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Screening of selected medicinal plants for urease inhibitory activity

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Abstract
In this study, we have investigated antiurease activity of eleven Et-OH and five Me-OH extracts of medicinal plants collected from the State of Kashmir. Selection of the plants was made on the basis of their uses by local people and herbalists for the treatment of different ailments including stomach problems. In the study Et-OH extracts of *Sussuria lappa, Malva parviflora, Solanum nigrum,* and *Melia azadirachta* were found inactive or showed low activity at final of concentration 200µg/ 5ml. While Et-OH extracts of *Taraxacum officinale, Achillea millefolium*, *Aristolachia bracteata*, *Eucalyptus globules, Adhatoda zeylanica, Cuscuta reflexa* and *Mentha longifolia* showed stronger action against urease activity. The IC_{50} values for these extracts were 33.33*, 94.24, 68.62, 66.91, 83.33, 89.19 and 57.47*µg/ 5ml. Among Me-OH extracts, *Achillea millefolium* and *Aristolachia bracteata* demonstrated stronger antiurease activity IC_{50}=60.29* and 58.73*µg/5ml. *Mentha longifolia, Solanum nigrum* and *Melia azadirachta* showed no action against urease activity at 200µg/ 5ml. Results of this study illustrate that most of the studied extracts exhibited reasonable antiurease activity, however, ethanolic extracts of *Taraxacum officinale*, *Mentha longifolia* and methanolic extracts of *Achillea millefolium* and *Aristolachia bracteata* showed significant inhibition potential. Our findings may help to explain the beneficial effects of these plant extracts against stomach infection associated with pathogenic strains of *H. pylori*. The crude extracts may be purified for the isolation of compounds with antiurease activity. These results also validate the traditional use of these herbs for the treatment of stomach infection.

Keywords: Urease; Inhibition; Plant extract; Stomach infection.

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Introduction

Stomach infection with pathogenic strains of *H. pylori* causes severe gastro duodenal diseases in a large number of patients worldwide (Montecucco et al., 1999). The *H. pylori* infection breaks up in early childhood, persists lifelong if not eradicated, and is associated with chronic gastritis and an increased risk of peptic ulcer and gastric cancer (Clyne et al., 1993). Across population of children, *H. pylori* prevalence ranges from under 10 to over 80% in developed and developing countries, respectively (Torres and Drumm, 2000).

Urease is the most prominent protein component of *H. pylori* (Dunn et al., 1997). Urease hydrolyzes urea, releasing ammonia, which neutralizes acids of stomach and thus enables survival and initial colonization of the pathogen (Blaser, 1990; Segal et al., 1992). Most of the urease is found in bacterial cytoplasm, although up to 10% appears on the surface (Bode and Mauch, 1993). It is the cytoplasmic fraction of the urease, which is required for the acid resistance. Urease also serves as a virulence factor in human and animal infections of urinary and gastrointestinal tracts (Andrews et al., 1984). Formation of urinary struvite stones in urinary tract is commonly associated with urea-splitting bacterium such as *Ureaplasme urealyticum. H. pylori* whole cell can stimulate an oxidative burst in human neutrophils (Suzuki et al., 1992). Hydrogen peroxide from the oxidative burst oxidizes chloride ions, which react with ammonia liberated by *H. pylori* urease to give the highly toxic product monochloramine (Mai et al., 1991). It is also reported that by increasing level of ammonia in the body some neurological disorders are also produced leading to Parkinson´s disease (Amtul et al., 2002). In recent studies, *H. pylori* infection is also suspected to be associated with coronary artery and ischemic heart diseases (Danesh et al., 1998; Mendall et al., 1994; Tougas et al., 1999).

Killing *H. pylori* with antibiotics can cure most patients specifically with duodenal ulcer (Parente et al., 1996; Boer et al., 2000). Many antibiotic-linked treatments have been recommended for eradication of *H. pylori* infection but the appearance of antibiotic resistance makes the treatment more complicated and the infection is persistent at higher levels when the drug treatment is stopped (Parente et al., 1996; Spengler et al., 2004). It is

also reported that above 15% of the patients undergoing drug treatment experience therapeutic failure (Jodi et al., 1998). Recently, screening of natural products has gained much interest for the safe inhibition of urease as potential new antiulcer drugs.

