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In vitro Antioxidant, Antimicrobial and *in vivo* Peripheral Analgesic Activities of Methanol and Petroleum Ether Extracts of Whole Plant of *Uraria lagopoides* DC

Md. Monir Hossain[1*], Md. Nur Alam[1], Nizam Uddin[1], Mohammad Burhan Uddin[1,2] and A. Y. Sk. Ferozuddin A. Chowdhury[1,2]

[1]Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh.
[2]Department of Pharmacy, Primeasia University, Dhaka-1213, Bangladesh.

Authors' contributions

This work was carried out in collaboration between all authors. Author MMH performed the experimental study and wrote the first draft of the manuscript. Authors MMH, MNA and NU wrote the protocol and managed the analyses of the study. Author MMH collected and extracted the whole plant. Author MBU managed the literature searches. Author AYSFAC designed and supervised the study as well as finalized the final drafting of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To study and evaluate *in vitro* antioxidant, antimicrobial and *in vivo* peripheral analgesic activities of both methanol and petroleum ether extracts of whole plant of *Uraria lagopoides* DC (Family-Fabaceae).
Study Design: Preliminary phytochemical screening, evaluation of antioxidant, antimicrobial and peripheral analgesic activities.
Place and Duration of Study: Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342 and Department of Pharmacy, Primeasia University, Dhaka-1213. The studies were carried out during February- September 2013.

**Corresponding author: Email: monirjupharmacy@gmail.com;*

Methodology: Phytochemical constituents were identified by qualitative analysis. *In vitro* antioxidant activity of the extracts were studied using DPPH radical scavenging assay, total phenol, total flavonoid content and total antioxidant capacity determination assays. Antimicrobial activity was investigated by disc diffusion technique and acetic acid induced writhing test was used for the evaluation of *in vivo* peripheral analgesic activity of the whole plant extracts.
Results: Phytochemical screening for both extracts showed positive results for carbohydrates, flavonoids and glycosides while only methanol extract showed positive results for alkaloids, saponins, steroids and tannins. Methanol extract of the plant showed higher total phenolic content and as well as higher DPPH free radical scavenging activity than petroleum ether extract. On the other hand, petroleum ether extract showed higher total flavonoid content and total antioxidant capacity than methanol extract. In disc diffusion technique among six bacterial species, methanol extract showed concentration dependent activity against one Gram positive (*Staphylococcus aureus*) and three Gram negative bacteria (*Escherichia coli, Salmonella typhi* and *Klebsiella pneumoniae*) while petroleum ether extract showed concentration dependent activity against only one Gram negative bacteria (*Salmonella typhi*). In acetic acid induced writhing test both methanol and petroleum ether extracts of 250 mg/kg and 500 mg/kg (per oral) both doses showed significant ($P < 0.001$) analgesic activity.
Conclusion: The results demonstrate that methanol and petroleum ether extracts of whole plant of *U. lagopoides* can be used as a potential source of antioxidant, antimicrobial and analgesic agent.

Keywords: Uraria lagopoides; antioxidant; phenolic; flavonoids; DPPH; antimicrobial; analgesic.

1. INTRODUCTION

Uraria lagopoides DC (*U. lagopoides*) (Family-Fabaceae) locally known as Chakuley, is a prostrate and ascending woody small shrub, 30-90 cm long. Leaves 2.5-5 cm long; leaflets solitary or 3-foliolate; oblong, rhomboid, rounded, hairy beneath. Racemes, dense, oblong, 2.5-6.3 cm long, 2 cm through. Flowers are white. Pods are 3.8 cm long and 2 cm wide. It has a large geographical distribution from tropical Africa, to southern and south east Asia and Australia. In Bangladesh it is distributed in Sal forests of Gazipur and Tangail. The plant is abortifacient, aphrodisiac; used in rheumatism, fever, bleeding piles, catarrh and scorpion-sting, asthma, diarrhea, dysentery and for the treatment of inflammation in the chest. *In vitro* antioxidant potential and free radical scavenging activities of aqueous and ethanolic root extract of *U. lagopoides* was evaluated [1]. Anti-inflammatory and analgesic activities of alcohol and aqueous extracts of aerial parts of *U. lagopoides* were determined earlier [2]. Various extracts of the aerial parts of the plant were tested for antimicrobial activity [3]. To the best of our knowledge no scientific study was done on the whole plant. So, this study was aimed, for the first time, to investigate *in vitro* antioxidant, antimicrobial and *in vivo* peripheral analgesic activities of methanol and petroleum ether crude extracts of whole plant of *U. lagopoides*.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Folin-Ciocalteu reagent, Methanol, Ethanol, Sodium Phosphate (Na_3PO_4) and Ammonium molybdate were purchased from Merck, Germany. Sodium carbonate, Potassium Acetate and Concentrated H_2SO_4 (98%) were purchased from Merck (India) Limited. Gallic acid, Quercetin and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemicals, USA. Aluminium Chloride and Ascorbic acid were purchased from SD Fine Chem. Ltd., Biosar, India. Ibuprofen was purchased from Beximco Pharmaceuticals Ltd, Bangladesh. All chemicals used were of analytical reagent grade.

