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Original Research

In vitro anti-ulcer potential of *Raphanus sativus* L. seeds

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Abstract

Background: *Raphanus sativus* L. is an indigenous plant that is traditionally used to treat peptic ulcer. Objective: This study assessed anti- *Helicobacter pylori,* antioxidant, and anti-urease activities of *R. sativus* seeds. Methods: The study was conducted by using in-vitro model. The antioxidant activity of the methanol extract and *n*-butanol, aqueous, *n*- hexane and chloroform fractions of *R. sativus* seeds was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH). Moreover, the inhibitory effects of the extract and fractions on the activity of urease and antibacterial activity against *Helicobacter pylori* were also evaluated. Results: The crude extract and *n*-butanol fraction exhibited higher (IC_{50}: 45 µg/mL and 31 µg/mL respectively) DPPH inhibition activity and inhibition of urease (IC_{50}: 75.0 µg/mL and 55.9 µg/mL respectively) while aqueous extract inhibited *H. pylori* strongly (MIC_{50}: 15 µg/mL).Conclusion: Collectively, our data show that *R. sativus* extract and fractions possess antioxidant, anti- *H. pylori* and anti-urease potential. The traditional uses of *R. sativus* as anti-ulcer, might be due to its anti-urease activity.

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Introduction

Peptic ulcers are usually triggered by *H. pylori* that can't grow and like acidic environment to live in rather alkaline mucus is favorable for it and is motile.[1] It can't survive in acidic environment and so it produces urease enzyme for the protection from acidic environment of stomach. Urease changes urea in to ammonia that turns the acidic environment of stomach to alkaline. In this way, survival of *H. pylori* is possible in stomach. If urease enzyme is inhibited, *H. pylori* could be removed from the body [2].

Various antibiotics are used to control *H. pylori* infections [3]. But drug resistance and side effects of allopathic medicines turn the World towards natural medicine [4]. These medicines act through prophylactic or therapeutic means. Antioxidant activity provides its prophylactic approach while therapeutic effects include anti-*H. pylori* effects [5]. Urease inhibition can cause the removal of *H. pylori* [6]. So, plants with antioxidant effects, urease and *H. pylori* inhibitory activity could be used as best antiulcer agents.

R. sativus (Brassicaceae) commonly known as "moli" is traditionally used in medicine and all parts of it including roots, leaves and seeds have important medicinal value. Leaves are used as diuretic and laxative. Roots are useful in gastric ailments, hemorrhoids and as anti-urolithic while seeds have carminative, laxative and diuretic properties [7]. Many studies showed in vivo antiulcer effect of different parts of *R. sativus* in alcohol induced, drug induced or stress induced peptic ulcer models [7-9]. *R. sativus* is explored for anti-urease, anti- *H. pylori,* and antioxidant.

Material and Methods

Plant Material and Chemicals: *Raphanus sativus* (Moli) seeds were collected from the herbalist and recognized by Dr. Sarwar, from department of Botany, The Islamia University Bahawalpur and voucher specimen (Voucher NO. 2205/L. S) was deposited in I.U.B herbarium. 2, 2 di phenyl 1, picrylhydazyl (DPPH) and enzyme Urease (sigma Aldrich, Germany), thiourea and Urea (Merck, Germany), Phenol reagent (Pak Land scientific productions), Na2[Fe(CN)5NO] • 2H2O (BDH laboratory supplies Poole, BH 15 ITD, England) were used.

Equipments: Synergy HT Bio Tek® USA micro plate reader, Glass wares - Pyrex, Japan & Iwaki, Japan, Digital electronic weighing balance- Precisa Instruments, Switzerland. Sonicator- Elmasonic, Germany, Rotary evaporator 4000 efficient HB digital of Heidolph Laboratory, Germany.

