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Anti-inflammatory and antimicrobial activity of Shikonin derivatives from *Arnebia hispidissima* (Lehm.) DC.

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Abstract

Arnebia hispidissima roots have been used traditionally for the treatment of ulcers, boils, cuts, heart ailments and headache, fever, tongue and throat troubles. The purpose of this study was to characterize the most potent phytochemicals present in the ethyl acetate extract having anti-inflammatory and antimicrobial activity using carrageenan and CFA model. The observed results revealed that shikonin isovalerate showed most potent anti-inflammatory effects (60.5%) at the dose of 5.0 mg/kg at 6 h after carrageenan injection. Moreover, the maximum inhibition rate was observed of isovaleryl shikonin (61.2%) at the dose of 10 mg/kg at 8 day after CFA injection. The strong antibacterial activity was showed by shikonin isovalerate at 20 μg/ml dose against *E. cloacae* and β,β-dimethylacryl shikonin demonstrated maximum antifungal activity against *C. albicans* at the dose of > 65 μg/ml.

Keywords: *Arnebia hispidissima*; anti-inflammatory activity; antimicrobial activity; shikonin derivatives; carrageenan and CFA models;

Introduction

Arnebia hispidissima is a perennial grass widely distributed in India, Persia, Pakistan and drier parts of Rajasthan (India). Roots are used in ulcers, boils, cuts, for heart ailments, headache, fever, water extract of flowering shoot is known for tongue and throat troubles, cardiac complaints, while the whole plant was used as a stimulant, tonic, diuretic and expectorant (Chopra et al., 1956; Kirtikar and Basu, 1967; Anonymous, 1985; Annon, 1979; Jain and Defilipps, 1991; Trivedi, 2005; Boktapa and Sharma, 2010). The phytochemical studies revealed that the roots contain a dl-alkannin, a crystalline red solid, from *A. euchroma* (Romanova et al., 1968; Fu et al., 1984; Sharma et al., 2009; Song et al., 2010), arnebin derivatives have also been isolated from *A. nobilis* (Shukla et al., 1973), β-hydroxyisovalerate and alkannin from *A. hispidissima* (Khan et al., 1983; Singh et al., 2004), and shikonin production has well documented by hairy root cultures (Choudhary and Pal, 2010; Pal and Choudhary, 2010). The ethanolic extract of *A. euchroma* provided tormentic acid, 2α-hydroxyursolic acid

(Yang et al., 1992), β-sitosterol (Nigam and Mitra, 1964). A fresh flowers of *A. hispidissima*, yielded a flavonoid namely vitexin (Hamdard et al., 1988). Several pyrrolizidine alkaloids viz. echimidine, monocrotaline, supinine, heliotrine, 7,9-tigloyl retronecine, O^9–angeloyl retronecine, rinderine and others had been isolated from *Arnebia* species by various research-ers (Gamila et al., 1987; Roeder and Rengel-Meyer, 1993; El-Dahmy and Ghani, 1995; Srivastav et al., 1999).

Pharmacologically and biologically the *Arnebia* species has antibacterial (Jain et al., 1999; El Sayed, 2010), antitumor (Sankawa et al., 1977; Mao et al., 2008; Xiong et al., 2009; Deng et al., 2010), anti-inflammatory (Tanaka et al., 1986; Kaith and Kaur, 1996), antifungal (Gao, 1986), antiviral–HIV (Kashiwada et al., 1995) and prostaglandin inhibitory activity (Yao et al., 1983, 1991a, b). However, there are no reports in literature on the anti-inflammatory activities of shikonin derivatives from *A. hispidissima* hence the study was carried out.

Materials and methods

Plant material

Arnebia hispidissima (Lehm.) DC. (Boraginaceae) was collected (Feb., 2007) from fields of the Agricultural Research Station Durgapura, Jaipur and authenticated by Professor R.S. Mishra, voucher specimens were deposited in the Herbarium, Department of Botany, University of Rajasthan, Jaipur, India, used for present investigation (sheet no. 19441).

General experimental conditions

The melting points of purified compounds were recorded on capillary Toshniwal melting point apparatus and are uncorrected. The spectral data were obtained on the following instruments: ir, Perkin-Elmer, 283; ms, Hewlett Packard HP 5930 A; gc-ms, equipped with a HP 5933 data system , direct inlet at 70 eV; uv, Perkin -Elmer, model - 200 ; nmr, JEOL PS 100 at 6.1 - 3.6 MHz; hplc, Waters, 600 Controller, Waters Delta 600 pump and adsorbents for (silica gel 60, 230 - 400 mesh, Merck) for column chromatography and silica gel G used for TLC (Merck); TLC solvent system – acetonitrile: MeOH: CH_3COOH (60–20–20, v/v).

Extraction and characterization

Shade-dried powdered plant roots (10.0 kg) were percolated with hexane for 36 h, filtered, the extract concentrated by removing hexane (19.563 g). The resultant residue was Soxhlet extracted with ethyl acetate (21.435 g) for 36 h, filtered and concentrated. The ethyl acetate extract (15.935 g) was subjected to thin layer chromatography (TLC) with standard shikonin derivatives. These TLC plates were then developed in an organic solvent mixture (acetonitrile: MeOH: CH_3COOH; 60–20–20, v/v; Kirimer et al., 1995; Staniforth et al. 2004; Papageorgiou et al., 1999) which demonstrated four colored spots corresponding to the reference compounds viz. β,β-dimethylacryl shikonin (**I**); isovaleryl shikonin (**II**); β-hydroxyisovaleryl shikonin (**III**) and shikonin isovalerate (**IV**)

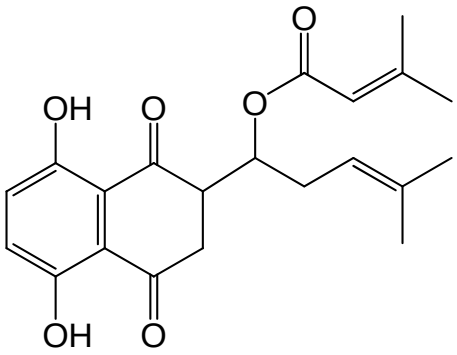
Estimation of shikonin derivatives

The quantification of shikonin derivatives was carried out by using preparative thin lay-

