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Original Research Article

GC-MS metabolic profiling and anti-urease activity of non-polar fractions of *Calligonum Polygonoides* L. (Polygonaceae) and *Crateva Adansonii* DC. Prodr. (Capparaceae)

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Abstract

Purpose: *To determine the urease-inhibitory activity and chemical constituents of fractions of Calligonum polygonoides and Crateva adansonii separated by physical properties.*
Methods: *The anti-urease activities of different fractions of the plants (methanol, n-hexane, $CHCl_3$, n-butanol) were evaluated using a standard procedure. The chemical constituents of the extracts with the highest urease-inhibitory activity were determined by gas chromatography-mass spectrometry.*
Results: *The n-hexane fractions of both plants had higher urease-inhibitory activity and a lower half-maximal inhibitory concentration (IC_{50}) than the other extracts. GC-MS evaluation revealed that n-hexane fraction of C. polygonoides was rich in fatty acids (39.36 %), sterols (22.29 %), long chain alkanes (98.5 %), and a few volatiles (5.26 %), while the n-hexane fraction of C. adansonii had high levels of alkanes (35.03 %), sterols (10.46 %), fatty acid esters (46.82 %), and triterpenes (23.76 %).*
Conclusion: *The n-hexane fractions of the plants demonstrate high urease-inhibitory activity. Thus, these plant-based anti-urease fractions can potentially serve as a starting point for the development of novel antibacterial agents with enhanced efficacy and reduced antibiotic resistance in the treatment of pathological conditions and infections associated with urease.*

Keywords: *Calligonum polygonoides, Crateva adansonii, Urease, Natural products, Antibiotics, Helicobacter pylori*

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INTRODUCTION

Many traditional medicines were derived from natural sources and contain a wide variety of biologically active compounds [1,2] with diverse pharmacological activities [3]. Along with their derivatives, these naturally derived compounds constitute more than half of the drugs that have been approved by the U.S. Food and Drug Administration. It is believed that natural sources still hold a rich collection of products with pharmacological potential, that is, undiscovered drugs [4]. In this study, we have assessed two desert plant species for their potential to serve as natural sources of biological compounds capable of inhibiting urease activity. The latter has been

implicated in a variety of diseases and pathological conditions.

Urease (urea amidohydrolase: EC 3.5.1.5) catalyzes the hydrolysis of urea to yield ammonia and carbon dioxide [5-8], and has been implicated in the pathology of several urinary diseases, including pyelonephritis, urolithiasis, and the formation of infection-induced urinary stones [9,10]. Urease is a major factor in gastritis and peptic ulcers that result from *Helicobacter pylori* infections, which also cause chronic inflammation that increases the risk for gastric cancer [11,12]. Thus, urease inhibitors may be an effective treatment of these conditions. Many of the urease inhibitors prescribed at present, for example, phosphorodiamidates, hydroxamic acid derivatives, and imidazoles, are not ideal clinical choices, due to either high toxicity or instability [13]. Therefore, there is great interest in the identification of novel urease inhibitors, and natural sources hold promise. Particularly, medical researchers hope to identify compounds with low toxicity, few side effects, high bio-availability, and great stability [14,15].

Two different plant species were studied as a possible potential source of natural urease inhibitors. *Calligonum polygonoides* Linn. (common names are Phog or Phogra) is a leafless slow-growing woody shrub that has smooth stems with branches mostly found in the Cholistan region of Pakistan. Its juice is used in the eye as an antidote for scorpion sting; the latex is used to treat eczema, dog bites, and to induce abortion. Decoction of the roots is used, in combination with catechu, as a gargle to relieve sore gums [16]. *Crateva adansonii* DC, of the family *Capparidaceae* is commonly called “Barna” or “Burma” in the Indian Subcontinent and “garlic pear” in English [17,18]. Its powdered bark is used in the treatment of urinary, renal tubules, gastro-intestinal, and uterine infection [18]. In ethno-medicine, the plant is used in conditions associated with inflammation, specifically asthma, snakebites, and as an astringent. Its roots are used in the treatment of syphilis, jaundice, and yellow fever [19]. This plant is known for its antimicrobial and anti-trypanosomal activities [20,21].

EXPERIMENTAL

Collection and drying of plant materials

The entire aboveground portions of *C. adansonii* and *C. polygonoides* were collected near Hasilpur Road in the Cholistan desert region of Pakistan during March and April of 2016. The identity of the plants was authenticated by Prof Ghulam Sarwer of the Botany Department of Islamia University, Bahawalpur. Voucher specimens were deposited in the Herbarium of the Department of Life Sciences, Islamia University, Bahawalpur with the voucher numbers: 471/LS for *C. polygonoides* and 472/LS for *C. adansonii*. Plant materials were air dried, pulverized to a coarse powder, and hermetically sealed in polyethylene bags until extraction [22,23].

