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Bioactive potential of leaf extracts from *Urtica dioica* L. against fish and human pathogenic bacteria

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The localization of bioactive phytochemical(s) may be one of the important approaches for the containment of antibiotic resistance. This study evaluated the antibacterial activity and toxicity of crude extracts and fractions from *Urtica dioica* L. Successive extraction of the leaves with hexane, chloroform, ethyl acetate and methanol; and their screening for antibacterial activity by disc diffusion assay against five strains each of fish and human bacteria, was assessed. Hexane extract showed good antibacterial activity; hence it was further fractionated using silica gel column chromatography into 30 sub fractions which were pooled together according to their thin layer chromatography (TLC) profile to give overall 5 sub fractions. Among the 5 sub fractions, fraction-2 (HF2) showed the highest antibacterial activity with minimum inhibitory concentration (MIC) values ranging from 31.25 to 250 µg/mL against both the fish and human pathogens, determined by serial tube dilution method. The toxicity tests with *Artemia salina* showed that all the solvent extracts of *U. dioica* along with HF2 showed a higher margin of safety with LC₅₀ value of >1000 µg/mL each. Gas chromatography–mass spectrometry (GC-MS) analysis of HF2 showed neophytadiene (19.96%), 2,6,10,15-tetramethylheptadecane (12.82%), heptadecyl ester (9.45%), hexyl octyl ester (6.31%), 2,7,10-trimethylldodecane (5.60%), butyl tetradecyl ester (4.73%), octadecan-1-ol (4.45%), 1,2-benzenedicarboxylic acid (4.38%), 4,6-di-tert-butyl-m-cresol (4.32%) and 2,4-ditert-butylphenol (4.30%), constituting 75.36% of the total peak area percentage, as the major constituents. Our results showed that the leaves of *U. dioica* are an interesting source of biologically active compounds that may be applied for the treatment of infectious diseases in both human as well as veterinary animals.

Key words: *Artemia salina*, gas chromatography–mass spectrometry (GC-MS), minimum inhibitory concentration (MIC), toxicity.

INTRODUCTION

The adoption of same antibiotics in both human and veterinary medicine aggrandizes the emerging problem of multidrug resistance phenomenon in which the therapy becomes quite difficult. Due to the use of a wide variety of antibiotics, aquaculture has been implicated as potential environment to the development and selection of resistant bacteria and a source of these pathogens to other animals and humans (Hatha et al., 2005). Problems including solubility, palatability, toxicity, cost effectiveness, delivery and governmental restrictions have limited the use of available antibiotics, especially in fish farms. In the aquatic environment, competition for space and nutrients leads to evolution of antibacterial defense strategies.
This, along with possibly adverse effects on the ecosystem and human health problems, has resulted in restrictions on the use of commercial antibiotics and chemicals in the aquatic environment (Serrano, 2005). The problem is worsened by antibiotic resistances coupled with the emergence of new pathogens with the potential for rapid global spread (Walsh, 2003), boosting the search for new antibacterial agents for human as well as aquacultural purposes. An alternative for searching new effective drugs for infectious disease eradication are natural products, especially those of vegetable origin (Hemaïswarya et al., 2008). Use of herbal medicines in Asia represents a long history with several applications against various diseases (Duraipandiyan et al., 2006). The practice of traditional medicine is widespread in China, India, Japan, Pakistan, Sri Lanka and Thailand. However, among the estimated 250,000-400,000 plant species, only 6% have been studied for biological activity, and about 15% have been investigated phytochemically. This shows a need for phyto-pharmacological evaluation of herbal drugs (Goyal et al., 2007). Furthermore, medicinal plants as the alternative agents are effective to treat the infectious diseases and mitigate many side effects that are associated with synthetic antimicrobials. In addition, plant-derived phytotherapeutics provide a cheaper source for treatment and greater accuracy than chemotherapeutic agents in this field (Punitha et al., 2008). Although they are used in folk medicine, some plants may also exhibit a high toxicity degree. The belief that herbal medicines are free from risk to health is part of the population cultural background used to take them. However, the natural character of such products is not warranted to be free of adverse reactions and other problems caused by such medicine. Therefore, tests that assess the natural product toxicities that are essential for these products can be safely used (Parra et al., 2001).