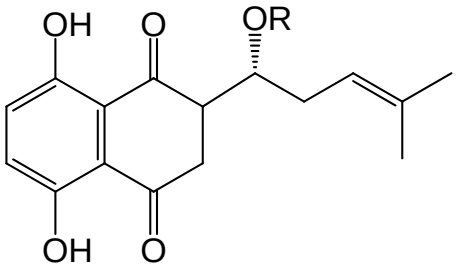
er chromatography (PTLC). The ethyl acetate soluble fraction was spotted on silica gel G coated glass plates (20×20 cm; wet thickness 0.5 mm) and developed in above mentioned solvent system. These spots were located to coinciding reference compounds and separately scrapped, eluted with ethyl acetate and concentrated, crystallized with $CHCl_3$ (Salim et al., 1996) and quantified viz. β,β-dimethylacryl shikonin (**I**, 563 mg), isovaleryl shikonin (**II**, 438 mg), β-hydroxyisovaleryl shikonin (**III,** 639 mg) and shikonin isovalerate (**IV**, 648 mg). These crystallized compounds (Figure 1) were dried *in vacuo* and subjected to various physical and spectral studies (Khan et al., 1983; Kirimer et al., 1995).

Tissue culture

The unorganized callus tissue of *A. hispidissima* was induced by seedlings. The seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ solution for 3-4 min and then rinsed three times with sterilized distilled water. These sterilized seeds were then aseptically inoculated onto Murashige and Skoog (MS; 1962) medium supplemented with 5.0 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 2.5 mg/l benzyl amino purine (BAP) and 3.0% sugar. The seeds took 10-15 days to germinate and the seedlings thus started differentiated tissue formation after 23-27 days of inoculation. These cultures were incubated at 25±1°C with 60% relative humidity under room light conditions (300 Lux). The callus tissue sample

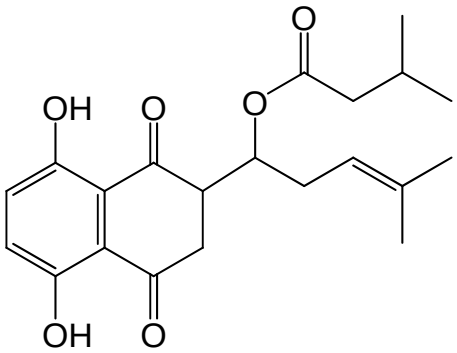


β, β- dimethylacryl shikonin

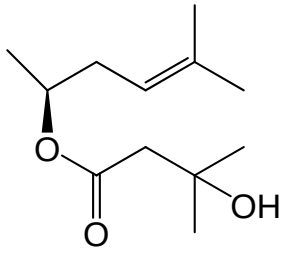


R=$OCOCH_2$ $CH(CH_3)_2$

Shikonin isovalerate



Isovaleryl shikonin



β-hydroxyisovaleryl shikonin

Figure 1. Shikonin derivatives isolated from *A. hispidissima.*

was transferred onto the fresh MS medium after 4-5 weeks intervals. The callus tissue was harvested at the transfer age of 2, 4, 6, 8 weeks and the growth index was calculated (GI = Final dry weight of callus – Initial dry weight of callus / Initial dry weight of callus).

The fresh tissue samples (1.0 kg each) were first dried at 100°C for 15 min to inactivate the enzymatic activity and then at 40°C until the weight in each sample became constant. Each of dried tissue samples were powdered, refluxed in Soxhlet apparatus with ethyl acetate for 24 h, filtered and dried (yield : 6.491 g, 1.37%; 10.461 g, 2.74%; 16.201 g, 3.16%; 16.102 g, 3.04%) *in vacuo* and taken up separately for quantification and characterization studies.

HPLC analysis

The HPLC determination of shikonin derivatives in *A. hispidissima* (roots) was achieved on a semi-preparative model HPLC (Waters HPLC system equipped with a Waters 600 Controller, Waters Delta 600 pump and 2487 Dual λ absorbance detector). A 5 μm C_{18} column (250 × 10 mm Merck) was used with two solvent system, acetonitrile – water (80: 20, v/v) (A) and MeOH (B). Elution was performed as following: 0-8 min, A: B = 95:5.0 (isocratic); 8-15 min, 95-80% A to B (linear gradient); 15-40 min 80-50% B (linear gradient) with a flow rate of 3.5 ml/min with the detection wavelength set at 254 nm, molecular weights of these compounds as determined by mass spectroscopy (Staniforth et al., 2004).

Animals

Male Wistar albino rats, 4-6 weeks old, 150-200 g, were used for carrageenan-induced inflammation studies and CFA-induced arthritis experiments were performed with male Swiss albino mice, 4-6 weeks old, 25-30 g each, were obtained from Animal Centre, Hamdard University, New Delhi. All animals were housed in air conditioned room at 223 and fed with standard laboratory diet and tap water throughout the experiments.

Carrageenan-induced paw oedema in rats

The anti-inflammatory activity was performed by using the method of Winter et al. (1962). The oedema was induced in left hind paw by injecting 0.05 ml of 1.0% (w/v) carrageenan (Sigma St. Louis, MO) in saline into the footpad, subcutaneously. The paw volume of each rat was measured before carrageenan injection and then at hourly intervals up to 10 h with Plethysmometer 7150 (UGO, Basil, Italy). The drug test groups were treated with ethyl acetate extract (25, 50, 100 mg/kg body weight, s.c.), and isolated compounds (5.0, 10, 20 mg/kg body weight, s.c.) 1 h before carrageenan injection. The animals of control group received saline only. Another group of animals was administered with acetyl salicylic acid (5.0, 10, 20 mg/kg body weight, s.c.) in carboxymethyl cellulose (CMC) as standard compound. The oedema rate and rate of inhibition of each group were calculated by the following formula:

$$\text{Oedema rate (\%)} = V_t - V_0 / V_0 \times 100$$
$$\text{Inhibition rate (\%)} = E_c - E_t / E_c \times 100$$

Where V_0 is the paw volume before injection of carrageenan (ml); V_t is the volume at *t*

h after carrageenan injection (ml); E_c is the rate of oedema of control group and E_t is the oedema rate of treated group.

