

PHYTOCHEMICAL ANALYSIS AND *IN VITRO* INVESTIGATION OF ANTI-INFLAMMATORY AND XANTHINE OXIDASE INHIBITION POTENTIAL OF ROOT EXTRACTS OF *BRYOPHYLLUM PINNATUM*

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ABSTRACT

The aim of present study is to emphasize the phytochemical and biological studies of root extracts of *Bryophyllum pinnatum*, as little work was documented on the roots. Phytochemical analysis of powder and extracts were evaluated by the standard methods. UV/ Visible scanning, FTIR and atomic absorption spectroscopy were also performed for the purposes of standardization. *In vitro* anti-inflammatory investigation was done by protein denaturation method and xanthine oxidase inhibition assay was performed to confirm anti-gout effect. Phytochemical analysis results revealed the presence of primary metabolites (%w/w); carbohydrates (48.54±0.6) > proteins (21.06±0.6) > lipids (1.32±0.001). Secondary metabolites (mg/g) were also isolated; methanol extract has higher concentrations of flavonoids (95.81±0.026) and glycosaponins (39.9±0.103) while in water extract polysaccharide (139.4 ± 0.026) and polyphenols (114.93±0.0234) were found to be in maximum quantities as compared to the other extracts. UV/Visible and FTIR scanning results have shown the presence of various significant functional groups. *In vitro* anti-inflammatory evaluation showed that aqueous extract (87.46%) was found to be the most active with IC₅₀ (570.24ug/ml) as compared to the reference drug diclofenac sodium. *In vitro* xanthine oxidase inhibition activity was performed and methanol extract (88.24%) found to be the most effective. The findings confirm the plant nutritional value and rationale behind its use in traditional medicine for the treatment of inflammation and gout.

Key words: *Bryophyllum pinnatum*, anti-inflammatory, gout, xanthine oxidase, phytochemical analysis.

INTRODUCTION

Globally, medicinal plants are extensively used for the cure of various disorders. Drugs obtained from medicinal herbs are low-priced, conveniently accessible and have lesser side effects as compared to the synthetic drugs (Mekuria *et al.*, 2017). *Bryophyllum pinnatum* (family: Crassulaceae) is also known as *Bryophyllum calycinum* or *Kalanchoe pinnatum* (Sadhana *et al.*, 2017). The word "*Bryophyllum*" is derived from the Greek word which means "sprout leaf" (leaf cuttings can grow) and "*pinnatum*" is coined from the Latin word which means "quill" (Nagaratna and Hegde, 2015). It is a perennial herb about 3 to 5 meters high and having divergent glabrous leaves (Afzal *et al.*, 2012). It has a sour taste, sugary post digestive effect and hot strength. Herb contains a wide range of valuable chemicals that are responsible for various beneficial therapeutic effects (Jaiswal and Sawhney, 2006; Latif *et al.*, 2019). *Bryophyllum pinnatum* grows naturally and is native to Madagascar. It is found in temperate regions, including: Asia, Galapagos, Polynesia, New Zealand, Melanesia, West Indies, the Pacific and Caribbean,

Hawaii and Australia (Zamora *et al.*, 1998; Naz *et al.*, 2009).

Several significant therapeutic active constituents and secondary metabolites of plant have been isolated and documented. Among these the most remarkable are flavonoids and bufadienolides (Fürer *et al.*, 2016). The major bufadienolides isolated from the plant leaves are Bryophyllin B and A (Potterat *et al.*, 2013). The herb is an outstanding reserve of vitamins such as niacin, thiamine and ascorbic acid. Minerals are also found in plant which includes: calcium, magnesium, sodium, phosphate, zinc and potassium (Milad *et al.*, 2014).

Worldwide, *Bryophyllum pinnatum* is used for the treatment of conjunctivitis, edema, cholera, cuts, eczema, piles, chest colds, epilepsy, constipation, asthma, chicken pox, menstrual disorders, and fever (Majaz *et al.*, 2011a). It is frequently used for the cure and management of blisters, burns, cough suppression, rheumatoid arthritis, psychiatric disorders, insect bites and abdominal discomforts. Moreover, it acts as a tocolytic agent to prevent premature labor (Gupta *et al.*, 2016; Sadhana *et al.*, 2017) and also employed for the treatment of leg

edema, gout, fever, otitis, abscesses and palpitations (Afzal *et al.*, 2012).

