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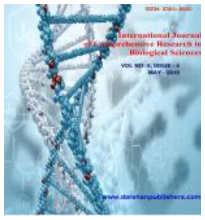

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Research Article

BIOLOGICAL EVALUATION OF ETHANOLIC EXTRACT OF LEAVES OF *Spinacia oleracea* LINN.

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Abstract

Spinacia oleracea Linn**.** belongs to the Family Chenopodiaceae and is extensively used as common vegetable in Indo Bangladesh sub-continental region. The present evaluating study was carried out to illustrate possible antimicrobial, analgesic and antidiarrheal activity of ethanolic extract of leaves of *Spinacia oleracea* Linn**.** The crude leaf extract was found to show strong analgesic activity(51.77%) at the dose of 500mg/kg-body weight using Swiss Albino mice and Diclofenac Sodium as standard chemical. Antimicrobial activity of this extract was performed against both gram positive and gram negative bacteria using Kanamycin as standard and the result showed moderate activity against *Staphylococcous aureus, Shigella sonnie, Shigella flexneri, Shigella boydii, Sarcina lutea, Escherichia coli , Shigella dysenteriae, Streptococcus pyogenes* and *Salmonella typhi*. Castor oil inducing antidiarrheal activity of ethanolic extract of *Spinacia oleracea* Linn**.** leaves in Swiss Albino mice was evaluated using Loperamide as standard chemical. But the extract didn't show remarkable antidiarrheal activity.

Keywords: *Spinacia oleracea*, antibacterial, analgesic, antidiarrheal.

Introduction

Since human race begins, man is dependent on plants to meet his hunger. Plants have been of Paramount importance not only for providing foods but also for various medicines as well. Subcontinental soil and climate are suitable for extensive growth of vegetables and local people are used to taking these indigenous vegetables in their daily dietary meal. These vegetables unknownly provide medicinal value. *Spinacia oleracea* Linn. is such an important vegetable which meets food demands and bestows medicinal values on human diet. Drugs obtained from different extracts of *Spinacia oleracea* Linn. have always been a subject of interest for scientists**.** Researches showed that *Spinacia oleracea* Linn. contains a plenty of bioactive chemical constituents such as phenolic compounds-anthocyanin-flavon, pigments, lutein, quercetin, bioflavonoid , Vitamin A, Vitamin C, Folic Acid, Oxalic Acid (Guha D and Das S., 2008)n-hentriacontanol and β-sitosterol, 20-hydroxyecdysone (Arch. Insect Biochem. Physiol.), Spinatoside-4- glucoside, Patuletin-3-gentiobioside, Patuletin-3-glucosyl-(1-6), dehydroascorbic acid, 5, 3,4-trihydroxy-3-methoxy- 6:7- methylenedioxyflavone-4-

glucuronide.(J. Agric. Food Chem, 2005.) which may be responsible forreduction of fatality of malignant colon disease, astringent , anti-inflammatory properties in easing the pain of arthritis, analgesic, antimicrobial and cytotoxic, anti-oxidant and CNS depressive activity. The leaves are cooling, emollient, anti-pyretic, hypoglycaemic, diuretic, digestible, anthelminthic, sore throats, flatulence throat (V. Gomathi *et al*,.2010).Different works have been done using different solvent systems of *Spinacia oleracea* Linn. but the motto of present study was to evaluate *in vitro* antibacterial, *in vivo* analgesic andantidiarrheal activity of ethanolic extract of leaves of *Spinacia oleracea* Linn**.**

Materials and Methods

Plant Material Collection and Identification

Disease free fresh leaves of *Spinacia oleracea* Linn. were collected during winter in the month of December, 2014 from Rupsha, Khulna, Bangladesh. The plant part was identified by the expert of Botany Department, University of Rajshahi

Preparation of Extract

After separating from undesirable materials or plants or plant parts clean leaves were shade dried and the dried leaves were cut into small pieces and ground into a coarse powder with the help of asuitable Laboratory grinder. 300gm powder of *Spinacia oleracea* Linn. leaves was macerated in 800 ml of 90% ethanol at 37°C for 14 days accompanying occasional shaking and stirring. The whole mixture was then underwent a coarse filtration by clean, white cotton, followed by a filtration through Whatmann filter paper. The filtrate (ethanol extract) was then evaporated through rotary evaporator followed by desiccation to get the dried crude extract . The extract was stored in an airtight container and kept in a cool, dark and dry place for subsequent evaluation of biological activities.

Antibacterial activity

Microorganisms and media

The test microorganisms used in this study were both gram positive and gram negative bacterial strains including *Staphylococcous aureus, Sarcina lutea, Shigella flexneri, Shigella boydii, Shigella sonnie, Escherichia coli, Shigella dysenteriae, Streptococcus pyogenes* and *Salmonella typhi*. The microorganisms were collected as pure cultures from Microbiology Laboratory, Department of Pharmacy, University of Rajshahi, Bangladesh. The bacterial isolates were first sub cultured in a nutrient agar and incubated at 37°C for 18 h.