Adjuvant-induced arthritis in rats

Experimental arthritis was induced in rats by adopting the protocol of Newbould (1963). The left footpad of each rat was injected (s.c.) with 0.05 ml of complete Freund's adjuvant (CFA, 1.0% suspension in olive oil, Difco). Rats in the drug test groups were treated with ethyl acetate extract (25, 50, 100 mg/kg body weight, p.o.) and isolated phytochemicals (5.0, 10, 20 mg/kg body weight, p.o.) 24 h before the injection of CFA and then with daily treatment until 16 days after CFA challenge. The animals in control groups received saline only. Another group of rats was administered with acetyl salicylic acid (5.0, 10, 20 mg/kg body weight, p.o.) in 1.0% CMC as a standard reference drug. The oedema and inhibition rates were measured with the same method as describe above.

Statistical analysis

The data were expressed as mean, SD and statistically assessed by one analysis of variance (ANOVA). The difference between drug treated groups and control group was evaluated by Dennett's *t*-test. $P < 0.005$ was considered significant.

Sources of microorganisms

Pure cultures of bacteria, *Escherichia coli* (ATCC-5922), *Eterobacter cloacae* (ATCC-25924), *Klebsiella pneumonie* (ATCC-59008) *Staphylococcus aureus* (ATCC-25923), *Bacillus subtilis* (ATCC-10031), *Streptococcus pneumoniae* (ATCC-10032) (obtained from S.M.S. Medical College, Jaipur) were grown on nutrient agar culture medium at 37°C for 24 h and fungi, *Aspergillus niger*, *A. flavus*, *Rhizoctonia phaseoli*, *Penicillium chrysogenum* (from Seed Pathology Laboratory, Department of Botany, University of Rajasthan, Jaipur), were grown on potato dextrose broth medium at 27°C for 48 h and *Candida albicans* (obtained from Superior Diagnostic Center, Jaipur) was cultured on Sabouraud dextrose broth medium at 30°C for 5 days (Chang and Cury, 1998).

Antimicrobial activity

The antifungal and antibacterial activity was evaluated by minimum inhibitory concentration (MIC) determined by microdilution method (Jones and Barry, 1987). The organisms to be tested were grown in nutrient broth (Difco Co.) at 37°C for bacteria and Sabouraud dextrose broth (Difco Co.) at 30°C for fungi, respectively. After 24 h, 1 ml of culture broth from culture was transferred to 10 ml of the same medium and further incubated for 6 h and each culture was adjusted with nutrient broth or Sabouraud dextrose broth to obtain 0.1 ml of cell culture was inoculated in tubes with 0.9 ml of nutrient broth or Sabouraud dextrose broth supplemented with different concentrations of the crude extract, isolated and standard compounds which were dissolved in dimethyl sulphoxide. Culture with dimethyl sulphoxide (0.5%) was used as solvent control, and culture supplemented with tetracycline, gentamycin and nystatin was used as positive control, respectively. The MIC was defined as the lowest concentration able to inhibit and visible microbe growth and was

determined by measure of cell growth OD after 48 h cultivation. All data are presented as mean values of triplicate of each microorganisms.

Results and discussion

The TLC of the ethyl acetate fraction showed the presence of four shikonin derivatives (**I** - **IV**) as colored spots (**I**, 563 mg; **II**, 438 mg; **III**, 639 mg; **IV**, 763 mg). The growth indices of *A. hispidissima* tissue cultures showed an increase in growth up to a period of 6 weeks old, but declined up to 8 weeks ($0.74 < 2.46 < 5.09 > 4.99$). For estimation of shikonin derivatives, ethyl acetate extract (25.315 g) of 6 weeks old callus was used. The following quantities of shikonin derivatives were observed viz. (**I**), 235 mg; (**II**), 336 mg; (**III**), 534 mg; (**IV**), 558 mg. The total protein and carbohydrate values increased slowly up to day 18-20 but later reached a sharp peak at day 38-40 day and then gradually diminished to the initial inoculum value. The microscopical observations suggest that much increase in cell dry weight (6 weeks old) is due to accumulation of starch grains which subsequently disappear during the growth cycle (Shimomura et al., 1991).

In the present investigation, the footpad of rats became oedematous soon after carrageenan injection. The left footpad's oedema rate reached its peak at 8 h (63.5%). Administration of ethyl acetate crude extract, isolated shikonin derivatives and acetyl salicyl-ic acid (reference drug) significantly inhibited the development of swelling from 1 to 10 h after carrageenan injection ($P < 0.005$, Table 1). The observed results revealed that shikonin isovalerate showed most potent anti-inflammatory effects (60.5%) at the dose of 5.0 mg/kg at 6 h after carrageenan injection. Acetyl salicylic acid (5.0, 10, 20 mg/kg) also showed more effectiveness than isolated shikonin derivatives.

In the carrageenan model, the early phase (1-2 h) is mainly mediated by histamine, serotonin and the increase of prostaglandin synthesis in the surroundings of the damaged tissues (Vinegar et al., 1987), while the late phase is mainly mediated by bradykinin, leukotriens and polymorphonuclear cells and prostaglandins produced in tissue macrophages (Antonio and Souza Brito, 1998). In the histamine-induced acute-inflammation, the isolated compounds and reference drug showed inflammatory effects, especially in small dose. The effects of histamine are outward passage of plasma proteins and fluid into the extracellular spaces, an increase in the flow of lymph and protein content and formation of oedema. H_1 receptors are clearly important for the response. Increased permeability results mainly from actions of histamine on postcapillary venules, where histamine causes the endothelial cells to contract and separate at their boundaries (Goodman-Gilman, 1990), by mediating mostly the H_1 receptors.