Preparation of Plant extract

R. sativus powder (2kg) was placed in methanol (2L) for 15 days and shaked after intervals followed by filtration. Methanol was evaporated by use of rotary evaporator [10]. Fractions were made by method of Ahmad et al [11]. The extract/fractions were collected in small glass bottles and stored at 4℃ until next use. Stock solutions of plant extract/fractions (0.5mg/mL) were prepared and serial dilutions (250µg/mL, 125µg/mL, 62.5µg/mL and 31.25µg/mL) were made from the stock solution for anti-*H. pylori*, anti-urease and antioxidant activities.

DPPH radical scavenging Assay

The antioxidant activity was checked against 2, 2 diphenyl 1, picryl hydra Zyl radical by following Meléndez methodology with some modifications [12]. Vitamin C was standard control of study. 90µL of DPPH and 10µL of tested sample were placed in micro plate. The procedure was repeated 3 times. The microplate was placed for 30 minutes at 37° C in oven. The absorbance was measured at 517nm by using Bio-tech ELISA micro plate reader. The %I was measured by following formula:

Antioxidant activity % = [100-(As / Ac)] * 100

Where,

Ac = Negative control absorbance

As = Sample extract absorbance

IC_{50} was calculated by using Microsoft excel.

Anti-urease Assay

Anti-urease activity was performed by following the methodology of [13]. Urea was the substrate. Potassium phosphate buffer (pH:

7) was used. Phenol, sodium hypochlorite are used as reagents. Temperature adjustment was done on 37°C. IC_{50} was calculated by using Microsoft excel.

%I = [100-(Absorbance of sample/ Absorbance of control) x 100]

Antibacterial Activity

Inoculum preparation: First fungal culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of The Punjab, Lahore, Pakistan (accession number: 14) was paid for *Helicobacter pylori*. 24 h culture was preferred to pick up colonies of *H. pylori* for inoculum preparation nutrient broth medium 10ml. 0.5 McFarland standard was fixed for turbidity of suspension[14].

Broth Micro dilution Method: The method of Rehman et al, 2017 was followed for antibacterial activity with modifications[15]. Each well of 96 well micro plate contained total amount of 150µL i.e., 75µL of extract solution (0.5mg/mL) and 75µL of inoculum. For pre-read ELIZA recorded absorbance at 540nm [Reference]. Post incubation of plate at 37℃ for 24 h, same wave length was selected for after read. Pre and after read difference was indicative of bacterial growth. The whole procedure was repeated 3 times. MIC_{50} was taken by serial dilutions. Ceftriaxone and distilled water were positive and negative controls respectively. %I was taken by following formula.

Inhibition (%) = (Absorbance of sample/ Absorbance of control) *100

Statistical Analysis

Analysis of Variance followed by post hoc test was used for statistical analysis ($p \leq 0.05$).

Results

The IC_{50} value of antioxidant potential of *R. sativus* extract (seed) / fractions is given in Table 1. *n*- butanol fraction possess least IC_{50} for 31.0 µg/mL DPPH, that was noticeably lower compared to other fractions. Moreover, it was similar to the standard control. Fig. 1 depicted the antioxidant ability of *R. sativus* seed extract/ fractions on DPPH free radical. The percentage inhibition of *n*- butanol fraction was higher significantly ($p < 0.05$) than *n*- hexane as well as aqueous fractions for all tested concentrations. At 125 and 62.5 µg/ mL concentrations, insignificant difference among *n*- hexane and aqueous fractions.

The MIC_{50} value for antibacterial action of *R. sativus* extract/ fractions against *H. pylori* is described in Table 2. Aqueous extract depicted least MIC_{50} for *H. pylori* (15 µg/mL). The least active fraction against *H. pylori* was *n*-hexane.

Fig. 2 demonstrated the inhibitory effects of *R. sativus* seed extract/ fractions against *H. pylori*. In the broth micro dilution assay, aqueous fraction showed highest percentage inhibition than crude methanol extract, *n*-butanol and *n*- hexane fractions. At all tested concentrations, there was no significant difference between crude methanol extract and *n*- butanol fraction.

The IC_{50} value for the urease inhibitory activity of *R. sativus* seed extract/ fractions is presented in Table 3. *n*- butanol fraction had the lowest IC_{50} for urease (55.9 µg/mL). Figure 3 described the effects of different fractions and dosages of *R. sativus* against urease.