This study is aimed to investigate the phytochemical composition of the plant root extracts and *in vitro* anti-inflammatory and xanthine oxidase inhibition effects. The roots were selected because there is little scientific documentation on them and there is also need to explore the therapeutic value of the plant (Majaz *et al.*, 2011).

MATERIALS AND METHODS

Plant material and extraction: *Bryophyllum pinnatum* whole plants were collected in July, 2017 (Figure 1). The plants were obtained from the botanical garden of University of the Punjab, Lahore, Pakistan and identified by taxonomist Prof. Dr. Zaheer-ud-Din Khan, Department of Botany, Government College University (GCU) Lahore, Pakistan. A specimen was deposited in a GCU herbarium (after authentication, voucher no. 3369 was issued). The roots were separated from the plant (Figure 2), cleaned and then dried at the room temperature under the shade for 20-25 days. The dried roots were then crushed into fine powder and stored in airtight plastic bag. Sequential extraction was carried out by using Soxhlet. During the extraction procedure, solvents of different polarity (n-hexane, chloroform, methanol and water) were used. Keeping the temperature less than the boiling point of the respective solvent, all extracts were dried by using a rotary evaporator except aqueous extract which was dried by freeze drying. All extracts were collected in clean, tarred and labeled bottles, and allowed to dry in oven at 40 °C.

Chemicals: Aluminum nitrate crystals, Bovine serum albumin, Chloroform, Copper sulphate, Glucose, Methanol sodium bicarbonate, Sodium hydroxide, Potassium tartrate (Merck, Germany), Gallic acid (Sinochem, China), n-Hexane (BDH, England), Quercitrin (Sigma life sciences, Germany), Anthrone reagent (Sigma life sciences, Germany), Folin and Ciocalteu's phenol reagent (Unichem Chemicals, Ireland), Triton X (Unichem chemicals, Ireland), Allopurinol TCI (Tokyo Chemical Industry), DMSO (Reagent Densen), Xanthine oxidase (Sigma).

Proximate analysis: The total ash, acid soluble ash, water insoluble ash, sulphated ash, moisture content and alcohol and water soluble extractive values were determined according to standard procedures (USP 2015).

Phytochemical analysis: *Bryophyllum pinnatum* root powder and extracts were evaluated for the levels of metabolites (primary and secondary metabolites). These were estimated by the standard methods described as: total proteins (Lowry *et al.*, 1951), total lipids (Besbes *et*

al., 2004), carbohydrates (Al-Hooti *et al.*, 1998), total polyphenols (Slinkard and Singleton, 1977), total flavonoids (Chang *et al.*, 2002), total polysaccharides and total glycosaponins (Hussain *et al.*, 2008).

Mineral content analysis: Atomic absorption spectroscopy and flame photometer were used to determine the mineral content of the plant. 20% w/v nitric acid was employed to clean volumetric flasks and then rinsed with deionized water. 1 g of root powder was taken into the beaker. The sample was treated with 15 ml of digestion mixture which comprised of concentrated nitric acid and hydrochloric acid in 1:3 (v/v). The mixture was heated in fuming hood till no more smoke appeared. At room temperature, contents were cooled and filtered. Deionized water was used to make a final volume up to 100 ml and stored in a stoppered bottle for analysis. Analysis of the digested sample was carried out by using the atomic absorption spectrophotometer and flame photometer. Working solution of standard (concentrations; 0, 1, 5, 10, 15, 20, 25 and 100 ppm) was prepared from standard stock solution (1000 ppm). The tests were performed in triplicates and concentrations of minerals in the sample were determined by the standard plot (Ahmad *et al.*, 2014).

UV/Vis spectroscopy: Extracts stock solutions (1 mg/ml) were prepared in methanol for the UV/Vis scanning. Methanol was added in stock solution to make the volume up to 10 ml and the same solvent was used as blank. In UV/Vis region (200-800 nm) scans were taken and for each extract maximum wave length (λ_{max}) was recorded.

FTIR spectroscopy: Root powder and extracts were subjected to FTIR analysis for the purposes of standardization. Material (1mg) was mixed with 100 mg of potassium bromide (KBr). Then the mixture was transferred to die which was compressed in a hydraulic press to prepare the pellets. FTIR spectra were obtained in the range 4000-400cm⁻¹ (Pavia *et al.*, 2008).