The effects of isolated shikonin derivatives, acetyl salicylic acid (reference drug) and time course of oedema and inhibition rate was assessed in rats. The left footpad of rats injected with CFA (Table 2) became swollen gradually for 16 days and reached its maximum peak at 12 day (70.5%) and thereafter declined at 16 day (68.3%). The administration of ethyl acetate extract (25, 50, 100 mg/kg body weight) and isolated phytochemicals at dose of 5.0, 10, 20 mg/kg significantly inhibited the development of joint swelling induced by CFA. The maximum inhibition rate was observed of isovaleryl shikonin (61.2%; Table 2) at the dose of 10 mg/kg at 8 day after CFA injection and maintained until the experiment was term-

Table 1. Effects of isolated shikonin derivatives and acetyl salicylic acid on carrageenan induced paw oedema in rats.

Active sub-fraction or isolated compounds	Dose (mg/kg)*	Oedema rate (%) 1 h	2 h	6 h	8 h	10 h
Active Sub- fraction						
Control	-	38.4 ± 0.04	46.3 ± 0.06	59.4 ± 0.01	63.5 ± 0.07	61.4 ± 0.03
EtOAc	25	20.2 ± 0.04[a] (21.7)	26.5 ± 0.05[a] (35.4)	41.1 ± 0.06[a] (42.3)	48.3 ± 0.07[a] (49.5)	50.3 ± 0.02[a] (40.3)
	50	24.3 ± 0.03[a] (19.5)	30.5 ± 0.06[b] (26.5)	45.4 ± 0.03[a] (45.6)	56.5 ± 0.03[a] (40.5)	59.4 ± 0.05[b] (43.5)
	100	26.4 ± 0.02[a] (22.5)	38.3 ± 0.08[a] (28.5)	48.3 ± 0.07[a] (39.5)	59.3 ± 0.04[a] (46.3)	60.2 ± 0.06[a] (44.4)
Isolated compounds						
β,β-dimethylacryl shikonin	05	23.3 ± 0.04[a] (26.5)	35.6 ± 0.05[a] (30.5)	50.5 ± 0.06[a] (40.5)	56.4 ± 0.08[a] (34.3)	59.4 ± 0.05[a] (45.6)
	10	26.5 ± 0.05[a] (20.3)	41.4 ± 0.07[a] (29.6)	46.2 ± 0.09[a] (54.3)	55.3 ± 0.07[a] (36.7)	54.5 ± 0.03[a] (46.5)
	20	24.6 ± 0.01[a] (33.4)	40.5 ± 0.01[a] (36.3)	49.5 ± 0.04[a] (55.4)	50.5 ± 0.06[a] (49.5)	48.3 ± 0.04[a] (31.7)
Isovaleryl Shikonin	05	28.4 ± 0.01[a] (24.5)	35.4 ± 0.05[a] (45.5)	56.3 ± 0.06[a] (39.8)	43.6 ± 0.05[a] (50.8)	46.3 ± 0.01[a] (35.4)
	10	29.3 ± 0.05[a] (31.2)	38.3 ± 0.06[a] (49.4)	49.3 ± 0.08[a] (40.5)	54.3 ± 0.03[a] (46.2)	50.5 ± 0.08[a] (40.2)
	20	26.5 ± 0.08[a] (38.7)	31.5 ± 0.06[a] (34.6)	45.4 ± 0.07[a] (46.3)	55.5 ± 0.02[a] (45.3)	52.4 ± 0.06[a] (39.5)
β-hydroxyisovaleryl shikonin	05	31.4 ± 0.03[a] (34.1)	39.4 ± 0.05[b] (40.3)	40.5 ± 0.05[a] (48.3)	46.3 ± 0.03[a] (36.5)	44.3 ± 0.07[a] (30.5)
	10	29.2 ± 0.05[a] (21.5)	30.3 ± 0.06[a] (39.1)	41.4 ± 0.09[a] (50.5)	49.5 ± 0.04[a] (38.4)	46.4 ± 0.08[a] (34.3)
	20	27.5 ± 0.08[a] (26.5)	34.3 ± 0.07[a] (35.7)	48.5 ± 0.07[a] (39.4)	40.3 ± 0.03[a] (45.3)	40.6 ± 0.04[a] (39.4)
Shikonin isovalerate	05	29.5 ± 0.04[a] (24.3)	40.5 ± 0.08[a] (45.3)	50.5 ± 0.05[a] (60.5)	56.8 ± 0.07[a] (49.5)	58.4 ± 0.07[a] (35.5)
	10	31.3 ± 0.02[a] (30.5)	39.4 ± 0.07[a] (49.4)	52.4 ± 0.03[a] (58.3)	59.5 ± 0.06[a] (50.8)	60.5 ± 0.04[a] (36.4)
	20	26.5 ± 0.01[a] (29.6)	35.3 ± 0.06[a] (36.3)	49.3 ± 0.04[b] (50.5)	51.8 ± 0.05[a] (54.3)	50.5 ± 0.08[a] (39.8)
Acetyl salicylic acid	05	18.3 ± 0.02[a] (61.5)	19.4 ± 0.07[a] (65.4)	23.4 ± 0.03[a] (60.3)	29.5 ± 0.01[a] (59.8)	27.3 ± 0.07[b] (56.6)
	10	22.5 ± 0.04[a] (59.4)	25.5 ± 0.04[a] (73.2)	28.5 ± 0.04[a] (59.4)	32.4 ± 0.04[a] (54.4)	30.5 ± 0.04[a] (51.4)
	20	14.4 ± 0.08[a] (65.6)	20.5 ± 0.05[a] (66.4)	30.1 ± 0.00[a] (56.4)	33.5 ± 0.02[a] (50.5)	35.6 ± 0.06[a] (55.3)

*mg/kg, body weight; values represent the mean ± SD of six animals for each groups; each value in parentheses indicate the percentage inhibition rate. Statistically significant from control [a] $P < 0.01$ and [b] $P < 0.05$ (Dunnett's't' - test).

Table 2. Effects of isolated shikonin derivatives and acetyl salicylic acid (standard drug) on CFA-induced paw oedema in rats.

