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ASSESSMENT OF ANTIOXIDANT, CYTOTOXIC AND ANTIBACTERIAL ACTIVITIES OF DIFFERENT FRACTIONS OF CRUDE EXTRACT OF *STEPHANIA JAPONICA* STEM

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ABSTRACT: The different fractions of crude methanolic extract of stem of *Stephania japonica* (Thunb.) Miers was evaluated for antioxidant, cytotoxic and antibacterial activities. The various fractions of *S. japonica* Stem i.e. Ethyl Acetate (EAJS), $CHCl_3$ (CFJS), CCl_4 (CTJS) and Petroleum ether (PEJS) were subjected to free radical scavenging activity. In this investigation, CFJS showed the most significant free radical scavenging activity with IC_{50} value of 119.0µg/ml for *S. japonica* stem. Cytotoxic activity was investigated by brine shrimp (*Artemia salina*) lethality assay. The LC_{50} value of sample CTJS (Carbon Tetrachloride fraction of *S. japonica* Stem), was 3.0µg/ml is highly most significant. Antibacterial activity was tested by disk diffusion method. The Carbon Tetrachloride soluble fraction showed good antibacterial activity against different species of bacteria at different doses. The Ethyl Acetate soluble fraction shows good activity only against *E. coli* at different doses.

INTRODUCTION: The history of medicinal plants in remedy of different diseases is well established. Various species of different family of plant and other sources contribute in the development of present therapeutic processes.

Stephania japonica (Thunb.) Miers (Menisperm aceae) locally known as Aknodi, Akonadi, Akondi, Fuit pata, etc., is a climbing shrubs distributed in widespread north from Eden, South Coast. also Qld and from India, throughout Asia to southern Pacific etc. Leaves and roots are bitter and astringent; used in fever, diarrhoea, urinary diseases and dyspepsia. Leaves are mounted on abscess and kept for bursting. Leaves are macerated in a glass of water and are taken after mixing with molasses to cure urethritis. Leaves are also given for gastritis in Khagrachari. Root paste is taken for vertigo and dysentery; root mixed with root juice of *Flemingia stricta* is taken for asthma; root paste is warmed and rubbed in hydrocele. Ethanolic extract of the leaf possesses wide range of good antibacterial and antifungal properties. However, no other biological activity has yet been reported. The objective of the present study was to investigate the antioxidant, cytotoxic and antibacterial activities of the different fraction of the crude extracts of stem of *Stephania japonica* (*S. japonica*).

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MATERIALS AND METHODS:

Plant material collection and extraction: The stem of *Stephania japonica* was collected from Savar road side in December, 2010.

One voucher specimen has been deposited in Bangladesh national Herbarium (DACB accession No. 35492). The plant sample (after cutting into small pieces) was sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature for better grinding.

The dried plant material was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, and University of Dhaka.

About 900 gm of the powdered sample was taken in a clean, round bottomed flask (5 liters) and soaked in 4.5 liters of methanol. The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 14 days accompanying routine shaking and stirring. The whole mixture was then filtered through cotton followed by Whitman No.1 filter paper and the filtrate thus obtained was then air dried to solid residue in different beaker. The weight of the crude extract obtained from the stem of *S .japonica* was found 14 gm.

Solvent-Solvent Partition of Crude extract: Modified Kupchan Partition [1]:

1. **Preparation of Mother Solution:** 10 gm of dried methanol extract was triturated with 90 ml of methanol containing 10 ml of distilled water. The crude extract was dissolved completely. This is the mother solution, which was partitioned off successively by four solvents of different polarity. In subsequent stages each of the fractions was analyzed separately for the detection and identification of compounds having different biological & pharmacological activities.

2. **Partitioning with Petroleum ether:** The mother solution was taken in a separating funnel. 100 ml of the petroleum ether was added to it and the funnel was shaken and then kept undisturbed. The organic portion was collected. The process was repeated thrice; petroleum ether fractions were collected together evaporated in Rota evaporator.

3. **Partitioning with Carbontetrachloride:** To the mother solution left after washing with petroleum ether, 12.5 ml of distilled water was added and mixed. The mother solution was then taken in a separating funnel and extracted with CCl_4 (100 ml X 3). The CCl_4 fractions were collected together and evaporated. The aqueous fraction was preserved for the next step.