Urtica dioica L., commonly known as stinging nettle or common nettle, belonging to Urticaceae family, is widely spread from Europe to Asia. The whole plant is used in folk medicine to treat allergies, kidney stones, burns, anemia, rashes, internal bleeding, diabetes, etc. (Dar et al., 2012). However, only a few of these pharmacological activities have been experimentally proved (Lourdes et al., 2008). The known phytochemicals of U. dioica include flavonoids, lignans, fatty acids, sterols, polysaccharides, glycoproteins, carotenoids, plastocyanins, and lectins (Sajírová et al., 2005). The aim of this study was to evaluate the antibacterial activity of extracts and fractions of U. dioica against both the fish and human pathogens along with their toxicological evaluation.

MATERIALS AND METHODS

Plant material

The fresh leaves of U. dioica were collected from the undisturbed fields of Aishmuqam (Distt. Anantnag), J & K, India. Voucher herbarium specimen has been deposited in the Kashmir University Herbarium (KASH 28100), Centre of Biodiversity & Taxonomy (CBT), Department of Botany, University of Kashmir.

Extract preparation

The leaves of U. dioica were shade dried at 25°C for 7 days. After being macerated to fine powder, 1000 g leaves were extracted successively with hexane, chloroform, ethyl acetate and methanol for 16 h using Soxhlet apparatus (Singh et al., 2012). The extracts were filtered through a Buchner funnel using Whatman no. 1 filter paper, and all the extracts were concentrated to dryness under vacuum using Heidolph rotary evaporator, causing the hexane, chloroform, ethyl-acetate and methanol crude extracts, yielding 68, 73, 44 and 79 g respectively. All the extracts were stored at 4°C in air tight glass bottles before use.

Microbiologic material

The micro-organisms used as standard strains for testing antibacterial activity comprised of both the fish and as well as of human pathogens. The fish bacterial strains include Aeromonas hydrophilia ATCC 19570, Aeromonas salmonicida subsp. salmonicida ATCC 14174, Flavobacterium columnare ATCC 23463, Vibrio salmonicida ATCC 43839 and Yersinia ruckeri ATCC 29493. The human pathogenic strains include Pseudomonas aeruginosa (ATCC 27853), Staphylococcus aureus (ATCC 25923), Salmonella typhi (ATCC 19430), Klebsiella-pneumoniae (ATCC 15380) and Enterococcus faecalis (ATCC 29212).

Antibacterial activity

The agar diffusion assay was performed according to European Pharmacopoe (1997). One loopful of each test organism was suspended in 3 ml 0.9% NaCl solution separately. Nutrient agar (Difco™ Tryptic Soy Agar, Becton Dickinson and Company, USA) was inoculated with this suspension of the respective organism and poured into a sterile Petri dish. Sterile filter paper discs with 6 mm diameter impregnated with 2000 μg (dissolved in 8 μL DMSO) of each extract, were transferred onto these prepared Petri dishes as per standard procedures (Chandrasekaran and Venkatesalu, 2004), gentamycin (30 μg/disc, Merck) was used as a positive, the solvent of each extract (DMSO) as a negative control. A pre-diffusion for 3 h was guaranteed. After pre-diffusion and incubation the Petri dishes were sprayed with a coloring solution (p-iodo nitrotetrazolium violet, 5% in 50% aqueous ethanol). Living bacteria on Petri dishes produce a red coloured compound; the inhibition zone appears colourless (Brantner, 1997). All experiments were carried out five times and results were recorded by measuring the zones of growth inhibition around the discs after 18 h incubation at 26°C.

