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Hypouricaemic and Anti-Inflammatory Effect of Ethanol Extract from *Balanophora fungosa* subsp. *indica* (Arn.) B. Hansen

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ABSTRACT

The parasitic plant *Balanophora fungosa* subsp. *indica* (Arn.) B. Hansen (family Balanophoraceae) had been used in Vietnamese folk medicine for a long time. This study aimed to investigate the hypouricaemic and anti-inflammatory effects of ethanol extract of *B. fungosa* subsp. *indica* (Arn.) B. Hansen (EBF) by potassium oxonate model in mice, carrageenan and monosodium urate models in rats, respectively. The total polyphenol content of EBF was determined using Folin-Ciocalteu method. High performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) analyses of the extract were also carried out.. Results showed that EBF possessed 250.23 ± 0.19 mg GAE/g of total polyphenol content. Gallic acid, caffeic acid and cinnamic and some lignans were identified in the extract by HPLC and HPTLC analyses. The extract decreased 28.32% of serum uric acid level in potassium oxonate-induced hyperuricaemic rats at the dose of 900 mg/kg. The extract at the dose of 500 mg/kg and 350 mg/kg reduced acute inflammation in the carrageenan-induced paw edema and significantly reduced the symptom of inflammation in the urate-induced synovitis. These results suggested that the ethanol extract of *B. fungosa* subsp. *indica* (Arn.) B. Hansen whole plant could be a promising remedy for the treatment of hyperuricemia and inflammation.

Keywords: *Balanophora fungosa* subsp. *indica*, Carrageenan, Monosodium urate, Potassium oxonate.

Introduction

The plant *Balanophora fungosa* subsp. *indica* (Arn.) B.Hansen (syn. *B. indica* (Arn.) Griff., *B. simaoensis* S.Y.Chang & P.C.Tam), a member of family Balanophoraceae Rich., is a widely distributed species in Indian and Indo-Chinese subcontinents, Malaya, Sumatra, Pacific island, Australia.[1] The whole plant of this species was used in Vietnamese folk medicine to treat abdominal pain, body pain and to strengthen bones and muscles.[2] Literature showed that the main constituents of this species are hydrozyable tannins, phenylpropanoids, lignans, triterpenes and sterols.[3] Although some biological potentials of *B. fungosa* subsp. *indica* including anti-inflammatory effect, cytotoxicity, antioxidant capacity were studied *in vitro* previously,[4-6] there were not so much *in vivo* study on biological effects of this parasitic plant. Our preliminary screening also confirmed that the ethanol extract of *B. fungosa* subsp. *indica* possesses significant *in vitro* xanthine oxidase inhibitory activity and inhibitory activity of nitric oxide production.
This study evaluated the hypouricaemic and anti-inflammatory activities of the ethanol extract of *Balanophora fungosa* subsp. *indica* (Arn.) B.Hansen *in vivo*.

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Materials and Methods

Collection and authentication of plant material
The flowering plants of *B. fungosa* subsp. *indica* was collected at Sapa City, Laocai Province, Vietnam in January 2017. The plant was identified and authenticated by Prof. Phan Ke Loc and Msc. Nguyen Anh Duc of Faculty of Biology, VNU University of Science, Vietnam National University. A voucher specimen (HNU 024069) was deposited in the Herbarium of Faculty of Biology.

Extraction of plant material
The collected whole plant of *B. fungosa* subsp. *indica* (Arn.) B.Hansen was carefully washed and cut into pieces. The material was air-dried and then ground into powder. The powdered material (300 g) was refluxed with ethanol 70% (1200 mL x 2 hours x 3 times). The total solution was filtered and concentrated *in vacuo* to obtain about 100 g of the brownish ethanol extract (EBF). The extract was stored in coloured bottle and kept at 4-8ºC.

Experimental animals
Male Swiss mice (20 ± 2 g) and male Wistar rats (140-180 g) used in the study were obtained from the National Institute of Hygiene and Epidemiology and Vietnam Military Medical University, respectively. The experimental animals were housed in plastic cages at controlled temperature (25ºC) and light/dark cycle and fed with standard rodent pellets. They were acclimatized to laboratory conditions for at least a week before the start of the experiment. Animals were used and processed according to the international regulations concerning laboratory animal welfare. The experimental protocol was approved by the Ethics Committee of Hanoi University of Pharmacy, Hanoi, Vietnam (1205/QĐ-DHN).

