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Phytochemical Determination and Antibacterial Activity of *Urtica dioica* Leave Extracts against Isolated Food Borne Bacteria

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A B S T R A C T

Aim of this study was to analyze phytochemical constituents and antibacterial activity of *Urtica dioica* leave extracts against isolated food-borne pathogens from different food samples. The phytochemical analysis showed the presence of carbohydrates, alkaloids, saponins, tannins and protein. Total 75 different isolates of bacteria were isolated from different food samples. Out of 75twenty-four were found to be positive for *Klebsiella*, twenty-six for *Staphylococcus* spp. and twenty-five for *E. coli*. Maximum zone of inhibition was recorded by ethyl acetate extract against *E. coli* with 2.36±0.26 mm diameter followed by *Klebsiella* (2.28±0.16 mm) *Staphylococcus* spp. (1.96±0.28 mm) respectively. Methanol extract showed maximum inhibitory zone of 1.91±0.26 mm against *Klebsiella* followed by 1.82±0.33 mm zone against *Staphylococcus* spp. and 1.5±0.17 mm inhibitory zone against *E. coli*. Aqueous extract not showed inhibition against any isolated bacteria.

Introduction

Current national and international trends in antibiotic resistance are becoming a public health crisis. Multidrug resistant organisms are most prevalent in hospital setting and alarmingly are now being identified in the community. Prevalence of several microorganisms in different food samples are responsible for different food-borne diseases. Many times these diseases are fatal and lead to a hazardous condition.

Urtica dioica (family *Urticaceaeis*) is a high value medicinal plant, recommended for treating osteoarthritis and urinary tract infections, rheumatoid arthritis, Alzheimer's disease, allergies, asthma, bronchitis, bursitis, cough, gout, gingivitis, hair growth and baldness, prostate enlargement, sciatica,

tendinitis, kidney stones (Banso and Adeyemo 2006, Bandow,*et al.,* 2003). New antimicrobial agents are required to treat human and animal diseases caused by drug resistant microorganisms. Interest has been increasing in plant-derived drugs, mainly due to the belief that "green medicine" is harmless and more reliable than costly manmade drugs (Belyakova *et al.,* 2002; Benkeblia, 2004). *Urtica dioica* is stated to possess antihaemorrhagic and hypoglycemic properties.

Natural products provide boundless prospects for new drug leads because of the availability of chemical diversity. There is a continuous and urgent need to discover new antimicrobial compounds with varied chemical structures and new mechanisms of action for new infectious diseases (Cowan, 1999). Hence, researchers are progressively turning their consideration to traditional medicine, looking for novel leads to develop improved drugs against microbial infections (Benkeblia, 2004; Banso and Adeyemo, 2006; Bandow *et al.,* 2003; De Boer *et al.,* 2005). So the main objective of the present study is to investigate the phytochemical constituents in *Urtica dioica* leave extracts and their antibacterial effect on isolated food-borne bacteria.

Materials and Methods

Sample collection

The samples including leaves of *Urtica dioica* were collected from local area around Dehradun (Uttarakhand) during February.

Methanolic and ethyl acetate extract preparation

The plant materials were collected, washed and dried for few days in a shade at ambient temperature (31 ºC). Dried plant leaves were crushed by a laboratory blender and weighed before extraction. Organic solvent extraction of nettle was carried out by using ethanol (95%) and ethyl acetate (95%) which is considered as very effective in extracting the active ingredients of the plant according to method described by Ilhami *et al.,* (2004) and Inouye *et al.,* (2001). This was done by using Soxhlet assembly, which consists of a heating element, which is used for heating the solvent taken in a round bottom glass flask that fitted to an extraction chamber. The extracting unit contains the solvent and cellulose (thimble) located inside it that contains the dry plant powder. A bulb type condenser is fitted on to the extraction unit. The soxhlets extractor acts on the principle of continuous heat percolation process. Small volume of hot solvent is passed through the material filled in the cylinder a number of times till active constituents of the material get exhausted. For condensation of vapor solvents, 20 gm. of powdered plant material (leaves) powder was taken in the thimble and 200 ml solvents (i.e. methanol ethyl, acetate) were placed inside the flask. The extraction was carried out for 8 to 10 hours by heating temperature that kept the solvent at 70 ◦C (methanol) and 75 ◦C (ethyl acetate). After that, the extract was dried by using an electric oven at temperature 40-45 ◦C until dry extract was obtained. The dry extract was placed in an incubator under 38-40 ◦C for complete dryness of the sample. The dried extracts are dissolved in Dimethyl sulphoxide (DMSO) to prepare concentration of 100 mg/mL that is used for testing its antibacterial activity.

