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Full Length Research Paper

Bioassay guided isolation and identification of anti-inflammatory and anti-microbial compounds from *Urtica dioica* L. (Urticaceae) leaves

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The present study describes the anti-inflammatory, anti-microbial activity and lipophilic profile with acute toxicological studies of *Urtica dioica.* Successive extraction of the leaves with organic solvents of increasing polarity and their screening for anti-inflammatory and anti-microbial activity was assessed. Hexane extract showed good anti-inflammatory and anti-microbial activity; hence it was further fractionated using open silica gel column chromatography into 19 sub fractions which were pooled together according to their thin layer chromatography (TLC) profile to give an overall 5 fractions. Among the 5 fractions, fraction-II (FII) at a dose of 200 mg/kg body-weight (bw) exhibits equipotent anti-inflammatory activity (48.83% after 3 h) as that of the standard drug indomethacin (53.48%) in Wistar rats. FII also showed a potent anti-microbial activity against all the tested bacterial strains and its minimum inhibitory concentration (MIC) value was 125, 15.62, 31.25, 250, 31.25, 125 and 7.81 µg/ml against *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Shigella flexneri* and *Salmonella typhi*, respectively which was determined by serial tube dilution method. FII was subjected to gas chromatography-mass spectrometry (GC-MS) analysis in search of potent anti-inflammatory and anti-microbial compound(s). 2,4-Di-t-butylphenol (4.56%), neophytadiene (26.97%), butyl tetradecyl ester (9.53%), dibutyl phthalate (7.45%), bis(2-ethyl hexyl) maleate (8.80%), 1,2-benzenedicarboxylic acid (9.89%) and 2-tert-butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl)phenol (3.19%) were the major constituents responsible for both anti-inflammatory and anti-microbial activity of hexane extract of *U. dioica*. Sub-acute oral toxicity of crude n-hexane extract of *U. dioica* was carried out in Wistar rats at doses of 250, 500, 1000 and 2000 mg/kg bw to assess the safety index. Hematological parameters from blood and other biochemical parameters from serum confirmed its safety at tested concentrations. Our results corroborate the anti-inflammatory and anti-microbial activity of *U. dioica*, and could justify its use in folk medicine for the treatment of rheumatic arthritis and other infectious diseases.

Key words: *Urtica dioica*, anti-inflammatory, anti-microbial, minimum inhibitory concentration (MIC), gas chromatography-mass spectrometry (GC-MS), toxicity.

INTRODUCTION

Inflammatory diseases including different types of rheumatic diseases are very common and affects 1% of the adult population worldwide (Gabriel, 2001). It leads to significant disability and a consequent reduction in quality of life, which have a substantial socio-economic impact (Buch and Emery, 2002). Inflammatory diseases are currently treated with steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) (Langman, 1998).

Unfortunately, both of these widely prescribed drug

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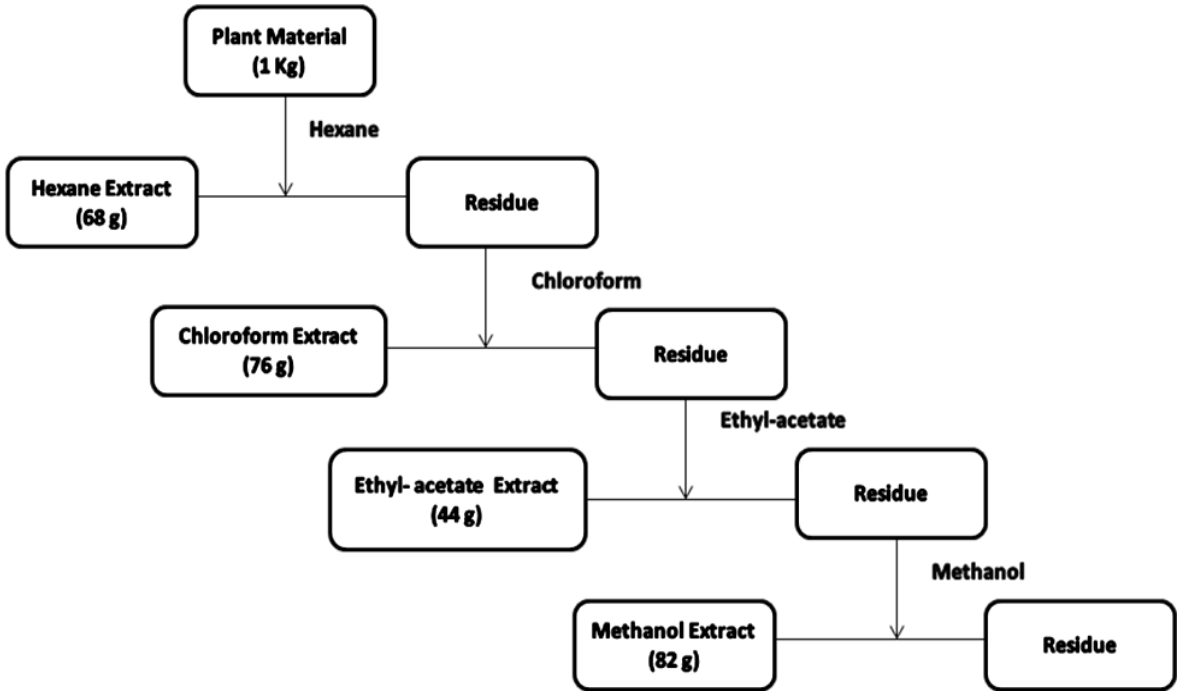


Figure 1. The process of plant extraction.

classes have significant negative side effects, reducing their use in certain segments of the population (Juni et al., 2005; Pathak et al., 2005). Besides, infectious diseases are the main cause of morbidity and mortality particularly in immunocompromised patients (Black et al., 1982). In the last decade, the clinical efficacy of many synthetic antibiotics has been threatened by the emergence of multi- drug resistant pathogens (Eldeen et al., 2005).