2.2 Plant Materials

The whole plants of *Uraria lagopoides* DC (*U. lagopoides*) (Family-Fabaceae) were collected in November-December 2012 from the botanical garden, Department of Pharmacy, Jahangirnagar University, Savar, Dhaka, Bangladesh. The plants were identified by the taxonomist of the National Herbarium of Bangladesh (Acc. No. 38618) where the voucher specimen has been deposited for future investigation.

2.3 Extraction

The whole plants were thoroughly washed with water and placed into a dryer having a good air circulating system and a temperature-controlling thermostat. Then all parts were dried in hot air oven at 60℃. The dried whole plants were ground to coarse powder with a mechanical grinder (Grinding Mill).The weight of the total dry powder was 800 g. 500 g powdered plant materials were used for extraction. Extraction was performed with two different solvent, methanol and petroleum ether. The powders were kept in 800 mL of petroleum ether for 5 days in sealed container accompanying occasional shaking and string. Then extracts were filtered through fresh cotton bed. Then solvent was completely removed by heating in a water bath at temperature of 40±2ºC. After petroleum ether extraction the powders were dried and soaked into 800 mL methanol. The methanol extract was obtained by same procedure of petroleum ether extract obtained. Yield obtained using petroleum ether was 22.5 g and methanol was 39.2 g. Both extracts were stored at 4ºC until use.

2.4 Phytochemical Screening

Phytochemical screening of the crude extracts were carried out to reveal the presence (or absence) of chemical constituents such as alkaloids (Mayer's, Hager's, Wagner's and Dragendorff's test), carbohydrates (Molisch's test), glycosides (General test and Test for Glucoside), flavonoids (General test), saponins (Frothing test), steroids (Libermann-Burchard's test) and tannins (Lead acetate test) [4].

2.5 Antioxidant Activity Evaluation

2.5.1 Determination of total phenol content

The content of total phenolic compounds in plant methanol and petroleum ether extracts was determined as described by Velioglu et al. [5]. 1.0 mL of plant extract (200 µg/mL) or standard of different concentration (200, 100, 50, 25, 12.5, 6.25 µg/mL) solution was taken in a test tube. 5 mL of Folin-Ciocalteu and 7.5% Sodium carbonate solution (4 mL) was added to the same test tube and mixed well. Test tubes were incubated to complete the reaction and absorbance of the solution was measured at 765 nm using a spectrophotometer against blank. The total content of the phenolic compounds of the plant extracts were expressed in mg/g, gallic acid equivalent (GAE).

2.5.2 Determination of total flavonoid content

Total flavonoid was determined using the aluminum chloride colorimetric method described by Wang and Jiao [6]. 1.0 mL of plant extract (200 µg/mL) or standard of different concentration (100, 50, 25, 12.5, 6.25 µg/mL) solution was taken in a test tube. 3 ml of methanol was added to the test tube. Then 200 µL of 10% aluminum chloride solution was added into the same test tube, followed by the addition of 200 µL of 1M potassium acetate solution into the test tube. Finally, 5.6 mL of distilled water was mixed with the reaction mixture. The reaction mixture then incubated for 30 minutes at room temperature to complete the reaction and absorbance of the solution was measured at 415 nm using a spectrophotometer against blank. The total content of flavonoid compounds in plant methanol and petroleum ether extracts were expressed in mg/g, quercetin equivalent (QE).