Disc Diffusion Method

The antibacterial assay was performed by disc diffusion technique (Bauer *et al*., 1966, Reiner, R. 1980&1982). The sample solution of the extract to be tested was prepared by dissolving a definite amount of extract in appropriate solvent to attain a concentration of 50mg/ml. 10 µl of such solution was applied on sterile disc (5mm diameter, filter paper) and allowed to dry off the solvent in an aseptic hood. Thus the discs contain 500µg of crude extract. Standard antibiotic disc (Kanamycin 30µg/disc) and blank discs (impregnated with solvents) were used as a positive and negative control respectively. The test discs and standard disc were placed in a Petri dish seeded with particular bacteria and then left in a refrigerator at 4°C for 12-18 hrs in order to diffuse the material from the discs to the surrounded media in the Petri dishes. The Petri dishes were then incubated at 37°C for overnight to allow the bacterial growth. The antibacterial activity of ethanolic extract of leaves of *Spinacia oleracea* Linn. was then determined by measuring respective zone of inhibition in mm.

Analgesic activity (Whittle 1964)

The test consists of injecting the 0.7 % acetic acid solution intraperitoneally and then observing the animal for specific contraction of body referred as

'writhing'. A comparison of writhing was made among control, positive control (Diclofenac Sodium), and test sample. Diclofenac Sodium was used as the positive control in this method that acts by inhibition of prostaglandin synthesis which has been reported to be responsible for pain sensation (Rang HP, 1993).

Experimental animal

Young Swiss-albino mice aged 4-5 weeks, average weight 25-30 gm were purchased from the Animal Research Branch of the International Centre for Diarrhoeal Disease and Research, Bangladesh(ICDDR,B) for the experiment. They were kept in standard environmental condition (RH 55% to 60%, room temperature 25± 2^{o}C and 12 h light/ dark cycle)for one week for adaptation after their purchase andfed ICDDR,B formulated rodent food and water.

Preparation of Sample

To prepare suspension of test samples at the dose of 500mg/kg-body weight 125 mg of samples was measured. The extract was triturated in unidirectional manner by the addition of small amountof tween-80 with distilled water to make the final volume of about 2.5 ml which reserve in separatecontainer. To stabilize the suspension, it was shaken well by vortex mixer**.** For the preparation of Diclofenac Sodium at the dose of 25 mg/kg-body weight, 6.25 mg of Diclofenac was taken and a suspension of 2.5 ml was made.

Methodology

Experimental animals were randomly selected and divided into three groups denoted as group-I, group-II, and group-III consisting of 5 mice in each group. Each group received a particular treatment i.e. control, positive control and the leaf extract. Each mouse was weighed properly and the doses of the test sample and control materials were adjusted accordingly. Test sample, control and Diclofenac Sodium were given orally by feeding needle. 30 minutes was given to ensure proper absorption of the administered substances. Then the writhing inducing chemical, acetic acid solution (0.7 %) was administered intraperitoneally to each of the animals of selected groups. After an interval of 5 minutes, which was given for absorption of acetic acid, number of squirms (writhing) was counted for 15 minutes and % of writhing inhibition was calculated as follows:

% Inhibition of writhing = 100 – (Treated mean/control mean) × 100

Antidiarrheal activity

Experimental animal

Young Swiss-albino mice aged 4-5 weeks, average weight 25-30 gm were purchased from the Animal Research Branch of the International Centre for Diarrheal Disease and Research, Bangladesh (ICDDR,B) for the experiment. They were kept in standard environmental condition (RH 55% to 60%, room temperature 25± 2^{o}C and 12 hour light/ dark cycle) for one week for adaptation after their purchase and fed ICDDR,B formulated rodent food and water.

Castor oil induced antidiarrheal activity

Castor oil induced diarrheal model was followed for this experiment. The employed mice were screened initially by giving 0.5 ml of castor oil and only those showing diarrhea were selected for the final experiment. The test animals were selected randomly and divided into three groups having five mice in each group. The experimental animals were accurately weighed & properly marked .As group-I or the control received only distilled water containing 1% Tween-80 (25 ml/kg). Group-II or the positive control received standard anti-motility drug, Loperamide (50mg/kg) as oral suspension. The test group III was treated with suspension of leaf extract of *Spinacia oleracea* Linn.at the oral dose of 500 mg/kg-body weight. Test sample, control and Loperamide were given orally by means of a feeding needle. The mice were fed with the

sample, control and Loperamide 1 hour prior to 0.5ml per mouse. Individual animals of each group were placed in separate cages having adsorbent paper beneath and examined for the presence of diarrhea every hour in four hours study after castor oil administration.