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Phytochemical screening
The phytochemical screening of the ethanol extract of *B. fungosa* subsp. *indica* (EBF) was carried out. The presence of alkaloids, flavonoids, coumarins, tannins, saponins, phenolic acids and triterpenes/sterols were tested using previously described methods.[7,8]

Determination of total polyphenolic content (TPC)
The TPC of EBF was determined using the method previously described by Singleton with some minor modifications.[9] Gallic acid was dissolved in methanol to five different concentrations (20, 40, 60, 80 and 100 µg/mL). EBF was also dissolved in methanol to compatible concentration for determination of TPC. To a 10 mL volumetric flask, 1 mL of test sample or standard solutions of gallic acid and 2.5 mL of 10% Folin-Ciocalteu reagent were added and left for 5 minutes at 25ºC. Then 5.0 mL of 2% sodium carbonate and was added, and made up to volume with distilled water. After incubation of the mixture for 60 min at room temperature, the absorbance was read at 754.5 nm using a spectrophotometer HITACHI U-1900 model (Japan).

Thin layer chromatography
The TLC analysis was developed using a HPTLC system (CAMAG, Switzland). The ethanol extract from *B. fungosa* subsp. *indica* (EBF) and reference compounds including pinoresinol, isolariciresinol and epipinoresinol-4-*O*-*β*-D-glucopyranose were dissolved in methanol. The samples were applied to the same TLC silica gel 60 F_{254} plate using Linomat 5 (CAMAG) and developed with the compatible eluent system. After development, the plate was visualized under ultra violet lamp before derivatization with anisaldehyde-sulfuric acid (AS) reagent and observed at white light and λ 366 nm. The chromatogram was photographed in a TLC Visualizer (CAMAG) and analyzed using visionCATs software version 2.5.

HPLC-UV/DAD analysis
HPLC analysis was performed using a UFLC equipped with a binary pump LC-20AD, an autosampler SIL-20AC, a diode array detector SPD-M20A and C18 column Agilent (250 mm x 4.6 mm; 5 µm). To assign compounds to the peaks, the retention time and UV spectra of standards eluted using the same chromatographic conditions as the EBF were used. The extract was dissolved in methanol to yield a concentration of 10 mg/mL while concentrations of standards (gallic acid, caffeic acid and cinnamic acid) in the standard mixture were 1 mg/mL. The samples were filtered through 0.45 µm syringe filters. The injected volume was 5 µL. Extract and standard mixture were eluted in a gradient system with acidified water (0.01% phosphoric acid) and methanol, starting with 10% of methanol, reaching 50% of methanol in 5 min, 55% of methanol in 8 min and kept for 7 min, 60% of methanol in 20 min and kept for 5 min, 100% of methanol in 30 min and kept for 5 min. The flow rate was kept constant at 0.5 mL/min. The separation temperature was 35ºC. The UV-DAD detector was set to record between 200 and 400 nm, and UV spectra were recorded at 280 nm.

Hypouricaemic studies of ethanol extract of B. fungosa subsp. indica
The potassium oxanate (PO) induced hyperuricemic mice model with some minor modifications was used to evaluate the hypouricaemic effect of EBF.[10,11] EBF and allopurinol were dissolved in a 0.5% sodium carboxymethyl cellulose (Na-CMC). PO was used to induce hyperuricemia in mice. Group I was treated with 0.5% Na-CMC alone, to serve as a normal control; group II was treated with PO (500 mg/kg b.w.), to serve as a model group; group III was treated with allopurinol (10 mg/kg b.w.) and PO (500 mg/kg), to serve as a positive control. Groups IV, V, and VI were treated with PO and EBF at doses of 300, 600, 900 mg/kg b.w., respectively based on the usage dose of *B. fungosa* subsp. *indica* in folk medicine. All groups were administered orally once a day for 5 consecutive days. On the fifth day of the experiment, PO (500 mg/kg) was injected intraperitoneally one hour before the administration of EBF and allopurinol to the experimental animals except the normal control group to induce hyperuricemia. Two hours after the administration, rats in all the groups were sacrificed to obtain blood which was then allowed to clot for 1 hour at room temperature and then centrifuged at 3500-4000x g for 10 minutes to obtain the serum. Serum was kept at -20^{o}C before determination of uric acid at 520 nm using a TC -3300 Plus (Teco Diagnostics, USA).

Anti-inflammatory studies of ethanol extract of B. fungosa subsp. indica
Carrageenan-induced rat paw oedema
The modified method of Winter was used.[12] The animal were either administered the ethanol extract (350, 500 mg/kg p.o), diclofenac (20 mg/kg) and negative control (Na-CMC 0.5%). One hour after treatment, inflammation was induced by injection of carrageenan (0.1 mL, 1% w/v in saline) into subplantar tissue of the right hand paw. At the time points (1h, 3h, 5h and 7h), the oedema was calculated by subtracting the initial paw volume from the paw volume after the injection of carrageenan. The inhibitory effect of oedema was expressed as percentage.

