it is used for treating skin diseases in the form of paste by the Gaddi tribe in Kangra valley and is locally known as "Patharshali." The paste is prepared by mixing the fresh herb with water and then applied topically 2 times a day for 1 week for the treatment of burns, boils, and blisters on the body.[6] Luteolin, 3′,4′,5,7-tetrahydroxyflavone, is a common flavonoid that exists in many types of plants including fruits, vegetables, and medicinal herbs. Plants rich in luteolin have been used in Chinese traditional medicine for treating various diseases such as hypertension, inflammatory disorders, and cancer.[7] The present study is designed to examine the biological properties of *P. appendiculatum* extracts, which will provide a scientific clarification for the ethnopharmacological use chiefly the ones associated with the inflammatory process.

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Materials and Methods

Collection of Plant Materials

P. appendiculatum grows as carpet on walls where shade and humidity exist. The sample was collected from the roadside wall of D.S.B. Campus Nainital, hill region of Kumaun Himalaya in the month of September. The clump was carefully sampled keeping all the plant parts intact. Clumps collected from different colonies of a single population were considered for replica analysis. After sampling, the sample was transferred in an air-tight zip-lock bag and brought to Botanical Survey of India (BSI), Dehradun, for identification. Dr. S. K. Singh, Director, BSI, identified the sample as *P. appendiculatum* Lehm. and Lindenb (Family Aytoniaceae) and kept the sample preserved with accession number 578 in BSI herbarium. In laboratory, sample was cleaned thoroughly to remove adhered soil and dirt. After cleaning, sample was dried in shade and kept at ambient temperature before further analysis.

Extraction of Phytochemicals

Dried biomass was extracted with different solvents of ascending polarity, like petroleum ether, chloroform, methanol, and water using the cold maceration method. The extracted material was then centrifuged and the supernatant was evaporated to dryness. The dried extracts were then kept for further analysis.

In Vitro Anti-inflammatory Activity

Protein denaturation assay

Methanolic extract was selected for the evaluation of anti-inflammatory activity, as the literature suggests that methanolic extract is the good source of various phytochemicals in *P. appendiculatum*.[8] The anti-inflammatory effect of *P. appendiculatum* was studied by the protocol described by Elias and Rao; Leelaprakashan and Dass,[9,10] with small modifications. Different concentrations (50, 100, 200, 400, and 800 µg/ml) of methanolic extract and standard indomethacin were homogenized with 1 ml of the aqueous solution of bovine serum albumin (1%), pH of the reaction mixture was adjusted with 1 N hydrochloric acid. The reaction mixtures were incubated at 27°C for 15 min. The control tube constituted a mixture of distilled water and bovine serum albumin. Denaturation of the proteins was initiated by introducing the blend in a water bath for 10 min at 70°C. The mixture was then cooled inside the ambient room temperature, and the activity of each mixture was measured at 660 nm. Each test was done 3 times. The percent inhibition of protein denaturation was calculated as follows: Percent inhibition = (Abs Control − Abs Sample) × 100/Abs control
Where, Abs = Absorbance.

In Vivo Anti-inflammatory Activity

Animals

Male Wistar rats weighing 150–200 g were used for the study. Animals were served with a standard pellet regimen and water *ad libitum*. All animals were adjusted for 1 week before the experiment. The Institutional Animals Ethics Committee guidelines were followed for all the experimental procedures. (Institutional Animals Ethics Committee approval number: KUDOPS/43 on dated October 22, 2016).

For the anti-inflammatory activity against the acute inflammation, animals were divided into five groups each containing six rats. Group I: Control (Distilled water), Group II: Test drug (*P. appendiculatum* methanolic extract [PAME] 50 mg/kg), Group III: Test drug (PAME 100 mg/kg), Group IV: Test drug (PAME 150 mg/kg), and Group V: Standard drug (Indomethacin 10 mg/kg).

Carrageenan-induced rat paw edema assay

The animals were pre-treated with oral medications 1 h before administration of carrageenan injection. Carrageenan (0.1 ml of 1%) was injected into subplanter surface of the right hind paw of each rodent. Paw edema was measured by IMCO plethysmograph at various time intervals, that is, at − 60 min (1 h preceding carrageenan injection at the time of drug administration), 0 h (at the time of carrageenan injection), and at 1, 2, and 3 h after carrageenan injection. Percentage inhibition (protection) edema development was carried as a record of acute anti-inflammatory activity, calculated as follows:

Percentage inhibition = {(V2-V1) control — (V2-V1) treated} × 100/(Vt-V0) control

Where V1 – Mean paw volume in ml before injection of carrageenan at 60 min.