Discussion

Natural sources of medication are appreciable due to eco and bio friendly habbits [16]. Current study demonstrated *in vitro* antibacterial, anti-urease and antioxidant activities of *R. sativus* seeds. The use of DPPH is the preferred method to find antioxidant potential due to its simplicity to perform and low cost. Moreover, it measures total antioxidant capacity of sample [17].

The methanolic *R. sativus* seeds extract and its various fractions showed a concentration dependent inhibition of DPPH. Though the DPPH radical scavenging activity was lower than standard ascorbic acid but all extracts/ fractions showed sufficient inhibition of DPPH that is statistically significant ($p < 0.05$).

Many naturally occurring antioxidant compounds in different plants exhibited multi potent effects. These multi potent antioxidants participated in preventing and treating different diseases. Free radicals are usually

involved in pathogenesis of different diseases and diseases are caused by multiple pathogenic factors [18].

WHO declared *H. pylori* as class 1 carcinogen, so research studies for its eradication are being carried out [6]. In this experiment, *R. sativus* extracts/fractions exhibited anti-*H. pylori* activity.

Broth micro dilution assay is the antibacterial susceptibility testing along with minimum inhibitory concentration determination [19,20]. MIC results have shown that all the tested extract/fractions displayed anti-*H. pylori* activity. Furthermore, aqueous fraction was found to be more active than all other extract/ fractions. This is depicted by the lowest IC_{50} generated with respect to the other fractions and is lower than ceftriaxone (standard). This is in accordance with previous studies that an ideal antiulcer agent should have a strong inhibitory activity against *H. pylori* [5]. The strongest inhibition by aqueous fraction could be susceptible to possess active phytocehemicals than *n*-butanol, *n*-hexane fraction and crude methanolic extract. It is controversial that medicinal plants could eradicate *H. pylori* [6], however it is believable that their active metabolites may quash the *H. pylori* virulence by urease inhibition.

This study revealed that *R. sativus* extract/ fractions were able to display urease inhibition. The tested extract/fractions possess urease inhibition particularly fraction of *n*. butanol fraction and crude methanolic extract, however, *n*. hexane fraction showed least inhibition. Regarding mechanism of urease inhibition, further studies are required. However, a previous study showed that *R. sativus* contain flavonoids, phenol, anthocyanins and sulphurated constituents [7]. Many polyphenols and flavonoids exhibited maximum inhibitory activity against free radicals and *H. pylori* urease [20]. So, antiulcer potential of *R. sativus* presumably attributed to its phenolic and flavonoids constituents.

R. sativus depicted traditional use for management of piles, gastric ailments and urolithiasis in eastern subcontinent [7]. The results produced by current study authenticate the practice of *R. sativus* in gastric illnesses. Moreover, *R. sativus* showed antiulcer effects in many studies in ulcer induced animal models [7-9]. This study validated the antiulcer effect of *R. sativus* through urease and *H. pylori* inhibition in an in vitro study.

Conclusion

The present study provides scientific validation to traditional uses of *R. sativus* in peptic ulcer. A significant anti-urease potential displayed by the tested extract/fractions points out towards its mechanism of action. It is, therefore, concluded that *R. sativus* can be further explored for its antiulcer uses in animal and other models.

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Table 1 IC_{50} values for the antioxidant activity of different extract/fractions of *R. sativus* seeds.

Samples/fraction	IC_{50} DPPH (µg/mL)
Crude methanolic extract	45.0 ± 1.0^{a}
n- butanol	31.0 ± 0.7^{b}
n- hexane	130.0 ± 0.9^{c}
Aqueous	312.0 ± 1.7^{d}
Ascorbic acid	22.7 ± 0.001^{e}

Results are stated as mean ± S.E.M of 3 readings. Different alphabets showed statistical difference ($p \leq 0.05$) with each other.