Active sub-fraction or isolated compounds	Dose (mg/kg)*	Oedema rate (%) 1 day	2 day	4 day	8 day	12 day	16 day
Active Sub- fraction							
Control	-	40.5 ± 0.01	49.4 ± 0.05	55.5 ± 0.04	61.3 ± 0.08	70.5 ± 0.05	68.3 ± 0.06
EtOAc	25	23.5 ± 0.05[a] (43.5)	29.3 ± 0.06[a] (37.3)	38.4 ± 0.05[a] (35.4)	48.5 ± 0.06[a] (41.3)	60.3 ± 0.04[a] (46.3)	56.4 ± 0.05[a] (45.5)
	50	26.6 ± 0.06[a] (46.5)	31.4 ± 0.08[b] (29.5)	45.6 ± 0.06[a] (38.3)	46.6 ± 0.08[a] (37.5)	62.4 ± 0.03[b] (50.5)	54.3 ± 0.05[b] (36.4)
	100	20.4 ± 0.02[a] (40.2)	26.5 ± 0.03[a] (30.3)	49.3 ± 0.07[a] (40.5)	50.3 ± 0.04[a] (45.3)	59.2 ± 0.06[a] (45.4)	51.4 ± 0.06[a] (33.3)
Isolated compounds							
β,β-dimethylacryl shikonin	05	34.3 ± 0.04[a] (31.3)	29.6 ± 0.05[a] (31.5)	36.5 ± 0.01[a] (49.3)	40.3 ± 0.06[a] (44.3)	46.4 ± 0.05[a] (34.6)	40.4 ± 0.03[a] (50.6)
	10	36.5 ± 0.05[a] (29.6)	30.4 ± 0.07[a] (33.6)	38.2 ± 0.04[a] (40.5)	49.3 ± 0.07[a] (39.7)	56.5 ± 0.03[a] (40.1)	54.5 ± 0.06[a] (30.5)
	20	39.6 ± 0.01[a] (30.5)	35.5 ± 0.01[a] (36.4)	45.4 ± 0.03[a] (35.4)	55.5 ± 0.09[a] (37.5)	59.3 ± 0.02[a] (46.7)	48.3 ± 0.04[a] (31.7)
Isovaleryl shikonin	05	30.4 ± 0.01[a] (34.5)	34.4 ± 0.05[a] (50.5)	39.3 ± 0.06[a] (39.8)	42.6 ± 0.05[b] (41.8)	46.5 ± 0.01[a] (44.3)	45.3 ± 0.01[a] (36.4)
	10	32.2 ± 0.05[a] (41.2)	37.3 ± 0.06[a] (45.4)	40.5 ± 0.08[a] (55.5)	46.3 ± 0.03[a] (61.2)	56.3 ± 0.03[a] (37.2)	54.5 ± 0.08[a] (48.2)
	20	27.5 ± 0.08[a] (39.7)	41.5 ± 0.05[a] (44.6)	46.4 ± 0.05[a] (50.3)	52.5 ± 0.02[a] (48.3)	59.4 ± 0.07[a] (40.5)	60.4 ± 0.06[a] (39.5)
β-hydroxyisovaleryl shikonin	05	31.4 ± 0.03[a] (30.1)	43.4 ± 0.05[b] (35.3)	47.5 ± 0.05[a] (50.3)	55.3 ± 0.03[a] (44.5)	63.3 ± 0.03[a] (31.5)	64.3 ± 0.02[a] (36.5)
	10	33.2 ± 0.05[a] (35.5)	40.3 ± 0.06[a] (46.1)	51.4 ± 0.09[a] (42.5)	53.5 ± 0.04[a] (52.4)	60.4 ± 0.08[a] (35.3)	56.4 ± 0.03[a] (35.3)
	20	35.5 ± 0.01[a] (46.5)	44.3 ± 0.07[a] (39.7)	47.5 ± 0.07[a] (47.4)	50.3 ± 0.03[a] (49.3)	55.6 ± 0.04[a] (49.4)	54.6 ± 0.04[a] (38.4)
Shikonin isovalerate	05	31.5 ± 0.04[a] (45.3)	39.5 ± 0.01[a] (45.5)	42.5 ± 0.05[a] (48.5)	47.8 ± 0.04[a] (33.5)	54.4 ± 0.07[a] (41.5)	46.4 ± 0.07[a] (44.5)
	10	36.3 ± 0.02[a] (38.5)	42.4 ± 0.04[a] (40.4)	47.4 ± 0.03[a] (41.3)	56.5 ± 0.03[a] (36.8)	53.5 ± 0.04[a] (45.4)	55.5 ± 0.04[a] (46.4)
	20	40.5 ± 0.01[b] (40.6)	44.3 ± 0.06[a] (45.3)	43.3 ± 0.04[b] (40.5)	60.8 ± 0.05[a] (34.3)	56.5 ± 0.08[a] (49.8)	51.5 ± 0.08[a] (47.8)
Acetyl salicylic acid	05	24.3 ± 0.02[a] (59.5)	26.4 ± 0.07[a] (61.4)	30.4 ± 0.03[a] (60.5)	36.5 ± 0.01[a] (67.8)	39.3 ± 0.07[b] (55.6)	40.3 ± 0.07[b] (56.5)
	10	23.1 ± 0.04[a] (54.4)	32.5 ± 0.04[a] (70.2)	33.5 ± 0.04[a] (56.4)	35.4 ± 0.04[a] (64.4)	37.5 ± 0.04[a] (56.4)	38.5 ± 0.04[a] (68.4)
	20	29.4 ± 0.08[a] (50.6)	36.5 ± 0.05[a] (65.4)	35.1 ± 0.00[a] (66.4)	37.5 ± 0.02[a] (60.5)	38.6 ± 0.06[a] (53.3)	36.6 ± 0.06[a] (49.3)

*mg/kg, body weight; values represent the mean ± SD of six animals for each groups; each value in parentheses indicate the percentage inhibition rate. Statistically significant from control [a] $P < 0.01$ and [b] $P < 0.05$ (Dunnett's't' – test)

Table 3. MICs of sub-fraction, reference compounds and isolated shikonin derivatives against selected bacteria and fungi.