4. **Partitioning with Chloroform:** To the mother solution that left after washing with petroleum ether and CCl_4, 16 ml of distilled water was added and mixed uniformly. The mother solution was then taken in a separating funnel and extracted with $CHCl_3$ (100 ml X 3). The $CHCl_3$ soluble fractions were collected together and evaporated. The aqueous methanolic fraction was preserved as aqueous fraction.

5. **Partitioning with Ethyl acetate:** To the mother solution that left after washing with petroleum ether, CCl_4 and $CHCl_3$, was then taken in a separating funnel and extracted with Ethyl acetate (100 ml X 3). The Ethyl acetate soluble fractions were collected together and evaporated. The aqueous methanolic fraction was preserved as aqueous fraction

Microorganisms: Five species of both gram positive and gram negative bacteria were used for antibacterial test. The bacterial strains were collected from the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). The bacterial strains used for the investigation are Gram negative (*Shigella dysenteriae, Escherichia coli, Salmonella paratyphi, Salmonella typhi,)* and Gram positive (*Staphylococcus aureus*) .

Preparation of Sea Water: Pure NaCl (20g) and table salt (18g) was weighed accurately, dissolved in distilled water to make one liter and then filtered off to get a clear solution.

Antioxidant activity: The anti-oxidant potential of the methanolic extract was determined on the basis of their scavenging activity of the stable 2, 2-diphenyl-1-picryl hydrazyl (DPPH) free radical. DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis [2]. The aliquots of the different concentrations (1.57-400 μg /ml) of the extract were added to 3 ml of a 0.004% w/v solution of DPPH. Absorbance at 517 nm was determined after 30 min, and IC_{50} (Inhibitory concentration 50%) was determined. IC_{50} value denotes the concentration of sample required to scavenge 50% of the DPPH free radicals [3].

The formula used for % inhibition ratio is-

% inhibition =

{(Blank absorbance - Sample absorbance) / Blank absorbance} X 100

At first, 9 test tubes were taken to make aliquots of 9 concentrations (1.57, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00, 200.00, 400.00 µg/ml) for various fractions of plant extract and another 9 test tubes were taken to make aliquots of 9 concentrations (1.57, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00, 200.00, 400.00 µg/ml) for ascorbic acid and a test tube for blank preparation. Test tubes were washed properly and rinsed with ethanol.

10 mg plant extract and 10 mg ascorbic acid were weighed accurately and dissolved in 20 ml ethanol to make the required stock solutions having concentrations 400 µg/ml in case of both plant extract and ascorbic acid. 1.57, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00, 200.00 µg/ml concentrations were made by dilution technique. Here ascorbic acid was taken as positive control. DPPH was weighed and dissolved in ethanol to make 0.004% (w/v) solution.

To dissolve homogeneously vortex mixer was used. After making the desired concentrations 6 ml of 0.004% DPPH solution was applied on each test tube containing 2ml of each concentration of extract and ascorbic acid by pipette. Test tubes were kept for 30 minutes in dark to complete the reactions.6 ml 0.004% DPPH solution was also applied for the preparation of blank at the same time where only 2 ml ethanol was taken as blank. After 30 minutes, absorbance of each test tube was determined by UV spectrophotometer at 517 nm.

Cytotoxic activity: The brine shrimp lethality bioassay was used to predict the cytotoxic activity of the crude extracts [4-5]. For the experiment, 50 mg of the extracts was dissolved in dimethylsulfoxide (DMSO) and solutions of varying concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 µg/ml) were obtained by the serial dilution technique using simulated seawater. The concentration of DMSO in these test tubes did not exceed 10µl/ml. The solutions were then added to the pre-marked vials containing 10 live brine shrimp nauplii in 5 ml simulated seawater. After 24 h, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. The mortality endpoint of this bioassay was defined as the absence of controlled forward motion during 30s [6]. From this data, the percent of lethality of the brine shrimp nauplii for each concentration and control was calculated. The lethal concentration LC_{50} of the test samples after 24 hr was obtained by a plot of percentage of the shrimps killed against the sample concentration (toxicant concentration) and the best fit line was obtained from the curve data by means of regression analysis.

