

Preliminary Study on TNF α -Blocker Activity of *Amygdalus lycioides* Spach Extracts

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Abstract: *Badam Talkh kuhi* is an endemic species of Iran commonly used by native people as anti-inflammatory and antimicrobial remedy. The present study was aimed at investigating the folk claims of this plant, identified by us as *Amygdalus lycioides* Spach. Several extracts were prepared using various extraction procedures combined with solvents of different polarity. A decoction was firstly prepared according to the procedure of the folk medicine. Maceration (ME), Ultrasound-Assisted Extraction (USAE) and Microwave-Assisted Solvent Extraction (MASE) were then experimented using pure methanol (MeOH) and *n*-hexane-acetone (*n*-Hex-Ac, 50% v/v). Concerning biological investigation, the antimicrobial potential of all extracts was firstly evaluated by employing the growth inhibition test. Then, the *free radical scavenging* effect of each extract was measured, given that reactive oxygen species (ROS) are involved in TNF α -induced signaling pathways associated with inflammation. Finally, the anti-TNF α activity of the most active extracts was evaluated using *in vitro* and *in vivo* assays. Regarding the anti-inflammatory activity, decoction and MASE *n*-Hex/Ac extract inhibited the production of TNF α with IC₅₀ in the range of 120-390 μ g/ml and 6-20 μ g/ml, respectively. Interestingly, after oral administration in mice, MASE *n*-Hex/Ac extract was effective in reducing TNF α of about 60%. Moreover, treated animals did not show any signs of toxicity. Results herein reported clearly evidenced that *Amygdalus lycioides* Spach could be a valuable source of TNF α -blockers effective against chronic inflammatory diseases.

Keywords: *Amygdalus lycioides* Spach, extraction procedures, anti-inflammatory properties, antimicrobial activity, free radical scavenging properties.

1. INTRODUCTION

Inflammation is an essential protective process preserving the integrity of organisms against physical, chemical and infective insults. However, repeated injuries of various aetiologies may induce chronic inflammation leading to prolonged damage of normal tissues. Chronic inflammation is thought to play a central role in the development of various diseases, including some cancers, atherosclerosis and chronic obstructive pulmonary disease [1]. Despite of the wide choice of drugs conventionally used in the treatment of chronic inflammation, there is an established need of new and safe anti-inflammatory agents, due to the discouraging toxicity profile of currently used drugs [2]. The discovery of endogenous molecules involved in the inflammatory process provides new targets for controlling inflammation. Following the discovery of the cytokine IL-1 β [3, 4] several different cytokines, termed pro-inflammatory cytokines [5], have been described to have a pivotal role in inflammation; among them one of the most important in sustaining inflammation is tumor necrosis factor alpha (TNF α) [6]. TNF α blockers represent a major advance in the treatment of

chronic inflammatory diseases, such as rheumatoid arthritis, bowel diseases and psoriasis [7].

Recently, the drug discovery process has been frequently focused on the screening of plant extracts commonly used in traditional medicines as source of novel therapeutic agents. In this context, the object of the present study was to evaluate the folk claims of *Badam Talkh kuhi*, an endemic Iranian species commonly used by native people as folk remedy in the treatment of various diseases since ancient time. In detail, its stem and branchlets decoction have been used for managing pain and inflammatory conditions, inflammatory skin disorders and infectious diseases [8].

To achieve our goal, different extracts were prepared, applying various extraction procedures combined with solvents of different polarity, and their biological properties were investigated.

2. MATERIALS AND METHODS

2.1. Chemicals

All solvents were purchased from Carlo Erba (Milan, Italy) and were of analytical grade.

2,2-Diphenyl-1-picrylhydrazyl radical and ascorbic acid were purchased from Sigma Chemicals (St Louis, MO,

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USA). Ampicillin and amphotericin B were purchased from Sigma–Aldrich S.r.l. (Milan, Italy) and kept at 4°C in dessicator.

2.2. Plant Material

Stems and branchelets of *Amygdalus lycioides* Spach were collected in September 2007 at an altitude of 2400 m in an area near Teheran. A voucher specimen was deposited in the Herbarium of the University of Pavia (PAV 2007 11/15/01), Department of Territorial Ecology, University of Pavia, Via S. Epifanio, 14 - 27100 Pavia, Italy. Freshly cut plants were sorted out, dried in a drying room with active ventilation at room temperature (about 20-22 °C) until they reached constant weight. The material was stored in dark conditions, into air tight polyethylene container. Branches were cut in small size, just before performing the extractions.