Numerous studies have concentrated on the eradication of *H. pylori* infection using herbal medicines. Garlic and pteleopsis extracts exhibited weak and modest, anti-*H. pylori* activity, respectively (Cellin et al., 1996; Germanò et al., 1998). Fifty-four Chinese herbs were also screened for anti-*H. pylori* activity (Bae et al., 1998). Anti-*H. pylori* compounds from the Brazilian medicinal plants have also been successfully isolated (Ohsaki et al., 1999). Extracts from seven Turkish plants are also demonstrated to draw out anti-*H pylori* activity (Yesilada et al., 1999). Extracts form fifty Taiwanese folk medicinal plants were examined and screened for anti-*H pylori* activity. About half of Taiwanese folk medicinal plants tested demonstrated to possess higher anti-*H. pylori* activity (Wang and Huang, 2005). In most studies, the strategy couples the benefit to inhibit urease, as without action of urease in stomach, *H. pylori* would not survive. Jack bean urease, however, has been used as a substitute for the bacterial enzymes because of the ready commercial availability of purified plant enzyme and its well-predicted amino acid sequence. The enzyme shares more than 50% similarity with that of the pathogenic enzyme (Ganga-Zandzou et al., 1999).

Materials and Methods

Chemicals

Crescent urea diagnostic kit (Crescent Cat No. CS 612) was purchased from Dia Sys., containing reagent 1 (R1) (Phosphate buffer 120mmol/l, Sodium salicylate 60mmol/l, Sodium nitroprusside 5mmol/l, EDTA 1mmol/l, Urease 5KU/l) and reagent 2 (R2) (Phosphate buffer 120mmol/l, Sodium hydroxide 400mmol/l, sodium hypochlorite, 10mmol/l). Urea standard 80mg/dl was used in the study. All reagents used were of analytical grade.

Plant Materials

Eleven medicinal plants were collected from the State of Kashmir on the basis of their medicinal use by local people and traditional herbalists for common ailments. All plants were identified by a taxonomist at the Department of Botany, University of Azad Jammu and Kashmir, Muzaffarabad.

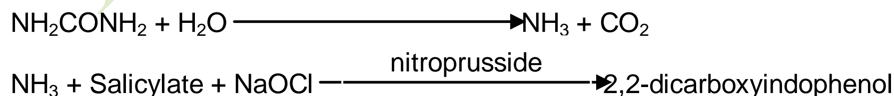
Preparation of Extracts

The plants were air dried under shade for several weeks before extraction. Dried plants were chopped into small pieces and grounded to a fine powder. The material of each plant was soaked in absolute ethanol/ methanol for six weeks with occasional shaking, at room temperature. Both the Et-OH and Me-OH soluble materials were filtered and the filtrate was concentrated under vacuum. Chlorophyll was removed by successive solvent extractions using n-hexane. The remaining solvent was evaporated to dryness at room temperature. The weighed amount of concentrates was dissolved in phosphate buffer (25ml) and stored at 4°C until further use.

Principle

In the present study, phenol hypochlorite method described by Weatherburn (Weatherburn, 1967) was adopted. The method is based on the following principle:

Urease catalyzes the conversion of urea to ammonia, which reacts, with a mixture of salicylate, hypochlorite and nitroprusside to yield a blue-green dye (indophenol) with λ_{max} 625nm. The intensity of the color is proportional to the concentration of ammonia produced by the urea.



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Urease Activity and Inhibition Study

The enzyme activity and inhibition was measured through catalytic effects of urease on urea by measuring change in absorbance in the absence and in the presence of inhibitor at 625nm, using (Schamadzu-1601) spectrophotometer. Absorbance of blank test mixture, prepared by taking buffer (500 µl), R1(2.5ml) and R2 (2.5ml) was set at zero by turning the spectrophotometer at auto-zero. The reaction mixture cocktail for the enzyme assay containing urea (15µl) and R1 (2.5ml), was incubated for 5min at 37°C followed by the addition of R2 (2.5ml) and 10min incubation time. Enzyme inhibition study was carried out by the addition of various concentrations of sample (substrate) followed by rest of the enzyme assay procedure. Protocol for enzyme activity and inhibition study is given in Table 1.

Results and Discussion

Like most developing countries, rural population of Pakistan heavily relies on valuable heritage of medicinal plants (Kattak et al., 1985). It is therefore of high interest to determine the reasons for efficacy of indigenous medicinal plants which are commonly used by local population and traditional practitioners. Therefore, current study presents investigation of enzyme inhibition studies of eleven indigenous medicinal plants collected from the State of Kashmir, Pakistan. The present study will provide scientific information, which may lead purification of high quality products at affordable prices. In the study, Et-OH extracts of *S. lappa, M. parviflora, S. nigrum,* and *M. azadirachta* were found inactive or showed low activity at final of concentration 200µg/ 5ml. While Et-OH extracts of *Taraxacum officinale, Achillea millefolium*, *Aristolachia bracteata*, *Eucalyptus globules, Adhatoda zeylanica, Cuscuta reflexa*, *Mentha longifolia* and methanolic extracts of *Achillea millefolium* and *Aristolachia bracteata* showed stronger action against urease activity. The IC_{50} value of each extract was calculated from straight-line equation given in Table 2 and brief description of botanical name, collection period, parts used, extraction medium, %activity and IC_{50} values are given in Table 3.