Extraction and fractionation

Pulverized samples of *C. adansonii* (700 g) and *C. polygonoides* (900 g) were thoroughly extracted, separately, with 15 L methanol, and the extracts were filtered through Whatman No.1 filter paper and concentrated under reduced pressure to yield 144 g of a highly viscous residue. The residue was suspended in 500 mL of distilled water and sequentially fractionated with n-hexane (6 x 1000 mL), $CHCl_3$ (6 x 1000 mL), and n-butanol (6 x 1000 mL) to furnish the following fractions: *C. polygonoides* [n-hexane (19 g), $CHCl_3$ (125 g), n-butanol (400 g)], and *C. adansonii* [n-hexane (25 g), $CHCl_3$ (79 g), n-butanol (175.5 g)].

Urease inhibition assay

The reaction mixture (300 µL) was prepared by mixing 100 µL Jack bean urease 200 µL assay buffer (100 mM KH_2PO_4, pH 6.8), 0.0007 µg/µL urea, and 100 µL of plant fraction in a 96-well plate and incubating for 30 min at 37°C. The ammonia released was quantified with the phenol–hypochlorite method [24] as follows: 500 µL of solution I (containing 5.0 g phenol and 25 mg of sodium nitroprusside) and 500 µL of solution II (containing 2.5 g sodium hydroxide and 4.2 mL of sodium hypochlorite in 500 mL of distilled water) were added to the wells at 37°C for 30 min, after which the absorbance due to production of blue coloured indophenol was measured at 625 nm against the control. Thiourea (0.5 mmol) was used as standard inhibitor. All assays were performed in triplicate in a final volume of 1 mL. Urease-inhibitory activity was expressed as in Eq 1.

$$H\ (\%) = \{(Ac - As)/Ac\} \quad \ldots\ldots\ldots\ldots \quad (1)$$

where Ac and As are the absorbance of control and test samples, respectively.

Gas chromatography-mass spectrometry (GC-MS) profiling

Sample volumes of 1 µL were injected into the GC-MS in the splitless mode using a hot-needle

technique. The GC-MS system consisted of an *Agilent* 7693A ALS (Automatic Liquid Sampler), a GC 7890A gas chromatograph, and an Agilent 5975C quadrupole mass spectrometer (Agilent, Santa Clara, CA 95051, USA). Gas chromatography was performed on a 30-m capillary column HP-5 MS of 0.25 mm internal diameter with an integrated guard column and a 0.25 µm film (Agilent, Santa Clara, CA 95051, USA). The injection temperature was 230 °C, the interface was set to 250 °C, and the ion source was adjusted to 200 °C. The carrier gas was helium at a constant flow rate of 1 mL/min. The temperature program was 2 min isothermal heating at 60 °C, followed by a 5 °C/min oven temperature ramp to 80 °C and a final 5-min heating at 10 – 310 °C. The system was then temperature equilibrated for 6 min at 70 °C prior to injection of the next sample. Mass spectra were recorded at two scans/s with an *m/z* of 50 – 650 scanning range. The chromatograms and mass spectra were evaluated using the *Agilent MSD* ChemStation *software*. Processed data were checked manually and corrected when necessary before subjecting to further data analysis. Metabolite peaks were quantified by calculating the percentage of peak area after comparing with the sum of peaks of other compounds. Metabolites were identified by comparison of retention times recorded with different mass spectrum data libraries (NIST, Wiley Registry).

Statistical analysis

Data are expressed as mean ± SEM of at least three independent experiments. Statistical analysis of data was performed using one-way analysis of variance (ANOVA) followed by Fischer's least significant difference (LSD) post-hoc test. Level of significance was set at $p \leq 0.05$. SPSS version 20.0 was used for all statistical calculations.

RESULTS

Urease inhibition

Table 1 shows the percentage inhibition of urease and the half maximal inhibitory concentration (IC_{50}) values of different fractions of *C. polygonoides* and *C. adansonii*. The n-hexane fraction of both plant species had the greatest inhibition of urease: this extract of *C. polygonoides* inhibited urease activity 93.25 % and had an IC_{50} value of 12 ± 0.68 µg/mL These values for *C. adansonii* were 95 % and 10 ± 0.55 µg/mL, respectively. These values for these two extracts were not significantly different ($p = 0.55$ for percentage inhibition and $p = 0.56$ for IC_{50}), but the values for the percentage inhibition and IC_{50} values of the other fractions were significantly different ($p \leq 0.05$).