***In vitro* anti-inflammatory activity:** *In vitro* anti-inflammatory potential of extracts was determined by the protein denaturation method. The reaction mixture (5 ml) comprised of 2.8 ml of phosphate buffer saline (pH 6.4), egg albumin (from fresh hen's egg) 0.2 ml and 2 ml of the extracts (1%). Control composed of a similar volume of water (double distilled). Mixtures were incubated for 15 minutes at the temperature 37 ± 2 °C and then heated for five minutes at temperature 70 °C. After cooling the mixtures, absorbance was measured at 660 nm wave length by using the vehicle as a blank. Diclofenac sodium and aspirin were used as the standard drugs and treated likewise. Percentage inhibition of protein denaturation was calculated by the following formula:

$$\% \text{ Inhibition} = (A_{\text{control}} - A_{\text{test}} / A_{\text{control}}) \times 100$$

Where, A is the absorbance.

50% inhibition (IC₅₀) of the most active extract and reference drug was determined through the plotting dose response curve by regression line equation where $y=50$ (Alhakmani *et al.*, 2013).

In vitro xanthine oxidase inhibition activity: *In vitro* xanthine oxidase inhibition assay was used to evaluate anti-gout potential of the plant. To prepare plant extract solution, extract (2 mg) was dissolved in 1 ml 5% DMSO in water solution. In this mixture, 2.9 ml phosphate buffer (1.15 M, pH=7.5) and 0.1 ml of xanthine oxidase enzyme solution (purchased from Sigma) were added. After 15 minutes of mixture pre incubation at 25 °C temperature, the reaction was started by adding a substrate solution (2 ml). Then after 30 minutes, the reaction was stopped by adding 1 ml of IN HCl. Absorbance was measured at 290nm. Likewise, blank was prepared but enzyme was being added to assay mixture after addition of the HCl. Xanthine oxidase activity was calculated by following formula and expressed as the percentage inhibition of xanthine oxidase.

% Inhibition of xanthine oxidase = $1 - (A_{\text{extract}} / A_{\text{no extract}}) \times 100$

Where,

A is the absorbance (Guerrero *et al.*, 2011).

RESULTS AND DISCUSSION

Proximate analysis: Proximate analysis was done for standardization of the root powder. The findings of the analysis are summarized in Table 1.

Table 1. Proximate analysis of *Bryophyllum pinnatum* root powder.

Physicochemical property	Contents (% w/w)
Moisture Content	6.25
Total Ash	13
Acid soluble ash	4
Water insoluble ash	9.5
Sulphated ash	16.75
Water soluble extractives	12.75
Alcohol soluble extractives	10.5

Table 3. Estimation of secondary metabolites.

Extracts	Total Polyphenols (mg/g)	Total Flavonoids (mg/g)	Total Glycosaponins (mg/g)	Total Polysaccharides (mg/g)
n-Hexane	1.96 ± 0.014	1.36 ± 0.016	4.9 ± 0.018	15.4 ± 0.023
Chloroform	10.93 ± 0.017	6.72 ± 0.001	18.9 ± 0.192	34.1 ± 0.039
Methanol	103.84 ± 0.022	95.81 ± 0.026	39.9 ± 0.103	100.7 ± 0.008
Water	114.93 ± 0.034	76.11 ± 0.03	24.8 ± 0.035	139.4 ± 0.026

Analyses of extracts' secondary metabolites have proven that the roots of *Bryophyllum pinnatum* contained the polyphenols in larger amount in aqueous

The moisture content was determined according to the USP 2015 prescribed method for botanical origin. The testing of moisture content is an important physicochemical parameter. Moisture content should be less so that the powder may be stable for a longer period of the time. The moisture content of the plant under investigation was found to be within specified range which is not greater than 14% w/w (Munir *et al.*, 2014). Ash value testing is of great significance to identify the presence of any foreign matter like silica, oxalate etc. To evaluate the quality, purity and polarity of the root powder different ash values and extractive values were estimated. The total ash value of the plant under test was found to be 13% which indicates it is within official range (less than 20%) (Kadam *et al.*, 2012). High alcohol and water soluble values have suggested the presence of polar substances like tannins, phenols, glycosides etc. (Baravalia *et al.*, 2011).

Phytochemical analysis: Phytochemical analysis comprised of primary and secondary metabolites estimation. The primary metabolites evaluation included: total proteins, total lipids and total carbohydrates and their content are shown in Table 2. Assessment of the secondary metabolites was included: total polyphenols, total polysaccharides, total flavonoids and total glycosaponins and the outcomes of which is summarized in Table 3.

Evaluation of the primary metabolites has revealed that the plant root powder contained varied amount of carbohydrates, lipids and proteins. Current findings have suggested that the carbohydrates were present in larger amount than lipids and proteins. Occurrence of the primary metabolites has indicated substantial nutritional value of the plant (Odhav *et al.*, 2007).