2.3. Extraction Procedures

Decoction

Dried stems and branchelets (5 g) were boiled in tap water (100 ml) for 1 hour under mechanical stirring. The procedure was repeated three times. After each extraction, solvents were filtered, combined and dried under reduced pressure (Laborota 4000, Heidolph Instruments, Schwabach, Germany, temperature 40-45°C).

Maceration Extraction (ME)

ME was performed at room temperature and in dark condition. Plant material (2 g) was macerated in the opportune solvent (40 ml), under mechanical stirring for 36 h.

Ultrasound-Assisted Extraction (USAE)

USAE was conducted in an ultrasonic bath (ELMA® Transsonic T420 Morfelden-Walldorf, Germany), at room temperature, for 2 h. The appropriate extraction solvent (40 ml) was added to plant material (2 g).

Microwave-Assisted Solvent Extraction (MASE)

MASE was performed on a multimode Microwave apparatus using a closed-vessel system (MARSX press, CEM Corporation Matthews, NC, USA). The appropriate solvent (20 ml) was added to plant material (1 g) and the samples heated at 120°C for 20 min with a power of 800 W. Samples were cooled at room temperature before opening the vessels.

ME, USAE and MASE extraction procedures were performed either with or without drug pre-treatment with petroleum ether 10% (w/v) for 30 min at room temperature under mechanical stirring [9]. Each extract was separated by filtration and solvent was evaporated to dryness under vacuum to 35°C.

All dried samples obtained were kept at room temperature in dessicator.

2.4. Antimicrobial Activity

To evaluate antimicrobial properties of prepared extracts the following reference strains were used: *Clostridium sporogenes* ATCC 3584, *Enterococcus hirae* ATCC 10541, *Staphylococcus aureus* ATCC 6538, *Streptococcus mutans* ATCC 25175, *Streptococcus pyogenes* ATCC 19615, *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa*

ATCC 10145, *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404.

Growth Inhibition Test

Antimicrobial activity was evaluated employing the agar disc diffusion method [10]. Stock sample solutions were prepared dissolving dried samples in DMSO in order to obtain a final concentration of 100 mg/ml of extracts. Stock standard solution of ampicillin and amphotericin B (concentration of 20 µg/ml in DMSO) was used as a positive control for bacteria and fungi, respectively. In both cases DMSO was used as a negative control.

The prepared suspension of bacterial and fungal strains was spread on Petri plates dispensed with Tryptone Soy Agar (TRS, Oxoid, Basingstoke, U.K.) for bacteria and Sabouraud Dextrose Agar (SAB, Oxoid, Basingstoke, U.K.) for fungi. The bacterial plates were then kept at 4°C for 2 h and afterwards were incubated for 24 h at 37°C. The fungal plates were incubated at 25°C for 48 h. Filter paper discs (12 mm in diameter) were impregnated with either 100 µl of stock sample solution or 100 µl of stock standard solution and placed onto the agar plates, previously inoculated with the tested microorganisms. Results were expressed as mean \pm SE of inhibitory diameter zone (IZD) of three independent experiments.

Minimum Inhibitory Concentration (MIC) Determination

MICs were determined by the agar dilution method [11]. A series of two fold dilution of each sample, ranging from 300 to 0.85 mg/ml was prepared, at 50°C, in TSA for bacteria and SAB for fungi and poured in a Petri plate. Plates were dried at 37°C for 30 min and then suspensions of bacterial and fungal strains were spotted onto the agar plates (10 µl). The final CFU inoculated onto the agar plates was approximately 10^5 for each strain. The bacterial plates were incubated for 24 h at 37°C while the fungal plates were incubated at 25°C for 48 h. Antibiotic control of ampicillin and amphotericin B were also prepared to obtain concentration ranges of 7×10^{-3} - 40 µg/ml. MICs were defined as the lowest concentration of plant extract or antibiotic control inhibiting visible growth of each microbial strain on Petri plate. All determinations were performed in triplicate and two growth controls consisting of medium without plant extracts were included.