Bioassay guided isolation of active compound(s)

The only active hexane extract of U. dioica (HEUD, 50 g) was fractionated using silica gel 60 (0.063-0.200 mm) column chromatography (CC). Solvents were distilled prior to use. The column was eluted with a solvent gradient of hexane-ethyl acetate (EtOAc) in 100:0 and 0:100 ratios to give 30 fractions (each of 250 mL). 30 fractions were collected, analyzed by thin layer chromatography (TLC) on silica gel 60 PF254 (Merck) aluminium sheets and pooled together due to similarity in TLC profile to give overall 5 sub fractions: HF1, HF2, HF3, HF4 and HF5. The sub fractions were tested for anti-microbial activity against both the fish and human pathogens by standard assays (European Pharmacopoe, 1997).
Minimum inhibitory concentration (MIC)

The MIC values were determined by standard serial broth microdilution assay (European Pharmacopoeia, 1997). 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 incubated aerobically at 37°C for 12-18 h; after which 50 μL of 0.2 mg/mL 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium 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.

GC-MS conditions

GS-MS analysis was carried out on Agilent 6890N Network GC system combined with Agilent 5973 Network Mass Selective Detector (GC-MS). The capillary column used was an Agilent 19091N-136 (HP Innowax Capillary; 60.0 m × 0.25 mm × 0.25 μm). Helium was used as carrier gas at a flow rate of 3.3 ml/min with 1 μL injection volume. 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 heating ramp without hold and increased to 215°C with 5°C/min heating ramp for 7 min. Then the final temperature was increased to 240°C with 10°C/min heating ramp for 15 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 50.0 psi. Run time was 35 min. Temperature and nominal initial flow for flame ionization detector (FID) were set as 250°C and 3.1 ml/min, correspondingly. MS parameters were as follows: scan range (m/z): 35-450 atomic mass units (AMU) under electron impact (EI) ionization (70 eV). 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.

Toxicity test with Artemia salina

The extract toxicity tests of U. dioica were held according to the method described by Meyer et al. (1982) using A. salina. All the extracts and fractions of U. dioica were dissolved in DMSO solution and subsequently in synthetic seawater. The concentration of the extracts and fractions were ranging from 1000 to 200 μg/mL and each of them were put in spiked plate between 6 and 12 nauplii, followed by further incubation in a water bath (20 to 25°C) for 24 h. A solution of potassium dichromate at concentrations of 400, 600 and 800 μg/mL was used as a positive control and the solvent used to dissolve the samples was taken as negative control. The criteria for evaluation was based on the LC50 of fractions and extracts above 250 μg/mL were considered as low toxic, LC50 ranging from 80 to 250 μg/mL were considered moderately toxic and LC50 of less than 80 μg/mL were considered toxic (Parra et al., 2001). For the final calculation of LC50, the Probit analysis method was used.

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.

RESULTS

Antibacterial activity

All the solvent extracts of U. dioica namely hexane, chloroform, ethyl acetate and methanol were tested for antibacterial activity against five strains each of fish and human pathogens. The HEUD showed good antibacterial activity, hence it was further fractionated using CC into 30 sub fractions each of which were pooled together according to their TLC profile to give overall 5 sub factions: HF1, HF2, HF3, HF4 and HF5. The results of antibacterial activity of all the extracts of U. dioica along with the sub fractions of hexane are shown (Tables 1 and 2). The antibacterial activity produced by the sub fraction HF2 of HEUD was comparable to that of the standard antibiotic gentamycin.

Minimum inhibitory concentration (MIC)

The MIC values of the most active sub fraction (HF2) of HEUD were 125, 125, 250, 62.5 and 31.25 μg/mL against the fish pathogens Aeromonas hydrophila, Aeromonas salmonicida subsp. salmonica, Flavobacterium columnare, Vibrio salmonicida and Yersinia ruckeri respectively (Table 1). Similarly the MIC values of HF2 were 250, 31.25, 7.81, 31.25 and 125 μg/mL against the human pathogens Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi, Klebsiella-pneumoniae and Enterococcus faecalis respectively (Table 2).

