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Hina Zahid
Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, University Road Karachi-75270, Pakistan.
Email: hinazahid.zh@gmail.com

Ghazala H. Rizwani
Professor,
Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, 75270 Pakistan.
Email: Rizwanix2@yahoo.com

Huma Shareef
Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, 75270 Pakistan.
Email: Phr_huma@hotmail.com

S. Tahir Ali
Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, 75270 Pakistan.
Email: drtahir2@gmail.com

Correspondence:
Hina Zahid
Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, University Road Karachi-75270, Pakistan
Email: hinazahid.zh@gmail.com
Tel: 0092-021-99261300-07

Antioxidant and urease inhibition activity of methanol extract of *Hibiscus schizopetalus* (Mast) Hook.

Hina Zahid, Ghazala H. Rizwani, Huma Shareef, S. Tahir Ali

ABSTRACT

The present study was aimed at measuring the antioxidant and urease inhibition capacities of methanolic extracts of flower and leaves (HFE and HLE) of *Hibiscus schizopetalus* (Mast) Hook (Malvaceae) an ornamental plant of this region. These extracts were screened for their scavenging properties using DPPH radical scavenging and nitric oxide antioxidant assay. Research on urease inhibitions from natural source yielded several imperative therapeutic drugs that are useful in gastric and urinary tract infections. Radical scavenging activity of extracts were evaluated at a concentration of 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml using ascorbic acid as standard antioxidant. IC_{50} values for ascorbic acid and extracts (HFE, HLE) were 28 ± 0.04 µg/ml, 38.2 ± 0.08 µg/ml and 58.9 ± 0.13 µg/ml respectively. While 10, 25, 50, 100, 150 µg/ml contractions of extracts were used for nitric oxide scavenging activity. The maximum activity of extracts (HFE, HLE) was observed at 150 µg/ml (80.70% and 75.2%) respectively, whereas ascorbic acid exhibited 81.02% urease inhibition at the same concentrations. The percentage urease inhibition of standard Thiourea was found to be 98.2%, IC_{50} 88.2 ± 0.01 µg/ml. HFE extract showed maximum urease activity with percentage inhibition of 55.5%, IC_{50} 80.1 ± 0.87 µg/ml. While least inhibitory activity was observed in HLE extract i.e. 22.2%.

This is the first report of mention research on *Hibiscus schizopetalus* (Mast) Hook in Pakistan. The results obtained in this study clearly showed that methanolic extracts of flower and leaves of plant could be proved as a good antioxidant and antiurease agent.

Keywords: *H. schizopetalus* (Mast) Hook, Antioxidant, DPPH, Nitric oxide, Urease inhibition, *H. pylori*.

1. Introduction

Living cells generate free radicals and other reactive oxygen species (ROS) by-products as a result of physiological and biochemical processes. The potentially reactive derivatives are continuously generated inside the human body as a consequence of exposure of exogenous chemicals in our ambient environment and a number of exogenous metabolic processes involving redox enzymes and bioenergetics electron transfer. Under normal circumstances, the free radicals are detoxified by the antioxidants present in the body and making equilibrium between the ROS generated and the antioxidants present. However, overproduction of ROS and inadequate antioxidant defence can easily affect and persuade oxidative damage to lipids, proteins and DNA which may eventually lead to various diseases such as cancer, diabetes, aging and other chronic degenerative diseases [1].

In recent years, there has been an increasing interest in finding natural antioxidants, which can protect the human body from free radicals and retard the progress of many chronic diseases. Natural antioxidants such as α – tocopherol, ascorbic acid, resveratrol, flavanols, carotenoids etc. are widely used because they are regarded as safer and causing fewer adverse reactions [2-3]. Therefore, there is considerable interest in finding safer antioxidants from natural sources to replace the synthetic ones.

Urease is an enzyme that catalyzes the hydrolysis of urea to produce ammonia and carbon dioxide, and the most crucial role is to protect the bacteria in the acidic environment of the stomach [4]. The urease inhibitors can play a vital role to counter effect the negative function of urease in living organisms.

Urease inhibitors are effective against several serious infections caused by the secretion of urease by *Helicobacter pylori* which include gastric tract syndromes and urinary tract infection. Research on urease inhibitions yielded several vital therapeutic drugs [5]. Moreover, it has been demonstrated that *H. pylori* lacking urease activity are incapable of causing infection in animal models. Thus, it is most likely that urease is essential for bacterial colonization and perhaps the pathogenesis of related disease in vivo. World Health Organization has categorized *H. pylori* as a class I carcinogen [6].