Medicinal plants have been a source of wide variety of biologically active compounds for many centuries and have been used extensively as crude material or as pure compounds for treating various disease conditions. According to World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (Anonynous, 1993). It is estimated that today, plant materials are present in, or have provided the models for 50% Western drugs (Baker et al., 1995). The primary benefit of using plant-derived medicine is that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatments. The search for better alternative anti-inflammatory and anti-microbial drugs from the bounties of our vegetation is thus a worthwhile venture.

Urtica dioica L., known as “stinging nettle, common nettle, or greater nettle” in English and “Soi” in Kashmiri, is a medicinal herb with a long history of use. *U. dioica* has been used in homeopathy since the end of the 19th century for allergies, kidney stones, burns, anemia, rashes, internal bleeding, diabetes, etc. (Vavilova, 1994). However, only a few of these pharmacological activities have been experimentally proved (Lourdes et al., 2008). Recently, anti-oxidant (Abdullin et al., 2002), anti-hyperglycemic (Bnouham et al., 2003), anti-proliferative (Durak et al., 2004) and anti-dandruff (Hadizadeh et al., 2009) activity of *U. dioica* were reported. The known phytochemicals of *U. dioica* include lignans, flavonoids, fatty acids, sterols, polysaccharides, glycoproteins, carotenoids, plastocyanins, and lectins (Sajfrtova et al., 2005).

In the present study, we summarize the anti-inflammatory activity by carrageenan-induced inflammation in the rodent paw which presents a classic model of edema formation and hyperalgesia, as well as the anti-microbial activity of leaf extracts and column eluted n-hexane sub fractions of *U. dioica*.

MATERIALS AND METHODS

Collection and extraction of plant material

U. dioica leaves were collected from the local hills of the University of Kashmir, India. Voucher herbarium specimen was deposited in the Kashmir university Herbarium (*KASH* 28100), Centre of Biodiversity and Taxonomy (CBT), Department of Botany, University of Kashmir. The leaves were air dried, grinded to obtain coarse powder and subjected to solvent extraction as per Figure 1 (Singh et al., 2012). Briefly, the powder (1 kg) was extracted successively with hexane, chloroform, ethyl acetate and methanol in Soxhlet apparatus for 24 h. The extracts were filtered over

Whatman No. 1 paper and the organic solvent extracts were concentrated under vacuum using Heidolph rotary evaporator. All the extracts were stored at 4°C until used for the anti-inflammatory and anti-microbial assay.

Antimicrobial susceptibility test

All the solvent extracts were screened against a total of seven bacterial strains. The test organisms were *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Klebsiella-pneumoniae* (ATCC 15380), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Shigella flexneri* (ATCC 29903) and *Salmonella typhi* (ATCC 19430). The disc diffusion method (Bauer et al., 1966) was used to screen the anti-microbial activity. Sterile filter paper discs with 6 mm diameter were impregnated with 2000 µg (dissolved in 8 µl DMSO) of each extract as per standard procedures (Chandrasekaran and Venkalesalul ., 2004). Gentamycin (30 µg/disc) was used as a positive control and a paper disc impregnated with DMSO (8 µl) was used as negative control. The nutrient agar plates were prepared for disc diffusion assay and each bacterial strain at a concentration of 1.5×10^6 cells/ml (adjusted to the 0.5 McFarland turbidity standards) was used (NCCLS, 2005). All experiments were performed in triplicates and results were recorded by measuring the zones of growth inhibition around the discs.

Bioassay guided isolation of active compound(s)

The hexane extract of *U. dioica* (*HEUD*, 50 g) was fractionated using silica gel 60 (0.063 to 0.200 mm) column chromatography (CC). The column was eluted with a solvent gradient of hexane-ethyl acetate (EtOAc) in 100:0 and 0:100 ratios to give 19 fractions (each of 250 ml) as follows:

10% EtOAc – hexane (7 fractions)
20% EtOAc – hexane (5 fractions)
30% EtOAc – hexane (2 fractions)
50% EtOAc – hexane (3 fractions)
100% EtOAc (2 fractions)