V2 – Mean paw volume in ml at different time periods after carrageenan administration.

Acute toxicity

All animals which are used in the inflammatory experiments were observed for 48 h and at the end of the observation period, mortality of animals was recorded in each group.

Statistical Analysis

All results are expressed as mean ± S.E.M. Statistical evaluations were made using analysis of variance followed by *t*-test (GraphPad InStat software) and $P < 0.001$ was considered significant. Data are represented as mean ± S.E.M.

Isolation of Luteolin by Column Chromatography

Preparation of extract

The thallus was dried and powdered coarsely (500 g) was defatted by petroleum ether (2000 ml) by cold maceration method. The obtained marc was dried and extracted with 2000 ml methanol. The extract was dried under reduced pressure using a rotary vacuum evaporator (Popular traders, PT 49), and then partitioned between water and ethyl acetate in a separating funnel. The ethyl acetate layer was collected and concentrated under reduced pressure and the ethyl acetate fraction was used for column chromatography.

Loading of sample

5 g ethyl acetate fraction was dissolved in 50 ml ethyl acetate, and the clear solution was saturated with 10 g silica gel (#60–120). To get free-flowing powder, the solvent was then removed under reduced pressure using a rotary vacuum evaporator. To prepare silica gel slurry, 100 g silica gel (#60–120) was mixed with 500 ml n-Hexane. The slurry was packed in a column and the column was eluted initially with *n*-hexane for 24 h at the flow rate of 10 mL/min to get a close bed of silica gel. The sample was then loaded over the silica gel bed.

Elution from column

The fractionation of the sample was done by eluting fractions using *n*-hexane, *n*-hexane-ethyl acetate, and ethyl acetate-methanol in different concentrations as mobile phases. The flow rate was maintained at 10 mL/min. Fractions were united based on similar thin-layer chromatograms, to acquire five fractions (F1–F5). Thin layer chromatograms were obtained on pre-coated silica gel plates by means of different solvent systems and visualized in ultraviolet chamber (254/366 nm).

Characterization of constituent isolated using spectroscopic studies

The infrared (IR) spectrum of an isolated constituents from *P. appendiculatum* was run on PerkinElmer Spectrometer, USA. The

Table 1: Effect of *Plagiochasma appendiculatum* methanolic extract on carrageenan-induced paw edema in rats

Groups	*Dose Orally (mg/kg)*	*Change in mean paw volume (ml)*				*Percent inhibition at 3 h*
		30 min	*1 h*	*2 h*	*3 h*	
Control	----------	0.7138±0.056	0.7585±0.075	0.7872±0.042	0.871±0.0058	--------
Group I	50	0.695±0.0224	0.715±0.0584	0.704±0.0451	0.607±0.0487	31
Group II	100	0.565±0.0845	0.612±0.285	0.562±0.0847	0.522±0.0457	40
Group III	150	0.532±0.821	0.562±0.1569	0.501±1.085	0.465±0.0602	47
Indomethacin	10	0.402±0.0568	0.455±0.218	0.347±0.0568	0.289±0.0568	67

Each value represents the mean±SD. *n*=6 in each group

Table 2: Results of column chromatography of ethyl acetate fraction of *Plagiochasma appendiculatum*

Fraction	*Eluent Composition*	*Yield (mg)*	*Constituent isolated and yield*
F1	Petroleum ether Petroleum ether: ethyl acetate (90:10) Petroleum ether: ethyl acetate (80:20) Petroleum ether: ethyl acetate (70:30)	985	--
F2	Petroleum ether: ethyl acetate (60:40) Petroleum ether: ethyl acetate (50:50) Petroleum ether: ethyl acetate (40:60)	856	--
F3	Petroleum ether: ethyl acetate (30:70)	873	--
F4	Petroleum ether: ethyl acetate (20:80)	674	--
F5	Petroleum ether: ethyl acetate (10:90) ethyl acetate	159	Yellow powder in crystalline form (45 mg)
F6	ethyl acetate: methanol (99:1) ethyl acetate: methanol (98:2)	919	--
F7	ethyl acetate: methanol (97:3)	834	--
F8	ethyl acetate: methanol (95:5)	765	--

1H-nuclear magnetic resonance (NMR) and 13C-NMR spectra were run on NMR spectrophotometer Bruker spectrometer, Singapore at 400 MHz using deuterated DMSO as NMR solvent to dissolve constituent.