Fortunately, its eradication with antibiotics can result in ulcer healing, prevent peptic ulcer recurrence and reduce the prevalence of gastric cancer in high-risk populations. However, it is not always successful because of its resistance to one or more antibiotics and other factors such as poor patient compliance, undesirable side effects of the drugs and significant cost of combination therapy [7]. Inhibition of urease was extensively studied because of their potential uses like therapy against bacterial urease e.g. *H. pylori* that induced pathogenic conditions i.e. urinary stone formation, peptic ulcer pyelonephritis and hepatic coma. Urease inhibitor dissolves crystals and struvite kidney stones and prevents new crystal formation in urine [8-9]. Many plants are known to possess urease inhibition activity such as *Hypericum oblongifolium*, *Taraxacum officinale*, *Achillea millefolium*, *Aristolochia bracteata*, *Eucalyptus globules*, *Adhatoda zeylanica*, *Cuscuta reflexa* and *Mentha* longifolia etc. [10-11].There is a growing interest all over the world for discovering the untapped reservoir of medicinal plants. Hence, the present study was aimed at measuring the antioxidant and urease inhibition capacities of *H. schizopetalus* (Malvaceae). This plant is a common ornamental shrub cultivated in Pakistan. The plant also found in various region of the world [12]. *H. schizopetalus* is the allied specie of *H. rosa – sinensis* sharing the same genera *Hibiscus* but the difference in both plant is the direction of flower. Leaves of plant are alternate, ovate to lanceolate, often with a toothed or lobed margin; resemble those of *H. rosa – sinensis* leaves. Various parts of the plant used in cold, cough and to reduce fever [13-15]. According to the current literature methanolic extract of flower and leaves found significant analgesic and antipyretic potential [16].

2. Materials and Methods

2.1. Plant Material and Extraction Procedures

Hibiscus schizopetalus (Mast) Hook (flowers and leaves) was collected from the premises of University of Karachi, Pakistan, in the month of July, 2009. The plant was identified and authenticated by Prof. Dr. Suriya, Ex. Chairperson Department of Botany, University of Karachi, Pakistan. Voucher specimen (No. 082) was deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, Pakistan. For the preparation of methanolic extract, 1000 g of leaves and 500 g of flowers were soaked in methanol for 7 to 10 days. After percolation period, the solvents were removed under reduced pressure and control temperature (40±2 °C) on a rotary evaporator (Buchi, Switzerland). The yield of obtained were 8.02% and 7.38% for methanolic extract of flower (HFE) and leaves (HLE) respectively.

2.2. Chemicals

The chemicals used were Methanol (Merck, Germany), DPPH (B.D.H. laboratory supplies, UK), ascorbic acid (Sigma Chemical, USA), sodium nitroprusside (B.D.H. laboratory supplies, UK), Phosphate buffer (pH 7.4), Sulfanilic acid reagent (B.D.H. laboratory supplies, UK), Naphthyl ethylene diamine dihydrochloride (B.D.H. laboratory supplies, UK), sodium hydroxide (Merck, Germany), Thiourea (Sigma-Aldrich, USA), sodium hypochlorite (Sigma-Aldrich, USA).

2.3. Antioxidant Assay

Antioxidant assay includes DPPH Radical scavenging activity, Nitric oxide scavenging activity [17-18].

2.3.1. DPPH Radical scavenging activity

For free radical scavenging capacity of the plant extracts (HFE and HLE) was determined by DPPH (2, 2-diphenyl- 1-picrylhydrazyl). DPPH solution was prepared in methanol (0.004% w/v). HFE, HLE extracts were mixed with 95% methanol to prepare the stock solution of 10 mg/100 ml or 100 µg/ml respectively. From stock solution 2 ml, 4 ml, 6 ml, 8 ml and 10 ml of this solution were taken in five test tubes and by serial dilution with same solvent was made the final volume of each test tube up to 10 mL whose concentration was then 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/mL and 100 µg/ml respectively. Freshly prepared DPPH solution was added in each of test tubes and after 10 min, the absorbance was taken at 517 nm using spectrophotometer. Ascorbic acid was used as reference standard and dissolved in distilled water to make a stock solution of 100 µg/ml. Following equation was used for measuring the scavenging activity

$$\text{Radical scavenging (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The relationship between percentage inhibition and sample concentration was plotted to determine the IC_{50} value.

