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Antibacterial and Phytochemical Analysis of Ethnomedicinal Plants.

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ABSTRACT

Aqueous and ethanolic extracts of leaves and stems of *Asparagus densiflorus, Erythrina blakei, Swertia chirata, Tinospora cordifolia* and *Ziziphus mauritiana* were screened for their antibacterial activity against *Enterobacter aerogenes, Clostridium perfringens* and *Salmonella typhimurium.* Out of five plant species tested, *T. cordifolia* showed the maximum zone of inhibition against *C. perfringens* (10mm) while, *S. chirata*, showed the maximum zone of inhibition against *E. aerogenes* and *S. typhimurium* (12mm, 10.34mm). The phytochemical analysis of the aqueous and ethanolic extracts were carried out for the presence of flavonoids, tannins, phenolics, saponins, cardiac glycosides, terpenoids, quinones, amino acids, carbohydrates and alkaloids. *T. cordifolia and S. chirata* were found to contain high amount of flavonoids (1100 µg/ml and 1000 µg/ml, respectively) and phenolics (580 µg/ml and 603 µg/ml, respectively). The results suggest that crude extracts from these plants can be used for therapeutic purposes as potent antioxidants and antimicrobials due to presence of various phtyochemicals in them.

Keywords: Antibacterial, *Asparagus densiflorus*, *Erythrina blakei,* phytochemical, *Swertia chirata, Tinospora cordifolia, Ziziphus mauritiana,*

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INTRODUCTION

Plants are nature's "Chemical factories" providing the richest source of organic chemicals on Earth. The current microbiological experiments and techniques have shown that medicinal plants to exhibit tremendous activity against human bacterial and fungal pathogens [1]. Many plants have been investigated scientifically for antimicrobial activity and a large number of plant products have been shown to inhibit the growth of pathogenic microorganisms. Plants are rich resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several plants having medicinal properties [2]. The beneficial products of plants result from combination of different secondary metabolites like alkanoids, saponins, tannins, anthraquinolones etc. Exploitation of these phytochemicals produced by certain plants can serve as a solution to the problem of ever increasing antibiotic resistance in microorganisms [3]. Moreover, phytomedicines have shown great promise in the treatment of intractable infectious diseases including opportunistic AIDS infections. Many plants have been found to have certain types of phytochemicals such as saponins, alkaloids, volatile oils, flavonoids and anthraquinones which can affect many diseases such as cancer, stroke or metabolic syndrome. Phytochemicals also provide certain antioxidant and anti-inflammatory properties; therefore, these are commonly used in medicines. About 80% of individuals from developed countries use traditional medicines, which has compounds derived from medicinal plants. However, such plants should be investigated to better understand their properties, safety, and efficiency [4]. This work was undertaken in order to carry out preliminary investigative studies on phytochemical analysis of five ethnomedical plant extracts viz *Tinospora cordifolia, Ziziphus mauritiana, Swertia chirata, Erythrina blakei and Asparagus densiflorus* and also find their antimicrobial activity against pathogenic microorganisms.

MATERIALS AND METHODS

Plant collection

Erythrina blakei leaves were collected from Forest Research Institute (FRI), Dehradun, India, leaves of the *Swertia chirata* were collected locally From Garo hills Meghalaya, while leaves of *Asparagus densiflorus* and *Ziziphus mauritiana* as well as mature stem of *Tinospora cordifolia* were collected locally from herbal garden at Lovely Professional University, Punjab, India.

Plant extract

Aqueous extract

Plant parts (leaves and/or stems) were washed thoroughly in tap water 3-5 times and homogenized with 100ml of double distilled water followed by centrifugation at 10,000 rpm for 5 min to separate the liquid medium from debris. The supernatant was collected in falcon tubes and was stored below ambient temperature according to the method of Sandhu and co-workers [5].

Ethanol extract

5 g of the air dried, coarsely powdered plant parts were shaken with 100 ml of ethanol in a flask for twenty-four hours. The flasks were shaken frequently for six hours and the allowed to stand for eighteen hours. The mixture was filtered and the filtrate was evaporated to dryness in a flat bottomed shallow dish, followed by drying at 105ºC. The sample was weighed and the percentage of extract was calculated.

Preparation of sterile disc

Whatman's No.1 filter paper was punched into 5mm disc form, sterilized and dipped in extracts. Precautions were taken to prevent the flow of the solvent extract from the discs to the outer surface. The condensed extracts were applied in small quantities on discs and they were allowed to dry in air.

