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Original Research Article

Antioxidant, Phytotoxic and Antiurease Activities, and Total Phenolic and Flavonoid Contents of *Conocarpus lancifolius* (Combretaceae)

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Abstract

Purpose: *To evaluate the antioxidant, phytotoxic and anti-urease properties of dichloromethane and methanol extracts of Conocarpus lancifolius in correlation with total phenolic and flavonoid contents.*
Methods: *The whole plant (dried aerial parts and root) of Conocarpus lancifolius was extracted successively with dichloromethane, methanol and water at room temperature. Antioxidant activity was determined by DPPH, Nitric oxide scavenging and FRAP methods. Phytotoxicity was performed by Lemna minor assay and analyzed relative to control with effective dose (ED_{50}) to determine FI_{50} values (concentration necessary to inhibit 50 % frond proliferation) and 65 % confidence intervals. Urease inhibitory activity was assessed at a concentration of 125 µg/mL by Berthelot reaction with slight modification. Total phenolic contents were calculated with reference to gallic acid equivalent and confirmed by Folin and Ciocalteau's phenol method. Total flavonoid was determined with reference to quercetin.*
Results: *The DPPH and hydroxyl radical scavenging activities of the methanol extract were 93.35 %. The phytotoxicity of the methanol extract was 90 % growth regulation while the anti-urease inhibitory activity was 91.1 % with half-maximal inhibitory concentration (IC_{50}) of 49.1 ± 1.3 µg/mL. Total flavonoid contents of dichloromethane extract was 629.4 ± 1.57 µg/mL. The phenolic content of the extract calculated with reference to quercetin, gallic acid, chlorogenic acid, ferulic acid and 4-hydroxy 3-methoxy benzoic acid equivalent was 45.772, 9.779, 70.304, 74.93 and 57.80 ppm, respectively.*
Conclusion: *The results confirm that Conocarpus lancifolius extracts possess some antioxidant, phytotoxicity and anti-urease potentials due to its phenolic and flavonoid contents.*

Keywords: *Antioxidant, Phenolics, Falvonoids, Phytotoxicity, Anti-urease, Conocarpus lancifolius*

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INTRODUCTION

Unexplored drug resources in medicinal plants have great therapeutic potential. These are helpful in achieving new drugs with lead targets and unique mechanism of action. The latest study by World Health Organization (WHO) estimates that for the primary healthcare needs, four-fifth of the total population still repose their confidence in plant medicine [1].

The genus *Conocarpus* belongs to the family Combretaceae and consists of only two species [2]. *Conocarpus lancifoliusis*, an ornamental tree and native to coastal and riverine areas of East Africa and found in some areas of Pakistan [3]. The mature leaves are glossy in appearance with relatively fewer trichomes on both surfaces [4].

Among the medicinal benefits of plants, antioxidant properties have received increasing attention due to their role in preventing or down regulating myriads of oxidative damages caused by free radicals in the body [5]. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxyl toluene (BHT) are supposed to be responsible for carcinogenesis and liver damage in laboratory animals. These changes contribute to cancer, atherosclerosis and cardiovascular diseases [6]. Phytotoxicity is an important attribute in determination of allelopathic potential of a plant species. Determination of phytotoxicity of a plant species helps in the formulation of natural plant growth regulators or biological herbicides. Losses caused by weeds are well documented in many studies [7].Urease is also indispensable for colonization of human gastric mucosa by *Helicobacter pylori*. The ammonia produced was proposed to damage the gastric epithelium via its interaction with the immune system by stimulating an oxidative burst in human neutrophils [8]. The antioxidant, phytotoxic and antiurease capacities of *Conocarpus lancifolius* consumed locally in Pakistan have not been presented. This study is aimed to evaluate the antioxidant, phytotoxic and anti-urease activities of *Conocarpus lancifolius* in selected *in vitro* assay systems along with total phenolics and flavonoids contents.