Aqueous extraction

Plant leaves were washed under tap water and then shade-dried in room temperature. The plant material was chopped into small pieces and then grinded by an electric blender into powder. The powdered mixture (50 gm.) was mixed with 100 ml distilled water. Aqueous extract was carried out according to Inouye *et*

al., (2001) Joanne *et al.,* (2002) by using magnetic stirrer at 60◦C for 3 hours, then filtrate was kept in incubator till complete drying. The yield of dry powder was 15 gm.

The dry powder was dissolved in DMSO to prepare concentration of 100 mg/mL that was used for testing antibacterial activity.

Phytochemical screening (Brinda and Sarswatih, 1981)

All the extracts (methanol, ethyl acetate and aqueous) were individually subjected to qualitative analysis of the active compounds present in them. The compounds screened are carbohydrate, alkaloids, saponins, flavonoids, tannins and proteins.

Test for flavonoids

Few drops of extract were taken in test tube to which 3-4 drops of $FeCl_3$ was added. The green color indicated the presence of phenolics in the sample.

Test for alkaloids

1 mL of Wagner's reagent was added to 1 mL of extract. Brown precipitate indicated the presence of alkaloids.

Test for saponins

To 1 ml of extract was added 2 mL of distilled water in graded cylinder and then shaken vigorously for 15 mins. A 1 cm layer of foam indicates the presence of saponins.

Test for tannins

1 ml of extract was taken separately in few ml of alcohol. To this few drops of freshly prepared $FeCl_3$ was added. Development of greenish violet colour indicated the presence of tannins.

Carbohydrates

To 1 ml of Fehling's reagent was added to 1ml of water in a test tube, mixed thoroughly and kept in water bath. The formation of red precipitate (cuprous oxide) in the bottom of the tube indicates the presence of carbohydrates.

Proteins

Add 1 mL of concentrated HNO_3 to 2 mL extract. A white precipitate formed. Boiled the solution and colour changes to yellow. Cool the test tube and 2 mL 0f 20% NaOH to make it alkaline. The colour changes to orange indicated the presence of proteins.

Food Sample collection

Food samples (cooked) were collected from local market, Dehradun (India) in sterile plastic bags and further used for microbial analysis.

Isolation of food borne pathogens

The samples are rinsed thoroughly with distilled water and used for isolation of bacteria on specific media nutrient agar and MacConkey agar. For cultivation of *Klebsiella* the sample rinsate was inoculated into Mac Conkey agar at 37^0C for 24 hours. For the isolation of *Staphylococcus spp.* and *E. coli,* the rinsate was incubated into nutrient agar media at 37^0C for 24 hours.

Morphological and biochemical characterization

Characterization of the colony isolates was achieved by initial morphological examination of the colonies in the plate for colonial appearance, size form, elevation, color and pigmentation and the results were recorded. Biochemical characterization of bacteria was done by performing specific tests such as

indole, Methyl Red, Voges Proskauer, citrate, glucose, sucrose and urease test.

Determination of Antimicrobial Activity-Disc diffusion method (Maruzzella and Henry, 1958)

Nutrient Agar (NA) plates were seeded with 8 h broth culture of different bacteria. The disc was made up of Whatman no 1 filter paper having diameter of 6 mm. The discs were sterilized before use. 20 uL of different extract solution (ethyl acetate, methanol and aqueous) were taken in the disc with the help of micropipette and forceps. Discs injected with 20 uL of DMSO served as control. The discs were injected with extracts were placed on solid agar medium by pressing gently. The bacterial plates were incubated at 37˚ C for 24 hrs. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone with the help of the ruler. The experiment was carried out in triplicate and the mean of the diameter of the inhibition zones was calculated.

Statistical analysis

Three replicates of each sample were taken and experiments were repeated thrice. The statistical analysis was done by ANOVA and significance of differences between replicates were measured at 5% ($P<0.05$).