Table 2. Estimation of primary metabolites.

Primary metabolite	% Content (%w/w)
Total protein	21.06 ± 0.6
Total lipids	1.32 ± 0.001
Total carbohydrates	48.54 ± 0.6

extract while the n-hexane has the lowest value. Appreciable amounts of polyphenols were present in methanol extract as well. Total flavonoids were present in

highest amounts in the methanol extract while n-hexane has the lowest quantity. Methanol extract has high quantity of glycosaponins while n-hexane has shown the minimum value. In aqueous extract, polysaccharides were found to be in abundance when compared to the others. Before the recognition of their significance in science, secondary metabolites were thought to be the surplus product of the plants. Later, their worth was recognized and it was determined that these metabolites play a crucial role (Verpoorte, 1998). Variety of secondary metabolites chemicals library was produced by the medicinal plants that displayed the inclusive array of the therapeutic effects and biological activities (Wink, 2015). Polyphenols constitute one of the diverse and abundant group of the secondary metabolites which displayed noticeable anti-inflammatory i.e. provide shielding against the arthritis (Bravo, 1998), anti-tumors, gout reduction (Oliviero *et al.*, 2018) and cardiovascular protective effects (Manach *et al.*, 2005). Phenolic contents were found in considerable amount and could serve as a natural source of potential anti-oxidant (Dudonné *et al.*, 2009). It has been recognized that the flavonoids are quite proficient against inflammation and also have anti-cancer properties (Middleton *et al.*, 2000). The presence of the glycosaponins in the plant has indicated that medicinal herb possesses hemolytic activity (Okwu and Okwu, 2004). Scientific studies have shown that saponins exhibit anti-microbial, anti-tussive, immunomodulation and anti-diabetic properties (Upadhyay *et al.*, 2018).

Mineral content analysis: The mineral content analysis has revealed the presence of various important minerals in the plant (Table 4).

Table 4. Mineral content analysis of *Bryophyllum pinnatum* roots

Element	Quantity (%)
Fe	2.4
Cu	0.01
Cr	0.004
Zn	0.0074
Pb	0.0026
Mg	0.81
Mn	0.036
P	0.025
Na	0.174
K	0.41
Ca	7.18

Results have indicated that the plant is a rich source of the valuable minerals. Sample of root powder contains the maximum amount of sodium, potassium, phosphorous, zinc, copper, calcium and iron. Deficiency of the minerals in the human body may lead to various

pathological conditions (Gharibzahedi and Jafari, 2017). Calcium is a ubiquitous macro element that is found in plants. Calcium plays a crucial role in blood coagulation and wound healing (Okwu and Josiah, 2006). Phosphorous is also a macronutrient. It is associated with the nucleic acid synthesis and hence essential for the plant growth (Schachtman *et al.*, 1998). Potassium helps in reducing high blood pressure (Whelton *et al.*, 1997). Magnesium is an important component of plant chlorophyll (Szulc and Waligóra, 2010) and iron is vital for the production of hemoglobin (Laurell, 1952). Presence of zinc indicates that plant may be helpful in the treatment of diabetes (Okaka and Okaka, 2001).

UV Visible scanning: The findings (Figure 3) indicate that lambda maximum for all extracts were present between the range 200-300 nm. n-hexane, chloroform, methanol and water have a maximum peak at the wavelength of 210 nm, 262 nm, 257 nm and 231 nm respectively. These scans suggest the presence of secondary metabolites i.e. polyphenols, flavonoids etc. (Gierschner *et al.*, 2012).

FTIR scanning: FTIR spectra of *Bryophyllum pinnatum* root powder (Figure 4) show a strong and broad peak at the 3275.1 cm^{-1} (OH stretching and H-bonded) which indicates the presence of alcohol and phenols. Peaks between 3000-2850 cm^{-1} indicate the presence of alkanes (medium, C-H stretch). At the 1735.0 cm^{-1} there is C=O stretch which showed the presence of esters and saturated aliphatic functional groups. Peak at the 1606.2 cm^{-1} showed the presence of an aromatic group (C-C stretch). Peaks between the range of the 1320-1000 cm^{-1} (strong, C-O stretch) indicate the presence of alcohol, carboxylic acids, esters and ethers. Extracts overlay (Figure 5) has exhibited the presence of alkanes, hydroxyl and aromatic groups. The presence of various functional groups has suggested that the plant is enriched with various bioactive chemicals i.e. flavonoids, polyphenols etc.

