EVALUATION OF ANALGESIC AND ANTIMICROBIAL ACTIVITY OF DIFFERENT FRACTIONS OF CRUDE METHANOL EXTRACT OF *TINOSPORA CRISPA* STEM.

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Abstract

Tinospora crispa, a plant belonging to the family of Menispermaceae is a well-known traditional medicinal plant used in Bangladesh. This study has carried out to evaluate the analgesic (by acetic acid induced writhing test) activities by using Swiss albino mice as a test animal. At a dose of 400 mg/kg body weight, the crude methanol extract and its other fractions of stem significantly ($p<0.05$) produced inhibition of writhing compared to the Standard (Diclofenac Sodium). Among all the fractions, Petroleum ether soluble fraction showed most significant analgesic activity (51.94%) compare with standard (65.12%). In addition the crude extract and its fraction showed no significant antimicrobial activities against five gram-positive, eight gram-negative bacteria and three fungi compare to the standard (kanamycin).

Keywords: Analgesic, antibacterial, antifungal, *Tinospora crispa*, Menispermaceae.

INTRODUCTION

The use of non-steroidal anti-inflammatory drugs (NSAIDs), like aspirin as pain killers/relievers have not been successful in all cases due to adverse effects such as gastric lesions and liver damage [1]. On other hand the frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality in immunocompromised patients in developing countries (2). Although huge numbers of antimicrobial agents have been discovered, the pathogenic microorganisms are developing resistance against these agents day by day. In third world countries likeBangladesh, Nepal, and Nigeria, irrational use of antimicrobial agents is a major cause of such resistance

(3).The strategy should therefore be the search for new and clinically useful analgesics which have negligible adverse effects. Plants produce wide array of bioactive principles and constitute a rich source of medicines. In many developing countries, traditional medicine is one of the primary health care systems [4-6]. They have been used to cure various diseases for centuries in many cultures all over the world and should be considered as new sources of analgesic and antimicrobial drugs. Substances such as alkaloids, flavonoids and terpenoids synthesized from plants have recently been discovered to have commendable biological properties [7].

Large scale evaluation of the local flora exploited in traditional medicine for various biological activities is therefore necessary. Isolation and characterization of the bioactive molecules ultimately lead to new drug development. In view of this, our attention has been focused particularly in *Tinospora crispa;* known by various vernacular names such as 'akar patawali' or 'akar seruntum' is a climber plant belonging to the family of Menispermaceae. *T. crispa* is an indigenous plant and can be found distributed from the southwestern part of China to Southeast Asia, including Malaysia[8]. Traditional folklore attributes various therapeutic uses to its stem for treatment of fever, jaundice, hyperglycemia,[9] hypertension, wounds, intestinal worms and skin infections. It is also used to treat tooth and stomach aches, cough, asthma and pleurisy[10]. Two tri-terpenes are extracted from the stem of *T. crispa* namely cycloeucalenol and cycloeucalenone[12]. *T.* crispa stem contains: flavones O-glycosides (apigenine), picroretoside, berberine, palmatine, picroretine and resin. Flavonoids are naturally occurring polyphenolic compounds ubiquitously found in plants. The health benefits of flavonoids attributed to polyphenols is usually linked to two properties namely inhibition of certain enzymes such as xanthine oxidase and antioxidant activity [12]. They have been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, thrombolytic, antiviral and anticarcinogenic activities[13-14].

Based on the traditional uses of *T. crispa*, there has been a substantial increase in the sales of this part of the plant by certain pharmaceutical companies as capsules containing this natural product to treat fever, hypertension, as a diaphoretic tonic and antihyper-glycemic agent. To date, there are numerous studies on this plant emphasizing its antioxidant, antidiabetic, antimalarial and cosmetic effects. However, limited information is available regarding analgesic potential of *T. crispa*. Information from different articles showed that the plants belong to family of Menispermaceae has analgesic and anti-inflammatory activity[15-17]. Against these backgrounds, the present study was undertaken to evaluation of analgesic and antimicrobial activity of different fractions of crude methanol extract of *Tinospora crispa* stem.