EXPERIMENTAL

Collection and extraction of plant material

The plant material was collected from surroundings of Pattoki and Lahore (Pakistan). The plant was identified as *Conocarpus lancifolius* by Professor Dr. Altaf Ahmad Dasti, a taxanomist and a specimen voucher (no. WCL-291) was deposited in Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan. The plant material was shade-dried for 15 days. The dried plant material was grounded in blender and weighed. The extraction of the powdered material was carried out by simple maceration. The weighed amount of plant material (1 kg) was taken in an extraction jar and dichloromethane (2.5 L) was added to it. Extract was filtered out after 24 h of addition of solvent. The process was repeated three times with dichloromethane. The extraction of the marc was done by using methanol and water in the same manner. Dichloromethane, methanol and water extracts were concentrated separately under reduced pressure by using rotary evaporator. The residues were collected in separate sample bottles and designated CLAD, CLAM and CLAW respectively for arial part extracts and CLRD, CLRM and CLRW for root extracts.

Chemicals

DPPH (1,1-diphenyl-2-picryl hydrazyl) radical and Rutin were purchased from Sigma Aldrich Chemical Company, USA; Folin and Ciocalteau's Phenol reagent and Tri-chloroacetic acid (TCA) from Qualikems Fine Chemical Pvt. Ltd., New Delhi, India; Gallic acid monohydrate from Kem Light Laboratories Pvt. Ltd., Mumbai, India. Solvents and other chemicals used for this study were of analytical grade, while water was glass distilled.

1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay

Antioxidant activity of *Conocarpus lancifolius* extracts was measured as ability to scavenge stable DPPH radical [9]. The DPPH radical had been extensively used to evaluate the reducing substances [10]. Concentrations 0.5 µg/mL of test extracts were prepared in methanol. 2.5 mL solution of each extract was added to 1 mL of 0.3 mM of freshly prepared DPPH solution in methanol and allowed to react in the dark at room temperature for 30 min. Absorbance of the resulting solution was measured at 518 nm. Methanol (1 mL) added to 2.5 mL of each extract concentration was used as blank, while 1 mL of 0.3 mM DPPH solution added to 2.5 mL of methanol served as a negative control. Gallic acid solution prepared in the same concentrations as the test extracts, was used as reference (positive control) [11].

Percentage scavenging activities of the extracts and reference were determined using the formula.

Scavenging activity (%) = $100 - [(A_s - A_b)/A_c \times 100]$

Where A_s=Absorbance of sample (extract or reference standard), A_b = Absorbance of blank and A_c = Absorbance of negative control [12].

Nitric oxide scavenging assay

Solution of phosphate buffer saline contained sodium nitroprusside (0.5-0.6 ml of 10 mM), was added in samples of *Conocarpus lancifolius* extracts and then incubated for 1/2 to 2 h. The mixture (0.5 - 0.6 mL) was taken and mixed with 1.5 mL of sulfanilic acid. 5-6 min incubation, 1-2 mL of naphthyl ethylene diamine dihydro chloride was poured. Wavelength of 541 nm, absorbance

was noted and quercetin was employed as a reference.

Evaluation of ferrous reducing activity power (FRAP)

A sample (25 µL) of *Conocarpus lancifolius* extract was added with 25 - 27µL of phosphate buffer (pH 7.2) and 50 - 55µL of 1 % potassium ferrecyanide solution, whole solution was incubated (50 - 55 °C for 10 - 15 min.) and then 25 - 30 µL of trichloroacetic acid and 100 µl distilled water had been mixed and observed the absorbance at 541 nm. In the end, 25 – 27 µL of ferric chloride solution was poured and observed the absorbance at 700 nm [13].

Phytotoxicity assay

Phytotoxicity was done by using *Lemna minor* assay. The phytotoxicity bioassay such as *Lemna minor* is a useful primary screen also bears an advantage enabling researchers to envisage growth stimulating effect of test sample. Apart from swift measurement of phytotoxicity of sample, the other advantage that *Lemna* assay offers is requirement of small quantities of crude extract, column fraction or pure compound in range of 1-1000 ppm. Number of fronds in each test vial will be counted and subsequently recorded on third and seventh day. The data obtained will be analyzed as percent of control with ED_{50} computer program to determine FI_{50} values and 65 % confidence intervals [14].

