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Bioassay- guided Isolation of New Urease Inhibitory Constituents from *Monotheca buxifolia* (Falc.) Fruit and Their Molecular Docking Studies

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Abstract: The aim of the study is to explore the inhibitory potential of extract/fractions, and compounds of *Monotheca buxifolia* fruit against urease enzyme. Crude hydro-ethanolic extract showed a mild inhibitory activity against urease, among fractions ethylacetate fraction was more active followed by *n*-butanol fraction while there was no inhibitory activity in *n*-hexane soluble fraction. Ethylacetate fraction was subjected to activity guided isolation yielding four pure compounds, among them two were new i.e. buxifoline-A (**1**) (First time isolated from natural sources) and buxilide (**2**) while the other two were first time isolated from the fruit that are isoquercetin (**3**) and oleanolic acid (**4**). Their structures were elucidated using spectroscopic and spectrometric techniques. Among the isolated compounds compound **3** showed maximum inhibition. In order to understand the binding interactions of the compound **3**, it was docked into the active site of urease enzyme. Our study validates the traditional use of the fruit in the treatment of gastritis and urinary tract infections, which is strongly supported by the isolated compound isoquercetin (**3**).

Keywords: *Monotheca buxifolia;* urease inhibitor; isoquercetin; bio assay guided isolation; buxifoline A; buxilide; molecular docking.

1. Introduction

Monotheca buxifolia (Falc.) A.D is a sole member of the genus *Monotheca*, belongs to family Sapotaceae and is native to Pakistan [1-2]. In Pakistan it is found in Dir, Swat, Buner, Darra Adam Khail and along the border of Afghanistan [3]. In traditional medicine fruit of *Monotheca buxifolia* is used as laxative, purgative, vermidical, hematenic, antipyretic, and also used in the management of urinary tract infections, eye infections, and gastritis [4-5].

Urease is an enzyme that catalyzes the hydrolysis of urea lead to the production of ammonia it is naturally produced by various pathogens especially *Helicobacter pylori* [6]. Production of ammonia

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has many roles in the survival of *H. pylori*, it makes the environment favorable for it in the stomach, also help in weakening the mucus membrane to enable the pathogen to get penetrated, while at kidney level, it makes the environment basic, leads to the precipitation of salts such as calcium oxalates led to the formation of kidney stone [7]. Studies suggested that urease inhibitors solubilise the kidney stones [8]. Keeping in view the traditional use of *Monotheca buxifolia* the current study is designed to evaluate the extract/fractions, and isolated compounds for urease inhibitory potential.

2. Materials and Methods

2.1. Plant material

Fruit of *Monotheca buxifolia* were collected from the northern areas of Pakistan in the month of August and were authenticated by a taxonomist at the Department of Botany, University of Peshawar. A specimen has also been deposited in the herbarium of the University of Peshawar, reference number: Bot. 20061 (PUP).

2.2. Extraction, fractionation, isolation and structure elucidation

Fruit of *Monotheca buxifolia* were collected and washed with distilled water to remove dust. Seeds were separated and the collected fleshy pulp was dried under shade in a well ventilated place at ambient temperature. The dried pulp was crushed to powder. Dry powder was subjected to extraction, added sufficient hydroethanolic (30:70) solvent, shaken occasionally for 15 days, and filtered through Whatman-1 filter paper. The solvent was evaporated under reduced pressure in a rotary evaporator (BUCHI Rotavapor R-200, Switzerland) at 40^{o}C. The hydroethanolic extract (2.304 kg) was mixed with 2.5 L distilled water and soaked overnight, extracted successively with *n*-hexane (3 × 5 L), chloroform (3 × 5 L), ethylacetate (3 × 5 L), and *n*-butanol (3 × 5 L) to get the respective solvents soluble fractions, the remaining was considered as aqueous fraction [9]. The Ethylacetate fraction (30 g) was eluted using normal phase column chromatography on silica gel (Merck Silica gel 60 (0.063-0.200 mm), 5 → 60 cm), using hexanes, hexanes-ethylacetate, ethylacetate, ethylacetate-methanol and methanol with increasing polarity. Based on TLC, 18 sub-fractions were obtained (IR-A→R).