Active sub-fraction or isolated compounds	MIC (µg / ml)										
	Ec	Ecl	Kp	Sa	Bs	Sp	An	Af	Rp	Pc	Ca
Active sub- fraction											
EtOAc	>80	>100	50	40	>80	50	80	>100	70	>60	50
Standard compounds											
Tetracycline	1.75	2.50	2.75	2.00	2.75	3.50	-	-	-	-	-
Gentamycine	2.50	4.50	3.75	4.00	3.00	3.75	-	-	-	-	-
Nystatin	-	-	-	-	-	-	7.00	7.50	6.00	8.70	6.75
Isolated compounds											
β,β-dimethylacryl shikonin	50	>80	40	>35	55	>60	>85	90	75	60	>65
Isovaleryl shikonin	45	>60	60	>70	>80	>90	80	75	>100	90	>80
β-hydroxyisovaleryl shikonin	60	70	>80	>90	40	50	90	>100	80	>70	>80
Shikonin isovalerate	>40	20	40	30	>60	40	>100	100	90	>90	70

Used microorganisms: Ec = *Escherichia coli*; Ecl = *Enterobacter cloacae*; Kp = *Klebsiella pneumonie*; Sa = *Staphylococcus aureus*; Bs = *Bacillus subtilis*; Sp = *Streptococcus pneumoniae*; An = *Aspergillus niger*; Af = *Aspergillus flavus*; Rp = *Rhizoctonia phaseoli*; Pc = *Penicillium chrysogenum*; Ca = *Candida albicans*.

nated on on day 16.CFA-induced arthritis has been used extensively in studies of behavioral pain responses (Choi et al., 2002; Akkol et al., 2009). It seems that bacterial peptidoglycan and muramyl dipeptide are responsible for its induction (Crofford and Wilder, 1993). However, superoxide released by PMNs or IL-1 released by activating macrophages play an important role in the development of chronic arthritis (Yoshikawa et al., 1985; Jadot et al., 1986; Tsai and Lin, 1999). Our results showed that isolated compounds significantly inhibited the devel-opment of chronic joint swelling induced by CFA through stop the release of IL-1 by activation of macrophages.

The MICs of ethyl acetate extract, isolated compounds to the selected micro-rganisms were determined and compared with commercially available antibiotics (Table 3). Tetracycline showed antibacterial activity at all tested concentrations (1.0 – 5.0 μg/ml) and nystatin was most potent at 5.0 to 10.0 μg/ml. The isolated compounds were tested at various doses from 10 to 100 μg/ml. The strong antibacterial activity was showed by shikonin isovalerate at 20 μg/ml against *E. cloacae* and simultaneously β,β-dimethylacryl shikonin demonstrated maximum antifungal activity against *C. albicans* at the dose of > 65 μg/ml. The moderate antibacterial and antifungal activity was exhibited by the all isolated compounds.

The present work was undertaken to evaluate *in vitro* antimicrobial activity of different isolated compounds from *A. hispidissima*. The tested compounds showed antibacterial and/or antifungal activity with varying degrees of potency, suggesting that the antimicrobial activity of shikonin derivatives is most common. Since the compounds are more hydrophobic than the conventional naphthaquinones they may easily penetrate through the cell membrane. The naphthaquinone compounds like as shikonin has many biological activities (Farnsworth and Cordell, 1976). The cytoplasmic membrane of bacteria has two fold principal functions (a) barrier functions and energy transduction which allow the membrane to form ion gradients that can be used to drive various processes, and (b) formation of matrix for membrane embedded proteins (Ultee, 1999).

Exploring the healing power of plants is an ancient concept. For many centuries, people have been trying to alleviate and treat diseases with different plant extracts and formulations (Cowan, 1999). The medicinal plants constitute an effective source of both traditional and modern medicine. World Health Organization (WHO) encourages countries to examine traditional medicine with a view to identifying and exploiting aspects that provide safe and effective remedies for different diseases (Akinyemi et al., 2005). This study validates and documents in a systematic way, the antimicrobial properties of large number of plants used for many years by people of India. It also provides valuable information for shikonin and alkannin derivatives have been identified as bioactive constituents of *Alkanna tinctoria* and *Lithospermum erythrorhizon* (Papageorgiou et al., 1999; Chien Chang et al., 2002). In conclusion, our study has verified that shikonin derivatives **I**, **II**, **III** and **IV** isolated from *A. hispidissima* possess anti-inflammatory and antimicrobial activities. These novel bioactivities would provide greater insight into the medicinal value and therapeutic use of shikonin derivatives. There are still many *A. hispidissima* naphthaquinones and their derivatives, whose pharmacological activities have not yet been investigated. It is possible that they may contain beneficial pharmacological properties. Therefore, *in vivo* and *in vitro* investigations regarding their effects could provide insight into the benefits of *A. hispidissima* for future clinical management of many human diseases.

Conflict of interest

The authors declare no conflict of interest.

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References

Akineyemi KO, Oladapo O, Okwara CE, Ibe CC, Fasure KA. (2005). Screening of crude extracts of six medicinal plants used in South West Nigeria Unorthodox medicine for anti-methicillin resistant *Staphylococci aureus* activity. *Complementary and Alternative Medicine* 5, 6-9.

Akkol EK, Koca U, Pesin I, Toker G, Yesilada E. (2009). Exploring the wound healing activity of *Arnebia densiflora* (Nordm.) Ledeb. by *in vivo* models. *Journal of Ethnopharmacology* 124, 137-141.

Annon (1979). Traditional Chinese Dictionary. Sanghai Science and Technology, Sanghai, China.

Anonymous (1985). The Wealth of India: Raw Materials. CSIR and PID, New Delhi, India. Antonio MA, Souza Brito AR. (1998). Oral anti-inflammatory and multi-ulcerogenic activities of a hydroalcoholic extract and partitioned fractioned fractions of *Turnera ulmifolia*. *Journal of Ethnopharmacology* 61, 215-228.