2.5.3 Determination of total antioxidant capacity

Total antioxidant capacity was determined using the phosphomolybdenum method described by Prieto et al. [7]. 300 µL (200 µg/mL) of each plant extract or standard of different concentrations (200, 100, 50, 25, 5 µg/mL) solutions were taken into different test tubes and 3 ml of reagent solution was added into each of the test tubes. The test tubes were incubated at 95^0C for 90 minutes to complete the reaction. The absorbance of the solutions was measured at 695 nm using a spectrophotometer against blank after cooling at room temperature. The antioxidant activity was expressed as mg/g, ascorbic acid equivalent (AAE).

2.5.4 DPPH free radical scavenging assay

DPPH free radical scavenging assay was done according to the method described by Braca et al. [8]. 1 ml of each plant extracts or ascorbic acid of different concentrations (500, 200, 100, 50, 25, 5 µg/mL) solutions were taken in different test tubes and 2 ml of 0.004% DPPH solution in methanol was added to each test tube to make the final volume 3 ml. The mixtures were incubated in room temperature for 30 minutes in a dark place. Then the absorbance was measured at 517 nm using a spectrophotometer against blank. IC_{50} values were calculated using linear regression analysis.

2.6 Antimicrobial Activity of Plant Extracts

2.6.1 Microorganisms

Two Gram positive *Staphylococcus aureus (S. aureus), Succina lutea (S. lutea*) and four Gram negative *Escherichia coli (E. coli), Salmonella typhi (S. typhi), Vibrio cholerae (V. cholerae)* and *Klebsiella pneumoniae (K. pneumoniae*) bacteria were used for this investigation. The bacterial strains were collected as pure cultures from the Department of Pharmacy, Primeasia University, Dhaka.

2.6.2 Antimicrobial screening by disc diffusion technique

Antimicrobial activity of the plant extracts was investigated by disc diffusion technique [9]. Subcultures prepared from pure cultures of six microorganisms were used for the sensitivity test. In an aseptic condition under laminar air hood cabinet, the test organisms were transferred from the subculture to 5 mL of nutrient broth contained in screw-capped test tubes using a transfer loop. Plant extract of 375, 750 and 1500 µg/disc concentrations were used for this investigation. Standard disc of Azithromycin (30 µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control respectively. Bacterial cell suspension was spread throughout the nutrient agar plates by spread plate method, using sterile 'L' shaped spreader. Then the discs were placed in the nutrient agar plates and kept in a refrigerator at 4ºC for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37ºC for 24 hours. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

2.7 Evaluation of Peripheral Analgesic Activity

2.7.1 Animals

For this experiment Swiss albino mice of either sex, 6-7 weeks of age, weighing between 25-30 g, were collected from Pharmacology Laboratory, Department of Pharmacy, Jahangirnagar University, Savar, Dhaka. Animals were maintained under standard environmental conditions (temperature: 27.0±1.0ºC, relative humidity: 55-65% and 12 h light/12 h dark cycle) and had free access to feed and water *ad libitum*. The animals were acclimatized to laboratory condition for one week prior to the experiment.

2.7.2 Acetic acid induced writhing test

According to Koster et al. [10] method the experimental animals were randomly selected and divided into six groups denoted as Group I, Group II, Group III, Group IV, Group V and Group VI consisting of five mice in each group. Group I served as the control group and received 1% Tween 80 in normal saline. Group II and Group III were treated with the crude methanol extract suspension 250 mg/kg and 500 mg/kg body weight per oral (p.o.), respectively and Group IV and Group V were treated with the crude petroleum ether extract suspension 250 mg/kg and 500 mg/kg body weight per oral respectively. Group VI received Ibuprofen 100 mg/kg body weight per oral. Thirty minutes after drug treatment for proper absorptions each group was treated intraperitoneally (i.p.) with 0.7% v/v acetic acid (0.1 mL/10 g). Five minute after acetic acid administer, the number of writhes (abnormal contraction or stretches) were counted for the next twenty minutes and recorded.

