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CYTOTOXIC, α-CHYMOTRYPSIN AND UREASE INHIBITION ACTIVITIES OF THE PLANT *Heliotropium dasycarpum* L.

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Abstract

Background: The aim of this study was to investigate Cytotoxic, α-Chymotrypsin and Urease inhibition activities of the plant *Heliotropium dasycarpum*.
Materials & Methods: Dichloromethane and methanol extracts of the plant were evaluated for cytotoxic, α-Chymotrypsin and Urease inhibition by using *in vivo* Brine Shrimp lethality bioassay and *in vitro* enzymatic inhibition assays respectively.
Results: The methanol extract of the plant exhibited significant cytotoxic activity. Out of 30 brine shrimp larvae, 2 (6%), 26 (86%) and 28 (93%) larvae were survived at concentration of 1000µg/ml, 100µg/ml and 10µg/ml respectively with LD50; 215.837. Similarly 21 (70%), 25 (83%), 29 (96%) larvae were survived of dichloromethane plant extract with LD50; 6170.64. The methanol and dichloromethane extract exhibited 10.50±0.18% and 41.51±0.15% α-chymotrypsin enzyme inhibition respectively with IC_{50} values of greater than 500 µmol. The methanol extract showed 24.39±0.21% Urease enzyme inhibition with IC_{50} values of greater than 400 µmol. While dichloromethane extract has 11.46±0.09% enzyme inhibition with IC_{50} values of greater than 500 µmol.
Conclusion: The results clearly indicated that *Heliotropium dasycarpum* has cytotoxic potential and enzyme inhibition properties. Further study is needed to screen out antitumor and anti-ulcerative agents.

Key Words: Cytotoxicity, Brine Shrimp Lethality Assay, *Heliotropium dasycarpum,*

Introduction

The family, Boraginaceae is widely distributed and comprises of about hundred genera and over two thousand species (Ali, 1977). Heliotropium is one of important genus of family Boraginaceae. Many species of genus Heliotropium are used as medicine and are reported in Brazillian, Indian, Iranians, African and Ivorian folk medicine (Carballo et al., 1992; Nagarajuand and Rao, 1990; Barrett, 1994). In West Africa, *Heliotropium indicum* is used locally to treat inflammatory tumors, eczema and impetigo in children. The leaf infusion of *Heliotropium indicum* is used traditionally to treat sores, pimples, stings, poisonous bites, and the sap to gumboils, for healing ulcers, to the eyes for ophthalmia and to treat umbilical hernia in Nigeria and Ghana (Dawodu, 1964; Adegoke, 1968). The whole plant of *Heliotropium ellipticum* is used as emetic, for healing of ulcer and snake bite (Kirtikar and Basu, 1967; Chopra et al., 1956). *Heliotropium eichwaldi* is used for headache, earache and cleaning of ulcer (Bhakuni et al., 1969; Gupta and Mathur, 1972). The fresh extract of *Heliotropium dasycarpum* is used for eye diseases (Tareen et al., 2010).

Heliotropium dasycarpum is present commonly in Afghanistan, Iran, Turkmenistan, Brazil and Pakistan. The species is distributed in Southern Punjab, Blauchistan, Gilgit and Waziristan (Ali, 1977; Dasti et al., 2003).
The antimicrobial and phytotoxic activity are already reported in *Heliotropium dasycarpum* that support its traditional importance (Ghaffari et al., 2013).

The cytotoxic potential of the plant extract can be evaluated by a simple and easy *in vivo* Brine shrimp lethality bioassay. The assay gives front line information about cytotoxic and pesticidal activity. Brine shrimp larvae are utilized in variety of bioassays. Many studies has been reported on the use of this animal in screening of natural toxins, a general information about bioactive substances in extracts of plants and in environmental sciences (McLaughin et al., 1998; Meyer et al., 1982).
Plant protease inhibitors are important candidates of highly effective inhibitory activity against target proteases of human pathogens causing diseases such as emphysema, pancreatitis, arthritis, high blood pressure, cancer, AIDS and muscular dystrophy (Johnson and Pellecchia, 2006). These plant protease inhibitors (PIs) are responsible for inhibition against microbial or animal proteases which play a key role in pests or pathogens for digestion of food (Ryan, 1990). Protease inhibitors like α-Chymotrypsin and trypsin get attraction of researchers because they can retard many deteriorative processes so protect the food material from early spoilage (Baird-Parker, 2003). Now a days, food spoilage is a major problem in the world because 25% of the food material is lost due to microbial activity (Dunaevsky et al., 1998). Hence, screening of new serine protease inhibitors is urgent need to extend the shelf life of sea food and to avoid the pathogenic attack on animals and humans (Reppond and Babbitt, 1993).