Boktapa NR, Sharma AK. (2010). Wild medicinal plants used by local communities of Manali, Himachal Pradesh, India. *Ethnobotanical Leaflets* 14, 259-264.

Chang MR, Cury AE. (1998). Amphotericin - B- metronidazole combination against *Candida* species. *Revista Iberoamericana de Micologia* 15**,** 78-80.

Chaudhary A, Pal M. (2010). Induction of shikonin production in hairy root cultures of *Arnebia hispidissima* via *Agrobacterium rhizogenes* mediated genetic transformation. *Journal of Crop Science and Biotechnology* 13, 99-106.

Chien Chang S, Wan Jr. S, Shyh-Yuan L, Chia-Hung L, Gum-Hee L, Chang Ming S. (2002). Antimicrobial activities of naphthazarines from *Arnebia decumbens. Journal of Natural Products* 65, 1857-1862.

Choi J, Huh K, Kim SH, Lee KT, Lee HK, Park H J. (2002). Kalopanax saponin A from *Kalopanax pictus* a potent antioxidant in rheumatoidal rat treated with Freund's complete adjuvant reagents. *Journal of Ethnopharmacology* 79, 113-118.

Chopra RN, Nayar SL, Chopra IC. (1956). Glossary of Indian Medicinal Plants. CSIR, New Delhi, India.

Cowan MM. (1999). Plants products as antimicrobial agents. *Clinical Microbiology Review* 14, 564-584.

Crofford LJ, Wilder RL. (1993). Arthritis and Autoimmunity in Animals. Lea and Febiger, London, pp. 525-539.

Deng R, Tang J, Xie B, Feng GK, Hung YH, Liu ZC, Zhu XF. (2010). Syung-16, a newly synthesized alkannin derivative, induces tumor cells apoptosis and suppresses tumor growth through inhibition of PKB/AKT kinase activity and blockade of AKT/FOXO signal pathway. *International Journal of Cancer* 127, 220-228.

El-Dahmy S, Ghani AA, Al-Azhar, (1995). Alkaloids of *Arnebia decumbens. Journal of Pharmaceutical Sciences* 15, 24-28.

El-Sayed MA. (2010). Effect of *Arnebia hispidissima* and *Echium rauwolfii* ethanolic root extracts on growth, forage quality and two rhizospheric soil fungi of pigeon pea. *Journal für Verbracherschutz und Lebensmittelsicherheit* 5, 145-150.

Farnsworth NR, Cordell GA. (1976). A review of some biologically active compounds isolated from plants as reported in the 1974-1975 literature. *Lioydia* 39, 420-455.

Fu S, Shang T, Xiao P. (1984). Analysis of naphthaquinone pigments in some Chinese medicinal ″Zi Cao″. *Yaoxue Xuebao* 19, 921-926.

Gamila W, Bassem EM, Atta S, Gama M. (1987). Toxic pyrrolizidine alkaloids of certain Boraginaceous plants. *Acta Pharmaceutica Suecica* 24, 199-204.

Gao J. (1986). A general review of resources, chemistry, pharmacology and clinical application of Gromwell. *Zhongcaoyao* 17, 28-31.

Goodman-Gilman A. (1990). In: Hardman JG, Limbird LE. (eds.). The Pharmacological Basis of Therapeutics. McGraw Medical Publ. Division, New York, USA, pp. 575-582.

Hamdard ME, Badar Y, Khan MSY. (1988). Revised phytochemical study of *Arnebia hispidissima. Pakistan Journal of Pharmaceutical Sciences* 1, 19-23.

Jadot G, Michelson AM, Puget K. (1986). Anti-inflammatory activity of superoxide dismutase: Studies on adjuvant-induced polyarthritis in rats. *Free Radical Research Communications* 2, 27-42.

Jain SC, Singh B, Jain R. (1999). Arnebins and antimicrobial activity of *Arnebia hispidissima. Phytomedicine* 6, 474-476.

Jain SK, Defilipps RA. (1991). Medicinal Plants of India. Reference Publ. Inc., Michigan.

Jones RN, Barry AL. (1987). The antimicrobial activity of A-56268 (TE-031) and roxithromycin (RU 965) against *Legionella* using broth microdilution method. *Journal of Antimicrobial Chemotherapy* 19, 841-842.

Kaith BS, Kaur M. (1996). Anti-inflammatory effect of *Arnebia euchroma* root extracts in rats. *Journal of Ethnopharmacology* 55, 77-80.

Kashiwada Y, Nishizawa M, Yamagishi T, et al. (1995). Anti-AIDS agents. 18. Sodium and potassium salts of caffeic acid tetramers from *Arnebia euchroma* as anti-HIV agents. *Journal of Natural Products* 58, 392-400.

Khan HA, Chandrashekharan I, Ghanim A. (1983). Naphthazarines from *Arnebia hispidissima. Phytochemistry* 22, 614-615.

Kirimer N, Bozan B, Baser KHC. (1995). A new naphthaquinone from the roots of *Arnebia densiflora*. *Fitoterapia* 66, 499-402.

Kirtikar KR, Basu BD. (1967). Indian Medicinal Plants. Popular Book Depot, Bombay, India.

Mao X, Yu CR, Li WH, Li WX. (2008). Induction of apoptosis by shikonin through a ROS/JNK-mediated process Bcr/Abl–positive chronic myelogenous leukemia (CML) cells. *Cancer Research* 18, 879-886.

Murashige T, Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15, 473-497.

Newbould BB. (1963). Chemotherapy of arthritis-induced in rats by mycobacterial adjuvant. *British Journal of Pharmacology* 21, 127-136.

Nigam SK, Mitra CR. (1964). Coloring matter of *Arnebia hispidissima. Indian Journal of Applied Chemistry* 27, 34-36.

Pal M, Chaudhary A. (2010). High frequency direct plant regeneration, micropropagation and shikonin induction in *Arnebia hispidissima. Journal of Crop Science and Biotechnology* 13, 13-19.

Papageorgiou VP, Assimopoulou AN, Couladouros EA, Hepworth D, Nicolaou KC. (1999). The chemistry and biology of alkannin, shikonin and related naphthazarine natural products. *Angewandte Chemie International Edition* 38, 270-300.