Compound **1** was isolated from subfraction "B" through normal phase column chromatography using ethylacetate:hexane (2:8) as mobile phase and then purified by using recycling HPLC. Methanol:water (70:30) was used as mobile phase. Flow rate of the mobile phase was 4 mL/minute. Compound was detected using UV detector. The retention time of the compound **1** was 36 minutes. White needle like crystals (67 mg) were obtained by evaporating solvents. The EI-MS of compound **1** displayed the molecular ion peak (M^+) at *m/z* 314, while the HREI-MS showed the (M^+) at *m/z* 314.1228, corresponding molecular formula $C_{17}H_{18}N_2O_4$ (calcd 314.1267). IR spectrum displayed sharp peaks at 3329 (NH), 1703 (C=O) and 1529 (aromatic) cm^{-1}. Its UV spectrum showed maximum absorptions at 248, 232 and 210 nm. The ^{1}H-NMR spectrum showed two signals of four proton each at aromatic region, at δ 7.10 (4H, d, *J* = 8.5 Hz, H-2, 2', 6, 6') and 7.30 (4 H, dd, *J* = 8.5 Hz, H-3, 3', 5, 5'), ^{1}H-NMR also displayed two proton containing signal at δ 3.85 s, attributed to H_2-9 and signal for oxygenated methyl protons at δ 3.70 (6H, s) assigned for H_3-8 and H_3-8'. In ^{13}C-NMR the broad-band decoupled spectrum showed a total of 10 carbon signals. DEPT-90 and 135 spectra of compound **1** showed that there were one methyl, two methylene and four methane carbons. The remaining was found to be quaternary in nature. The 2D-NMR spectra helped to identify the structure of compound.

Compound **2** was isolated as off-white mild brownish crystals (16.2 mg) from sub-fraction "M" by further fractionated with normal phase column chromatorgraphy using ethylacetate:Hexanes (7:3→8:2) and then purified using normal phase preparative TLC. Mobile phase used were combination of 3 i.e. hexane:ethylacetate:methanol (70:30:1). Its molecular formula, $C_6H_6O_4$, was determined from HREI-MS spectrum, which showed molecular ion peak at *m/z* 142.0277 (calcd for $C_6H_6O_4$ = 142.0266) and ^{13}C-NMR values (BB, and DEPT). EI-MS spectrum displayed molecular ion peak at *m/z* 142.0 and fragments at *m/z* 113 and 69. Absorption band at 3388 and 1737 cm^{-1} in the IR spectrum showed the presence of hydroxyl and lactone groups in compound **2**. UV spectrum

displayed absorptions at 272 and 223 nm. ^{1}H- NMR spectrum showed two downfield signals at δ 6.49 s and 7.94 s which were attributed to H-3 and H-6 respectively and one two proton signal at δ 4.39 s for H_2-7. ^{13}C-NMR spectrum (BB and DEPT) displayed resonances for six carbons which include one methylene, two methine and three quaternary carbons. Structure of compound was further confirmed by using 2D-NMR spectra such as COSY, HSQC, HMBC and NOESY.

Compound **3** was purified from the sub-fraction "N". The sub-fractions were re-chromatographed using normal phase silica gel as stationary phase. Compound 3 purification was made possible using solvent systems ethylacetate:Hexane (8:2). Compound **3** was isolated as yellow amorphous powder (30.5 mg). Based on the physical and spectral data compound **3** was identified as isoquercetin. The data was unambiguously matched with the previously reported for isoquercetin [10].

Compound **4** was isolated from the sub-fraction "A". Compound was purified through repeated column chromatography using normal phase silica gel as stationary phase. Mobile phase used were ethylacetate: Hexane (1:9→1.5: 8.5).Compound 4 was isolated as white amorphous powder (93 mg) and was identified as oleanolic acid. Its physical and spectral data was exactly matched with the previously reported for oleanolic acid [11].