Results and Discussion

Qualitative Phytochemical Analysis

The presence of different compounds in extracts of *Urtica dioica* was determined by qualitative phytochemical analysis. The results of the phytochemical analysis are presented in Table 1. Leaf ethyl acetate possess only alkaloids and flavonoids. Carbohydrates saponins, tannins and proteins were absent in ethyl acetate extract. Carbohydrates, alkaloids, flavonoids and tannins were detected in the methanol leaf extract. Saponins and proteins were absent in leaf methanol extract. In aqueous leaf extract carbohydrates, alkaloids, saponins and flavonoids were present while tannins and proteins absent. Nettle has different constituents (i.e. alkaloids, phenols, flavonoids, tannins and saponins) which plays major role in antibacterial antimicrobial activity (Cowan, 1999; Le Grand *et al.,* 1988). Alkaloid, it is antibacterial activity may be due to its ability to react with carboxyl, amino, hydroxyl and sulfhydryl groups in bacterial nucleic acid as well as protein, it very reactive chemical compounds that associated with proteins to give intermolecular cross-links and intercalate with DNA (Onmetta-aree, 2005). Tannin is the another constituent which are precipitate gelatin from solution, a property is known as astringency. It has been reported to inhibit the development of microorganisms by precipitating microbial protein and making nutritional proteins unavailable for them (Gonzalez-Lamothe *et al.,* 2009; Palic *et al.,* 2002). Phenolic compound is mostly hydrophobic in nature which has a hydroxyl group (-OH). The antimicrobial activity due to this group is well known, the number and site(s) of -OH groups on the phenol group are thought to be related to their relative activity to microorganisms (Randall *et al.,* 2000; Rojas *et al.,* 2003). Abdeltawab *et al.,* (2012) also reported that *Urtica dioica* is a rich source of phytochemicals such as phenolic compounds and minerals which can be used as a potential source of useful drugs. Bameta *et al.,* (2017) documented the presence of phenol, steroids, saponins, tannins coumarin and reducing sugar in *Nerium oleander*.

Isolation of food born microorganisms

Out of total 33 samples, 75 total different isolates of bacteria were isolated from food samples. In that 25 were found to be positive for *E. coli*, 24 were found to be positive for *Klebsiella* and 26 were found to be positive for *Staphylococcus* spp. (Table 2). All the

samples were streaked on the surface of Mac Conkey agar as well as on the surface of nutrient agar for the growth after overnight incubation.

Morphological and biochemical characterization of different bacterial isolates

E. coli is seen as a gram negative, rod shaped, motile, flagellated bacteria (Figure 1a). The microscopic appearance of *Staphylococcus spp*. was of gram-positive cocci. The organism appears purple (Figure 1b). *Klebsiella* is seen as a non motile, Gram negative, bacteria (Figure 1c).

Klebsiella isolates grew in Mac Conkey were found to be Mucoid pink (Figure 1d). All the *E. coli* isolates grew on nutrient agar producing white colonies (Figure 1e) while *Staphylococcus spp.* producing golden yellow colonies (Figure 1e). Isolates belongs to *E. coli* were Indole, Methyl Red positive as shown by change in colour however, Voges Proskauer was negative for all the isolates.

They fermented glucose and sucrose with the production of acid gas. They were negative for other biochemical tests performed such as urease production and citrate utilization. *Staphylococcus spp.* isolates were found to be Methyl Red, Voges Proskauer, citrate, glucose, sucrose and urease test positive negative for indole test. All the isolates grew on Mac Conkey agar medium producing colonies of pink or light pink color. All the isolates belonging to *Klebsiella* were positive for Voges Proskauer, citrate utilization and urease production however, they were negative for indole and methyl red test as there was no change in color. Like *E. coli* they fermented glucose and sucrose with the production of acid gas (Table 3).

Table.1 Qualitative phytochemical analysis of *Urtica dioica* extracts

S. No.	Tests performed	Ethyl acetate	Methanol	Aqueous
1	carbohydrates	-	+	+
2	Alkaloids	+	+	+
3	Saponins	-	-	+
4	Flavonoids	+	+	+
5	Tannin	-	+	-
6	Proteins	-	-	-

Table.2 Number of isolates in food samples

S.No.	Samples	Total no of samples	Total no of isolates	Total number of *E. coli* isolates	Total number of *Klebsiella* isolates	Total number of *Staphylococcus spp.* isolates
1	Spoiled rice	04	22	09	06	07
2	Spoiled daal	13	18	04	08	06
3	Spoiled vegetables	16	35	12	10	13
		Total no of samples= 33	Total no of isolates =75	Total number of *E. coli* isolates= 25	Total number of *Klebsiella* isolates= 24	Total number of *Staphylococcus spp.* isolates= 26

Table.3 Culture and biochemical characteristics of different isolates

S. No.	Characteristics	Organisms		
		E. coli	***Klebsiella***	***Staphylococcus spp***
1	**Culture characteristics**	White colour colonies	Mucoid pink colour colonies	Golden yellow colonies
2	**Biochemical characteristics**			
	Indole	+	-	-
	Methyl Red	+	-	+
	Voges Proskauer	-	+	+
	Citrate	-	+	+
	Glucose	+	+	+
	Sucrose	+	+	+
	Urease	-	+	+