MATERIALS AND METHODS:

Plant materials collection and extraction: Plant samples of *Tinospora crispa* was collected from Tangail in March, 2010. One voucher specimen has been deposited in Bangladesh national Herbarium (DACB accession no. 35291). The stem-leaf (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, 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 Whatman 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 *T. crispa* was found 26 gm. A portion (10.0 g) of the methanol extract was fractioned with petroleum ether and chloroform by the modified Kupchan partitioning method.[16]

Preparation of Sample

Preparation of test materials for analgesic activity: In order to administer the extracts and different fractions at doses of 400 mg/kg body weight of mice, 100 mg of each test samples were measured respectively and were triturated unidirectional way by the addition of small amount of suspending agents Tween-80. After proper mixing of sample and suspending agent, normal saline was slowly added. The final volume of the suspension was made 3 ml. For the preparation of Diclofenac Na(Standard sample) at the dose of 50 mg/kg body weight, 25 mg of Diclofenac Na was taken and a suspension of 3 ml was made. Mixture of Tween-80 (1%), DMSO and the normal saline used as a control.

Preparation of sample discs with test samples of *T. crispa:* Measured amount of each test sample (8gm for 20 discs) was dissolved in specific volume of solvent (chloroform or methanol) to obtain the desired concentrations (400 µg/disc) in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

Animals and diet

Healthy Swiss-albino mice(weighed 16-23 gm) of either sex, aged 4-5 weeks, obtained from the Animal Resource Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh (ICDDR, B) were used for the investigating analgesic(mice weight was 16-23 gm) property. The animals were given standard ICDDR, B formulated food pellets and water and kept in the laboratory environment (12h dark/12h light cycle) for 3 days for acclimatization. The cleaning of the cages was done daily. In this study, all the animal experimentation was carried out according to the guidelines of Institutional Animal Ethics Committee (IAEC).

Microorganisms

Antibacterial activity was determined against five gram-positive bacteria (*Bacillus subtilis, Bacillus megaterium, Staphylococcus aureus, Bacillus subtilis and Sarcina lutea*) and eight gram-negative bacteria (*Escherichia coli, Shigella dysenteriae, Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi, Shigella boydii, Vibrio mimicus, Vibrio parahemolyticus*). Antifungal screening was carried out against three fungi (*Candida albicans, Aspergillus niger and Sacharomyces cerevacae*). The pure cultures of these microorganisms were collected from the Microbiological Laboratory of the Institute of Nutrition and Food Science (INFS) and Department of Microbiology, University of Dhaka, Bangladesh.

tudy of analgesic activity by acetic acid induced writhing method

The peripheral analgesic activity of the methanolic extracts of *T. crispa* stem bank and leaf was studied using acetic acid induce writing method in mice carried out by the slightly modified procedure previously described by Whittle, 1964 and Ahmed *et al*., 2001. Experimental animal were randomly selected and divided into four group denoted as control group, positive control group and test group-I(Crude extract of stem) and test group-II(Crude extract of leaf) consisting of five mice each group. Positive control group receive Diclofenac sodium at dose of 50 mg/kg body weight whereas both test group given the test sample orally at the dose of 400mg/kg body weight. A 30 min interval was given to ensure proper absorption of the administered substances. The writhing inducing chemical, acetic acid solution (0.7%) was administered intraperitoneally to each of the animals of a group. Five min was given for absorption of acetic acid and number of writhing was counted for 5 min. The animals did not always perform full writhing and the incomplete writhing was taken as a half writhing. So two half writhing were taken as one full writhing.

Antibacterial screening

In vitro antibacterial screening was carried out by disc diffusion method (21-23), which is a qualitative to semiquantitative test. Briefly, 20 ml quantities of nutrient agar were plated in petri dish with 0.1 ml of a 10^{-2} dilution of each bacterial culture (18 h old). Filter paper discs (6 mm in diameter) impregnated with various concentrations of plant extract were placed on test organism- seeded plates. Methanol was used to dissolve the extract and was completely evaporated before application on test organism-seeded plates. Blank disc impregnated with solvent methanol followed by drying off was used as negative control. The activity was determined after 18 h of incubation at 370 C. The diameters of zone of inhibition produced by the extract were then compared with the standard antibiotic kanamycin 30 µg/disc. Each sample was used in triplicate for the determination of antibacterial activity.