Antiurease assay

Dichloromethane and methanol extracts of *Conocarpus lancifolius* were checked for antiurease activity at concentration of 125 µg/mL with slight modification by Berthelot reaction. The plant extracts were tested in a concentration range of 0.25 to 0.5 mM. Thiourea was used as standard. The assay mixture contains urea (850 µL) and crude extract (135 µL) giving a total volume of 985 µL. The reactions were started by the addition of 15 µL of urease enzyme in phosphate buffer (100 mM, pH 7.4, 1 µg/mL). Urease activity was determined by measuring concentration of ammonia after 60 min of enzymatic reaction. The ammonia was determined using 500 µL of solution A (contained 0.5 g phenol and 2.5 mg of sodium nitroprusside in 50 ml of distilled water) and 500 µL of solution B (contained of 250 mg sodium hydroxide and 820 µL of sodium hypochlorite 5 % in 50 mL of distilled water) at the temperature of 37 °C for 30 min. The absorbance was checked at 625 nm. The cctivity of uninhibited urease was presented as the control activity of 100 % [15].

IC_{50} values determination

Enzymatic reaction was calculated as in Eq 1.

I (%) = 100 – 100 * (T/C) …… (1)

where I (%) is enzyme inhibition, T (test) is the absorbance of the tested sample (plant extract or positive control in the solvent) in the presence of enzyme, C (control) is the absorbance of the solvent in the presence of enzyme. Data are expressed as mean ± standard error deviation (SD). All the tests were were carried out at least three times.

Determination of total phenolic contents

TPC had been confirmed by the assay, 90.1 µL of Folin-Ciocalteu reagent added to 20.2 µL sample of *Conocarpus lancifolius*. The absorbance will be checked at 726 nm (pre read). The sample mixture will be incubated for 5-6 min at a temperature 25-26 °C. Sodium carbonate solution (90.5 µL) was added in the mixture and absorbance (726 nm) will be observed. Phenolic contents will be calculated with reference to gallic acid equivalent [16].

Determination of total flavonoid contents

Deionized water (80.5 µL) and 20.5 µL samples of *Conocarpus lancifolius* extract will be added with 16.5 µL sodium nitrate solution and incubated for 5 - 6 min (temp 25 - 26 °C). Absorbance at 510 - 520 nm was determined. 16.5 µL of aluminum chloride and 8.5 µL sodium hydroxide solution was added and absorbance at 510.5 nm was observed. Flavonoid was determined with reference to quercetin [17].

Quantification of phenolic contents by HPLC

Methanol extract (50 mg) of *Conocarpus lancifolius* was mixed 16 ml of double distilled water and then 24 ml of methanol was added. It was shaken for 5 min and 10 mL 6 M hydrochloric acid was added. The mixture was kept in an oven at 95 °C for 2 h and filtered with the aid of a filtration assembly.

Methanol extract of *Conocarpus lancifolius* was injected into HPLC system, Shimadzu Model 10A (Japan), equipped with UV-visible detector at 280 nm. The column of HPLC system was Shim-pack CLC-ODS (C-18), with 25 cm x 4.6 mm, 5 µm. The mobile phase was A (water: acetic acid, 94:6, pH = 2.28), B (acetonitrile 100 %) 0 - 16 min. =15 % B, 16 – 31 = 45 % B, 31 – 45 = 100 % B, at a flow rate of 1 ml/min. The whole procedure was performed at room temperature.

Diverse phenolic compounds, ferulic acid, quercetin, m.coumeric acid, gallic acid, sinapic acid, vitamin C, vanillic acid, chlorogenic acid, caffeic acid, 4-OH 3-methoxy benzoic acid, syringic acid, trans-4-hydroxy-3-methoxy cinamic acid and chromatotropic acid had been utilized for the analysis of the extracts of *Conocarpus lancifolius*.

Statistical analysis

The experimental results are expressed as mean ± standard deviation (SD) of three replicates. The data were subjected to one way analysis of variance (ANOVA) and mean values were compared by Duncan's multiple range tests using SPSS software, version 15 (SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Antioxidant activity

Diverse methods, DPPH, NO scavenging and ferrous reducing activity power, revealed the antioxidant potential of *Conocarpus lancifolius*. It was observed that methanol extract of the aerial part of *Conocarpus lancifolius* had highest inhibition, 92.10 ± 0.98 % by DPPH method. The dichloromethane and water extracts showed 28.1 ± 0.98 and 87.8 ± 0.56 % inhibition, respectively, with reference to quercetin 92.12 ± 0.49 %. By using ferrous reducing activity methanol extract had maximum inhibition is 93.2 ± 1.02. While dichloromethane and water extracts of *Conocarpus lancifolius* exhibited percentage inhibition 42.06 ± 0.64 and 93.1 ± 0.78 %, respectively with standard of quercetin 98.12 ± 0.46 %.