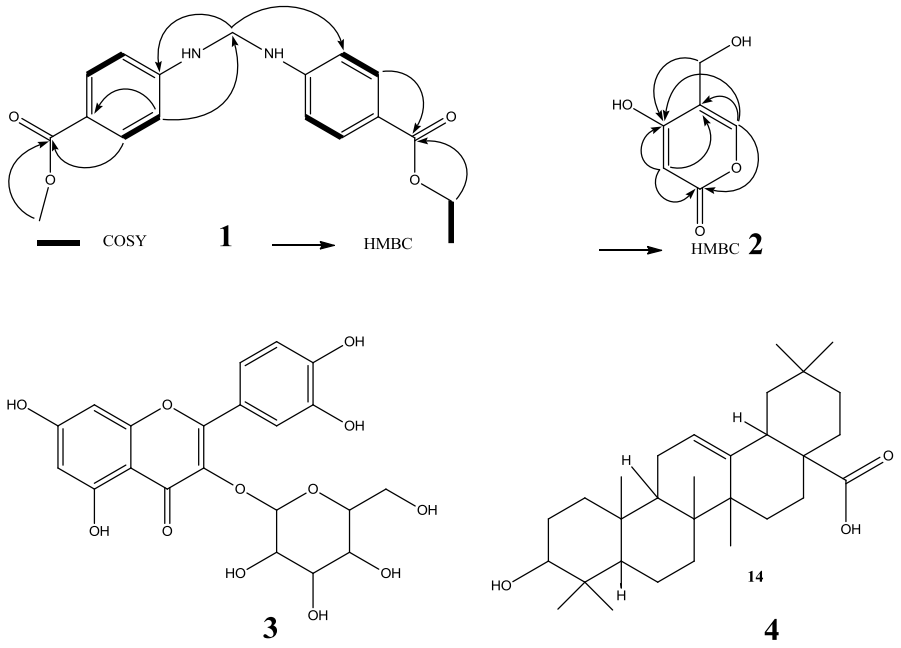

Figure 1. Chemical structures of isolated compounds (1-4) from *Monotheca buxifolia* fruit.

2.3. Urease inhibition assay

Previously reported method was used for the evaluation of urease inhibitory potential [12]. Briefly, solution for the reaction was comprised of urease enzyme (Jack bean source 25 µL), urea (100 mM) and buffer solution (55 µL). The reaction solution along with test samples (5 µL, 1mM) was incubated at 30˚C for 15 minutes in 96-well plate. Indophenol's procedure was adopted for measuring produced ammonia during reaction. Simply 45µL of 1% (w/v) phenol reagent, alkali reagent (70 µL) and sodium nitroprusside 0.005% (w/v) was added to each well. After 50 min of incubation, increase in absorbance was measured with the of micro plate reader at 630 nm. Absorbance change (per minute) was obtained with the help of Soft-Max Pro software. pH during the entire experiment was maintained at 8.2 using phosphate buffer (4 mM). Procedure was repeated for two more times. Thiourea was used as standard.

Percent inhibition of urease enzyme was obtained by using formula.

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

*2.4. Docking analysis of isoquercetin (compound **3**)*

To understand the binding mode of compound **3,** it was docked into the binding site of urease (PDB ID: 4ubp) enzyme using the software package MOE (Molecular Operating Environment). Protein molecule included in our study; urease (PDB ID 4ubp) was obtained from Protein Data Bank. The active site of Jack bean (*Canavalia ensiformis*) and *Bacillus pasteurri* urease is similar containing a bidentate nickel center [13]. Most macromolecular crystal structures contain little or no hydrogen coordinate data due to limited resolution and thus protonation was done prior to docking followed by energy minimization up to 0.05 Gradient using Amber 99 force field. The 3D structure of compound 3 was built using builder in MOE and was energy minimized to 0.05 Gradient using MMFF 94x force field. The structure was saved in mdb file format as input file for docking. The possible 30 conformations of ligand were allowed in the given docking protocol. The top ranked conformation was selected on the basis of docking score (S) for further analysis. The conformations are ranked by the scores from the GBVI/WSA binding free energy calculation in the S field which is the score of the last stage. The GBVI/WSA is a scoring function which estimates the free energy of binding of the ligand from a given pose. For all scoring functions, lower scores indicate more favorable poses. The unit for all scoring functions is kcal/mol. The docking results are well correlated with the experimental results as the compound showed good interactions with the active site residues and docking score (-14.9213).

3. Results and Discussion

3.1 Inhibitory effect of extract/ fraction and isolated compounds against urease

The results of the inhibitory potential of extract, fractions and isolated compounds of *Monotheca buxifolia* fruit against urease are showed in table 1. The tested samples were showing inhibitory potential against urease enzyme. The crude extract showed a mild inhibitory activity of 17.9%. Among fractions ethylacetate fractions showed a maximum inhibitory potential of 61.7% with IC_{50} value of 151.3 ± 1.40 µg/mL, n-butanol fraction inhibited the enzyme 35.3% while n-hexane fraction didn't show any significant inhibitory activity.

Among the compounds, isoquercetin (compound **3**) showed a maximum inhibition of 98.6% with IC_{50} value of 51.6±1.46 μM followed by Buxidilide (compound **2**) with inhibition of 40.9% while compound **1** and **4** didn't show any appreciable inhibition potential.