The percentage inhibition of writhing was calculated using the following formula:

> % Inhibition = (No. of control writhing- no. of sample writing) / no. of control writhing x 100

2.8 Statistical Analysis

The data are expressed as mean ± SD (Standard Deviation Mean) and mean ± SEM (Standard Error Mean). The results were statistically analyzed using one way analysis of variance (ANOVA) Dunnett's multiple comparison when compared against control in acetic acid induced writhing test. Pearson's correlation was performed to show positive or negative relationship among different antioxidant methods. Student's *t*-test was performed to determine variation between two data sets in antioxidant screening. Bonferroni multiple comparison (one way ANOVA) was performed to show significant variation among IC_{50} values. Fisher's LSD post hoc analysis (one way ANOVA) was performed to show difference among antimicrobial activity. Linear regression analysis was performed to calculate IC_{50} values. Values were considered as statistically significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$. Statistical programs used were SPSS

for windows (version 16.0, IBM corporation, NY, USA), Sigma Plot (version 12.0, Systat Software Inc., San Jose, California, USA), and Microsoft Excel, 2007.

3. RESULTS

3.1 Qualitative Phytochemical Screening of *U. lagopoides* Extracts

In phytochemical screening methanol extract of *U. lagopoides* showed (Table 1) the presence of alkaloids, carbohydrates, flavonoids, glycosides, saponins, steroids and tannins while petroleum ether extract suggested the presence of carbohydrates, flavonoids and glycosides.

3.2 Antioxidant Activity of Plant Extracts

3.2.1 Total phenol, total flavonoid content and total antioxidant capacity assay

Results of total phenol, total flavonoid content and total antioxidant capacity were presented in Table 2. Total phenol content of methanol and petroleum ether extracts of the plant was found to be 2.36±0.51 and 0.72±0.26 mg/g, GAE respectively. Total flavonoid content of methanol extract was 31.33±0.72 mg/g, QE and petroleum ether extract was 81.07±1.80 mg/g, QE. Total antioxidant capacity of methanol and petroleum ether extracts of *U. lagopoides* were 109.25±1.75 and 152±2.50 mg/g, AAE respectively. Two extracts differed significantly ($P < 0.05$) in total flavonoid and total antioxidant capacity assay but insignificantly ($P > 0.05$) in total phenol content.

3.2.2 DPPH free radical scavenging activity

The results of DPPH free radical scavenging activity of plant extracts and standard were summarized in above Table 3. DPPH free radical scavenging activity of both methanol and petroleum ether extracts of *U. lagopoides* were found to be increased with the increase of concentration of the extracts. IC_{50} values of the methanol and petroleum ether extracts were found to be 1046.59±44.47 and 1584±1.58 µg/mL respectively. Methanol extract has good property than petroleum ether extract. IC_{50} value of the reference compound ascorbic acid was found 44.47±1.27 µg/mL. The IC_{50} values were differed significantly ($P < 0.05$) from one another.

Table 1. Phytochemical constituents identified in the methanol and petroleum ether extracts of *U. lagopoides*

Phytochemicals	Name of the test	Observed changes	Result	
			ULM	ULP
Alkaloids	Mayer's test	Creamy white precipitate	+	–
	Hager's test	Yellow crystalline precipitate	++	++
	Wagner's test	Brown or deep brown precipitate	+	–
	Dragendorff's test	Orange or orange-red precipitate	–	–
Carbohydrates	Molisch's test	A red or reddish violet ring is formed at the junction of two layer and on shaking a dark purple solution is formed	++	+
Glycosides	General test	Yellow color	++	++
	Test for Glucoside	Production of brick-red precipitation (carried out with the hydrolyzed extract)	–	–
Flavonoids	General test	Red color	+	+
Saponins	Frothing test	Formation of stable foam	++	–
Steroids	Libermann-Burchard's test	Greenish color	++	–
Tanins	Lead acetate test	A yellow or red precipitate	+	–

[++ = Strong presence, + = Presence, - = Absence]; ULM = Uraria lagopoides methanol extract, ULP = Uraria lagopoides petroleum ether extract

Table 2. Total phenol, total flavonoid and total antioxidant capacity of different extracts of *U. lagopoides*