Antibacterial Testing

Three bacterial cultures viz. *Clostridium perfringens* MTCC No. 450, *Enterobacter aerogenes* MTCC No. 7325 and *Salmonella typhimurium* MTCC No. 3231 were procured from Microbial Type Culture Collection, Mohali and maintained on Nutrient Agar Medium, EMB Agar Medium and Tryptone Soya Agar medium, respectively. Antibiotics effective against the bacteria viz Clindamycin, Ciprofloxacin and Azithromycin (Effective against *C. perfringens*, *E. aerogenes,*and *S. typhimurium,* respectively) were used as positive controls. Both the aqueous and ethanolic extracts were subjected to screening for antimicrobial activity by standard Disk diffusion method [6]. When an extract impregnated disk is placed on the agar previously inoculated with the test bacterium, the extract diffuses radially outward through the agar producing a gradient. A clear zone around the disc indicated the antimicrobial effect of the plant extracts. The wider the zone surrounding a disk, the more susceptible the pathogen is to a particular extract.

Phytochemical Analysis

Various parts of the medicinal plants were subjected to preliminary phytochemical screening for the presence or absence of various both primary and secondary metabolites [7].

Alkaloids (Wagner's reagent): A small amount of extract was taken and treated with 3-5 drops of Wagner`s reagent and observed for the formation of reddish brown precipitate (or colouration).Wagner`s reagent was prepared by mixing 1.27g of iodine and 2g of potassium iodide in 100ml of water.

*Carbohydrates (Molisch`s test)*: To 2ml of the extract, few drops of Molisch`s reagent were added. This was followed by the addition of 2ml of conc. H_2SO_4 along the side of the test tube. The mixture was then allowed to stand for two-three minutes and the formation of a red or dull violet colour at the interphase of the two layers indicated a positive test.

Cardiac glycosides (Keller Kelliani`s test): To 5ml of extract, 2ml of glacial acetic acid and a drop of ferric chloride were added. The mixture was then carefully underlayed with 1ml of conc. H_2SO_4. A brown ring at the interface indicated the presence of deoxysugar characteristic of cardenolides.

Flavonoids (Alkaline reagent test): To the 2ml of each extract, a few drops of 20% NaOH solution were added. The formation of yellow colour which becomes colourless on addition of dilute HCl, confirmed the presence of flavonoids.

Phenols: A small amount of extract was treated with aqueous 5% $FeCl_3$ and observed for the formation of deep blue or balck colour.

*Amino acids***:** To 2ml of extract, 2-5 drops of ninhydrin solution was added and placed in water bath for 1-2 minutes. The formation of purple colour confirmed the presence of amino acids.

Saponins (Foam test): 2ml of extract was taken and added to 6ml of water in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirmed the presence of saponins.
Tanins (Braymer`s test): 2ml of extract, 10% alcoholic ferric chloride solution was added and observed for the formation of blue or greenish colour solution which confirmed the presence of Tanins.

Terpenoids (Salkowki`s test): To 1ml of chloroform, 2ml of extract was added followed by the addition of few drops of conc. H_2SO_4. The formation of reddish brown precipitate produced immediately confirmed the presence of terpenoids.

Test for Quinones: A small amount of extract was taken and treated with concentrated HCl and observed for the formation of yellow precipitates or colouration.

Estimation of Phytochemicals with the help of spectrophotometer

The plant extracts were subjected to quantitative estimation of flavanoids and phenolics which are two of the most important and potent phytochemicals with respect to antioxidant activity [8]. Quercetin was taken as standard for the estimation of flavonoids and phenolic content present in the extracts.

Estimation for the presence of Flavonoids

0.5 ml solution of extract in methanol were separately mixed with 1.5 ml methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with the help of UV-visible spectrophotometer.

Estimation for the presence of phenolic compound

Folin-Ciocalteu method was used for the estimation of phenolic compound. To 0.5 ml of extract, 2.5 ml of 1/10 aqueous dilution of FC reagent was mixed. After 5 minutes, 2 ml of Na_2CO_3 (7.5%) was added and incubated at room temperature for 120 min. The absorbance of the reaction mixture was measured at 765 nm by using UV-Visible spectrophotometer

RESULT AND DISCUSSION

Antimicrobial activity of plant extracts

The petriplates containing media were prepared on Nutrient Agar media, EMB Agar media, Tryptone Soya Agar media and the discs soaked in the filtrate were kept on the plates aseptically. The plates were incubated for 24-48 hours at 37^{O}C. The diameter of clear zone formed around the disc was an indicator of the antibacterial activity of the respective plant extract. It was observed that aqueous extract of all the plant parts had higher antimicrobial activity as compared to ethanolic extracts. However, specific conventional antibiotics (used as positive control) were found to have much better antibacterial activity as compared to the plant extracts as suggested by the zone of inhibition (Table 1). This can be attributed to the fact that the plant extracts used were crude plant extracts. It was also observed that aqueous extract of *Tinospora cordifolia* showed the maximum activity against *Clostridium perfringes* whereas *Swertia chirata* shows the minimal activity whereas *Swertia chirata* extract showed the maximum activity against *Enterobacter aerogenes* and *Salmonella typhimurium*.