Roeder E, Rengel-Meyer B. (1993). Pyrrolizidine alkaloids from *Arnebia euchroma*. *Planta Medica* 59, 192-196.

Romanova AS, Bankovskii AI, Boryaev KI. (1968). Extraction of shikonin. *United States of Soviet Replubic* 200, 737-743.

Salim ML, Ammar HA, Oriquat GA. (1996). Isolation and structure elucidation of 3, 6-dihydroxy-2-isovaleryl-1, 4-benzoquinone. *Bulletin of Faculty of Pharmacy* 34, 231-236.

Salman S, Kumbaser S, Ozgen U, Erdogan F, Suleyman H. (2009). Contraceptive effects of *Onosma armeniacum* on embryo implantation in rats. *Cell Membrane and Free Radical Research* 1, 90-94.

Sankawa U, Ebizuka Y, Miyazaki T, Isomura Y, Otsuka H, Sibata S, Inomata M, Fukuoka F. (1977). Antitumor activity of shikonin and its derivatives. *Chemical and Pharmaceutical Bulletin* 25, 2392-2397.

Sharma N, Sharma UK, Gupta AP, Sinha AK. (2009). Simultaneous densitometric determination of shikonin, acetyl shikonin and β-acetoxyisovaleryl shikonin in ultrasonic assisted extracts of four *Arnebia* species using reversed phase TLC. *Journal of Separation Sciences* 32, 3239-3245.

Shimomura K, Sudo H, Saga H, Kamada H. (1991). Shikonin production and secretion by hairy root cultures of *Lithospermum erythrorhizon*. *Plant Cell Reports* 10, 282-285.

Shukla YN, Tandon JS, Dhar MM. (1973). Arnebin-7, a new naphthaquinone from *Arnebia nobilis. Indian Journal of Chemistry* 11, 528-529.

Singh B, Sahu PM, Jain SC, Singh S. (2004). Estimation of naphthaquinones from *Arnebia hispidissima* (Lehm.) DC. *in vivo* and *in vitro*. I. Anti-inflammatory screening. *Phytotherapy Research* 18, 154-159.

Song Y, Yang XN, Yang SW, Geng DS, Chen HS. (2010). Three new compounds from *Arnebia euchroma. Journal of Asian Natural Products Research* 12, 286-294.

Srivastav A, Shukla YN, Kumar S. (1999). Chemistry and pharmacology of the genus *Arnebia* – a review. *Journal of Medicinal and Aromatic Sciences* 21, 1131-1142.

Staniforth V, Wang S-Y, Shyur L-F, Yang N-S. (2004). Shikonins, phytocompounds from *Lithospermum erythrorhizon*, inhibit the transcriptional activation of human Tumor Necrosis Factor ∝ promoter *in vivo*. *The Journal of Biolgical Chemistry* 279, 5877-5885.

Tanaka S, Kuwai Y, Staba M. (1986). A comparative study on anti-inflammatory activities of enantiomers, shikonin and alkannin. *Journal of Natural Products* 49, 466-473.

Trivedi PC. (2005). Ethnomedicinal Plants. Pointer Publishers, Jaipur, India.

Tsai CC, Lin CC. (1999). Anti-inflammatory effects of Taiwan folk medicine 'Teng-Khia-U' on carrageenan and adjuvant-induced paw oedema in rats. *Journal of Ethnopharamcology* 64, 85-89.

Ultee A, Kets EP, Smid EJ. (1999). Mechanism of action of carvacol on the food-borne pathogen *Bacillus cereus. Applied and Environmental Microbiology* 65, 4606-4610.

Vinegar R, Traux JF, Selph J H, Johnston PR, Vinable AH, Mckenzie RK. (1987). Pathway to carrageenan-induced inflammation of the hind limb of the rat. *Federation Proceedings* 6, 118-126.

Winter CA, Risley CA, Nuss CW. (1962). Carrageenan-induced oedema in hind paw of the rats as an assay for anti-inflammatory drugs. *Proceedings of Society for Experimental Biology and Medicine* 114, 544-551.

Xiong W, Luo G, Zhou L, Zeng Y, Yang W. (2009). *In vitro* and *in vivo* antitumor effects of acetyl shikonin isolated from *Arnebia euchroma* (Royle) Johnst. (Ruanzicao) cell suspension cultures. *Chinese Medicine* 4, 14-19.

Yang MH, Blunden G, O'Neil MJ, Lewis JA. (1992). Tormentic acid and 2∝-hydroxyursolic acid from *Arnebia euchroma*. *Planta Medica* 58, 227-234.

Yao, X.S., Ebizuka, Y., Noguchi, H., Kiuchi, F., Iitaka, Y., Sankawa, U., 1983. Structure of arnebinol, a new ansa type monoterpenyl benzoid with inhibitory effect to the prostaglandin biosynthesis. *Tetrahedron Letters* 24, 2407-2410.

Yao XS, Ebizuka Y, Noguchi H, Kiuchi F, Shibuya M, Iitaka Y, Seto H, Sankawa U. (1991a). Biologically active constituents of *Arnebia euchroma*: Structure of arnebinol an ansa type –

monoterpenyl benzoid with inhibitory activity on prostaglandin biosynthesis. *Chemical and Pharmaceutical Bulletin* 39, 2956-2961.

Yao XS, Ebizuka Y, Noguchi H, Kiuchi F, Shibuya M, Iitaka Y, Seto H, Sankawa U. (1991b). Biologically active constituents of *Arnebia euchroma*: Arnebinone and arnebifuranone. *Chemical and Pharmaceutical Bulletin* 39, 2962-2964.

Yoshikawa T, Tanaka H, Kondo M. (1985). The increase of lipid peroxidation in rat adjuvant arthritis and its inhibition by superoxide dismutase. *Biochemistry and Medicine* 33, 320-326.

Zhu F, Lu F, Xiang G. (1984). Isolation of shikonin and its derivatives by HPLC. *Sepu* 1, 131-136.