Solvents were distilled prior to use. Nineteen (19) fractions were collected, analyzed by thin layer chromatography (TLC) on silica gel 60 PF_{254} (Merck) aluminium sheets and pooled together due to similarity in TLC profile to give overall 5 sub fractions: FI, FII, FIII, FIV, and FV. The sub fractions were tested for both anti-inflammatory and anti-microbial activity by standard assays.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined by serial tube dilution technique (Rahman et al., 2000). Ten serial dilutions of stock, ranging from concentration of 1000 µg to 0.97 µg/ml were prepared in the test tubes. The tubes were inoculated with 100 µl of bacterial strain inoculums at a concentration of 10^6 cell/ml. Standard antibiotic gentamycin was included in the assay for comparison. Nutrient broth with the inoculums only was used as growth control. All experiments were carried out in triplicates. The tubes were incubated aerobically at 37°C for 12 to 18 h; after which 50 µl of 0.2 mg/ml 2-(4-iodophenyl)3-(4-nitrophenyl)-5 phenyl-tetrazolium chloride (INT) solution was added to each test tube, the tubes were tested for color change (Anders et al., 2002). The concentration at which a decrease in red color is apparent compared to the next dilution was taken as MIC value. Bacterial growth is indicated by the red color of INT reduced to formazan.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was carried out by using Perkin Elmer-Clarus 500 GC-MS with PE-5 (equivalent to DB-5) capillary column (length, 30 m) and helium as carrier gas (flow rate- 3.3 ml/min). Samples were analyzed with the column held initially at 100°C for 1 min after injection, then increased to 170°C with 10°C/min programme rate and increased to 215°C with 5°C/min heating ramp for 5 min. Final temperature was increased to 270°C, with 10°C/min heating ramp for 10.5 min. The injections were performed in split mode (30:1) at 250°C. Detector and injector temperatures were 260 and 250°C, respectively. Pressure was established as 50 psi. Run time was 60 min. Temperature and nominal initial flow for flame ionization detector (FID) were set as 250°C and 3.1 ml/min, correspondingly. The constituent compounds were determined by comparing their retention times and mass weights with those of authentic samples obtained by GC as well as the mass spectra from the Wiley and Nist database searches by GC-MS.

Biological studies

Animals

Wistar rats weighing 150 to 200 g were used in the experiments, taking into account international principles and local regulations concerning the care and use of laboratory animals. The animals had free access to a standard commercial diet and water *ad-libitum* and were kept in the animal house at an ambient temperature of 25°C and 45 to 55% relative humidity, with a 12 h light/dark cycle. Animal experimental protocols were in accordance with the recommendations of the institutional animal ethical committee (Olfert et al., 1993).

Anti-inflammatory assay

The anti-inflammatory activity of all the solvent extracts and hexane sub fractions were screened by rat paw edema anti-inflammatory assay (Winter et al., 1962). The rats of both sexes were divided into six groups of 6 rats each. Group I received normal saline and served as the control. Groups II, III, IV, and V were orally gavaged at 200 mg/kg body-weight (bw) of the hexane, chloroform, ethyl acetate and methanol extract of *U. dioica* respectively. Group VI received the standard drug indomethacin (10 mg/kg bw). Paw edemas were induced by subcutaneous injection of 100 µl of 1% carrageenan solution (Sigma) (w/v solution in saline, 0.9% NaCl) in the plantar aponeurosis of the right hind paw. One hour after carrageenan injection, edemas were measured first at zero and then at 1, 2, 3, and 24 h after administration of drugs by a volume displacement method using a digital plethysmometer (Ugo Basile, Italy). The percentage inhibition of edema compared with that of the control was taken as anti-inflammatory activity (Perez, 1996). The percentage inhibition of edema was calculated by the formula (Ahmed et al., 1993; Perez, 1996):

Percentage inhibition of edema = $[(A - B) / A] \times 100$

Where, A represents the paw volume of the control group at corresponding time and B represents the paw volume of the test group at the same time.

Acute toxicity studies

Thirty Wistar rats without any sex discrimination were used in the experiment. Animals were divided into five groups of six rats in each group. The first group served as a control group and the remaining four groups (A, B, C and D) received the doses of 250, 500, 1000

Table 1. Antibacterial activity of different solvent extract of leaves of *Urtica dioica.*

Microorganisms (DIZ, mm)	Extract treated					
	He	Ch	Ea	Mt	C	Sd
Enterococcus faecalis (ATCC 29212)	++	-	-	-	-	+++
Escherichia coli (ATCC 25922)	++	+	-	-	-	+++
Klebsiella pneumoniae (ATCC 15380)	++	+	-	-	-	+++
Pseudomonas aeruginosa (ATCC 27853)	++	-	-	-	-	+++
Salmonella typhi (ATCC 19430)	++	+	-	-	-	+++
Shigella flexneri (ATCC 29903)	++	+	-	-	-	+++
Staphylococcus aureus (ATCC 25923)	++	+	-	-	-	+++

He: Hexane extract, Ch: Chloroform extract, Ea: Ethyl-acetate extract, Mt: Methanol extract, C: Control (dimethyl sulphoxide, DMSO), Sd: Standard (gentamycin).The results were represented as inactive (-, inhibition zone diameter ≤ 7mm), moderate activity (+, inhibition zone diameter ≤ 10 mm), good activity (++, inhibition zone diameter ≤ 15 mm) and very good activity (+++, inhibition zone diameter > 15 mm).

and 2000 mg/kg bw respectively of *HEUD* for 14 consecutive days by gavaging (Mosaddik and Haque, 1999). Animals were sacrificed on the 15th day and blood was collected by cardiac puncture to perform various hematological and biochemical parameters. All the hematological and biochemical parameters were determined using standard procedures and reagents supplied by Diagnostic kit (Siemens Medical Solution Diagnostics Ltd, India).

Statistical analysis

Results were expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Dunnett's t test was applied for statistical analysis. $P<0.01$ was considered significant and $p< 0.001$ was considered highly significant.