In vitro anti-inflammatory activity: The *in vitro* anti-inflammatory activity and IC_{50} of the most active extract were investigated against the protein denaturation of the egg albumin and outcomes are summarized in Table 5 and Table 6 respectively.

Table 5. In vitro anti inflammatory activity assessment of root extracts of *Bryophyllum pinnatum*.

Standard/extracts	% Inhibition
Control	-
Standard Diclofenac	94.85 ± 0.06
Standard Aspirin	85.38 ± 0.38
n-hexane extract	23.71 ± 0.12
Chloroform extract	20.27 ± 0.06
Methanol extract	40.06 ± 0.1
Water extract	87.67 ± 0.69

Table 6. IC₅₀ of diclofenac and aqueous extract (most active) against protein denaturation.

Concentration (ug/ml)	Diclofenac (% inhibition)	Water extract (% inhibition)
Control	-	-
62.5	11.23	6.0
125	35.08	12.46
250	44.92	24.62
500	52.15	64.92
1000	68.46	70.77

The anti-denaturation egg albumin method was adopted to evaluate the anti-inflammatory activity of *Bryophyllum pinnatum*. Results (Figure 6) show that the aqueous extract has the highest value of percentage inhibition (87.46%), while chloroform extract has the lowest value of percentage inhibition (20.27%). The anti-inflammatory effect of aqueous extract is less than the standard drug diclofenac (94.85%) but is greater than the aspirin (84.96%). Percentage of inhibition of protein denaturation is actually the measure of protein stabilization against the control. Reference drug diclofenac and aqueous extract exhibited marked reduction in protein denaturation. Comparative evaluation of IC₅₀ (Figure 7) reveals that the water extract (IC₅₀= 570.24 ug/ml) effect was greater than the reference drug, diclofenac (IC₅₀= 625.24 ug/ml) (Chandra *et al.*, 2012). These findings have shown a concentration dependent inhibition of protein denaturation by the aqueous root extract and standard drug (diclofenac). The current investigation has confirmed anti-inflammatory activity of the plant and can therefore be helpful in the treatment of inflammatory conditions. The anti-

**Figure 1. *Bryophyllum pinnatum* plant**

inflammatory activity of the plant may be related with the presence of high phenolic contents (Alhakmani *et al.*, 2013).

***In vitro* xanthine oxidase inhibition activity:** Outcomes of the assay is summarized in Table 7 and demonstrated in Figure 8.

Table 7. % inhibition of xanthine oxidase of root extracts of *Bryophyllum pinnatum*

Standard/extracts	% inhibition of xanthine oxidase
Control	-
n-Hexane	7.34 ± 0.002
Chloroform	31.54 ± 0.005
Methanol	88.30 ± 0.004
Water	44.22 ± 0.04
Standard	97.1 ± 0.002

The *in vitro* xanthine oxidase inhibition model was used. Results have shown that the methanol extract has significant ability to inhibit the xanthine oxidase activity but the value of percentage inhibition is less than that of the allopurinol. Drugs that impede the activity of xanthine oxidase are helpful for the treatment of gout and liver ailments (Ashiq *et al.*, 2018). Xanthine oxidase belongs to the class of flavoprotein that transforms xanthine into uric acid and yields free radicals (Sahgal *et al.*, 2009). These findings have shown that the methanol extract has blocking effect on the xanthine oxidase and hence beneficial for the treatment and management of gout. Phytochemicals i.e. polyphenols, flavonoids and saponins are useful to lessen the levels of uric acid in serum (An *et al.*, 2010).