H. pylori are recognized for stomach infections and initiate oxidative burst in human neutrophils leading to production of Hydrogen peroxide (a free radical) that oxidizes chloride ions (Suzuki et al., 1992). Urease enzyme that is part of *H. pylori* protein component hydrolyzes urea into ammonia that neutralize stomach acid thus support the initial colonization of the *H. pylori* in human stomach (Dunn et al., 1997). This ammonia reacts with chloride ions to give a highly toxic compound monochloramine (Mai et al., 1991). Urease inhibitors can utilize to control *H. pylori* infections. Antibiotics therapy is usually used for the treatment of *H. pylori* infection. But there is increasing bacterial resistance and due to harmful side effects of antibiotics, it's a need to explore very effective urease inhibitors and anti-ulcerative agents to enhance efficacy against microbes and exhibiting less toxicity to human cells (Spengler et al., 2004; Parente et al., 1996). The urease inhibition assay is prominent method to check the ability of plant extract to inhibit urease enzyme by measuring its absorbance in UV spectrophotometer.

The present work emphasis on cytotoxic, α-Chymotrypsin and urease inhibition effect of the dichloromethane (HDWPD) and methanolic (HDWPM) extracts of the plant, *Heliotropium dasycarpum* based on traditional importance of genus Heliotropium.

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Material and Methods

Plant Collection

The plant was collected from desert area of Kot Mithan, District Rajanpur (Pakistan) on the basis of its literature survey indicating its traditional medicinal importance. The plant was indicated as *Heliotropium dasycarpum* by taxonomist at The Institute of pure and applied Biology of Bahauddin Zakariya University Multan and voucher number "Stewart 589(7)" was allotted.

Preparation of extracts of plant, *Heliotropium dasycarpum*

The *Heliotropium dasycarpum,* whole plant was shade dried for 15 days. The plant material was grounded by using grinding mill and then weighed it. The plant extraction was done by simple maceration process. The 200gm of grounded plant material was taken in extraction bottle by addition of dichloromethane. Then this mixture was shaken to get maximum extraction and homogenized it on ultrasonic bath. The mixture was filtered after 24 hours. The marc after filtration was macerated again by same dichloromethane solvent. After 3rd collection, the marc was treated with methanol. Both extracts of the plant were concentrated separately by using a rotary evaporator under reduced pressure. Both extracts were collected in separate bottles and weighed. Then they were named as HDWPD (dichloromethane extract) and HDWPM (methanol extract).

Cytotoxicity Bioassay

Brine-Shrimp Lethality Assay

Artificial sea water was prepared by dissolving 3.8 g of sea salt/liter in water and filtered. Tanks were filled with artificial sea water and shrimp eggs were added. The shrimp larvae (nauplii) were attracted through illuminated compartment, then hatched the shrimp eggs and mature within two days at 22-29 ºC. Testing vials were prepared and tested initially at 1000, 100 and 10µg/ml. Then three replicates of each fraction were prepared and weighed 20mg of sample and 2ml of organic solvent (20mg/2ml); from this solution was transferred to 500µl, 50µl or 5µl vials corresponding to 1000, 100 or 10µl/ml respectively. Polar insoluble material was dissolved in dimethyl sulfoxide (DMSO) and up to 50µl/5ml of artificial sea water was added. Etoposide was taken as standard drug. After two days, 5ml artificial sea water and ten shrimps for each vial (30 shrimps per dilution) was added. The vials were placed under illumination. After 24 hours, the number of surviving shrimps were counted and recorded. The Data was analyzed with Finney computer program (Probit analysis) (Meyer et al., 1982).