Extracts	Total phenol (mg/g, GAE)	Total flavonoid (mg/g, QE)	Total antioxidant capacity (mg/g, AAE)
Methanol extract	2.36±0.51[a]	31.33±0.72[a]	109.25±1.75[a]
Petroleum ether extract	0.72±0.26[a]	81.07±1.80[b]	152±2.50[b]

Values are the mean of duplicate experiments and represented as mean ± SD (Standard Deviation Mean). Values in same column with different superscripts are significantly different (P < 0.05). Student's t-test was performed to analyze this data set. GAE = Gallic Acid Equivalent, QE = Quercetin Equivalent, AAE = Ascorbic Acid Equivalent

Table 3. DPPH radical percent scavenging activity of different extracts of *U. lagopoides* and ascorbic acid

Extracts/ Standard	Concentrations µg/mL (DPPH% scavenging)						IC_{50} (µg/mL)
Methanol extract	5 (13.71±1.01%)	25 (16.86±0.90%)	50 (19.39±0.99%)	100 (20.68±0.54%)	200 (26.15±0.35%)	500 (31.47±0.89%)	1046.59±44.47[b]
Petroleum ether extract	5 (9.56±0.91%)	25 (11.42±1.53%)	50 (11.89±0.58%)	100 (15.50±0.99%)	200 (17.99±1.29%)	500 (22.61±0.72%)	1584±1.58[c]
Ascorbic acid	5 (12.57±0.61%)	25 (28.04±1.35%)	50 (49.22±1.16%)	100 (61.58±2.94%)	200 (95.05±1.34%)	500 (96.18±1.24%)	44.47±1.27[a]

Values are the mean of duplicate experiments and represented as mean ± SD (Standard Deviation Mean).
Values in same column with different superscripts are significantly different ($P < 0.05$).
Bonferroni multiple comparison was performed to analyze this data set

Table 4. Correlation co-efficient for DPPH, total phenol, total antioxidant and total flavonoid

	Total phenol	Total antioxidant	Total flavonoid
DPPH	-0.915	0.997[a]	0.997[a]
Total phenol		-0.909	-0.933
Total antioxidant			0.998[a]

Here, [a]P < 0.01, correlation among different antioxidant activity. Correlation was measured by Pearson's correlation analysis

3.3 Antimicrobial Activity of Plant Extracts

The results of disc diffusion method of *U. lagopoides* extracts are shown in Table 5. Among six bacterial species methanol extract showed activity against one Gram positive (*S. aureus*) and three Gram negative bacteria (*E. coli, S. typhi* and *K. pneumoniae*). The antimicrobial activity of methanol extract was increased with increasing concentration. The zone of inhibition at concentrations of 375, 750 and 1500 µg/disc were ranged from 0.00±0.00 to 8.50±0.71 mm,0.00±0.00 to 11.00±2.12 mm and 0.00±0.00 to 12.25±1.77 mm respectively. Furthermore, *S. typhi* (12.25±1.77 mm) appeared to be the most sensitive strain followed by *K. pneumoniae* (10.00±1.41 mm), *E. coli* (9.50±1.41 mm) and *S. aureus* (8.75±1.07 mm) at 1500 µg/disc. Petroleum ether extract showed concentration dependent activity against only one Gram negative bacteria (*S. typhi*). The zones of inhibition at concentrations of 375, 750 and 1500 µg/disc were 0.00±0.00, 8.25±0.35 and 11.50±0.71 mm respectively. While, the standard drug Azithromycin (30 µg/disc) showed antimicrobial activity against tested all microorganism with zone of inhibition ranged from 9.50±1.41 to 32.00±2.89 mm. These findings suggest that methanol extract showed better activity than petroleum ether extract.

3.4 Analgesic Activity

The results of analgesic activity are presented in Table 6. At the dose of 250 mg/kg both methanol and petroleum ether extracts significantly ($P < 0.001$) inhibited the number of acetic acid induced writhing by 33.44% and 36.88% respectively. While both extracts at 500 mg/kg dose also significantly ($P < 0.001$) reduced the number of acetic acid induced writhing by 52.50% and 64.69% respectively compared with Ibuprofen 100 mg/kg which caused 76.56% reduction in the number of writhing. Both extracts at doses of 250 mg/kg and 500 mg/kg inhibited writhing in a dose dependent manner (Table 6). From this result we may assume that petroleum ether extract was more potent than methanol extract.