Fig.1 *(a) E. coli* (b) *Staphylococcus* spp. (c) *Klebsiella* (d) Mac Conkey plates with lactose fermenting colonies (*Klebsiella*) (e1) Nutrient agar plate having *Staphylococcus spp*. (e2) Nutrient agar plate having *E. coli*

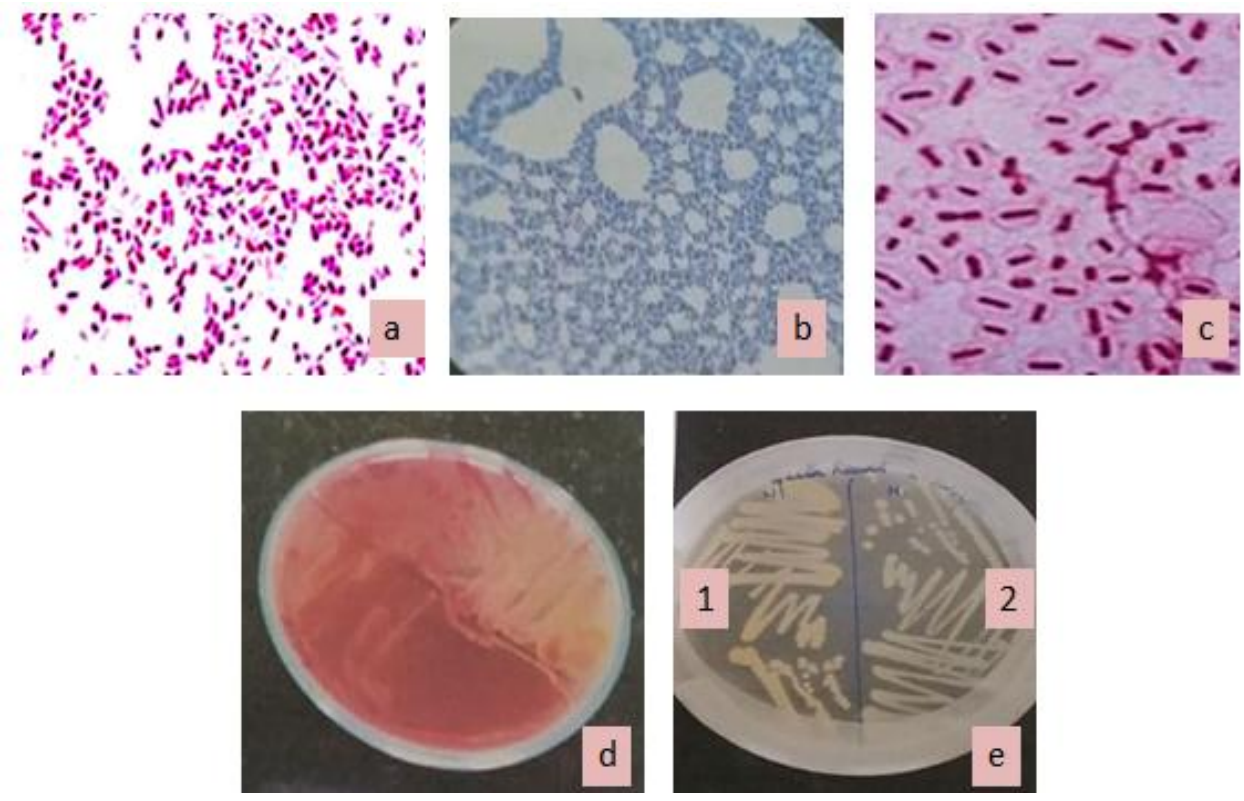
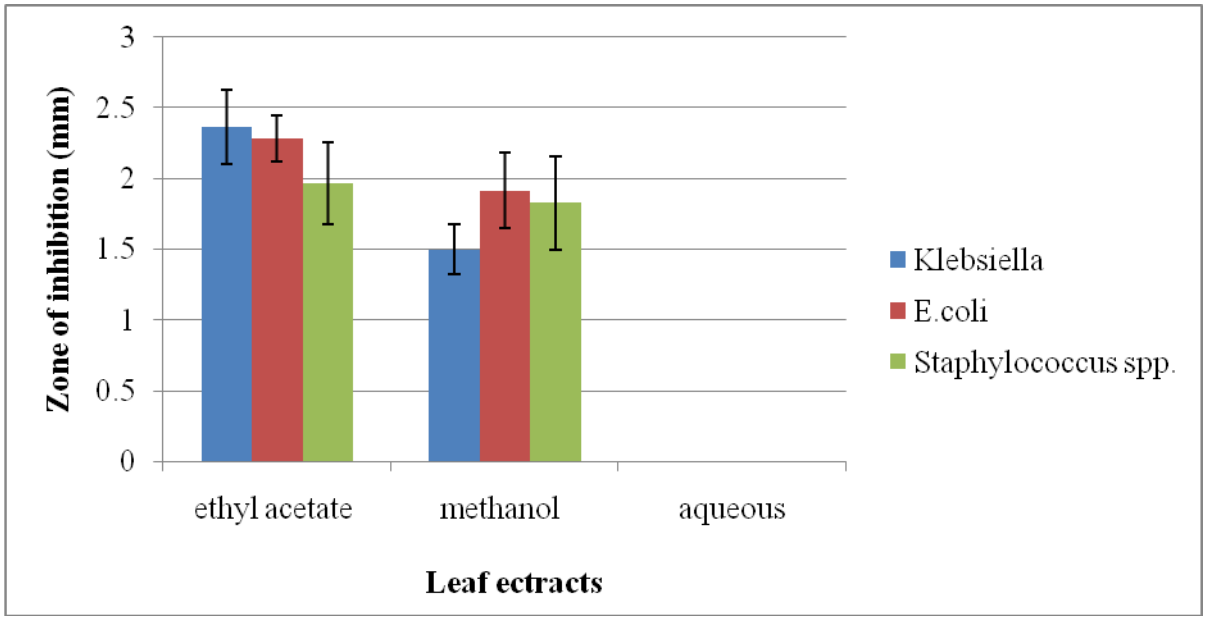