**Figure 2. *Bryophyllum pinnatum* plant roots**

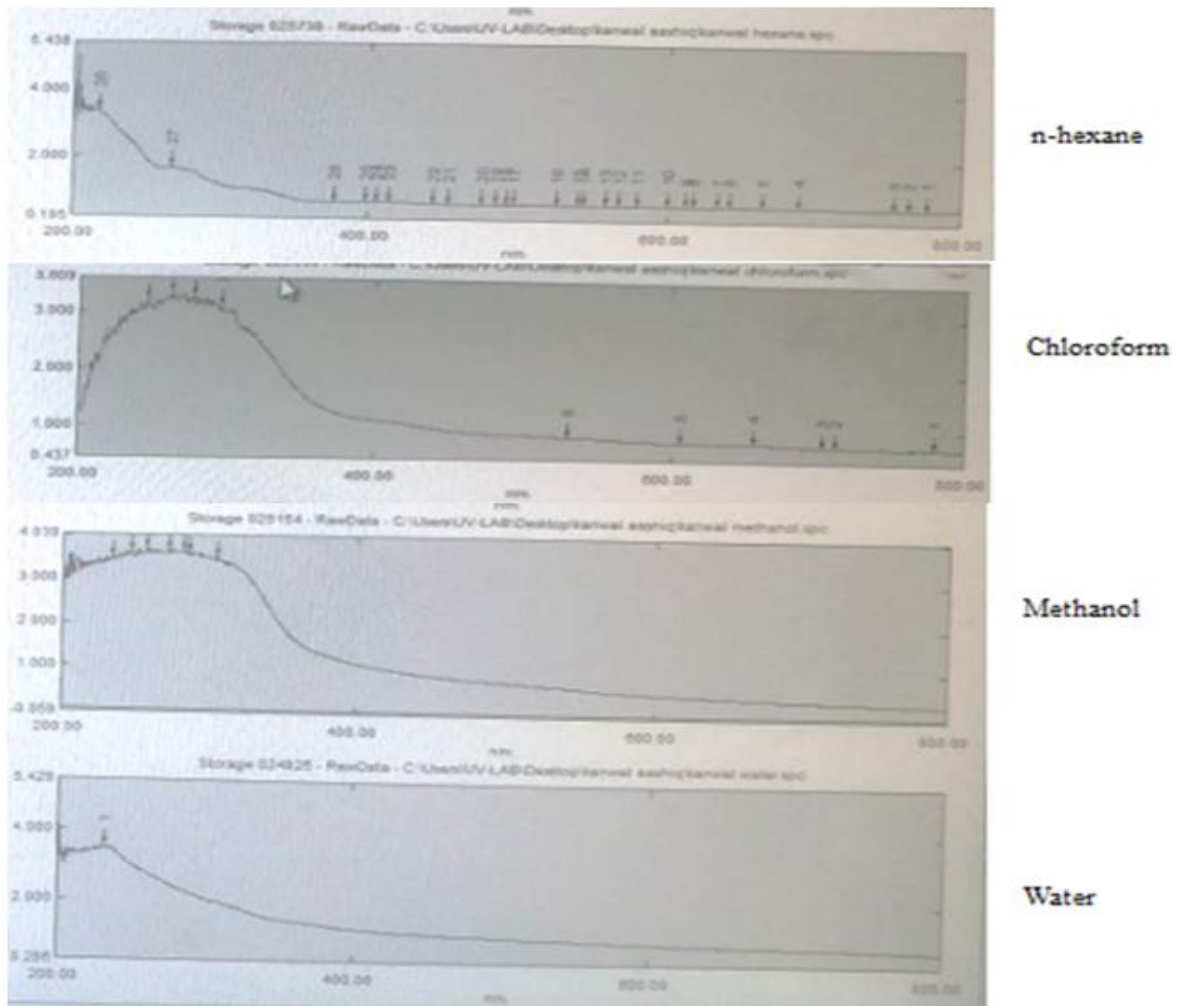


Figure 3. UV Visible spectra of n-hexane, chloroform, methanol and water

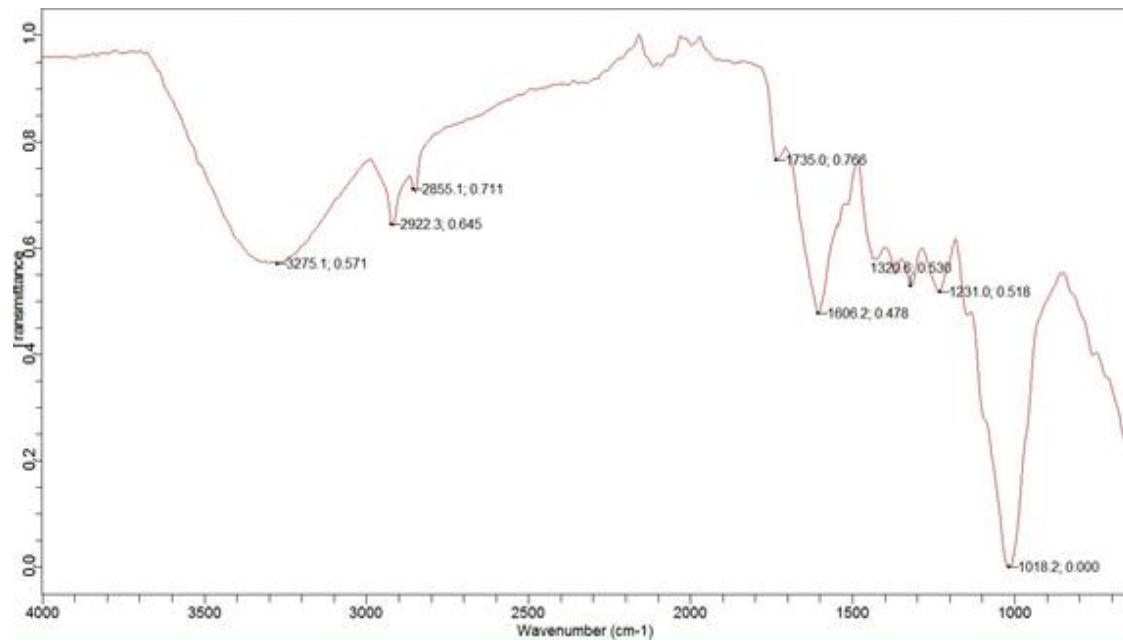


Figure 4. FTIR scan of root powder of *Bryophyllum pinnatum*