Table 1: Urease inhibition by different fractions of *C. polygonoides* and *C. adansonii* extracts and thiourea as a positive control

Sample	Urease (5 mg/ml) Inhibition (%)	IC_{50} (µg/ml)
Thiourea (0.5 mmol)	98	1.2 ± 0.08
Methanol-CP	58.5	20 ± 0.64
***n*. Butanol-CP**	63.8	30 ± 0.23
Chloroform-CP	45.8	25 ± 0.45
***n*. Hexane-CP**	93.25	12 ± 0.67
Methanol-CA	39.1	-
***n*. Butanol-CA**	41.26	-
Chloroform-CA	32.6	-
***n*. Hexane-CA**	95	10 ± 0.55

Samples are described by their extraction solvent-plant source (CP, *C. polygonoides;* CA, *C. adansonii);* percentage inhibition values are expressed as mean ± SEM; n = 3

GC-MS of n-hexane fraction of *C. polygonoides*

Thirty-five compounds were identified in the GC-MS spectrum of the n-hexane fraction of *C. polygonoides* (Table 2). Seven volatile compounds were discovered. We detected 2-hexenal (0.3 %), which is known to inhibit urease [25]. The n-hexane fraction of *C. polygonoides* contained fatty acids in the range of 9-26 C atoms. Six alkanes were found: nonacosane (3.7 %), dotriacontane (20.65 %), tritriacontane (4.55 %), tetratriacontane (12.66 % min), untriacontane (50.16 %), and pentatriacontane (2.53 %). Six sterols were identified. These were: cycloartenol (7.5 %), campesterol (1.8 %), stigmasterol (6.99 %), β-sitosterol (12.8 %), sitostenone (5.3 %), and β-amyrin (9.7 %).

GC-MS of n-hexane fraction of *C. adansonii*

Table 3 shows phytocompounds in the n-hexane (non-polar) fraction of *C. adansonii* from GC-MS. Fatty acid esters with 17-20 C atoms were detected, and the methyl ester of hexadecanoic acid was present at the greatest percentage (13.589 %). Five alkanes were identified, but more notably six medicinally important triterpenes were discerned: γ-sitosterol (4.206 %), ψ-taraxasterol (6.25 %), lupeol (5.036 %), lupenone (4.65 %), lupanol (10.52 %), and oleanolic acid (7.55 %).

DISCUSSION

The n-hexane fractions of *C. polygonoides* and *C. adansonii* were ascertained to have high

Table 2: Components of the n-hexane extract of *C. polygonoides* from GC-MS

Constituent	Retention time (min)	Molecular formula	Relative area (%)
3-Hexen-1-ol	10.22	$C_6H_{12}O$	1.5
1-Hexanol	10.66	$C_6H_{14}O$	0.85
Benzaldehyde	11.58	C_7H_6O	0.56
3-Pentanol, 2,4-dimethyl	12.12	$C_7H_{16}O$	0.03
Hexanoic acid	11.03	$C_6H_{12}O_2$	1.54
2-Hexenal	12.36	$C_6H_{10}O$	0.3
2-Octen-1-ol	14.45	$C_8H_{16}O$	0.77
Benzeneacetaldehyde	15.28	C_8H_8O	1.2
1-Octanol	16.11	$C_8H_{18}O$	0.35
Octanal	17.46	$C_8H_{16}O$	0.45
Nonanoic acid	18.79	$C_9H_{18}O_2$	1.89
Decanal	19.55	$C_{10}H_{20}O$	3.5
Decanoic acid	21.1	$C_{10}H_{20}O_2$	2.5
Myristic acid	22.2	$C_{14}H_{28}O_2$	0.633
Palmitoleic acid	23.16	$C_{16}H_{32}O_2$	10.8
Palmitic acid	24.05	$C_{16}H_{32}O_2$	12.5
Linoleic Acid	24.16	$C_{18}H_{32}O_2$	6.2
Margaric acid	24.25	$C_{17}H_{34}O_2$	15.5
α-linolenic acid	24.37	$C_{18}H_{32}O_2$	9.3
Nonadecylic acid	24.56	$C_{19}H_{38}O_2$	1.5
Heneicosylic acid	24.85	$C_{21}H_{42}O_2$	4
Cycloartenol	28.24	$C_{30}H_{50}O$	7.5
Cerotic acid	29.14	$C_{26}H_{52}O_2$	1.2
Campesterol	30.22	$C_{28}H_{48}O$	1.8
Stigmasterol	30.65	$C_{29}H_{48}O$	6.99
Nonacosane	32.57	$C_{29}H_{60}$	3.7
Squalene	32.89	$C_{30}H_{50}$	6.3
Sitostenone	33.09	$C_{29}H_{48}O$	5.3
β-Sitosterol	34.55	$C_{29}H_{50}O$	12.8
β-Amyrin	34.98	$C_{30}H_{50}O$	9.7
Dotriacontane	35.12	$C_{32}H_{66}$	20.65
Tritriacontane	36.11	$C_{33}H_{68}$	4.55
Untriacontane	37.58	$C_{31}H_{64}$	50.16
Tetratriacontane	38.55	$C_{34}H_{70}$	12.66
Pentatriacontane	39.99	$C_{35}H_{72}$	2.53