4. DISCUSSION

4.1 Phytochemical Screening of *U. lagopoides* Extracts

The chemical constituents present in the plants or crude extracts are known to be biologically active ingredients. Some chemical components are considered as secondary metabolites. They are responsible for different activity such as antioxidant, antimicrobial, anticancer and antifungal [11-13]. This study was designed to make logical and authentic approach in ascertaining the mentioned pharmacological properties of *U. lagopoides.* Phytochemical constituents found in methanol extract of whole plant (Table 1) is consistent with the previous findings shown the presence of alkaloids, saponins, glycosides, steroids, tannins and flavonoids in aqueous and ethanol root extracts of *U. lagopoides* [14] but differs with petroleum ether extract. This slight variation may be due to difference in solvent extraction, local climate, soil composition and the harvest time of the collected plant [12,15-16].

4.2 Antioxidant Activity of *U. lagopoides* Extracts

The total phenolic contents of two extracts were expressed as GAE. Between two extracts, methanol extract contained more amounts of phenol compounds than petroleum ether extract (Table 2). But their phenolic content does not vary significantly ($P > 0.05$). It has been stated that the health beneficial effects of polyphenols could result from their antioxidant functions e.g. by acting as modulators of cellular signaling processes [17-18]. Antioxidant properties of polyphenols arise from their high reactivity as hydrogen or electron donors which can stabilize and delocalize the unpaired electron (chain-breaking function) and from their potential to chelate metal ions (termination of the Fenton reaction) [19]. They have been shown to exert anticarcinogenic effects by modulating enzyme systems that metabolize carcinogens or pro-carcinogens to genotoxins by converting them to less reactive compounds before they react with

DNA. Polyphenols have been shown to inhibit the Cytochrome P_{450} super family of enzymes that metabolizes many pro-carcinogens to reactive compounds before they react with DNA and induce malignant transformation, thus reducing the formation of reactive intermediates [20]. Blockage of LDL oxidation, decrease of the formation of atherosclerotic plaques and reduction of arterial stiffness, stimulation of arteries for vasodilation are also major pharmacological activities of polyphenolic compounds [21-23]. In total flavonoid content assay both extract showed good flavonoid content. But petroleum ether extract contains higher amounts of flavonoids than methanol extract (Table 2). The values differed significantly ($P < 0.05$) than each other. Phenolic compounds or polyphenols, represented in majority by flavonoids, are a major area of research, because they are considered as potent antioxidants, anti-inflammatory, anti-bacterial, antiviral and anti-cancer agents [24-27]. Flavonoids play an important role in antioxidant system. The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper and inhibition of enzymes responsible for free radical generation [26]. Depending on their structure, flavonoids are able to scavenge practically all known ROS. The total antioxidant activity was estimated from their ability to reduce Phosphate/Mo (VI) complex to Phosphate/Mo (V). In total antioxidant content assay both extract showed very good total antioxidant content. But petroleum ether extract contains more amount of antioxidant than methanol extract. The values differed significantly ($P < 0.05$) than each other.The assay has been successfully used to quantify vitamin E in seeds and, being simple and independent of other antioxidant measurements commonly employed; it was decided to extend its application to plant extracts [7].

The DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical scavenging activities of medicinal plants. It accepts an electron or hydrogen radical to become a stable diamagnetic molecule [28]. The odd electron in the DPPH free radical gives a maximum absorption at 517 nm and it is purple in color. The color turns from purple to yellow when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H [29]. The more DPPH radical reduction will occur, the more will be the free radical scavenging property. Both methanol and petroleum ether extracts showed a concentration dependent DPPH free radical scavenging activity (Table 3). IC_{50} value of the methanol extract showed good activity than petroleum ether extracts. It has been stated that free radical scavenging property is due to phenolic compounds like flavonoids, polyphenols, and tannins [30]. Presence of such phytochemical constituents (Table 1) may be responsible for their free radical scavenging activity.