2.3.2 Nitric oxide scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generated nitric oxide which interacts with oxygen to produce nitrite ions determined by the use of Griess reagents. Two mL of 10 mM sodium nitroprusside dissolved in 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of plant extracts at various concentrations (10, 25, 50, 100, 150 µg/ml). The mixture was incubated at 25 °C after 150 min. From the incubated mixture 0.5 mL was taken out and added into 1.0 mL sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally 1.0 mL naphthyl ethylene diamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm was measured with a spectrophotometer. The nitric oxide radical scavenging activity was calculated.

$$\text{Nitric oxide scavenging (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.4 Urease inhibition activity

Urease is a nickel containing enzyme found in bacteria, fungi and plants. The enzyme converts urea into ammonia which neutralizes the acid around the microorganism. Urease inhibitory activity of the extracts of *H. schizopetalus* was determined according to the protocol reported by Lateef *et al*. [19], based on the method proposed by Weatherburn [20]. Urease (Jack bean) solution (25 µl) was mixed with the 5 µl each extracts (500 µg) and incubated at 30 °C for 15 min. Aliquots were taken and immediately transferred to assay mixtures containing urea (100 mM) in buffer (40 µl) and re-incubated for 30 minutes in 96 well plate. 50 µl each of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 µl of alkali reagent (0.5% w/v sodium hydroxide NaOH and 0.1% sodium hypochlorite NaOCl) were added to wells. Increase in

absorbance was measured after 50 min at 630 nm against blank (Spectramax Plus 384 Molecular Device, USA). Final volume of reaction is 200 µl at pH 8.2. All reactions were performed in triplicates. Thiourea was used as positive control. The percentage inhibitions were calculated by formula

$$\text{Urease Inhibition } (\%) = 100 - (OD_{\text{test well}}/OD_{\text{control}}) \times 100$$

The % inhibition was plotted verses the concentration of the samples and a regression curve was established to calculate the IC_{50} value for each sample which is the concentration of the given sample to inhibit the activity of urease by 50%.

2.5. Statistical analysis

All the values are expressed as Mean ± SEM. The data were analyzed by one way ANOVA followed by LSD multiple comparison tests. A level of $P < 0.05$ was considered as statistically significant. A level of significance was noted and interpreted accordingly.

2.6. Determination of IC_{50} values

IC_{50} values (half maximal inhibition concentration) were determined by investigating the HFE, HLE extracts inhibitory activity (DPPH, Nitric oxide and Urease) at their different concentrations in comparison to their individual positive control employing spectrophotometric measurement. IC_{50} values were obtained from curves obtained by linear regression.

3. Results

3.1. DPPH Radical scavenging activity

Scavenging activity for free radicals of 1.1-diphenyl-2-picrylhydrazyl (DPPH) has been widely used to evaluate the antioxidant activity of plant extracts. At a concentration of 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml ascorbic acid and HFE and HLE extracts of *H. schizopetalus* scavenged of DPPH radical were 43.41%, 55.24%, 76.43%, 86.36 and 91.88%, 33.01%, 53.04%, 74.14%, 82.80%, 90.46%; 24.41%, 37.89%, 47.90%, 68.78%, 72.00% respectively represented in Table 1. Significant activity was observed in HFE as compared to ascorbic acid. IC_{50} values for ascorbic acid and extracts (HFE, HLE) were 28 ± 0.04 µg/ml, 38.2 ± 0.08 µg/ml, 58.9 ± 0.13 µg/ml respectively showed in Figure - 1.

Table 1: *In vitro* DPPH radical scavenging activity of *H. schizopetalus*

Concentration (µg/ml)	DPPH		
	Ascorbic Acid	HFE	HLE
20	43.41	33.01*	24.41*
40	55.24	53.04*	37.89*
60	76.43	74.14*	47.9*
80	86.36	82.8*	68.76*
100	91.88	90.46*	72*

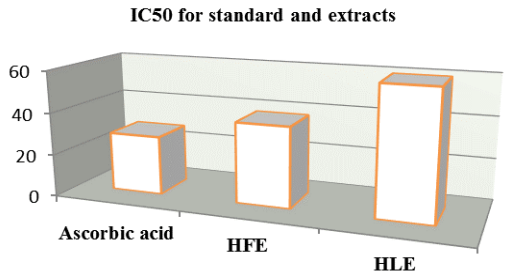

Fig 1: IC50 values for standard and extracts (HFE, HLE) in DPPH assay

3.2. Nitric oxide scavenging activity

The scavenging of nitric oxide by the extracts (HFE and HLE) was increased in dose dependent manner. Table 2 illustrates a significant decrease in the NO radical due to the scavenging ability of extracts and ascorbic acid. The methanolic extracts showed maximum activity of 80.70% and 75.2% respectively at 150 µg/ml, whereas ascorbic acid at the same concentration exhibited 81.02% inhibition. The IC_{50} values were found to be 12 ± 2.13 µg/ml, 40 ± 0.10 µg/ml and 30 ± 0.16 µg/ml for ascorbic acid and extracts respectively depicted in Figure - 2.