RESULTS

Anti-microbial susceptibility test

All four solvent extracts of *U. dioica*, that is, hexane, chloroform, ethyl acetate and methanol were tested for anti-microbial activity against seven strains of both Gram positive and Gram negative bacteria viz., *E. faecalis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Staphylococcus aureus*, *S. flexneri* and *S. typhi*. The anti-microbial activity produced by the *HEUD* was comparable to that of the standard antibiotic gentamycin (Table 1).

Bioassay guided isolation of active compound(s)

The *HEUD* showed good anti-microbial activity, hence, it was further fractionated using CC into 19 sub fractions (Figure 2) of 250 ml each which were pooled together according to their TLC profile to give overall 5 sub fractions- FI, FII, FIII, FIV and FV. The sub fractions were tested for anti-microbial activity by disc diffusion assay and FII showed potent anti-microbial activity as compared to other fractions (Table 2).

Minimum inhibitory concentration (MIC)

The MIC values of the most active sub fraction (FII) of *HEUD* were 125, 15.62, 31.25, 250, 31.25, 125 and 7.81 µg/ml against *E. faecalis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. flexneri* and *S. typhi* respectively (Table 3).

GC-MS analysis

The most active sub fraction (FII) of *HEUD* was subjected to GC-MS analysis in search of active compounds (Figure 3). GC-MS analyzed eight major compounds (Table 4) viz., tributyl ester (3.56%), 2,4-di-t-butylphenol (4.56%), neophytadiene or 2,4,10-trimethyl,14-ethylene-14-pentadecne (26.97%), butyl tetradecyl ester (9.53%), dibutyl phthalate (7.45%), bis(2-ethyl hexyl) maleate (8.80%), 1,2-benzenedicarboxylic acid (9.89%) and 2-tert-butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl) phenol (3.19%) which constitute 73.95% of the total peak area percentage.

Twelve minor compounds constituting 20.76% of the total peak area were also analyzed by the GC-MS which are 8-methylheptadecane (1.62%), 1-heptadecene (1.62%), eicosane (2.86%), 3, 7, 11,15-tetramethyl-2-hexadecyl ester (2.06%), 2,6,10,15-tetramethyl-heptadecane (0.90%), olean-18-ene (1.97%), 3,5-di-tert-butyl-ortho-benzoquinone (1.08%), 2,6,10,14-tetramethyl pentadecane (1.06%), heneicosane (2.00%), hexacosane (1.93%), nonacosane (2.30%) and pentacosane (1.36%).

Biological studies

Anti-inflammatory assay

The results of anti-inflammatory activity of all the four solvent extracts along the five column eluted sub

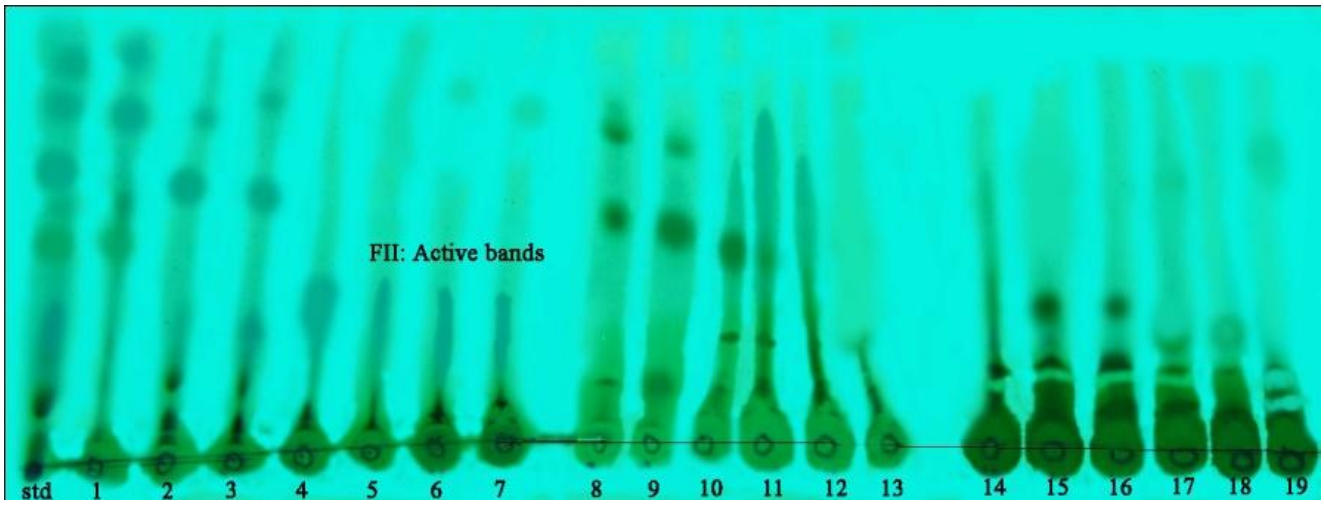


Figure 2. TLC separation of *HEUD* using hexane: EtOAc as mobile phase.

Table 2. Antibacterial activity of different column eluted fractions of hexane extract of *U. dioica* (*HEUD*).