GC-MS analysis

In order to find out the bioactive compounds, the most active sub fraction (HF2) of HEUD was subjected to GC-MS analysis which identified a total of 23 compounds (Table 3). HF2 showed ten major compounds and 13 minor compounds, with molecular weights ranging from 100 to 400 atomic mass units. The compounds were classified into primary, secondary and tertiary metabolites based on their retention times and mass weights.

Toxicity test with A. salina

The extract and fraction toxicities of U. dioica were evaluated using brine shrimp test, whose results can be seen in Table 4. All the extracts (hexane, chloroform, ethyl-acetate and methanol) of U. dioica along with the
Table 1. Antibacterial activity of different solvent extract and fractions of leaves of Urtica dioica against fish pathogens.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>DIZ (mm)</th>
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<tbody>
<tr>
<td></td>
<td>Aeromonas hydrophilia</td>
</tr>
<tr>
<td>Fish pathogens</td>
<td></td>
</tr>
<tr>
<td>Hexane extract</td>
<td>12±0.4</td>
</tr>
<tr>
<td>CH₃Cl extract</td>
<td>7±0.3</td>
</tr>
<tr>
<td>EtOAc extract</td>
<td>---</td>
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<tr>
<td>Methanol extract</td>
<td>---</td>
</tr>
<tr>
<td>HF1</td>
<td>---</td>
</tr>
<tr>
<td>HF2</td>
<td>16±0.5</td>
</tr>
<tr>
<td>HF3</td>
<td>9.66±0.3</td>
</tr>
<tr>
<td>HF4</td>
<td>8.3±0.4</td>
</tr>
<tr>
<td>HF5</td>
<td>7±0.3</td>
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<tr>
<td>Control</td>
<td>---</td>
</tr>
<tr>
<td>Standard</td>
<td>17.66±0.3</td>
</tr>
</tbody>
</table>

Minimal inhibitory concentration [µg/ml]

<table>
<thead>
<tr>
<th></th>
<th>HF2</th>
<th>125</th>
<th>125</th>
<th>250</th>
<th>62.5</th>
<th>31.25</th>
</tr>
</thead>
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DIZ: Diameter Inhibition Zone, EtOAc: Ethyl acetate, HF: Hexane fraction, Control: Dimethyl Sulphoxide (DMSO), Standard: Gentamycin.

Table 2. Antibacterial activity of different solvent extract and fractions of leaves of Urtica dioica against human pathogens.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>DIZ (mm)</th>
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<tbody>
<tr>
<td></td>
<td>P. a</td>
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<tr>
<td>Human pathogens</td>
<td></td>
</tr>
<tr>
<td>Hexane extract</td>
<td>11±0.5</td>
</tr>
<tr>
<td>CH₃Cl extract</td>
<td>---</td>
</tr>
<tr>
<td>EtOAc extract</td>
<td>---</td>
</tr>
<tr>
<td>Methanol extract</td>
<td>---</td>
</tr>
<tr>
<td>HF1</td>
<td>---</td>
</tr>
<tr>
<td>HF2</td>
<td>14±0.5</td>
</tr>
<tr>
<td>HF3</td>
<td>9±0.3</td>
</tr>
<tr>
<td>HF4</td>
<td>9±0.3</td>
</tr>
<tr>
<td>HF5</td>
<td>8.3±0.4</td>
</tr>
<tr>
<td>Control</td>
<td>---</td>
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<tr>
<td>Standard</td>
<td>18.66±0.3</td>
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</table>

Minimal inhibitory concentration [µg/ml]