An overview on the medicinal uses of the plants showing antiurease activity is given here which may predict their potential traditional use for stomach problems. *A. bracteata,* is most abundant throughout Kashmir in xerophytic environment. Its leaves and roots are useful in dyspepsia, dysentery and diarrhea. *A. millefolium*, is also abundant in Himalayas and Kashmir. Its leaves and flower heads are useful as carminative. *A. zeylanica,* is another popular herb of Kashmir which is commonly employed locally for the treatment of bilious disorders. *C. reflexa,* is traditionally used as a carminative and for bilious problems. *E. globules* is a popular plant, widely distributed throughout the world, having carminative and digestive properties. *A. millefolium,* is used for the treatment of dyspepsia and intestinal colic.

Conclusion

The present findings provide a scientific basis for the traditional use of some selected plants in stomach related diseases. The antiurease activity of the extracts of these plants may be associated to inhibit *H. pylori*, a key cause of stomach infections. The study is a step towards developing Flow Injection Analysis (FIA) method for enzymatic studies.

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Table 1. Protocol for the urease assay and inhibition study.

Test mixture	Test sample (µl)	Urea (80mg/ml)	Buffer (µl)	R1 (ml)	1st Incubation (37^0C)	R2 (ml)	2nd Incubation (25^0C)
Blank	--	--	500	2.5	--	2.5	00
Standard	--	15	485	2.5	5min	2.5	10min
Sample	--	15	485	2.5	5min	2.5	10min

Table 2. The slope, intercept and r^2 of % inhibition equation.

S. No.	Plant name	Equation for ethanolic / methanolic extracts	
		Slope	**r^2**
1	*Mentha longifolia*	0.6093	0.9844
2	*Achillea millefolium*	0.4881/0.876	0.9902/0.9934
3	*Aristolachia bracteata*	0.5101/0.8638	0.9291/0.9873
4	*Cuscuta reflexa*	0.3727	0.9949
5	*Adhatoda zeylanica*	0.3348	0.9929
6	*Eucalyptus globules*	0.6807	0.9779
7	*Taraxacum officinale*	1.2613	0.9934

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Table 3. Botanical name, collection period, area of collection, parts used, extraction medium, appearance of the extracts, % activity and IC_{50} values.

S. No.	Plant name	Part used	Solvent Used	Appearance of extract	% Activity 200µg/5ml	Conc. tested µg/5ml	MI ± S.E.M.	IC_{50} µg/5ml
1	*Mentha longifolia*	root, stem and leaves	Ethanol/Methanol	Powdered	A/NA	27 68 122	20 ± 2.2 46 ± 1.5 71 ± 0.99	57.4712*
2	*Achillea millefolium*	root, stem and leaves	Ethanol/Methanol	Powdered	A/A	24 122 196/ 24 96 122	15 ± 1.2 65 ± 1.5 92 ± 2.5/ 20 ± 2.1 65 ± 1.5 83 ± 1.8	94.243 60.2941*
3	*Solanum nigrum*	root, stem and leaves	Ethanol/Methanol	Powdered	NA/NA			
4	*Aristolachia bracteata*	stem and leaves	Ethanol/Methanol	Powdered	A/A	11 68 144/ 12 52 122	18 ± 0.55 40 ± 0.89 70 ± 2.3/ 9 ± 0.99 40 ± 1.5 75 ± 2.2	68.6274 58.7301*
5	*Melia azadirachta*	Leaves	Ethanol/Methanol	Powdered	NA/NA			
6	*Cuscuta reflexa*	Stem	Ethanol	Jelly like Paste	A	18 90 181	9 ± 2 36 ± 1.0 66 ± 2.0	89.1891
7	*Sussuria lappa*	root and stem	Ethanol	Jelly like Paste	NA			
8	*Adhatoda zeylanica*	stem and root	Ethanol	Jelly like Paste	A	32 80 160	8 ± 0.88 25 ± 0.56 55 ± 0.87	83.3333
9	*Eucalyptus globules*	leaves	Ethanol	Thick paste	A	13 81 136	18 ± 0.66 58 ± 0.15 90 ± 1.5	69.9111
10	*Taraxacum officinale*	root, and leaves	Ethanol	Powdered	A	12 51 70	20 ± 2.1 67 ± 1.5 97± 2.8	33.3333*
11	*Malva parviflora*	root, stem and leaves	Ethanol	Jelly like paste	NA			

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