Table 2 MIC_{50} values for the antibacterial activity of different extract/fractions of *R. sativus* seeds

Samples / fraction	MIC_{50} *H. pylori* (µg/mL)
Crude methanolic extract	49.5 ± 0.8^{a}
n- butanol	31.0 ± 0.9^{b}
n- hexane	212.0 ± 0.5^{c}
Aqueous	15.0 ± 0.7^{d}
Ceftriaxone	17.3 ± 0.01^{d}

Results are stated as mean ± S.E.M of 3 readings. Different alphabets showed statistical difference ($p \leq 0.05$) with each other.

Table 3 IC_{50} values for the anti-urease activity of different extract/fractions of *R. sativus* seeds.

Samples/fractions	IC_{50} Urease (µg/mL)
Crude methanolic extract	75.0 ± 1.5^{a}
n- butanol	55.9 ± 0.9^{b}
n- hexane	262.0 ± 0.5^{c}
Aqueous	261.5 ± 0.7^{d}
Thiourea	19 ± 0.01^{d}

Results are stated as mean ± S.E.M of 3 readings. Different alphabets showed statistical difference ($p \leq 0.05$) with each other.

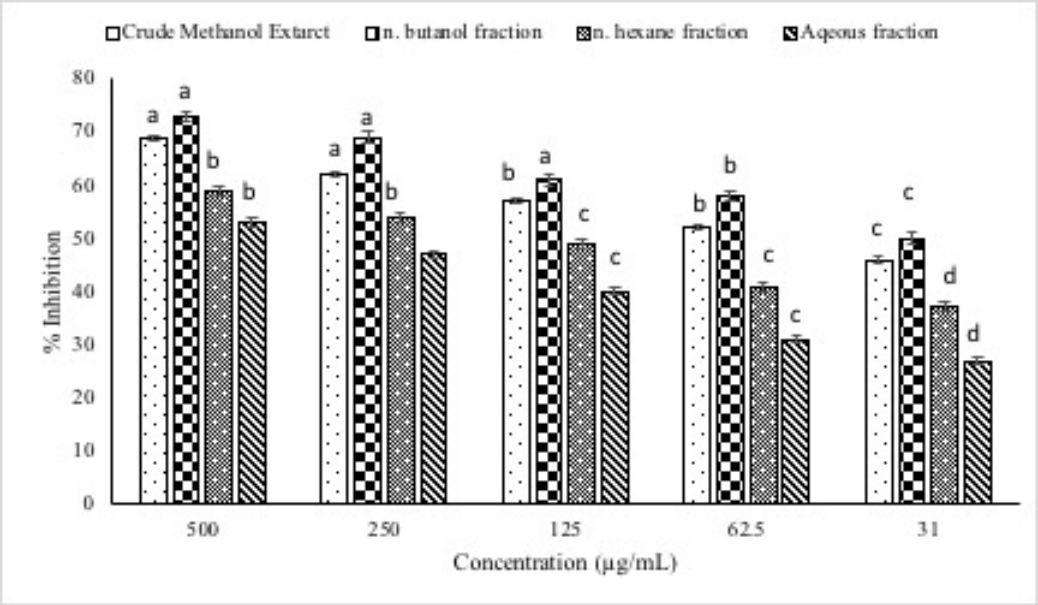

Fig. 1 Antioxidant activity of different extract/fractions of R. sativus against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Results are stated as mean ± S.E.M of 3 readings. Different alphabets for different concentrations showed statistical difference ($p \leq 0.05$) with each others.