α-Chymotrypsin Assay

The modified method of Rehman *et al* was adopted for α-chymotrypsin enzyme inhibition activity. The contents of Tris-HCl buffer of 60 µl, 0.9 units (15 µl) purified chymotrypsin enzyme and 10 µl test compound was taken in a 100 µl total volume assay mixture. The assay mixture was incubated at 37°C for 10min and visualized at 410nm wavelength. The 15 µl (1.3 mM) of N-succinyl phenyl-alanine-P-nitroanilide (substrate) was added to start the reaction and check its absorbance change. The Chymostatin (0.5 mM/well) was considered as standard. Each reactions was done for three times and inhibition (%) was calculated by the following formula.
Inhibition (%) = (Abs. of Control- Abs. of Test / Abs. of Control) $\times$ 100
IC50 values (concentration at which there is 50% enzyme catalyzed reaction occur) compounds were calculated using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc. Amherst, USA).

Urease Assay

The enzyme assay was the modified form of the commonly known Berthelot assay. A total volume of 85 µl assay mixture contained 10 µl of phosphate buffer of pH 7.0 in each well in the 96-well plate followed by the addition of 10 µl of sample solution and 25 µl of enzyme solution (0.1347 units). Contents were pre-incubated at 37ºC for 5 minutes. Then, 40 µl of urea stock solution (20 mM) was added to each well and incubation continued at 37ºC for further 10 min. After given time, 115 µl phenol hypochlorite reagent was added in each well (freshly prepared by mixing 45 µl phenol reagent with 70 µl of alkali reagent). For color development, incubation was done at 37ºC for another 10 min. Absorbance was measured at 625 nm using the 96-well plate reader. The percentage enzyme inhibition was calculated by the following formula:
Inhibition (%) = (Abs. of Control- Abs. of Test / Abs. of Control) $\times$ 100
IC50 values (concentration at which 50% enzyme catalyzed reaction occurs) of compounds were calculated using EZ-Fit Enzyme Kinetics Software (Perrella Scientific Inc. Amherst, USA) after making suitable dilution of test compound.

Results

Brine Shrimp Lethality Bioassay

Bioactive compounds cause toxicity to shrimp larvae. Eggs of brine shrimp (Leach) are available easily in pet shops. The eggs of brine shrimp are hatched within 48 hours in artificial sea water and are used to measure cytotoxicity of test samples. The Brine-Shrimp lethality bioassay is a quick, economical and in house method for screening, fractionation and monitoring of physiologically active natural products (Meyer et al., 1982)

The result showed that survival of brine shrimp larvae was maximum with less concentration of plant extracts. As concentration of plant extract was increased, number of deaths of brine shrimp larvae were increased as showed in the table 1. The methanol extract of plant exhibited significant cytotoxic effect to shrimp larvae (only 6% survived) at concentration of 1000µg/ml. But 86% and 93% larvae were survived at concentration of 100µg/ml and 10µg/ml respectively. So, HDWPM extract of the plant showed cytotoxic activity at highest level of dose with LD50; 215.837 while HDWPD extract showed no significant cytotoxic activity.

Table 1: Results of Brime-Shrimp Lethality bioassay of *Heliotropium dasycarpum*

Extract	Dose (µg/ml)	No. of shrimps	No of Survivors	LD50 (µg/ml)	Standard Drug	LD50 (µg/ml)
HDWPM	1000	30	02	215.837	Etoposide	7.4625
	100	30	26			
	10	30	28			
HDWPD	1000	30	21	6170.64		
	100	30	25			
	10	30	29			

α-Chymotrypsin Assay

The α-chymotrypsin inhibition assay was done by utilizing standard chymostatin that showed 93.27±0.23% inhibition. The methanol extract (HDWPM) and dichloromethane extract (HDWPD) of *Heliotropium dasycarpum* exhibited 10.50±0.18% and 41.51±0.15% enzyme inhibition respectively with IC_{50} values of greater than 500 µmol. The results of assay are given in the table 2.

Table 2: Results of α-chymotrypsin inhibition assay (mean ± SEM, n = 3) of *Heliotropium dasycarpum*

Extract	Conc. (mM)	Inhibition (%)	IC_{50} (µmol.)
HDWPM	0.5	10.50±0.18	>500
HDWPD	0.5	41.51±0.15	>500
Chymostatin (Standard)	0.5	93.27±0.23	8.24±0.11

Urease Inhibition Assay

In present study, urease inhibition assay of dichloromethane (HDWPD) and methanol (HDWPM) extract of *Heliotropium dasycarpum* was performed. The methanol extract (HDWPM) of the plant showed 24.39±0.21% enzyme inhibition with IC_{50} values of greater than 400 µmol. While dichloromethane (HDWPD) extract has 11.46±0.09% enzyme inhibition with IC_{50} values of greater than 500 µmol.