Majority of drugs present today are due to extensive research on their isolation from plants, characterization, derivatization and bioactivity screening. Bio assay guided isolation is well known methodology and led to the isolation of a variety of pharmacologically active compounds. Traditionally fruit of *Monotheca buxifolia* has been used for various ailments, including gastritis, and urinary tract infections without valid scientific rationale, in the current study it is shown that crude hydroethanolic extract, and subsequent fractions have a marked inhibitory potential against urease enzyme, while compound **3** (isoquercetin) showed potent inhibitory potential of 98.6% inhibition with an IC_{50} of 51.6 ± 1.46 μM. A number of polyphenolic flavonoids including isoquercetin and their derivatives were reported for urease inhibition [14-16].

3.2. Interactions detail of isoquercetin and protein

The docking studies of isoquercetin (compound **3**) clearly showed that this compound was bound correctly in the binding cavity of urease enzyme and making siginificant interactions with active site residues His 139, His 137, Asp 363, KCX 220, Ala 170, His 222 and Arg 339 (figure 2). The OH group of this compound interacts with basic His 137, His 139, KCX 220 Asp 363 and Ni ion of the protein. At the other end another two OH groups of pyran-triol moiety of compound are making bonds with Ala 170, His 222 and Arg 339. The docking study revealed that sufficient number of polar and electron rich substituents i.e. OH in compound **3** may lead to its high inhibition potential. The literature related to molecular docking studies of flavonoids showed that its inhibition against urease enzyme is due their OH groups, which may easily chelate with Ni ions and important residues in the

active site of urease enzyme [6, 15-17]. Our docking studies are also in close agreement with previous docking studies on urease enzyme.

Table 1. Urease inhibitory potential of the crude extract/fractions of *Monotheca buxifolia* fruit.

Extract/fractions/ compounds	Conc. (mg/mL for extract & fractions, mM for compounds)	% Inhibition	IC_{50}± S.E.M (μg/mL)
Crude	0.2	17.9	NA
n-Hexane	0.2	7.2	NA
Choloroform	0.2	19.7	NA
Ethylacetate	0.2	61.7	151.3±1.40
n-butanol	0.2	35.3	NA
Aqueous	0.1	14.4	NA
1	0.5	7.3	NA
2	0.5	40.9	NA
3	0.5	98.6	51.6±1.46
4	0.5	8.6	NA
Standard (thiourea)	0.5	98.2	21±0.11

NA= Not active, S.E.M = Standard Error Mean

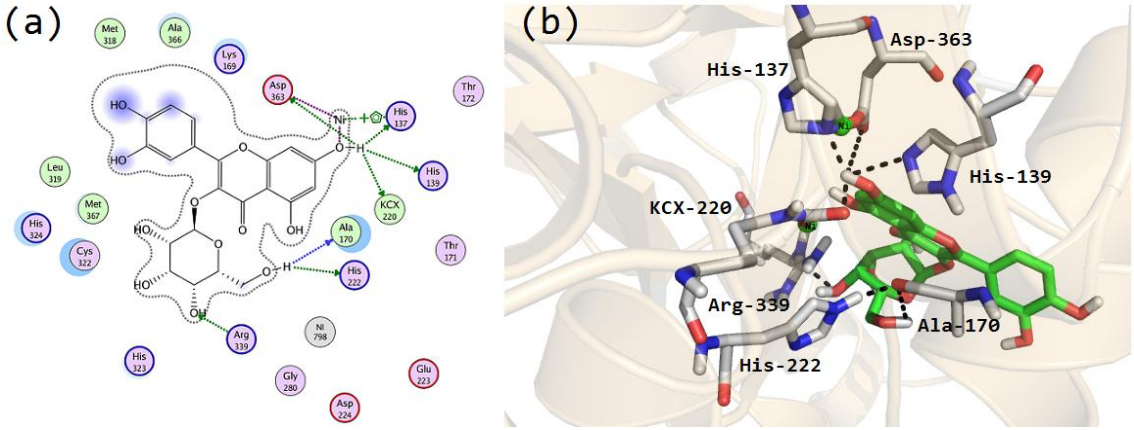

Figure 2. Binding mode of compound **3(a)** 2D conformation and **(b)** 3D conformation in the active site of urease enzyme.

Conclusion

In conclusion, the use of *Monotheca buxifolia* in folk medicine for the treatment of gastritis and urinary tract infection can be validated through this research work while urease inhibitory potential of *Monotheca buxifolia* fruit is due to the presence of above mentioned compounds.

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Conflict of Interest

There are no conflicts of interest of any authors with respect to this work.

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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