Pearson's correlation analysis was performed aforesaid four antioxidant methods (Table 4). In this study we have found a significant correlation ($P < 0.01$) between pairs of DPPH and total flavonoid (correlation coefficient r =0.997), DPPH and total antioxidant (correlation coefficient r = 0.997), total antioxidant and total flavonoid (correlation coefficient r = 0.998). While there was a negative correlation ($P > 0.05$) found between DPPH and total phenol (r = -0.915), total phenol and total antioxidant (r =-0.909), total phenol and total flavonoid (r = -0.933). However positive correlation indicates potential antioxidant capacity of both crude extracts.

4.3 Antimicrobial Activity of *U. lagopoides* Extracts

In disc diffusion technique, methanol extract showed concentration dependent activity against one Gram positive (*S. aureus*) and three Gram negative bacteria (*E. coli, S. typhi* and *K. pneumoniae*), petroleum ether extract showed concentration dependent activity against only one Gram negative bacteria (*S. typhi*),while standard drug Azithromycin showed antimicrobial activity against all the tested microorganisms (Table 5). These findings closely matched to the finding of earlier Sivakumar et al. [14] and Hamid et al. [3]. Both methanol and petroleum extracts at 1500 µg/disc showed good zone of inhibition (12.25±1.77 and 11.50±0.71 mm respectively) against *S. typhi* in comparison to the standard Azithromycin (15.50±0.71 mm), which gives support in favour of its traditional use in fever. In phytochemical screening both extract of *U. lagopoides* showed presence of different types of phytochemical compounds (Table 1). The alkaloids present in the extract may be responsible for the antibacterial activity [31-39]. The mechanism of antimicrobial effect given by different alkaloids is not clear. Cryptolepine, an indoloquinoline alkaloid, was studied in detail. Sawer et al. [40] reported that cryptolepine,

causes cell lysis and morphological changes of *S. aureus.* However antimicrobial effect of the alkaloid is thought to be through a different mechanism as the compound inhibits topoisomerase to intercalate DNA and to inhibit DNA synthesis [41-43]. The tannin contents of the plant extracts may also contribute to the antimicrobial activity [44-48]. The antimicrobial mechanisms of tannin might be due to their astringent property that can induce complexation with microbial enzymes or substrates, iron deprivation, hydrogen bonding or nonspecific interaction with microbial enzymes, toxic action on microbial membranes, complexation of metal ions [49-51]. The antimicrobial activity may also be due to the presence of flavonoids [34,52-55], saponins [56-58] and steroids [59-62]. Methanol extract showed presence of these five potential phytochemical components alkaloids, tannins, flavonoids, saponins and steroids and while petroleum ether extract contains only flavonoids (Table 1). May be for the presence of these phytochemical constituents methanol extract showed activity against both Gram positive and Gram negative bacteria while, petroleum ether extract showed activity against only one Gram negative bacteria. These findings suggest that antibacterial activity of methanol extract may be indicative of the presence of broad spectrum of antibiotic compounds or simply general metabolic toxin [63]. Further studies may be conducted to find out possible mechanism of antimicrobial activity of the phytochemical constituents.

4.4 Analgesic Activity of *U. lagopoides* Extracts

Both extracts of *U. lagopoides* showed significant ($P < 0.001$) analgesic activity in the acetic acid induced writhing test on mice at both 250 mg/kg and 500 mg/kg dose. Earlier Hamid et al. [2] reported that alcohol and aqueous extract of aerial parts of plant exhibit's marked analgesic activity in mice ($P < 0.01$), compared with acetylsalicylic acid (100 mg/kg) in acetic acid induced writhing test. So, our findings are consistent with the findings of Hamid et al. [2]. Plants that are effective in this test have peripheral analgesic activity [64]. In phytochemical screening test suggested that different phytochemical compounds were present in both extracts of *U. lagopoides* (Table 1). Acetic acid induced writhing model represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from tissue phospholipid [65]. The acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics. The response is thought to be mediated by peritoneal mast cells [66], acid sensing ion channels [67] and the prostaglandin pathways [68]. Flavonoid, a powerful antioxidant [69], shows analgesic activity [70-75]. Flavonoids primarily shows analgesic activity by targeting prostaglandins [76-77]. There are also reports on the role of tannins in antinociceptive activity [78]. This plant's extracts demonstrated good antioxidant action in the tested models. So it can be assumed that cyclooxygenase (COX) inhibitory activity together with antioxidant activity may reduce the production of free arachidonic acid from phospholipid or may inhibit the enzyme system responsible for the synthesis of prostaglandins and ultimately relive pain-sensation. The result suggests that flavonoid and tannin present in this plant may responsible for peripheral analgesic activity. In our investigation we found that petroleum ether extract showed more potent activity than methanol extract. Higher amount flavonoid content of petroleum ether extract (81.07±1.80 mg/g, QE) than methanol extract (31.33±0.72 mg/g, QE) may contribute to this better activity. Steroids also possess pain relieving activity [79-83]. Earlier four new