Urate-induced synovitis
Anti-inflammatory activity of EBF was evaluated by an experimental model of gouty arthritis as previously described by Faires and McCarty.[6] Monosodium urate crystals were synthesized using the procedure described by Faires and McCarty.[6] The crystals were suspended in 9% sterile saline (48 mg/mL) prior to use. Similar to the carrageenan model, each group was administered the ethanol extract EBF (350, 500 mg/kg p.o), diclofenac (20 mg/kg), and negative control (Na-CMC 0,5%) once a day for 5 consecutive days. On the fifth day, one hour after the treatment, a syringe containing the urate suspension was attached and volumes of 0.05 mL was injected into the joint (approximately 2.4 mg urate).
At time points (4h, 5h and 6h) after injection of urate, the anti-inflammatory activity of EBF in urate-induced synovitis was evaluated. A scoring system was adopted in which inflammatory symptoms ranging from tenderness, limping, occasional 3-legged gait to complete 3-legged gait were scored from 1+ to 4+.

Statistical analysis
Results were expressed as mean ± standard error of the mean (M ± SEM) of rats in each group. The data were analyzed by an analysis of variance (ANOVA) followed by Dunnett's test or LSD test, when appropriate. Kruskal-Wallis test was used to analyze non-parametric data, followed by Mann-Whitney U test where applicable. The values of $p < 0.05$ were considered to be statistically significant.

Results and Discussion

Phytochemical analysis
The phytochemical screening showed that tannins, phenolic acids, flavonoid and triterpenes were abundant in the extract of *B. fungosa* subsp. *indica*. Our data showed a relative high content of phenolic compounds in EBF (250.23 ± 0.19 mg GAE/g). The chemical composition of *B. fungosa* subsp. *indica* has been investigated in previous studies.[14,15] Four triterpenes (balanophorin A, balanophorin B, *β*-amyrin acetate and lupeol acetate) were identified in five Balanophora species including *B. spicata*, *B. laxiflora*, *B. dioica*, *B. polyandra* and *B. simaoensis* (a synonym of *B. fungosa* subsp.).[14] *indica* From ethyl acetate fraction of *B. fungosa* subsp. *indica* collected in Lao Cai prov. (Vietnam), pinoresinol, 7,9':7',9-Diepoxy-3-methoxy-3-methoxy-4,4'-lignandiol and balanophonin had been isolated.[15] Analysis performed on HPLC-UV/DAD compared retention time and UV spectra with standards and confirmed the presence of gallic acid (t_R = 9.286 min), caffeic acid (t_R = 12.766 min) and cinnamic acid (t_R = 23.481 min) in EBF (Figure 1).
The TLC analysis developed with the eluent system chloroform - toluene - methanol - 25% aqueous ammonia (10:3:6:1) (v/v/v/v) showed that chromatogram of EBF possessed equivalent tracks to pinoresinol (P) (R_f = 0.69), isolariciresinol (IL) (R_f = 0.60), epipinoresinol-4-O-*β*-D-glucopyranose (P') (R_f = 0.45) suggesting the presence of these lignans in EBF (Figure 2). These lignans had been

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isolated from some different species of genus Balanophora J.R. & G.Forst.[3]