Table 3: Components of the n-hexane extract of *C. adansonii* from GC-MS

Constituent	Retention time (min)	Molecular formula	Relative area (%)
Pentadecane	14.55	$C_{15}H_{32}$	0.6
2,6,10-Trimethyl Tetradecane	15.757	$C_{17}H_{36}$	0.785
6,10,14-trimethyl-2-Pentadecanone	18.479	$C_{18}H_{36}O$	0.66
Hexadecanoic Acid, Methyl Ester	19.348	$C_{17}H_{34}O_2$	13.589
Hexadecanoic Acid, Ethyl Ester	19.959	$C_{18}H_{36}O_2$	2.801
9,12-Octadecadienoic Acid (Z,Z)-,methyl ester	20.97	$C_{19}H_{34}O_2$	11.466
9,12,15-Octadecatrienoic Acid (Z,Z,Z)-,methyl ester	21.045	$C_{19}H_{32}O_2$	10.768
Phytol	21.154	$C_{20}H_{40}O$	3.379
Octadecanoic Acid, Methyl Ester	21.222	$C_{19}H_{38}O_2$	2.092
9,12-Octadecadienoic Acid (Z,Z)-,Ethyl ester	21.534	$C_{20}H_{36}O_2$	1.464
Octadecanoic Acid, Ethyl Ester	21.785	$C_{20}H_{40}O_2$	0.688
Erucic Acid	22.688	$C_{22}H_{42}O_2$	2.286
Docosanoic Acid, Methyl Ester	24.534	$C_{23}H_{46}O_2$	0.66
1,2-Benzene Dicarboxylic Acid, Mono(2-ethylhexyl)ester	24.758	$C_{16}H_{22}O_4$	3.944
Heptacosane	25.777	$C_{27}H_{56}$	1.288
Nonacosane	27.182	$C_{29}H_{60}$	2.941
Tetratriacontane	28.58	$C_{34}H_{70}$	20.652
17-Pentatriacontene	29.205	$C_{35}H_{70}$	0.549
Hexatriacontane	29.836	$C_{36}H_{74}$	8.216
γ-Sitosterol	30.366	$C_{29}H_{50}O$	4.206
Lupenone	31.125	$C_{30}H_{48}O$	4.65
Lupeol	31.106	$C_{30}H_{50}O$	5.036
ψ-Taraxasterol	33.32	$C_{30}H_{50}O$	6.25
Oleanolic acid	29.65	$C_{30}H_{48}O_3$	7.55
Lupanol	28.56	$C_{30}H_{52}O$	10.52

urease-inhibitory activity, greater than the other fractions we collected. We used GC-MS to characterize the components in the fraction, and to provide some information as to the identify components with anti-urease activity. Fatty acids and lipids inhibit urease and the activity of *H. pylori* [26, 27]. Alkanes such as n-heptacosane havealso shown moderate urease inhibition [28], and several terpenoids (mono-, di-, and tri-) have anti-urease activity [29]. Of particular interest is the fact that oleanolic acid, which has displayed remarkable urease inhibition, has also been identified in the n-hexane fraction of *C. adansonii* [30-32]. We conclude that urease-inhibitory activity of these non-polar fractions can be attributed to the alkanes, fatty acids, sterols, triterpenes, and terpenoids present in them. To address the issue of rapid development of antimicrobial resistance, plant-based anti-urease agents with in vivo activity could be a direction for the development or discovery of novel antibacterial agents with enhanced efficacy and reduced antibiotic resistance [32-35]. Plant-derived herbal formulations or secondary metabolites could also serve as cost-efficient antimicrobial agents with low toxicity and improved stability [36,37].

CONCLUSION

The findings of this study reveal the presence of bioactive compounds that have been shown elsewhere to have anti-urease activity. Therefore, both plants contain compounds that are capable of urease inhibition. Few compounds with urease-inhibitory activity have been isolated from plants and established as clinically effective drugs. Thus, the phytochemicals identified in the present work may serve as candidates for further development as anti-urease agents for clinically applications.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities concerned with the claims relating to contents of this article will be borne by the authors. The study was conceived and designed by Prof. Dr. Saeed Ahmad, plants were collected by Irfan Pervaiz, and samples were dried and pulverized by Abdul Basit. Experimental work was conducted by Irfan Pervaiz and Adeel Arshad. Data handling and statistics application was done by Umair Khurshid. Manuscript was written by Irfan Pervaiz and Saeed Ahmad. All authors read and approved the manuscript for publication.

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