Number of faeces or any fluid material that stained the adsorbent paper were counted at each successive hour during the 4-hours period and were noted for each mouse. The latent period of each mouse was also counted. At the beginning of each hour new papers were placed for the old ones. During an observation period, the total number of stool output including diarrheic faces excreted by the animals was recorded. A numerical score based on stool consistency was assigned as follows: normal stool=1 and watery stool=2 (Jebunnessa *et al*., 2009, Atta AH *et al* ., 2004, Nwodo *et al*., 1991).

Results

Antibacterial Activity

This study was performed to find out antibacterial effect of *Spinacia oleracea* Linn. the oral administration of castor oil at a dose of leaves extract. Antibacterial activity of ethanolic extract of *Spinacia oleracea* Linn. Leaves was evaluated using Kanamycin (30µg /disc) as standard by measuring the zone of inhibition in mm.Table-1exhibits that the ethanolic extract at a dose of 500µg/disc showed moderate antibacterial activity in comparison with standard Kanamycin (30µg /disc) against *Staphylococcous aureus, Shigella sonnie, Shigella flexneri, Shigella boydii, Sarcina lutea, Escherichia coli, Shigella dysenteriae, Streptococcus pyogenes* and *Salmonella typhi* with the zone of inhibition ranging from 6 to 14 mm. The highest zone of inhibition was observed against *Staphylococcous aureus* (14mm). The blank discs (30 µl /disc) didn't show any zone of inhibition.

Antibacterial activity of ethanolic extract of *Spinacia oleracea* Linn**.** leaves against different bacterial strains is tabulated in Table-1 in terms of diameter of zone of inhibition in mm.

Table-1: *In vitro* antibacterial activity of ethanolic extract of *Spinacia oleracea* Linn. leaves

Bacterial strains	Diameter of zone of inhibition in mm		
	Kanamycin(30µg /disc)	Ethanolicextract (500µg/disc)	Blank (30 µl /disc)
Shigella boydii	30	6	0
Streptococcus pyogenes	20	6	0
Shigella dysenteriae	28	6	0
Escherichia coli	28	6	0
Shigella flexneri	18	8	0
Salmonella typhi	30	6	0
Shigella sonnie	27	8	0
Staphylococcous aureus	30	14	0
Sarcina lutea	28	6	0

Analgesic activity

Each mouse of all groups was observed carefully to count the number of writhing that they had made in 15 minutes and tabulated in following Table-2.

Table-2:Evaluation of the results of analgesic activity of ethanolic extract of *Spinacia oleracea* Linn. leaves

Animal group	Total Writhing	Mean	% Writhing	Standard deviation(SD)	Standard error (SE)	%Inhibition
Control	141	28.2	100	3.70	1.85	
Diclofenac sodium (25mg/kg)	30	6.0	21.28	2.44	1.22	78.72
SOLE (500mg/kg)	68	13.6	48.23	2.97	1.48	51.77

SOLE= *Spinacia oleracea* leaf extract

Antidiarrheal Activity

At the dose of 500mg/kg-body weight, the ethanolic extract of *Spinacia oleracea* Linn. leaves compared to the control group, offered about 0.68hr of the mean latent period where as standard Loperamide provided 1.86hr of the mean latent period for diarrheal episode. Table -4 indicates that mean number of faeces of *Spinacia oleracea* Linn. leaves extract induced mice increases with time in comparison with standard Loperamide and control group. Antidiarrheal activity of *Spinacia oleracea* Linn. leaves in castor oil induced mice at the dose of 500 mg/kg as compared to the standard antidiarrheal agent Loperamide didn't show marked effect i.e. neither delayed the onset of diarrheal episode nor decreased the frequency of defecation.

The antidiarrheal activity of ethanolic extract of *Spinacia oleracea* Linn. leaves is showed in the following tables.

Table-3: Effect of *Spinacia oleracea* Linn. leaves extract on latent period of castor oil induced Diarrheal episode in mice

Group (dose)	Numbering Of mice	Latent period(hr)	Mean latent period(hr)	Standard Deviation(SD)	Standard Error(SE)
Control (25ml/kg)	M_1	0.84	0.82	0.108	0.054
	M_2	0.68			
	M_3	0.96			
	M_4	0.89			
	M_5	0.77			
Loperamide (50mg/kg)	M_1	1.95	1.86	0.1645	0.082
	M_2	1.76			
	M_3	1.84			
	M_4	1.68			
	M_5	2.10			
SOLE (500 mg/kg)	M_1	0.85	0.68	0.069	0.034
	M_2	0.73			
	M_3	0.75			
	M_4	0.62			
	M_5	0.62			

SOLE= *Spinacia oleracea* leaf extract

Table -4: Effect of ethanolic extract of *Spinacia oleracea* Linn. leaves on faeces of castor oil induced diarrheal episode in mice