2.5. Free Radical Scavenging Activity

The free radical scavenging activity (FRS) of the extracts was determined by using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) method [12]. Briefly, both dried extracts and standard (ascorbic acid) were dissolved in MeOH at a concentration of 1 mg/ml; stock solutions were then serially diluted in MeOH by two folds, giving test solutions at final concentration of 0.5, 0.25, 0.125, 0.06 mg/ml. Reaction mixture was prepared by adding 100 µl of each extract solution (or standard solution) to 3.9 ml of DPPH solution, freshly prepared dissolving DPPH in methanol/KH₂PO₄ and NaOH buffer (50/50 v/v) at a concentration of 6×10^{-5} M. After 20 min of incubation at room temperature, the absorbance was measured at 515 nm by a UV-Visible spectrophotometer (Lambda 25 UV/VIS spectrometer, Perkin Elmer instruments, Massachusetts, USA).

FRS was expressed as a percent compared with the control, consisting of 3.9 ml of DPPH solution and 100 μ l of methanol. The percent inhibition of the DPPH radical by the test solution was calculated using the following formula

$$\text{FRS}\% = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \times 100$$

Each solution was prepared in triplicate. The analyses were carried out in triplicate and results are expressed as mean \pm SE.

2.6. Anti-Inflammatory Activity

Inhibition of TNF α from human peripheral blood mononuclear cells (hPBMC) *in vitro* was carried-out as previously described by us [13]. Briefly, hPBMC were separated by Ficoll-Hypaque centrifugation by buffy-coat from healthy donors and resuspended in RPMI 1640 containing 5% of Foetal Bovine Serum (FCS) and maintained over night at 4°C. The next morning the cells were added to 96-well flat bottom microtiter plate in the presence of serial doses of decoction (from 800 to 12 μ g/ml) or MASE *n*-Hex-Ac extract (from 400 to 6.2 μ g/ml) or medium (control cells) and incubated for 60 min at 37°C. At the end of the incubation period, *E. Coli* lipopolysaccharides (LPS) was added at 10 ng/ml and the cells were incubated for 24 h at 37°C. The amount of TNF α released in the supernatant was, then, measured by enzyme-linked immunosorbent assays (ELISA) (h-TNF α DuoSet, R&D Systems, Minneapolis, MN, USA). The inhibition of TNF α production was expressed as inhibitory concentration 50% (IC₅₀) determined by using Graph-Pad 5.0 Software.

Cytotoxicity of the extracts was determined on parallel cell culture plate by using the Cell Titer 96 assay (Promega Italia, Milan, Italy) based on the use of the tetrazolium salt MTS and expressed as effective dose 50% (ED₅₀).

Inhibition of TNF α *in vivo* was carried-out as previously described [13]. Briefly, BALB/c mice (Charles River Italia, Calco, Italy) were treated by oral gavage with MASE extract suspended in 0.5% methyl-cellulose, in a final volume of 200 μ l/mouse. Vehicle treated mice received 200 μ l of water. After 60 min. mice were injected i.p. with *Salmonella E.* LPS (30 mg/kg) and 90 min later TNF α serum levels were measured by ELISA (m-TNF α DuoSet, R&D Systems, Minneapolis, MN, USA).

2.7. TLC Analysis

Thin Layer Chromatography (TLC, Kieselgel 60 F254, 0.2 mm, Merck, Germany) of extracts was performed using two different mobile phases [ethyl acetate-formic acid-water (90:5:5 v/v/v) or chloroform-methanol-water (61:32:7 v/v/v)] combined with FeCl₃ reagent (3% in water) or ammonia vapour exposure for the identification of condensed tannins and flavonoids respectively [14].

2.8. Statistical Analysis

Data were analyzed by analysis of variance (ANOVA). Means were separated with the LSD method at confidence levels of 99% and 95%.