Microorganism (DIZ, mm)	Extract treated						
	FI	FII	FIII	FIV	FV	C	Sd
Enterococcus faecalis (ATCC 29212)	-	+++	+	+	-	-	+++
Escherichia coli (ATCC 25922)	-	+++	+	+	+	-	+++
Klebsiella pneumoniae (ATCC 15380)	-	+++	+	+	+	-	+++
Pseudomonas aeruginosa (ATCC 27853)	-	++	+	-	-	-	+++
Salmonella typhi (ATCC 19430)	-	+++	+	+	+	-	+++
Shigella flexneri (ATCC 29903)	-	+++	+	+	+	-	+++
Staphylococcus aureus (ATCC 25923)	-	+++	+	+	+	-	+++

FI: Combined fractions 1-4, FII: Combined fractions 5-7, FIII: Combined fractions 8-10, FIV: Combined fractions 11-14, FV: Combined fractions 15-19, Sd: Standard; gentamycin and C: control; DMSO. The results were represented as inactive (-, inhibition zone diameter ≤ 7 mm), moderate activity (+, inhibition zone diameter ≤ 10 mm), good activity (++, inhibition zone diameter ≤ 15 mm) and very good activity (+++, inhibition zone diameter > 15 mm).

Table 3. The minimum inhibitory concentration (MIC) of FII of hexane extract of *U. dioica* (*HEUD*).

Tested microorganism	MIC range (µg/ml)	Gentamycin (µg/ml)
Enterococcus faecalis (ATCC 29212)	125	< 7.81
Escherichia coli (ATCC 25922)	15.62	< 7.81
Klebsiella pneumoniae (ATCC 15380	31.25	< 7.81
Pseudomonas aeruginosa (ATCC 27853)	250	< 7.81
Staphylococcus aureus (ATCC 25923)	31.25	< 7.81
Shigella flexneri (ATCC 29903)	125	< 7.81
Salmonella typhi (ATCC 19430)	7.81	< 7.81

fractions of *HEUD* were evaluated (Table 5). There was a gradual increase in edema paw volume of rats in the control group. However, in the test groups, hexane extract (200 mg/kg bw) showed a significant inhibition of 46.51% ($P<0.01$) in the edema paw volume after 3 h of treatment and was comparable to that of standard indomethacin (10 mg/kg bw) which showed a significant inhibition of 53.48% ($P<0.01$). There was no significant inhibition of inflammation ($P>0.05$) in case of test groups treated with chloroform and ethyl acetate extracts. Among the five column eluted fractions of *HEUD*, FII showed a significant inhibition of 48.83% ($P<0.01$) after 3

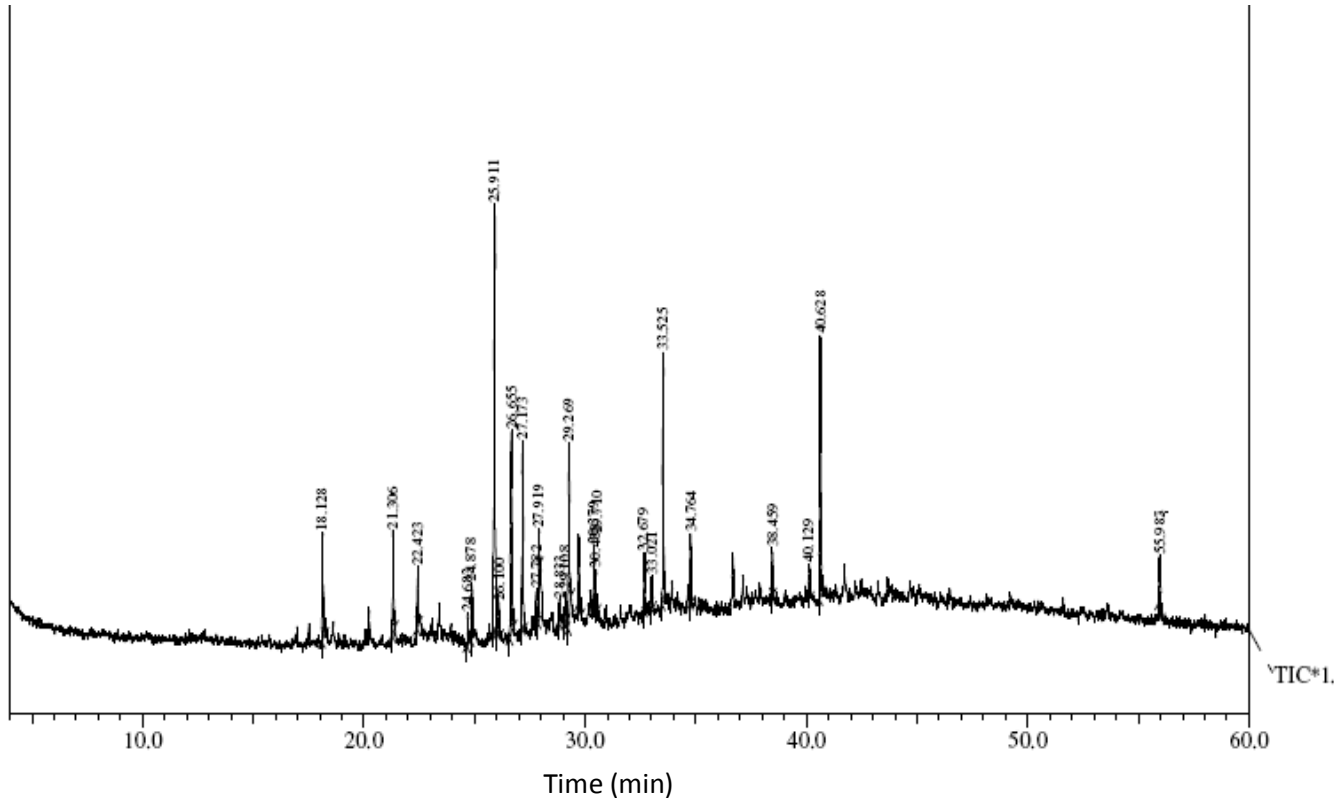


Figure 3. TIC chromatogram of FII of *HEUD*.