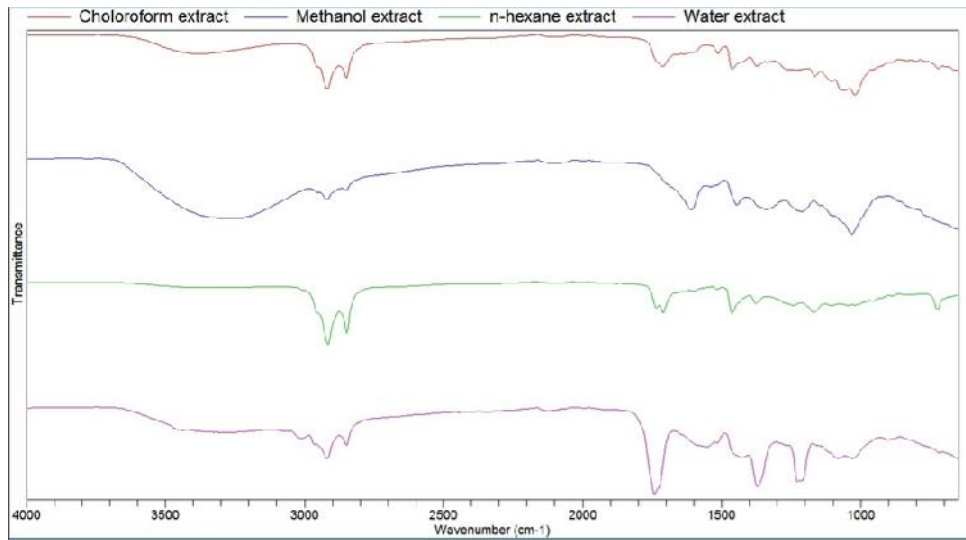


Figure 5. FTIR scan overlay of root extracts of *Bryophyllum pinnatum*

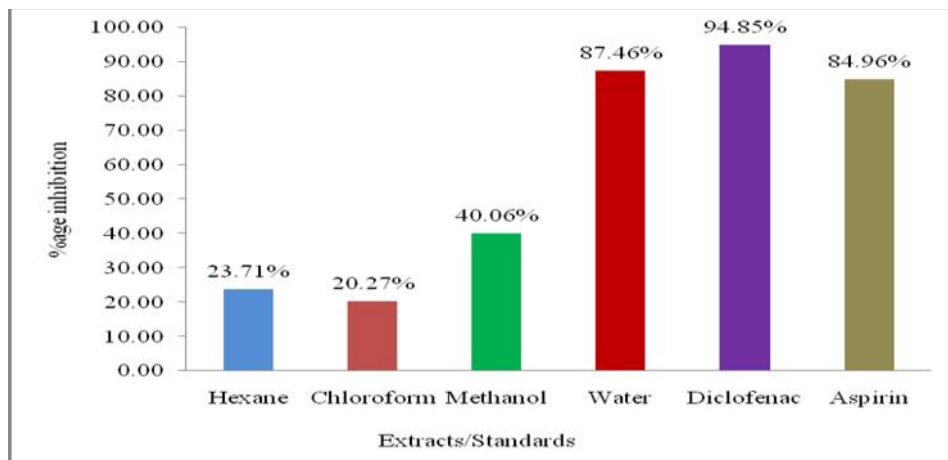


Figure 6. *In vitro* anti-inflammatory activity evaluation of root extracts of *Bryophyllum pinnatum*