3. RESULTS AND DISCUSSION

The first step of the present study was aimed at identifying the *Badam Talkh kuhi*'s plant material that was classified

by us as *Amygdalus lycioides* Spach [Family: *Rosaceae*, Genus: *Amygdalus*, Subgenus: *Dodecandra*, Species: *lycioides*; Syn.: *Prunus lycioides* C.K. Schneid], a very spiny, much branched, dense scrub about 1 m tall, with linear-lanceolate 1.5-3.0 cm long leaves which have sharp pointed apex and crenate-dentate margins [15, 16]. *Amygdalus lycioides* is an endemic species extending into south Anatolia, growing on steep dry slopes made of limestone, sandstone or even granite, as well as in clayish or gravelly habitats and covering a wide altitude range - from 200 to 2900 m [17].

Successively, different extracts of *Amygdalus lycioides* were prepared and their biological properties evaluated, applying the modern ethnobotanical approach which suggests firstly to perform preliminary bioassays on crude plant extracts and then to accomplish their bioactivity-directed fractionation aimed at obtaining the active compounds. To the best of our knowledge, no scientific evaluation of pharmacological activities of *Amygdalus lycioides* Spach extracts has been reported yet.

The decoction of *Amygdalus lycioides* was prepared essentially according to the procedure of the Iranian folk medicine. Maceration (ME), Ultrasound-Assisted Extraction (USAE) and Microwave-Assisted Solvent Extraction (MASE) were also experimented using two different extraction solvents, pure methanol (MeOH) and *n*-hexane-acetone (*n*-Hex-Ac, 50% v/v). In order to remove the fat from the drug just before performing the extraction, a protocol involving a drug pre-treatment with petroleum ether was also applied [9, 18]. The yield of each dried extract was calculated in terms of percentage of the original drug weight and reported in Table 1.

In order to evaluate the medicinal potential of *Amygdalus lycioides*, the antimicrobial and anti-inflammatory properties of prepared extracts were investigated.

Preliminary antibacterial activity screening against *S. aureus*, *E. coli* and *P. aeruginosa* was performed on each extract, employing the agar disc diffusion method. Results are reported in Table 1.

Generally, tested extracts resulted effective against the Gram-positive *S. aureus* (IZD ranging from 15.4 \pm 0.8 to 20 \pm 0.7 mm), with the only exception of methanolic extracts obtained applying USAE and ME methodologies. It can be outlined that the drug pre-treatment with petroleum ether gave rise to extracts showing enhanced antimicrobial activity with respect to those obtained without pre-treatment of the vegetable matrix. Moreover, a decrease of extraction yield was observed, proving the removal of biologically inactive components during the drug pre-treatment process. Regarding the activity against *E. coli* and *P. aeruginosa*, all tested extracts were found ineffective. As showed in Table 1, the highest antibacterial activity against *S. aureus* was observed for the extract obtained using *n*-Hex-Ac as extraction mixture and applying MASE methodology to pre-treated drug (IZD 20.0 \pm 0.7 mm). Thus, the antimicrobial potential of this extract was deeply investigated by performing Growth inhibition test as well as MIC determination on different bacteria (*C. sporogenes*, *E. hirae*, *S. mutans* and *S. pyogenes*) and fungi (*C. albicans* and *A. niger*) strains. Interestingly, MASE *n*-Hex-Ac extract showed maximum activity against *C. sporogenes* (IZD of 30.3 \pm 2.1 mm and MIC of 1

Table 1. Yield Extraction (%) and IZD of Different *Amygdalus lycioides* Extracts

Extraction Methodology	Extraction Solvent	% Yield (w/w)	IZD (mm) <i>S. aureus</i>
Decoction	Water	11.2	16.9 \pm 0.5
MASE	<i>n</i> -Hex-Ac	1.7	15.8 \pm 0.7
MASE	<i>n</i> -Hex-Ac ^a	1.5	20.0 \pm 0.7
MASE	MeOH	5.2	16.2 \pm 0.5
MASE	MeOH ^a	4.9	16.5 \pm 0.3
USAE	<i>n</i> -Hex-Ac	1.4	15.4 \pm 0.8
USAE	<i>n</i> -Hex-Ac ^a	1.2	16.0 \pm 0.9
USAE	MeOH	3.8	NI ^b
USAE	MeOH ^a	3.6	NI ^b
ME	<i>n</i> -Hex-Ac	2.7	16.7 \pm 0.3
ME	<i>n</i> -Hex-Ac ^a	2.4	17.0 \pm 0.3
ME	MeOH	7.7	NI ^b
ME	MeOH ^a	6.5	NI ^b

^adrug pre-treatment with petroleum ether.