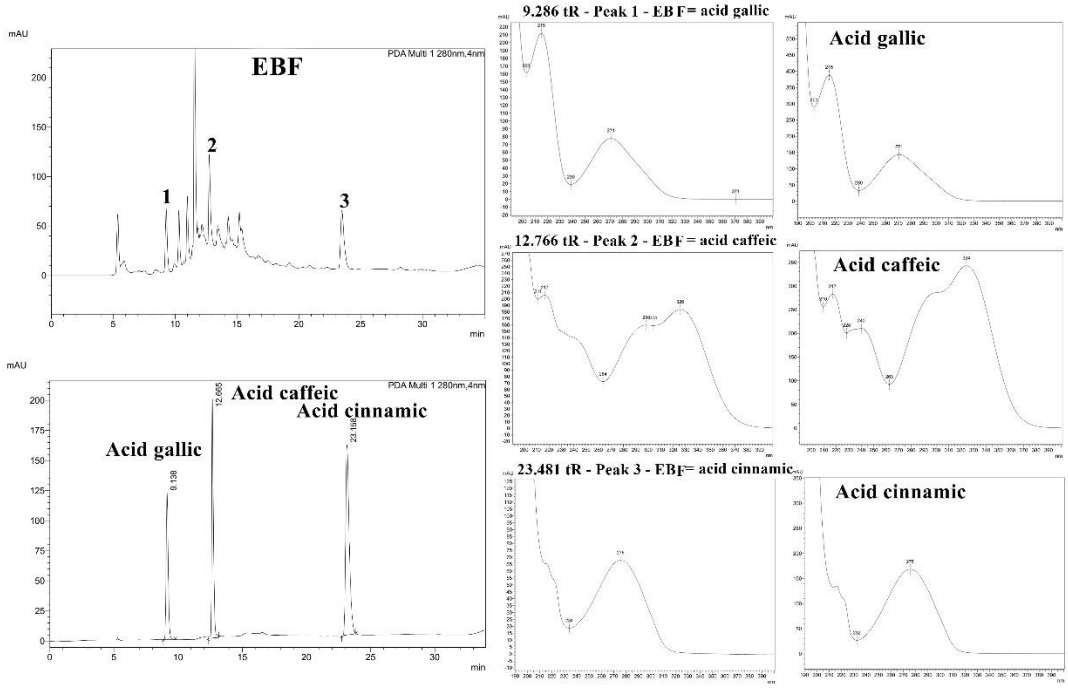

Figure 1: HPLC chromatogram of EBF at 280 nm and UV spectra of identified and standard substances gallic acid, caffeic acid and cinnamic acid

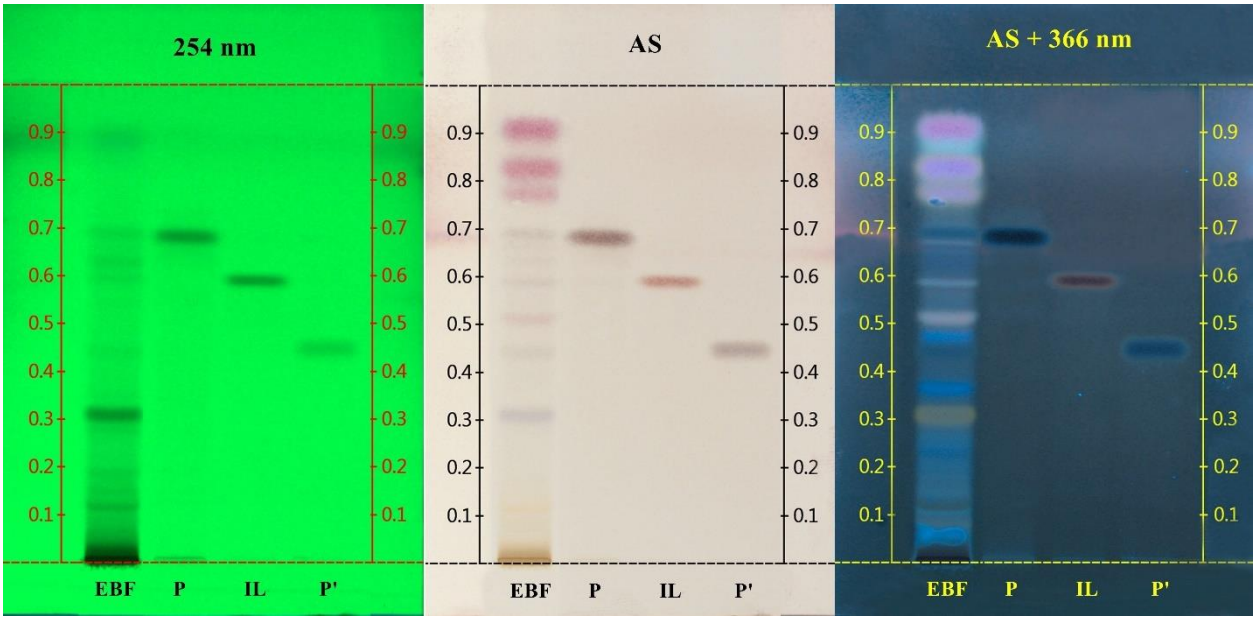

Figure 2: TLC chromatogram of EBF developed with the eluent system chloroform - toluene - methanol - 25% aqueous ammonia (10:3:6:1) and observed before derivatization (λ 254 nm) and after derivatization with AS reagent (at white light and λ 366 nm).