Table 5. Antimicrobial activity of methanol and petroleum ether extracts of *U. lagopoides* in disc diffusion method.

Micro-organisms	Zone of inhibition (mm)						
	Methanol extract			Petroleum ether extract			Azithromycin
	375 µg/disc	750 µg/disc	1500 µg/disc	375 µg/disc	750 µg/disc	1500 µg/disc	30 µg/disc
S. aureus	0.00±0.00^{a}	7.25±0.35^{b}	8.75±1.07^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	27.75±1.07^{d}
S. lutea	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	10.5±3.54^{ab}
E. coli	7.25±1.07^{b}	8.00±0.71^{b}	9.50±1.41^{bc}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	32.00±2.89^{d}
S. typhi	8.50±0.71^{b}	11.00±2.12^{c}	12.25±1.77^{c}	0.00±0.00^{a}	8.25±0.35^{b}	11.50±0.71^{b}	15.50±0.71^{b}
V. cholera	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	9.50±1.41^{a}
K. pneumonia	7.00±1.41^{b}	9.25±1.77^{bc}	10.00±1.41^{bc}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	21.75±1.77^{c}

Values are the mean of duplicate experiments and represented as mean ± SD (Standard Deviation Mean). Fisher's LSD was performed. Values in same column with different superscripts are significantly different (P < 0.05)

Table 6. Effect of methanol and petroleum ether extracts of *U. lagopoides* in acetic acid induced writhing test

Group	Dose (mg/kg)	Number of writhing	Inhibition (%)
Control	-	32.0±0.79	-
Methanol extract	250	21.3±1.04[a]	33.44
	500	15.2±0.93[a]	52.50
Petroleum ether extract	250	20.2±1.29[a]	36.88
	500	11.3±0.66[a]	64.69
Ibuprofen	100	7.5±0.35[a]	76.56

Values are presented in mean ± SEM (Standard Error Mean) for five mice per group.
[a]P < 0.001 compared to control (Dunnett's multiple comparison)

compounds, namely 7′-carboxylic-*n*.-hepta-cosanyl-glutarate, *n*.-nonacosan-6-ol-1,13-dioic acid, 4,12-dimethyl-*n*.-tetradeca-6,8,10-triene-1-ol-4-oic acid, and 8,14-13,17-diseco-stigmast-5,22-diene-3-α-ol, along with 2-hydroxytricontane and *β*-sitosterol glycoside, have been isolated from the aerial parts of *Uraria lagopoides* D.C. [84]. *β*-sitosterol (plant steroids) has been reported as an anti-inflammatory, analgesic agent [80]. Plant methanol extract exhibited analgesic activity may be due to the presence of steroids (Table 1). Further studies are required to determine the possible mechanism of action of these potential compounds.

5. CONCLUSION

On the basis of the findings of the present study it may be assumed that moderate total phenolic content and DPPH free radical scavenging activity were showed by the methanol extract and moderate total flavonoid content and total antioxidant capacity were showed by petroleum ether extract of the plant. Methanol extract exhibited antimicrobial activity especially against *S. typhi* which is comparable to the standard drug Azithromycin. Both of the extracts exhibited significant analgesic activity in acetic acid induced writhing test. The potential of these extracts as antioxidant, antimicrobial and analgesic agents may be due to the presence of different phytoconstituents. However, extensive further researches are necessary to search for active principles responsible for these activities.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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