Antibacterial activity: Antibacterial activity of *S. japonica* was tested by using the disc diffusion method [7-8]. In this method-measured amount of the test samples are dissolved in definite volumes of solvent to prepare solutions of desired concentration (µg/ml). The sterile Matricel (BBL, Cocksville, USA) filter paper discs are impregnated with known amount of test substances using micropipette and dried. Disk of sample, positive control and negative control are then placed in petridishes (120 mm in diameter) containing a suitable agar medium seeded with the test organisms using sterile transfer loop for anti-microbial screening.

The plates are then kept at 40^{o}C for facilitating maximum diffusion. The plates are then kept in an incubator for 12-18 hour to allow the growth of the microorganisms. If the test material has any anti-microbial activity, it will inhibit the growth of microorganism giving a clear, distinct zone called "zone of inhibition". The antibacterial activity of the test agent is determined by measuring the diameter of the zone of inhibition in term of millimeter and compared with the standard antibiotic. The experiments are carried out duplicate manner.

RESULTS:

Antioxidant activity: The various fractions of *S.japonica* Stem i.e. Ethyl Acetate (EAJS), $CHCl_3$ (CFJS), CCl_4 **(**CTJS**)** and Petether (PEJS) were subjected to free radical scavenging activity. Here, Ascorbic Acid was used as reference standard. In this investigation, CFJS showed the most significant free radical scavenging activity with IC_{50} value of 119.0 µg/ml for *S. japonica* stem. Other fractions EAJS, CTJS, PEJS also showed significant free radical scavenging activity having IC_{50} values of 174.0 µg/ml, 125.0µg/ml, 251.0µg/ml respectively (**Table 1**).

Cytotoxic activity: Following the procedure of Meyer [4] the lethality of the EAJS (Ethyl Acetate Soluble fraction of *japonica* Stem), CFJS (Chloroform soluble fraction *of japonica* Stem), CTJS (Carbon Tetrachloride fraction *of japonica* Stem), PEJS (Petroleum Ether fraction *of japonica* Stem), *Stephania japonica* stem were subjected to brine shrimp bioassay. The results are summarised in **Table 2**.

The LC_{50} values of sample VS (Vincristine Sulphate), EAJS (Ethyl Acetate Soluble fraction of *japonica* Stem), CFJS (Chloroform soluble fraction *of japonica* Stem), CTJS (Carbon Tetrachloride fraction *of japonica* Stem), PEJS (Petroleum Ether fraction *of japonica* Stem), were found to be 0.312 µg/ml, 4.0µg/ml, 12.5µg/ml, 3.0µg/ml, 6.25µg/ml (Table 2). Here, CTJS showed the most significant result.

Antibacterial activity: The Cabon Tetrachloride soluble fraction showed good antibacterial activity against different species of bacteria at different doses. The Ethyl Acetate soluble fraction showed good activity only against *E. coli* at different doses. The Chloroform soluble fraction shows medium activity against different organisms. But the Petroleum Ether soluble fraction shows no activity against any kind of bacteria. Average zone of inhibition of various fractions are shown in **table 3**.

TABLE 1: IC_{50} VALUES OF POSITIVE CONTROL AND VARIOUS FRACTIONS OF *S. JAPONICA* STEM

Sample Code	Test Sample	IC`1$_{50}$ (µg/ml)
Positive control	Ascorbic acid	23.0
EAJS	Ethyl Acetate soluble fraction of *S. japonica* stem	174.0
CFJS	$CHCl_3$ soluble fraction of *S. japonica* stem	119.0
CTJS	CCl_4 soluble fraction of *S. japonica* stem	125.0
PEJS	Petroleum Ether soluble fraction of *S. japonica* stem	251.0

TABLE 2: LC_{50} VALUES OF POSITIVE CONTROL AND VARIOUS FRACTIONS OF *S. JAPONICA* STEM

Sample Code	Test Sample	LC_{50} (µg/ml)
Positive control	Vincristine sulphate	0.312
EAJS	Ethyl Acetate soluble fraction of *S. japonica* stem	4.0
CFJS	$CHCl_3$ soluble fraction of *S. japonica* stem	12.5
CTJS	CCl_4 soluble fraction of *S. japonica* stem	3.0
PEJS	Petroleum Ether soluble fraction of *S.japonica* stem	6.25

TABLE 3: THE TABLE SHOWED THE AVERAGE ZONE OF INHIBITION AGAINST DIFFERENT BACTERIAL STRAINS OF VARIOUS FRACTIONS OF *S. JAPONICA* STEM.