Antifungal screening

In vitro antifungal screening was carried out by disc diffusion method (22,23). Here, 20 ml quantities of Sabouraud dextrose were plated in petri dish with 0.2 ml of a 10-2 dilution of each fungal culture (10 h old). Filter paper discs (6 mm in diameter) impregnated with various concentrations of the extract were placed on test organism-seeded plates. Methanol was used to dissolve the extract and was completely evaporated before application on test organism-seeded plates. Blank disc impregnated with solvent methanol followed by drying off was used as negative control. The activity was determined after 72 h of incubation at 30 ºC. The diameter of zone of inhibition produced by the extract was then compared with the standard antibiotic kanamycin 30 µg/disc. Each sample was used in triplicate for the determination of antifungal activity.

Statistical analysis

The experimental results are represented as mean ± SEM (standard error of mean). Statistical analysis of antidiarrheal and analgesic activity was performed using one way-ANOVA followed by Dunnet's Test on SPSS

16 statistical program. The significance of the difference between the mean of the control and treated groups was considered at $p<0.05$.

RESULTS AND DISCUSSION

After screening the analgesic activity, it was found that the percentage inhibition for the Standard (Diclofenac sodium), Methanolic crude extract of stem and its petroleum ether fraction ,Chloroform fraction were 65.12, 48.06,51.94 and 43.41 respectively (Table 1). The petroleum ether fraction of crude extract of (400mg/kg) *T. crispa* showed significant analgesic activity ($p<0.05$) as compared to the control and standard.
Traditionally *T. crispa* use in pain in the rural area of Bangladesh. From our study we can see the petroleum ether extracts of this plant have very good activity in pain. Further research required to find a new molecule which one can be very useful against pain.

Table 1: Effects of different extracts of *Tinospora crispa* (stem and leaf) and their respective % inhibition of writhing

Treatment	**Dose (mg/kg)[a]**	**Writhing [b] (Average ±SEM)**	**% Inhibition**
Control (1% Tween 80 in saline)	0.1 ml/10 gm of body weight	25.8±0.80	-
Standard(Diclofenac Na)	50	9±0.71*	65.12
Crude Methanolic Extract	400	13.4±0.58*	48.06
Pet. Ether Extract	400	12.4±0.67*	51.94
Chloroform Extract	400	14.6±0.68*	43.41
Glacial acetic acid (0.7%)	0.1 ml/ 10 gm of body weight	-	-

[a]Two test samples and the standard was administered orally. [b]Writhings are measured as the average ± SEM. *Significant for $P<0.05$

In other hand the results of antimicrobial activity of methanol extract of T. crispan stem and its petroleum ether fraction and chloroform fraction against the test bacteria and fungi are presented in Table 2.According to Rahman et al.,1999 extracts of T. crispa showed significant activity against malaria parasites but in we found no significant activity against bacteria and fungus. In comparison to reference standard kanamycin (30 µg/disc), the methanol extract exhibited insignificant antibacterial activity at 400 µg /disc. Chloroform fraction showed little antibacterial and antifungal activity whereas petroleum ether fraction showed no activity.

Table 2: Antimicrobial activity of different extracts of *T. crispa* stem.

Test microorganisms	Diameter of zone of inhibition (mm)			
	MEFTS	**PEFTS**	**CLFTS**	**Kanamycin**
Gram positive Bacteria				
Bacillus cereus	4.6	-	5.2	33.5
Bacillus megaterium	3.6	-	6.5	35.3
Bacillus subtilis	5.2	-	7.4	35.1
Staphylococcus aureus	7.9	-	8.8	34.2
Sarcina lutea	3.4	-	5.5	34.0
Gram negative Bacteria				
Escherichia coli	2.8	-	6.3	35.3
Pseudomonas aeruginosa	3.5	-	6.5	31.2
Salmonella paratyphi	5.0	-	8.2	30.6
Salmonella typhi	4.8	-	7.7	32.7
Shigella boydii	3.6	-	6.8	34.0
Shigella dysenteriae	5.2	-	6.7	32.5
Vibrio mimicus	4.0	-	5.8	32.4
Vibrio parahemolyticus	5.5	-	7.6	33.3
Fungi				
Candida albicans	5.5	-	11.5	31.4
Aspergillus niger	3.2	-	11.6	30.3
Sacharomyces cerevacae	6.5	-	7.4	34.0

CONCLUSION

In conclusion, it could be suggested that the various fractions of crude extract of *T. crispa* stem posse analgesic and limited antimicrobial effect. However, further extensive phytopharmacological studies are necessary to find out the active principles responsible for these activities.

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