Phytochemical Test Analysis

Phytochemical properties of aqueous and ethanolic extracts of *Tinospora cordifalia, Ziziphus mauritana, Swertia chirata, Erythrina blakei, Asparagus densiflorus* were studied and compared (Table 2). The crude plant extracts were screened for presence of ten phytochemicals viz. tannins, flavanoids, saponins, carbohydrates, cardiac glycosides, phenols, quinones, terpenoids, amino acids and alkaloids. *Swertia chirata* aqueous extract showed the presence of all the phtochemicals except amino acids. Aqueous extract of *Tinospora cordifolia* showed the presence of all phytochemicals except terpenoids and quinones. However, its ethanolic extract was additionally found to show the presence of terpenoids.

Quantitative Estimation of Flavonoids and Phenolics

Since flavanoids and phenolics are the most important phytochemicals with respect to their antioxidant properties, therefore, these two compounds were estimated quantitatively in the crude plant extracts. Since the aqueous plant extracts showed better antimicrobial activities, therefore only aqueous plant extracts were used for quantitative estimation of flavonoids and phenolics

For the estimation of flavonoids, the absorbance was read at 415nm in order to determine the concentration of flavonoids in different plant extracts with respect to quercetin as a standard (Table 3). In order to estimate the concentration of phenolics in the crude plant extracts, the absorbance was read at 765 nm. It was found that highest flavonoid concentration was present in *Tinospora cordifolia* (1100 µg/ml)

followed by *Swertia chirata* (1000 µg/ml) while the lowest content was present in *Erythrina blakei* (480 µg/ml). Highest phenolic content was found to be present in *Swertia chirata* and *Tinospora cordifolia* with a concentration of 603 and 580 µg/ml, respectively. Similar results have been obtained in our lab when flavonoid and phenolic contents of *Bacopa monnieri* were analysed indicating that plants rich in these compounds are potent antioxidants [9]. Different research groups have also found ethnomedicinal plants to contain these phytochemicals [10-13].

Table 1: Zone of inhibition produced by aqueous and organic plant extracts

Plant extracts	Zone of inhibition (in mm)*					
	Clostridium perfringens		*Enterobacter aerogenes*		*Salmonella typhimurium*	
	Aqueous	*Ethanol*	*Aqueous*	*Ethanol*	*Aqueous*	*Ethanol*
Tinospora cordifolia	10	8.1	11.3	8.4	9.7	7.7
Ziziphus mauritiana	8	5.7	8.7	5.2	10	6.2
Swertia chirata	7.7	5.2	12	8.7	10.3	5.9
Erythrina blakei	9	5.9	8.7	6.2	9.7	5.2
Asparagus densiflorus	7.7	ND	9.7	5.7	8.7	ND
Control	Clindamycin= 20		Ciprofloxacin= 18		Azithromycin= 30	
	Negative control = ND		Negative control = ND		Negative control = ND	

	CD (@5%)	**F-Ratio**
Strains	1.13	3.97
Extracts	1.13	8.09
Strains × Extracts	1.95	3.59

*Average of three replicates
ND: Not Detected

Table 2: Phytochemical Screening of five ethnomedical plants

	Tinospora cordifalia (Stems)		*Ziziphus mauritiana* (Leaves)		*Swertia chirata* (Leaves)		*Asparagus densiflorus* (Leaves)		*Erythrina blakei* (Leaves and stems)	
	Water	Ethanol	Water	Ethanol	Water	Ethanol	Water	Ethanol	Water	Ethanol
Tannins	+	+	+	+	+	-	+	+	-	-
Flavonoids	+	+	+	+	+	-	+	+	+	+
Saponins	+	+	+	-	+	-	+	+	-	-
Carbohydrate	+	+	+	+	+	-	+	+	+	+
Cardiac glycosides	+	+	-	+	+	+	+	+	+	+
Phenols	+	+	+	+	+	-	+	+	-	-
Quinones	-	-	-	-	+	-	+	-	+	+
Terpenoids	-	+	-	+	+	-	-	+	+	+
Amino acids	+	+	+	+	-	-	-	-	-	+
Alkaloids	+	+	-	-	+	-	-	+	+	+

+ : present
- : absent

Table 3: Estimated flavanoid and phenolic content of aqueous plant extracts

Plant extract	Flavanoid Content (µg/ml)	Phenolic Content (µg/ml)
Erythrina blakei	480	400
Swertia chirata	1000	603
Asparagus densiflorus	900	380
Tinospora cordifolia	1100	580
Ziziphus mauritiana	800	405

Flavonoids CD (@5%): 18.18 F-Ratio: 1700.40
Phenolics CD (@5%): 14.75 F-Ratio: 535.13

The results indicate that *Swertia chirata* and *Tinospora cordifolia* can be used for therapeutic purpose because of their antioxidant properties owing to the presence of flavanoids and phenolics in their extracts validating the importance of these ethnomedicinal plants.

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