^bNI: no inhibition was observed.

IZD values are expressed as mean \pm SE of three determinations.

mg/ml), followed by *S. mutans* (IZD of 28.0 \pm 1.7 mm and MIC of 2.5 mg/ml) and *S. pyogenes* (IZD of 26.5 \pm 1.7 mm and MIC 2.1 mg/ml).

Moving from antimicrobial to anti-inflammatory properties evaluation, our approach consisted in performing a preliminary screening of all extracts by testing their free radical scavenging effect (FRS), given that reactive oxygen species (ROS) are involved in TNF α -induced inflammation [19]. The most interesting extracts in terms of FRS effect were then selected to be further investigated concerning their TNF α -blocker properties, by both *in vitro* and *in vivo* assays.

The FRS activity was evaluated by the DPPH assay, using ascorbic acid as standard. The antioxidant potential of

extracts was initially evaluated at a stock concentration of 1 mg/ml. Successively, stock solutions were serially diluted into a range of 0.5-0.06 mg/ml and their corresponding FRS activity was determined. Basing on results obtained during the antimicrobial activity study, only the extracts prepared from pre-treated drug were considered. Generally, an interesting FRS activity was evidenced for all *Amygdalus lycioides* extracts (Table 2). Particularly, in the high doses range (\geq 0.25 mg/ml) all the extracts showed a significantly ($p < 0.05$) higher effect with respect to ascorbic acid. On the contrary, in the low doses range ($<$ 0.25 mg/ml) the strongest antioxidant effect was observed for the extract obtained applying MASE methodology and *n*-Hex-Ac extraction mixture. In fact, only MASE *n*-Hex-Ac extract showed a FRS profile similar to that of ascorbic acid (Table 2).

Table 2. FRS % of *Amygdalus lycioides* Extracts and STD (Ascorbic Acid)

Conc ^a	FRS %							
	<i>Amygdalus lycioides</i> Extracts							STD
	MeOH			Hex-Ac (50/50 v/v)			Water	Ascorbic Acid
	MASE	USAE	ME	MASE	USAE	ME	Decoction	
0.06	26.59 \pm 0.4	25.77 \pm 1.1	30.45 \pm 1.9	49.01 \pm 1.1	30.03 \pm 0.7	27.54 \pm 2.9	17.13 \pm 1.5	59.50 \pm 1.2
0.125	35.66 \pm 0.6	20.20 \pm 2.2	53.42 \pm 0.7	54.89 \pm 0.9	45.45 \pm 0.6	35.04 \pm 1.4	28.77 \pm 1.1	56.61 \pm 1.1
0.25	59.16 \pm 0.6	52.88 \pm 2.1	64.02 \pm 1.3	61.58 \pm 1.2	59.88 \pm 0.9	55.03 \pm 0.6	57.48 \pm 1.9	56.20 \pm 3.0
0.5	61.50 \pm 1.4	62.86 \pm 0.3	64.55 \pm 0.7	63.16 \pm 0.8	64.28 \pm 1.5	57.79 \pm 1.7	60.82 \pm 0.7	54.27 \pm 2.5
1.0	63.28 \pm 0.3	64.67 \pm 1.9	64.58 \pm 0.4	65.13 \pm 0.9	64.99 \pm 2.4	57.92 \pm 1.4	70.23 \pm 0.9	54.83 \pm 1.8

^amg/ml.

FRS % values are expressed as mean \pm SE of three determinations.

Table 3. TNF α Effect and Cytotoxicity of *Amygdalus lycioides* Extracts

Extraction Methodology	TNF α Inhibition (IC ₅₀ , μ g/ml)	Cytotoxicity (ED ₅₀ , μ g/ml)
MASE (<i>n</i> -Hex-Ac)		
Donor A	20.0	>>400
Donor B	< 6.2	\approx 100
Decoction		
Donor A	387.0	>> 800
Donor B	119.9	>> 800

Basing on these results MASE extract obtained from plant material pre-treated with petroleum ether and using *n*-Hex-Ac as extraction mixture (by now on called the MASE extract) was selected for the TNF α -activity evaluation. The anti-inflammatory efficacy of decoction was also assayed according to the traditional use of *Amygdalus lycioides*.