Table 4. The major compounds identified by GC-MS in the FII of *HEUD*.

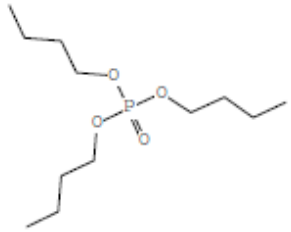
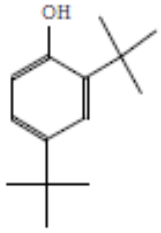
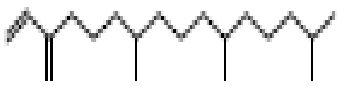
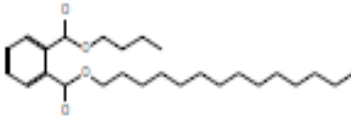
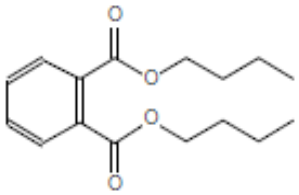
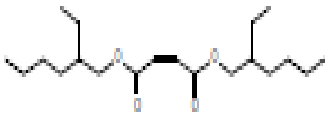
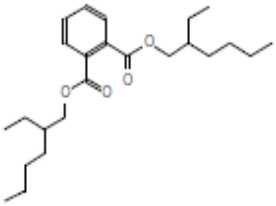
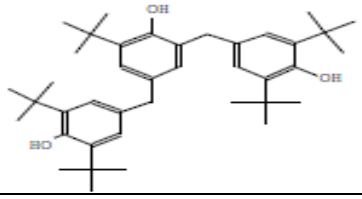
T_R (min)	Compound	Molecular formula	Structure	MW	Peak area %
21.30	Tributyl ester	$C_{12}H_{27}O_4P$		266	3.56
18.12	2,4-di-t-butylphenol	$C_{14}H_{22}O$		206	4.56
25.91	2,4,10-trimethyl,14-ethylene-14-pentadecne	$C_{20}H_{38}$		278	26.97
26.65	Butyl tetradecyl ester	$C_{26}H_{42}O_4$		418	9.53

Table 4. Contd.

29.26	Dibutyl phthalate	$C_{16}H_{22}O$		278	7.45
33.52	Bis(2-ethyl hexyl) maleate	$C_{20}H_{36}O_4$		340	8.80
40.62	1,2-benzenedicarboxylic acid	$C_{24}H_{38}O_4$		390	9.89
55.98	2-tert-Butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl)phenol	$C_{40}H_{58}O_3$		586	3.19

h and 51.28% (P<0.01) after 24 h of treatment.

Acute toxicity studies

The safety index of crude *HEUD* was assessed by carrying out the sub-acute oral toxicity for a period of 14 days in Wistar rats at doses of 250 (Group A), 500 (Group B), 1000 (Group C) and 2000 mg/kg bw (Group D). The hematological parameters of the tested and control groups were determined at the 15th day after treatment with *HEUD* by gavaging and were compared to check the hematological disorders (Table 6). There was a significant increase in red blood cell count (RBC, ×10^6 µl) in case of Groups A (P<0.05) and B (P<0.01); no noticeable changes were seen in the values of white blood cell count (WBC, ×10^6 µl), differential leucocyte count (DLC, %), hemoglobin percentage (Hb, g/dl), packed cell volume (PCV, %) and mean corpuscular hemoglobin concentration (MCHC, g/dl). However, there was a slight increase (P<0.05) in mean corpuscular volume (MCV, cµ) and mean corpuscular hemoglobin (MCH, pg) in Group D.

Liver function markers like alkaline phosphates (ALP), serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), bilirubin and total protein (Table 7) were found under the normal reference values. It was found that there were no significant changes in all the experimental groups with respect to the control group.

DISCUSSION

Phytomedicines are a major component of traditional system of healing especially in developing countries, which have been an integral part of their history and culture. Besides widespread use of natural products in developing countries, such products are becoming part of the integrative healthcare system of industrialized nations, known as complementary and alternative system of medicines. The world at large is currently engaged in discovering novel drugs for various diseases, one such possibility is the use of plant compounds in search of potent anti-inflammatory and anti-microbial agents. A number of pharmacological activities of *U. dioica* like anti diabetic, anti oxidant, anti dandruff, etc., are attributed. In this work, we evaluated the anti-inflammatory and anti-microbial activity of *U. dioica* by the carrageenan induced rat paw edema anti inflammatory assay and disc diffusion assay respectively.

HEUD showed good anti-microbial activity against all the tested bacterial strains as compared to other extracts. There are a number of reports of hexane extracts of plant

Table 5. Effect of *Urtica dioica* leaf extracts and sub fractions of *HEUD* on carrageenin induced hind paw edema in Wistar rats.