Table.4 Antibacterial effect of ethyl acetate, methonolic and aqueous extract of *Urtica dioica* against some bacterial isolate

S.No.	Isolates	Diameter of inhibition zone(mm)		
		Ethyl acetate (100 mg/mL)	Methanol extract (100 mg/mL)	Aqueous extract (100 mg/mL)
1	*E. coli*	2.36±0.26	1.5±0.17	0.0±0.0
2	*Klebsiella*	2.28±0.16	1.91±0.26	0.0±0.0
3	*Staphylococcus spp.*	1.96±0.28	1.82±0.33	0.0±0.0

Fig.2 Antibacterial activity of leaf extract



Antibacterial activity of different extracts against different isolated food born bacteria

On comparing the antimicrobial activity of ethyl acetate, methanol and aqueous extracts, results shows that ethyl acetate extract has maximum antimicrobial activity against *E. coli* (2.36±0.26) followed by *Klebsiella* (2.28±0.16) *Staphylococcus spp.* (1.96±0.28) respectively. Methanolic extract showed antibacterial activity against all bacterial isolates. Maximum zone of inhibition was displayed by *Klebsiella* (1.91±0.26) followed by *Staphylococcus spp.* (1.82±0.33) and *E.coli* (1.5±0.17). Aqueous extract was showed negative result against all bacterial strains (Table 4 and Figure 2).

The antibacterial activity of 95% ethanol and aqueous extracts of nettle leaf were tested against *S. aureus, E. coli, Klebsiella spp., B. subtillus, Proteus spp. Salmonella spp.* and *Pseudomonas spp.* (Salih *et al.*, 2014). *S. aureus, B. subtillus and Salmonella spp.* showed the highest susceptibility to *Urtica dioica* extracts antibacterial effect, while *Pseudomonas, E coli* and *Proteus* were less susceptible. Animal studies proved that nettle leaf extract may inhibit blood clotting, can decrease total cholesterol levels as well as enhance the overall liver function (EI Haouari *et al.*, 2006; Nassiri *et al.*, 2009).

Terpenes and phenols of *U. dioca* are one of the major groups associated with the inhibition

of microbial inhibitions and cancer (Dar *et al.*, 2012). The results of Chahardehi *et al.,* (2012) revealed that ethyl acetate, hexane and chloroform extracts showed highest inhibition against *B. cereus*, *Staphylococcus aureus* and *Vibrio parahaemolyticus*. The ethanol extract of nettle leaves did not inhibit the growth of *E. coli* ATCC 9837 (Sanchez, *et al.*, 2009), unlike the water extract, which exhibit considerable antibacterial activity (Gulcin *et al.,* 2004).

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