Table 2: *In vitro* Nitric oxide scavenging activity of *H. schizopetalus*

Concentration (µg/ml)	Nitric oxide		
	Ascorbic Acid	HFE	HLE
10	42.92	32.89*	37.2*
25	53.56	42*	48.12*
50	64.24	65.4*	64.3*
100	77.6	72.3*	69.5*
150	81.02	80.7*	72.5*

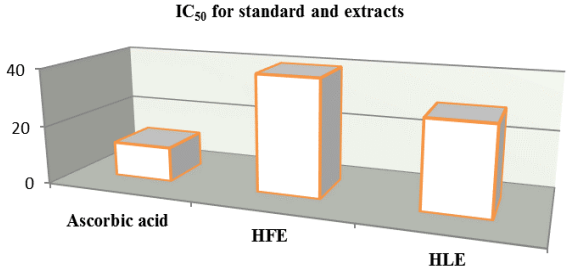

Fig 2: IC_{50} values for standard and extracts (HFE, HLE) in Nitric oxide scavenging assay

3.3. Urease inhibition activity

Urease inhibitory activity of *H. schizopetalus* was depicted in Figure - 3. The activity was comparable to that of Thiourea which was used as standard. The percentage urease inhibition of standard Thiourea was 98.2%, IC_{50} 88.2 ± 0.01 µg/ml. HFE extract showed maximum urease activity with considerable percentage inhibition of 55.5%, IC_{50} 80.1 ± 0.87 µg/ml. While in HLE extract least inhibitory activity was observed i.e. 22.2%.

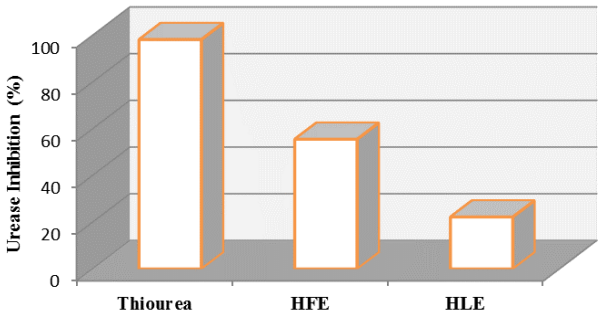

Fig 3: Urease inhibition activity of HFE and HLE

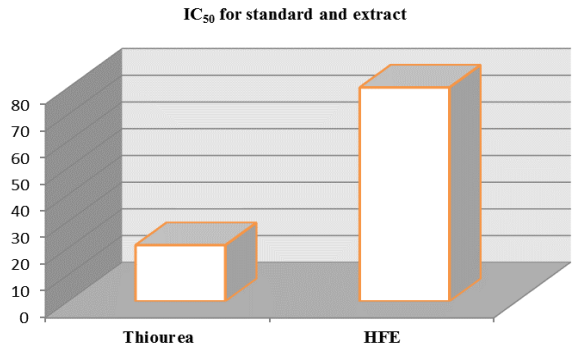

Fig 4: IC_{50} values for standard and extract (HFE) in Urease inhibition activity

4. Discussion

The effect of antioxidants on DPPH radical scavenging is to due hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. A freshly prepared DPPH solution exhibited a deep purple colour with a maximum absorption at 517 nm. The purple colour disappears when an antioxidant is present in the medium [21].

Nitric oxide is an essential bio-regulatory molecule required for several physiological processes like neural signal transmission, immune response, control vasodilatation and control of blood pressure [22-24]. However, during infections and inflammations formation of nitric oxide is elevated and may bring about some undesired deleterious effects [25]. The plant and plant products may have the property to counteract the effect of nitric oxide formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation.

According to chemical literature survey about 80-90% plants found in nature has great potential against large number of naturally occurring antimicrobial agents and these agents are the best sources for the treatment of *H. pylori* [26]. In developing countries, the application of antibiotics is still under a poor management as a whole and there is a growing need for finding new antiurease agents from nature that can hopefully eradicate the invasion and presence of survived *H. pylori* strains to avoid relapse of gastric ulcer.

5. Conclusion

This is the first report of mention research on *Hibiscus schizopetalus* (Mast) Hook in Pakistan. The results obtained in this study clearly showed that methanolic extracts of flower and leaves of plant have powerful antioxidant activity against various antioxidant systems *in vitro*. Moreover, these extracts can be used not only as source of natural antioxidants and but also useful in pharmaceutical applications. Significant urease inhibition was detected in HFE extract which could be potentially utilized in various disease conditions such as peptic ulcer, pyelonephritis and urinary stone formation. Therefore, after further focused research plant can be proved as a good antioxidant and antiurease agent for controlling mentioned disease conditions of the body.

6. Conflict of interest

The authors declare that they have no conflict of interest.

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