Table 3: Results of Urease Assay (mean ± SEM, n = 3) of *Heliotropium dasycarpum*

Extract	Conc. (mM)	Inhibition (%)	IC_{50} (µmol.)
HDWPM	0.5	24.39±0.21	>400
HDWPD	0.5	11.46±0.09	>500
Thiourea (Standard)	0.5	99.15±0.13	21.25±0.17

Discussion

In current study, Brine shrimp lethality bioassay was performed to evaluate toxic substances. Brine shrimp lethality assay is general bioassay that gives preliminary information about toxic substances and is predictive of pesticidal, weedicidal and cytotoxic activities. The species of genus Heliotropium have pronounced toxic effect due to presence of pyrrolizidine alkaloids. The pyrrolizidine alkaloids are responsible for hepatocytes damage due to formation of pyrrole metabolites in the liver. Human deaths were also reported due to accidental consumption of seeds of Heliotropium species in Afghanistan (Tandon et al., 1978). The phytotoxic studies on the methanol and dichloromethane extracts of *Heliotropium dasycarpum* showed 100% activity against *Lemna*

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minor (Ghaffari et al, 2013). The bioassay was performed on the basis of traditional importance and previous toxicity reports. The dichloromethane extract did not give significant results but methanol extract of plant showed valuable toxicity results (28, 26 and 2 larvae survived) in increasing dose interval (10, 100, 1000 µg/ml) with LD50; 215.837. The claim of toxicity of Heliotropium genus in previous data is substantiated by scientific studies. So, there is need to work on more specific cytotoxic bioassays like cell line (MTT assays) and to screen out the novel compounds that have chemotherapeutic use.

Enzyme inhibitors have got increasing role in recent years not only for enzyme mechanisms and structures but also increased attention in field of agriculture (Ahn et al., 2004) and pharmacology
(Imada, 2005). The epidemiological studies has demonstrated the plant protease inhibitors role in decreasing colon, prostate and breast cancers. The Bowman-Birk derived from soya bean is a protease inhibitor that is used to treat oral, liver, lung, esophageal and colon cancers. The researchers think that α-Chymotrypsin inhibition activity of protease inhibitor "Bowman-Birk" is responsible for its anti-cancerous activity (Witschi and Kennedy, 1989). The results (41.51±0.15% Enzyme Inhibition) of dichloromethane (HDWPD) and methanol (HDWPM) plant extracts (10.50±0.18% Enzyme Inhibition) will force the researchers to screen out the valuable protease inhibitors.

The importance of Urease inhibition assay is due to their extensive use in bacterial urease therapy (pathogenic activity of *H. pylori* such as peptic ulcer, stone formation, hepatic coma and pyelonephritis), analytical techniques to check enzyme inhibitors, protect from pH elevation of soil and control of urea hydrolysis due to nitrogen loss in urea fertilizer that used in soil (Upadhyay, 2012). The bacterial therapy (antibiotics) is usually employed for the eradication of *H. pylori* in stomach infections. Now a days, bacterial resistance is increasing worldwide, so another modes of therapy like urease inhibition assay has increased attention of the researchers. The folk medicine history on genus Heliotropium also targeted on ulcer treatment. The plants, *Heliotropium ellipticum* and *Heliotropium eichwaldi* are traditionally used for cleaning and healing of ulcer in African countries (Kirtikar and Basu, 1967; Bhakuni et al., 1969). The results of Urease inhibition assay of plant, *Heliotropium dasycarpum* validate the folk medicinal use for treatment of ulcer. These results will force the scientists to work on the further fractions of extracts to purify unique urease inhibitors for enhancement of the activity.

Conclusion

The above findings has clearly showed that the plant, *Heliotropium dasycarpum* has valuable cytotoxic potential and can be useful to study more specific cytotoxicity assays in future. The plants extracts also showed inhibition against both enzymes. The results are supportive to already available traditional and scientific data. So further study is required to screen out bioactive constituents that are utilized for the treatment of ulcerative diseases.

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