Hypouricaemic effect

Table 1 shows the hypouricaemic effect of EBF. The oral administration of EBF produced a statistically significant reduction of serum uric acid at the dose of 900 mg/kg (28.32%). The lower doses of EBF (300 and 600 mg/kg) did not show hypouricaemic effect. The reduction of serum uric acid could be due to increased excretion of uric acid or decreased uric acid formation through inhibition of XO. Caffeic acid and gallic acid could contribute to the hypouricaemic effect of EBF. Caffeic acid have shown *in vivo* and *in vitro* xanthine oxidase (XO) inhibitory effect[16,17] while gallic acid inhibit XO *in vitro* (IC_{50} = 28.47 ± 0.06 µg/mL).[18]

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Anti-inflammatory effect

The anti-inflammatory effect of EBF is presented in Table 2. EBF (500 mg/kg) showed a net anti-inflammatory effect at all time points after the induction of inflammation with inhibition of 58.02 – 77.55% of oedema. EBF at the dose of 350 mg/kg showed anti-inflammatory effect at 3 h and 5 h with inhibition of 23.16% and 19.69% of oedema, respectively. Isolariciresinol which modulate the production of inflammatory mediators could play an important role in this effect.[9] Beside, lupeol acetate which is a common triterpene in Balanophora species also exhibited significant inhibitory activities of NO production, iNOS and COX2.[20] Principally, this compounds also could contribute to the anti-inflammatory effect of EBF.

Table 1: Hypouricaemic effect of EBF in the experimental model of oxonate-induced hyperuricemia

Treatment	Serum uric acid (µmol/L)
Normal control (n=8)	108.90 ± 6.63
Normal control + PO (n=8)	235.38 ± 14.53
Allopurinol (10 mg/kg b.w.) + PO (n = 8)	44.67 ± 6.86**
EBF (300 mg/kg b.w.) + PO (n=7)	218.44 ± 24.21
EBF (600 mg/kg b.w.) + PO (n=7)	221.67 ± 15.90
EBF (900 mg/kg b.w.) + PO (n=7)	168.71 ± 17.89 **

*(**: p < 0,01)*

In the urate-induced synovitis model, EBF at a dose of 350 mg/kg showed anti-inflammatory effect on rats at 4 hours after the injection of urate while the higher dose of EBF (500 mg/kg) showed the effect at all-time intervals (4 h, 5h and 6 h) with the best effect observed at 6 hours after the injection (Table 3). This suggested a dose-dependent effect of EBF.

Conclusion

The study demonstrated that the ethanol extract from *Balanophora fungosa* subsp. *indica* possessed hypouricaemic and anti-inflammatory effects. Therefore, EBF could be used as a remedy in gout and inflammatory conditions.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 2: Effect of ethanol extract from *B. fungosa* subsp. *indica* (EBF) on carrageenan-induced paw oedema in rats

Treatment	Oedema 1h (mL) (% inhib.)	Oedema 3h (mL) (% inhib.)	Oedema 5h (mL) (% inhib.)	Oedema 7h (mL) (% inhib.)
Control (vehice) (n = 9)	14.77 ± 1.38	36.23 ± 2.05	32.09 ± 1.99	25.70 ± 2.04
Diclofenac 20 mg/kg (n = 9)	6.2 ± 0.65 ** (58.02%)	11.78 ± 1.12 ** (67.49%)	9.08 ± 1.17 ** (71.70%)	5.77 ± 1.11 ** (77.55%)
EBF (350 mg/kg. b.w.) (n = 9)	13.89 ± 1.91 (5.96%)	27.84 ± 1.42 ** (23.16%)	25.77 ± 1.20 * (19.69%)	21.57 ± 1.42 (16.07%)
EBF (500 mg/kg. b.w.) (n = 9)	12.59 ± 1.49 (14.76%)	23.80 ± 1.80 ** (34.31%)	19.64 ± 2.10 ** (38.80%)	12.47 ± 1.84 ** (51.48%)

**: Statistically significant. p < 0.05; **: p < 0.01*

Table 3: Effect of ethanol extract from *B. fungosa* subsp. *indica* on urate-induced synovitis

Treatment	Score		
	After 4 h	After 5 h	After 6 h
Control (n = 7)	2 (2-4)	3 (2-4)	3 (2-3)
Diclofenac (20 mg/kg b.w.) (n = 7)	1 (1-2) **	1 (1-2) **	1 (1-2) **
EBF (350 mg/kg b.w.) (n = 7)	2 (1-2) *	2 (1-3)	2 (1-3)
EBF (500 mg/kg b.w.) (n = 7)	2 (1-2) *	2 (1-2) *	1 (1-3) *

(: p < 0.05; **: p < 0.01)*

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