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<table>
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<tbody>
<tr>
<td></td>
<td>HF2</td>
<td>250</td>
<td>31.25</td>
<td>7.81</td>
<td>31.25</td>
</tr>
<tr>
<td>Standard</td>
<td>&lt;7.81</td>
<td>&lt;7.81</td>
<td>&lt;7.81</td>
<td>&lt;7.81</td>
<td>&lt;7.81</td>
</tr>
</tbody>
</table>

A. Abbreviations; P. a, Pseudomonas aeruginosa; S. a, Staphylococcus aureus; S. t, Salmonella typhi; K. p, Klebsiella pneumoniae and E. f, Enterococcus faecalis. DIZ, diameter inhibition zone; EtOAc, Ethyl acetate; HF, hexane fraction; Control, Dimethyl Sulphoxide (DMSO); Standard, Gentamycin.

**DISCUSSION**

Natural products are considered an important source of new antibacterial agents. Medicinal plants continue to be used world-wide for the treatment of various diseases and have a great potential for providing novel drug leads with novel mechanism of action (Singh et al., 2012). In the developed countries, high throughput screening tests are used for bioassay guided fractionation leading to the most active sub fraction (HF2) showed a higher margin of safety (LC50>1000 µg/mL).
isolation of active principles that may be developed into clinical agents either as the natural product or a synthetic modification or a synthesized analogue with enhanced clinical action or reduced adverse side effects. The main objective of this study was to evaluate the ability of different solvent extracts of *U. dioica* to inhibit the growth of both the fish and human pathogenic bacteria with the aim to use them in the future as alternatives to common antibiotics in human as well as in veterinary medicine including aquaculture.

The data showed that the *HEUD* contained ingredients that were active against both the fish and human pathogens. There are a number of reports of hexane extracts of plant possessing antibacterial activity (Elzaawely et al., 2005). These results indicate that the extracting solvent has a definite effect on bioactive principles. CC eluted HF2 of *HEUD* exhibited more antimicrobial activity as compared to other sub fractions in agar diffusion assay. Usually, the extract having large inhibition zone diameter with low MIC can be recognized as more potent drug than that of small inhibition zone diameter and high MIC (Semwal et al., 2009). The extracts and fractions can be considered actives when the MIC is less than 1 μg/mL (Rios and Recio, 2005). The MIC of less than 250 μg/mL of HF2 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).

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 toxicity test results showed that the HF2 of *HEUD*...
had a higher margin of safety (LC₅₀ > 1000 μg/mL) against A. salina larvae. The test is used to evaluate, in a preliminary way, the natural product toxicities. It is fast, easy and inexpensive, and can also be used to verify the anti-tumor activity, since this cytotoxicity assessment is related to possible activity against tumor cells, when the LC₅₀ values are less than 250 μg/mL (Parra et al., 2001). GC-MS analyzed ten major bioactive constituents in HF2 of HEUD. All these compounds are likely to possess potent anti-microbial activity. Essential oils rich in terpenes have been shown to possess good antibacterial activity (Taylor et al., 1996). HF2 showed the appreciable presence of the terpene (neophytadiene; 19.96%) which could explain its anti-microbial activity. Neophytadiene is already reported to possess antibacterial and antifungal properties (Russel, 1991). Our findings showed a pretty good percentage of fatty acid esters (24.86%) namely heptadecyl ester, hexyl octyl ester, butyl tetradecyl ester, and 1,2 benzenedicarboxylic acid which are reported to exhibit both the anti-inflammmatory (Li et al., 2004) and anti-microbial activity (Modupe 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 appreciable amount of phenols (2,4-ditert-butylphenol) might also have a critical part for action in anti-microbial activity. 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 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
The findings showed that the leaves of U. dioica is an interesting source of biologically active compounds that may be applied for prophylaxis and therapy, in both human as well as veterinary animals, which justifies their traditional use to treat infectious diseases and hence reinforce the importance of the ethnobotanical approach as a potential source of bioactive substances.

REFERENCES