Bacterial Strains	Average Diameter of zone of inhibition in mm								
	CTJS (250*)	CTJS (500*)	EAJS (250*)	EAJS (500*)	CFJS (250*)	CFJS (500*)	PEJS (250*)	PEJS (500*)	Kanamycin (30 *)
Shigella dysenteriae (-)	14	20	0	0	11	14	0	0	20
Escherichia coli (-)	09	16	12	15	09	12	0	0	21
Salmonella paratyphi (-)	07	20	0	0	12	16	0	0	19
Salmonella typhi (-)	08	19	0	0	13	18	0	0	17
Staphylococcus aureus (+)	06	19	0	0	10	13	0	0	18

* = µg/Disc, (-) = Gram Negative Bacteria, (+) = Gram Positive Bacteria, EAJS = Ethyl Acetate soluble fraction of *S. japonica* stem, CFJS = $CHCl_3$ soluble fraction of *S. japonica* stem, CTJS = CCl_4 soluble fraction of *S. japonica* stem, PEJS = Petroleum Ether soluble fraction of *S.japonica* stem.

DISCUSSION: Antioxidants have been widely used in the food industry to prolong shelf life. However, there is a widespread agreement that some synthetic antioxidants such as butyl hydroxyl anisole and butyl hydroxyl toluene (BHA and BHT, respectively) need to be replaced with natural antioxidants because of their potential health risks and toxicity. Thus, the search for antioxidants from natural resources has received much attention, and efforts have been made to identify new natural resources for active antioxidant compounds [9]. Phenolic natural products such as flavonoids are of particular interest because of their antioxidant activity through scavenging oxygen radicals and inhibiting peroxidation.

Antioxidants that scavenge free radicals play an important role in prevention of cardiovascular disease, aging, cancer, and inflammatory disorders [10]. In addition, these naturally occurring antioxidants can be formulated to give nutraceuticals, which can help to prevent oxidative damage from occurring in the body.

The brine shrimp lethality bioassay can be recommended as a guide for the detection of antitumour and pesticidal compounds because of its simplicity and low cost. It indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, pesticidal, antitumor, etc. of the compounds [7, 11].

An approximate linear correlation was observed when concentration versus percentage of mortality was plotted on the graph. The results tend to suggest its possible cytotoxic activity. Therefore, various fractions of crude methanolic extract of *S. japonica* stem might possess a significant cytotoxic activity. However, further investigations are necessary to isolate the active compound(s) responsible for the activity.

Antibacterial activity was tested by using the disc diffusion method. Disc diffusion method is widely acceptable for the preliminary screening of antibacterial activity. It is essentially a qualitative or semi qualitative test indicating the sensitivity or resistance of microorganisms to the test materials [12]. It is well known that plant containing various phytochemical constituents such as flavonids, saponins and steroids have antimicrobial activity [13]. Plant containing Quercetagetin-7-arabinosyl-galactoside, a flavonoid has been used extensively to treat infectious disease [14].

The flavone baicalein is reported to be largely responsible for antimicrobial effects [15]. Flavonoid rich plant extracts from species of *Hypericum* [16]. *Capsella* [19] and *Chromolaena* [17] have been reported to possess antibacterial activity. Many other phytochemical preparations with high flavonoid content have also been reported to exhibit antibacterial activity [18-26]. It has been reported that saponins have potent antimicrobial activity [27]. The antibacterial activity of various fractions of *S. japonica* probably due to the presence of flavonoids and saponins that revealed in phytochemical studies.

In conclusion, it can be suggested that the various fractions of crude methanolic extract of *S. japonica* may possess different range of antioxidant, cytotoxicity and antibacterial activity, which correlates well with the traditional uses of the plant. Therefore, further studies are essential to find out the active principle(s) responsible for these activities.

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