Group	Treatment	Mice no.	Number of faeces in 4 hr	Mean of defecation	SD
Control	Water containing 1% tween 80 (25ml/kg)	M_1	22	19.0	2.23
		M_2	16		
		M_3	19		
		M_4	18		
		M_5	20		
Positive control	Loperamide (50 mg/kg)	M_1	8	6.0	1.58
		M_2	5		
		M_3	4		
		M_4	6		
		M_5	7		
Test group	SOLE (500mg/kg)	M_1	15	17.2	1.92
		M_2	16		
		M_3	17		
		M_4	20		
		M_5	18		

SOLE= *Spinacia oleracea* leaf extract

Discussion

Antibacterial Activity

Since time immemorial, biodiversity of natural resources has introduced the researchers with a familiar way for exploration of new natural bioactive compounds as an alternative therapy to antibacterial resistance. Based on the present study, we can consider the leaves of *Spinacia oleracea* Linn. to be an important source of antimicrobial agent. However, Kishore *et al* reported that the presence of alkaloids, proteins, glycosides, saponins, flavonoids and tannins of crude extract might be responsible for antimicrobial activity. *Spinacia oleracea* Linn. leaves contain huge quantity of polyphenolic compounds (Andjelkovic M*et al*.,2008), and different types of flavonoids(Sultana B and Anwar F.,2008 and Annonymus.,2004 **)** which are accountable for antibacterial activities .

Antibacterial activities using methanolic extract (Kaiser Hamid *et al*., 2013) of *Spinacia oleracea* Linn. was previously reported mentioning significance of this plant for potential antibacterial activity. But this study represents moderate antibacterial activity of ethanolic extract of *Spinacia oleracea* Linn.leaves against both gram positive and gram negative bacteria indicating that this plant is going to be a pivotal source of antibacterial agent for upcoming decades due to the presence of diverse chemical group.

Analgesic activity

Algesia (pain) is an ill-defined warning signal, unpleasant sensation, usually evoked by an external or internal noxious stimulus. An analgesic neutralize pain sensation as a symptom, without affecting its cause. It selectively relieves pain by acting on CNS or on peripheral pain mechanisms, without significantly altering consciousness.

Intraperitoneal administration of acetic acid (0.7%) causes induction of writhing or algesia by liberation of eicosanoids (mainly prostacyclin (PGI2) and prostaglandin-E) from free arachidonic acid resulting from tissue phospholipid by the action of phospholipase A2 and other acyl hydrolases(Rang and Dale, 1993). Diclofenac sodium used as the positive control in this method acts by inhibition of prostaglandin synthesis. Ethanolic extract of leaves of *Spinacia oleracea* Linn. lowers the number of writhing will demonstrate analgesia by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition. According to Utpal *et al*.,2011theethanolic extract of leaves of *Spinaciaoleracea*Linn.produced55.61%inhibition where as this study showed 51.77% protection or writhing inhibition at orally doses of 500 mg/kg body weight of mice in comparison with the standard chemical Diclofenac sodium having 78.72% of writhing inhibition at the dose of 25mg/kg body weights of mice. However, this extract ensured the presence of plenty of Flavonoids (Sultana B and Anwar F.,2008 and Annonymus.,2004 **)** which is reported to inhibit the release of autacoids and prostaglandins leading to the relief from pain sensation. Sufficient abundance of vital chemical groups in leaves extract with significant bioactivities have attracted *Spinacia oleracea* Linn.a overwhelming attention as a potential analgesic compound in the field of medicinally important natural products.

Antidiarrheal activity

Ricinoleic acid is produced from triglyceride after mixing of orally administered castor oil with bile and pancreatic enzymes which is absorbed partially from the gastrointestinal tract and metabolized like any other fatty acid but most remains in the intestine where it occurs its antiabsorptive or secretory effect through producing soap or surfactant like ricinoleate salts with Sodium and Potassium in the lumen of the intestine(Shadid Hossain, *et al*., 2016). No much work have been done on antidiarrheal activity of *Spinacia oleracea* Linn.due to rapid onset of diarrheal episode and increased frequency of defecation of plant extract induced mice. This study showed that there was no decreased frequency of defecation and increased mean latent period of test group than that of positive control group. It claimed that ethanolic extract of *Spinacia oleracea*Linn. leaves possess no antidiarrheal activity. It demands further investigation to find out medicinally active chemical compounds responsible for this type of mechanism of action.

Conclusion

In fine, we can consider the ethanolic extract of *Spinacia oleracea* Linn. leaves to be good sources of antimicrobial and analgesic property due to abundance of significant bioactive constituents which has provided *Spinacia oleracea* Linn.an incredible medicinal value. Based on the present study, further investigations are required to explore the bioactive molecules which are responsible for the extracts' activities as well as their mechanisms of action.

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