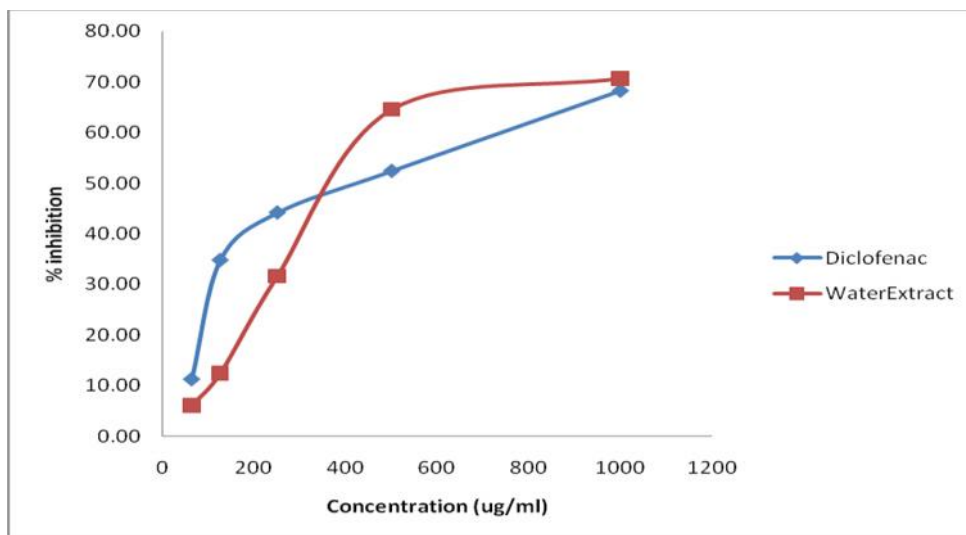


Figure 7. Comparison between IC₅₀ of diclofenac and water extract

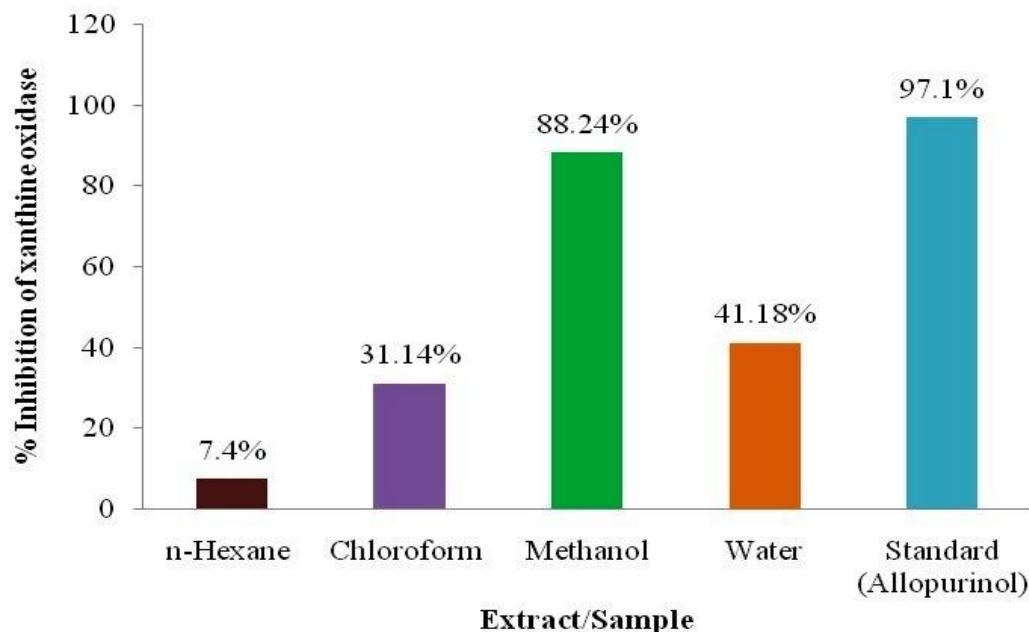


Figure 8. % inhibition of xanthine oxidase by root extracts of *Bryophyllum pinnatum*

Conclusion: The findings of current study show that the *Bryophyllum pinnatum* has potential effects against inflammation and gout. Moreover, the plant contains a significant amount of the important phytochemicals which are responsible for various pharmacological activities. The plant is enriched with various minerals that play vital roles in different physiological functions. There are however, needs for further studies to ascertain its safety and justify its efficacy.

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