TNF α production by hPBMC was induced *in vitro* by a treatment with *E. Coli* endotoxin (LPS) for 24 h in the presence of serial doses of both extracts. The TNF α levels in the cell supernatant was measured by ELISA and the inhibitory activity expressed as IC₅₀; hPBMC were obtained from 2 different donors and the results are reported in Table 3. To confirm that the inhibitory effect was specific and to exclude that it was due to cytotoxicity, the vitality of the cells was determined and expressed as ED₅₀.

MASE extract dose-dependently inhibits TNF α with an IC₅₀ of 20 μ g/ml on donor A, whereas on the second donor appear to be more potent with an IC₅₀ value lower than 6.2 μ g/ml (the lowest concentration tested). The inhibition was clearly due to a specific cellular effect on TNF α synthesis since MASE extract did not cause any toxic effect at the active concentrations. In fact, on donor A the cytotoxicity was negligible at the higher dose used (ED₅₀ higher than 800 μ g/ml), whereas on donor B cytotoxicity (ED₅₀ about 100 μ g/ml) was evident at concentrations significantly higher than the effective dose on TNF α (IC₅₀ < 6.2 μ g/ml). Decoction resulted similarly active as TNF α inhibitor but its potency was lower than that of MASE extract, in fact the IC₅₀ values were about 390 μ g/ml and 120 μ g/ml on donor A and B, respectively. As for MASE extract, the anti- TNF α activity of the decoction is specific since on the cells of both donors no signs of cytotoxicity were detected.

Due to the *in vitro* efficacy of the MASE extract, its *in vivo* activity (using a model of systemic inflammation, i.e. the LPS-induced TNF α production in the mouse) was also evaluated. In detail, MASE extract was administered orally at the dose of 50 and 100 mg/kg, 60 min before LPS administration. The reduction of circulating level of TNF α was measured 90 min later, corresponding to the time point of the circulating peak concentration of the cytokine. Results reported in Fig. (1) indicate that the lower dose (50 mg/kg) did not inhibit significantly the TNF α release, whereas the higher dose (100 mg/kg) is effective in reducing TNF α of about 60% (3.8 ng/ml vs. 9.8 ng/ml in the LPS control group). It should be noted that the treated animals did not show any signs of toxicity during the overall experimental period.

The overall anti-inflammatory results indicate that both *Amygdalus lycioides* extracts have anti- TNF α properties *in vitro* and the MASE extract is also orally effective *in vivo*.

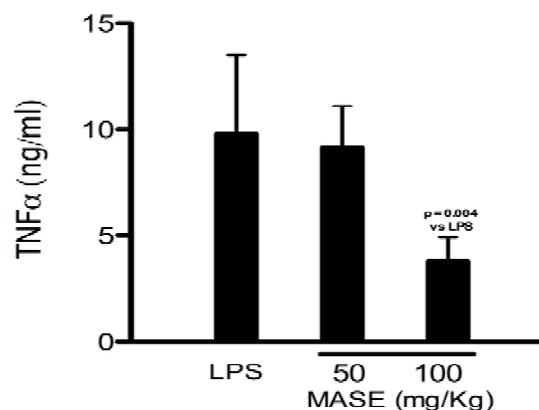


Fig. (1). Effect of MASE extract on LPS-induced TNF α production in mouse.

Following biological activity results, MASE extract was analyzed by TLC for a qualitative screening of its phytochemical composition. Interestingly, visual inspection of TLC plates under a UV lamp at 254 nm as well as coloration of the spots with specific reagents revealed the presence of condensed tannins and flavonoids.

4. CONCLUSIONS

In the present paper the ethnomedical use of *Amygdalus lycioides* Spach was assessed. Particularly, of high interest is the anti-inflammatory activity *via* TNF α -blocker mechanism of *n*-Hex-Ac MASE extract, which resulted effective both *in vitro* and *in vivo* at non toxic doses.

Results herein reported can be considered a useful starting point for the discovery of new anti-inflammatory compounds effective against chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel diseases.

Present efforts are directed to perform bioassay-guided purification of *n*-Hex-Ac MASE extract in order to isolate and to identify the active constituents, which belong to tannin and flavonoid chemical classes as evidenced by TLC analysis.

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