Treatment and dose	Time interval (h) and edema volume (ml)				
	0	1	2	3	24
Control	0.20±0.008	0.35±0.006	0.39±0.004	0.43±0.003	0.39±0.003
Hexane (200 mg/kg)	0.20±0.004	0.24±0.009 (31.42) *	0.23±0.011 (41.02) *	0.23±0.007 (46.51) **	0.20±0.008 (48.71) **
Chloroform (200 mg/kg)	0.22±0.005	0.30±0.004 (14.28)	0.35±0.005 (10.25)	0.40±0.009 (6.97)	0.42±0.009 (-7.69)
Ethyl acetate (200 mg/kg)	0.21±0.009	0.31±0.005 (11.42)	0.37±0.005 (5.12)	0.44±0.008 (-2.32)	0.45±0.005 (-15.38)
Methanol (200 mg/kg)	0.21±0.006	0.27±0.008 (22.85)	0.29±0.011 (25.64)	0.32±0.009 (25.58)	0.26±0.010 (33.33) *
Indomethacin (10 mg/kg)	0.17±0.007	0.21±0.009 (40.00) *	0.23±0.011 (41.02) *	0.20±0.010 (53.48) **	0.17±0.007 (56.41) **
Sub fractions of *HEUD*					
FI (200 mg/kg)	0.23±0.011	0.25±0.005 (28.57)	0.28±0.007 (28.20)	0.30±0.012 (30.23) *	0.30±0.009 (23.07)
FII (200 mg/kg)	0.19±0.014	0.22±0.008 (37.14) *	0.24±0.009 (38.46) *	0.22±0.010 (48.83) **	0.19±0.011 (51.28) **
FIII (200 mg/kg)	0.21±0.011	0.26±0.013 (25.71)	0.31±0.010 (20.51)	0.34±0.008 (20.93)	0.33±0.010 (15.38)
FIV (200 mg/kg)	0.18±0.010	0.23±0.009 (34.28) *	0.28±0.007 (28.20)	0.30±0.011 (30.23) *	0.25±0.009 (35.89) *
FV (200 mg/kg)	0.20±0.010	0.24±0.007 (31.42) *	0.27±0.013 (30.76) *	0.29±0.011 (32.55) *	0.24±0.007 (38.46) *

Values of edema are mean ± SEM from 6 animals in each group, while those in parenthesis represent percent inhibition of edema; One-Way ANOVA followed by Dunnett's t test is applied for statistical analysis. P values: *<0.05; **<0.01 ;***< 0.001.

possessing antibacterial activity (Elzaawely et al., 2005). These results indicate that the extracting solvent has a definite effect on bioactive principles. CC eluted FII of *HEUD* exhibited more anti-microbial activity as compared to other sub fractions in disc diffusion assay. Usually, the extract having large inhibition zone diameter with low MIC can be recognized as a more potent drug than that of small inhibition zone diameter and high MIC (Semwal et al., 2009). The MIC of less than 250 µg/ml of FII against the tested strains indicates its promising potential as an alternative for the treatment of infectious diseases caused by these strains, since most of them have developed resistance against the known antibiotics (Singleton, 1999).

The characteristic swelling that occurs in the rat paw model of inflammation is due to edema formation. In accordance with Marsha-Lyn et al. (2002), inflammation occurs throughout three distinct phases: an initial phase mediated by histamine and 5-hydroxytryptamine (up to 2 h); an intermediate phase involving the activity of bradikinin and a third phase (3 to 6 h) with prostanoid synthesis induced by cyclooxygenase (COX) (Di Rosa, 1972; Perez et al., 2001). Indomethacin is a non-steroidal anti inflammatory drug (NSAID) with strong anti-inflammatory activity that is effective in the treatment of rheumatic and non-rheumatic conditions. In experimental animals, indomethacin is able to totally inhibit acute and chronic inflammatory processes (erytema, edema, hyperalgesia and glaucoma) (Litter, 1992). It is also accepted that indomethacin inhibits COX, limiting therefore the biosynthesis of PGs, although it also has other activities that contribute to its therapeutic effects (Insel, 1996). In the carrageenan test, initially, the crude *HEUD* and then the isolated FII of *HEUD* significantly reduced the inflammation showing activity right from the first (1 h) to last (24 h) measurement. According to these results, it can be suggested that the mechanism of anti-inflammatory action of this extract occurs by interfering with the synthesis or liberation of histamine and PG mediators.

Increase in popularity and scarcity of scientific studies on the safety have raised concern regarding toxicity and adverse effect of herbal remedies (Saad et al., 2006). The hematopoietic system is very sensitive to toxic compounds (Mukinda and syce, 2007) and it ranks with liver, in pre clinical and clinical safety evaluation tests (Bloom, 1993). The analysis of hematological parameter shows relevance in risk evaluation as the changes in hematological system have a higher predictive value for human toxicity, when

Table 6. Effect of 14 days oral administration of *HEUD* on hematology of Wistar rats.