Results and Discussion

Protein Denaturation Assay

PAME showed a significant inhibition in protein denaturation in a dose-dependent manner. The percent inhibition of protein denaturation ranges between 17.8954 ± 2.15 and 74.854 ± 1.547. The highest value 74.854 ± 1.547 was observed at a concentration of 800 µg/ml while at the same concentration, standard drug indomethacin exhibited 79.238 ± 1.523 percent inhibition [Figure 1]. The physiology of inflammation involves protein denaturation as most biological proteins lose their biological function when denatured. In the denaturation process, proteins, on the application of external stress, such as strong acid or base, a concentrated inorganic salt, an organic solvent, or heat, lose their tertiary and secondary structure. The ability of plant extract to inhibit protein denaturation was studied, as a part of the investigation on the mechanism of the anti-inflammation activity.[11,12]

Carrageenan-induced Rat Paw Edema

The treatment with methanolic extract of *P. appendiculatum* produced a diminished inflammation in rat hind paw when challenged with carrageenan. On carrageenan-induced paw edema, the effects of PAME are shown in Table 1. A dose of 150 mg/kg elicited a greater percent inhibition of inflammation after 3 h than other groups. These results showed that test drug at the dose level of 150 mg/kg has the most potent anti-inflammatory activity. Non-steroidal anti-inflammatory drugs act by inhibiting cyclooxygenase (COX) and the production of prostaglandins. Indomethacin offers relief from inflammation by suppressing the production of prostaglandins and bradykinin.[13-15]

Acute Toxicity

The PAME was found devoid of mortality of all animals.

Isolation of Luteolin from *P. appendiculatum*

Thin-layer chromatographic study of the isolated compound showed that it was not homogeneous and contaminated with other minor constituents. Therefore, it was purified by preparative thin-layer chromatography, using silica gel and the same solvent system of column chromatography (Petroleum ether and ethyl acetate) [Table 2]. The compound was obtained crystalline yellow powder, melting point 254°C. The molecular formula of luteolin is C15H10O6 (molar mass 286.23g mol^{-1}) [Luteolin is a flavone, a type of flavonoid, with a yellow crystalline appearance. Luteolin is the principal yellow dye compound].

The IR spectrum revealed the presence of absorption bands at 3420 cm^{-1} (OH-stretching), 3065 cm^{-1} (=C-H-stretching) 1660 cm^{-1} (C=O group), 1279 cm^{-1} (C-O stretching). The 1H-NMR spectrum revealed the presence of phenolic compound and signals downfield region showing that the compound may be flavones of flavonoid. The presence of two olefinic protons as doublets at 6.33 and 6.10 ppm is characteristic peaks for the flavone series. The 13C-NMR spectrum indicated the presence of nine quaternary carbons and signal at 166 (C=O group).

Flavonoids comprise a wide variety of biologically active compounds, luteolin comes under flavone type flavonoids and is reported to possess anti-inflammatory activity, with the mechanism of having COX-2; interleukin; tumor necrosis factor as molecular target.[16-18] Thus, it can be concluded that the anti-inflammatory activity of *P. appendiculatum* is due to luteolin.

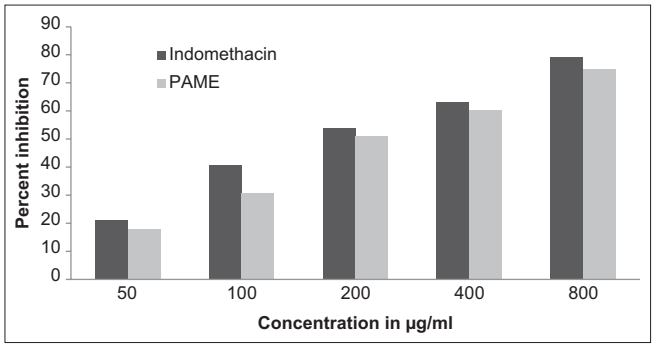

Figure 1: Percent inhibition of protein denaturation by indomethacin and *Plagiochasma appendiculatum* methanolic extract
Each value represents the mean ± SD. *n*=3, PAME: *Plagiochasma appendiculatum* methanol extract

Conclusion

The *in vitro* and *in vivo* data obtained from this study demonstrated for the 1st time that *P. appendiculatum* has potent anti-inflammatory activity. The result obtained revealed that the plant is endowed with an anti-inflammatory activity which supports its use in traditional medicines. Isolation of luteolin from the plant also supports the anti-inflammatory activity of the plant. These findings provide pharmacological evidence for the folk use of *P. appendiculatum* also. Further molecular and cellular experiments need to be carried out to explore the mechanism, including its active components.

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