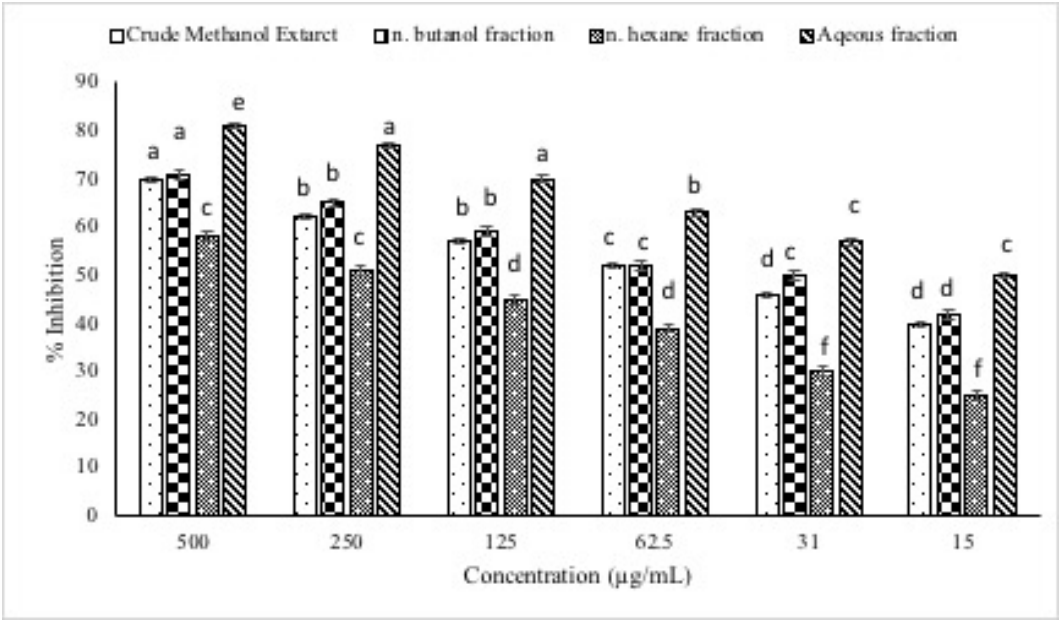

Fig. 2. Antibacterial activity of different extract/fractions of R. sativus against H. pylori. Results are stated as mean ± S.E.M of 3 readings. Different alphabets for different concentrations showed statistical difference ($p \leq 0.05$) with each other.

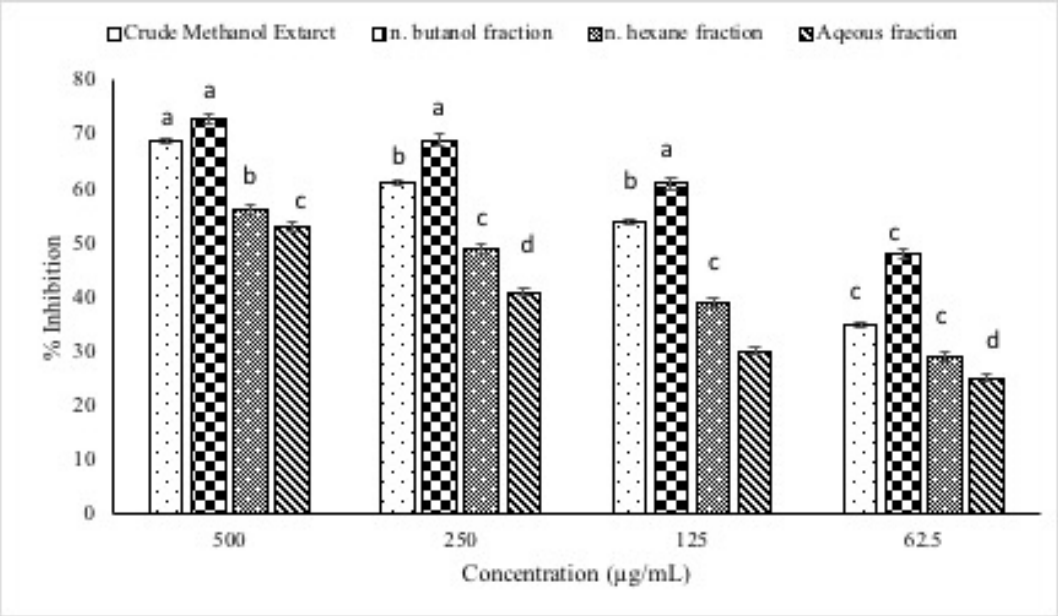

Fig. 3 Inhibitory effects of R. sativus seed extract/ fractions against urease. Results are stated as mean ± S.E.M of 3 readings. Different alphabets for different concentrations showed statistical difference ($p \leq 0.05$) with each other.