Parameter	Group				
	Control	Group-A	Group-B	Group-C	Group-D
RBC (10^6/µl)	7.62±0.1	8.00±0.0*	8.56±0.1 **	7.95±0.0	7.59±0.1
WBC (10^6/µl)	6.93±0.0	7.30±0.3	7.02±0.3	6.99±0.2	6.66±0.0
Neutrophils	58.96±0.8	61.22±0.4	59.95±0.7	58.72±0.6	61.61±0.9
Eosinophils	5.12±0.3	5.57±0.1	5.23±0.2	5.19±0.2	6.02±0.3
Basophils	0.13±0.1	0.24±0.1	0.27±0.1	0.47±0.2	0.34±0.2
Lymphocytes	26.22±0.4	24.94±0.3	26.05±0.2	26.35±0.3	23.94±0.6**
Monocytes	9.56±0.6	8.41±0.3	8.32±0.4	8.91±0.3	8.93±0.4
PCV	42.48±0.3	39.06±0.4 *	40.83±0.3	43.15±1.0	41.4±1.3
MCV	44.29±0.8	43.41±0.7	47.74±0.9	48.25±1.4	50.46±1.63*
MCH	15.39±0.5	16.02±0.2	17.37±0.3*	16.10±0.4	17.44±0.5*
MCHC	36.09±0.5	36.92±0.5	36.42±0.5	35.28±0.9	36.99±1.8)
Hb	14.91±0.2	14.41±0.1	14.86±0.1	15.20±0.3	15.20±0.3

*P<0.05, **P<0.01. RBC, Red bllod cell; WBC, white blood cell; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpucsular hemoglobin concentration; Hb, hemoglobin.

Table 7. Effect of 14 days oral administration of HEUD on liver function markers of Wistar rats.

Parameter	Group				
	Control	Group-A	Group-B	Group-C	Group-D
ALP (U/L)	(122.28±4.6)	(124.45±2.7)	(127.11±2.8)	(128.92±3.3)	(130.45±3.1) *
SGOT (U/L)	(10.01±2.0)	(11.96±3.6)	(6.55±1.2)	(6.61±0.9)	(14.15±3.4)
SGPT (U/L)	(4.71±0.9)	(5.96±1.2)	(4.35±0.4)	(3.58±0.5)	(2.92±0.5)
Bilirubin (mg/dl)	(0.34±0.0)	(0.48±0.0)	(0.37±0.0)	(0.36±0.0)	(0.32±0.0)
Total Protein (g/dl)	(6.84±0.1)	(6.43±0.1)	(6.73±0.2)	(6.40±0.1)	(6.27±0.2)

*P<0.05, **P<0.01. ALP, Alkaline phosphatase; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.

the data is translated from animal studies (Rhiouani et al., 2008). After 14 days treatment, no significant changes were observed in hematological parameter indicating that *HEUD* was safe up to the tested concentrations. However, RBC content showed an increase in Group A (P<0.05) and Group B (P<0.01) which could corroborate its use in traditional medicines for anemia. Liver and kidneys are the first sites to be affected by the toxic chemicals or herbs as they are primarily concerned with the function of detoxification and clearance of toxic substances from the body (Elvin-Lewis, 2001). Damage to these organs often results in elevationin clinical parameters. The most frequently used enzymes to assess hepato-cellular injury are alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase SGPT. In the present study, no variations were observed in ALP, SGOT, SGPT, bilirubin and total protein in all the experimental groups with respect to the control indicating that the extract is non hepatotoxic.

Eight major bioactive constituents were analyzed by GC-MS in FII of *HEUD*. All these compounds are likely to possess potent anti-inflammatory and anti-microbial activity. Essential oils rich in terpenes have been shown to possess good antibacterial activity (Taylor et al., 1996). FII showed the appreciable presence of the terpene (neophytadiene; 26.97%) which could explain both its anti-inflammatory as well as anti-microbial activity. Neophytadiene is already reported to possess anti-bacterial activity as well as helping in the treatment of headache, rheumatism and some skin disease (Suresh et al., 2010). Apart from the terpenes, phenols are one of the major groups of non-essential dietary components that have been associated with the inhibition of microbial infections, atherosclerosis and cancer, as well as for age-related degenerative brain disorders (Cheung et al., 2003; Wang et al., 2009). Presence of two new phenolic compounds, that is, 2,4-di-t-butylphenol and 2-tert-butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxy benzyl) phenol might also have a critical part for action in anti-inflammatory and anti-microbial activity. Many fatty acids are known to have antibacterial and antifungal properties (Russel,

1991). Fatty acid esters - phthalic acid, dibutyl ester, bis(2-ethyl hexyl) maleate and 1,2 benzenedicarboxylic acid were previously reported to be responsible for both the anti- inflammatory (Li et al., 2004) and anti-microbial activity (Modupe et al., 2010). Besides, the minor components might also be responsible for both anti-inflammatory and anti-microbial activity, possibly by producing a synergistic effect between other components. However, the composition of *U. dioica* in this study differs from that described by other authors (Lapinskaya and Kopyt'ko, 2008) because the composition of any plant essential oil is influenced by several factors such as planting, climatic, seasonal and experimental conditions (Daferera et al., 2000).

Conclusions

Our pharmaco-toxicological results corroborate that *U. dioica* present anti-inflammatory and anti-microbial effect with the absence of acute toxicity. These results support scientifically the use of *U. dioica in* popular medicine for the treatment of rheumatic and other illnesses. As only the *HEUD* showed potent anti-inflammatory and anti-microbial activity, the results also indicate that the extracting solvent has a definite effect on bioactive principles. Thus, compounds of various chemical natures (terpenes, fatty acid esters, hydrocarbons, etc.) were identified in the *HEUD*.

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