NO scavenging results showed that maximum percentage inhibited by methanol extract was 93.35 ± 0.61 which is significant followed by water and dichloromethane extracts showed inhibition 87.4 ± 0.32 and 44.3 ± 0.49 %, respectively, with reference to standard quercetin 99.34 ± 1.91 %.

The order of antioxidant potential by NO scavenging of *Conocarpus lancifolius* is CLAM > CLRM > CLRW > CLAW > CLRD > CLAD. The order of percentage inhibition by DPPH is CLAM> CLRW > CLRM > CLAW>CLRD> CLAD. The rank order of inhibition by FRAP is CLAM > CLAW> CLRW> CLRM > CLAD > CLRD. The detail of these results is given in Table 1.

Phytotoxic activity

Dichloromethane and methanol extracts of whole plant of *Conocarpus lancifolius* were tested for their phytotoxic potential by performing phytotoxic bioassay against *Lemna minor*. The dichloromethane extract showed good activity just at high dose while the methanol extract showed excellent significant activity.

Table 1: Antioxidant activity of dichloromethane, methanol and water extracts of root and aerial parts of *Conocarpus lancifolius*

Part	Extract	Code	Conc. (mg/mL)	Inhibition by DPPH method (%)	Inhibition by NO scavenging method (%)	Inhibition by FRAP method (%)
Arial	Dichloromethane	CLAD	0.5	28.1 ± 0.98	34.35 ± 0.46	42.06 ± 0.64
	Methanol	CLAM	0.5	92.1 ± 0.11	93.35 ± 0.61	93.2 ± 1.02
	Water	CLAW	0.5	87.7 ± 0.56d	78.9 ± 0.32	93.1 ± 0.78
Root	Dichloromethane	CLRD	0.5	38.35 ± 0.43	44.3 ± 0.49	24.1 ± 0.64
	Methanol	CLRM	0.5	85.4 ± 0.51	88.2 ± 0.43	87.2 ± 0.31
	Water	CLRW	0.5	91.5 ± 0.46	87.4 ± 0.32	91 ± 0.52
Standard		Quercetin	0.1	92.12 ± 0.49	99.34 ± 0.48	98.12 ± 0.46

Table 2: Phytotoxicity of the extracts

Extract code	Plant	Conc.of Compound (µg/mL)	No. of Fronds		Growth Regulation (%)	Conc.of Standard Drug (µg/mL)
			Sample	Control		
CLM	*Lemna minor*	1000	02	20	90	0.015
		100	20		20	
		10	20		20	
CLD		1000	09	20	55	
		100	20		0	

Table 3: Antiurease activity of the extracts

Extract code	Conc. (mM)	Inhibition (%)	IC_{50} ± SEM (µM)
CLM	0.25	52.7	229±2.11
CLM	0.5	91.1	49.1± 1.31
CLD	0.0625	Inactive	
STD Thiourea	0.5	96.9	21.8±1.62

Antiurease activity

Several classes of compounds have been reported as the agents having antiurease activity, among them hydroxamic acids are the best recognized urease inhibitors. Table 3 shows that methanol extract at concentration 0.5 mM shows significant inhibition 91.5 % with IC_{50} of 49.1 ± 1.31 and dichloromethane extract was inactive. Thiourea was used as standard.

Flavonoid contents

Flavonoids contents was calculated in dichloromethane, methanol and water extracts aerial and root parts of *Conocarpus lancifolius* by using quercetin as a standard 990.17 ± 2.84 mg/mL. The results illustrate that dichloromethane extract of aerial part of *Conocarpus lancifolius* contained maximum significant flavonoid contents 629.4 ± 1.57 while water extract of the root exhibited the least flavonoid content of 104.2 ± 0.06 with reference to quercetin.

Sonication assisted extraction (exposure time - 30, 40 and 60 min) of *Conocarpus lancifolius* was done for dichloromethane, methanol and water extracts. The results revealed that flavonoid contents obtained by sonication assisted extraction for 40 min for *Conocarpus lancifolius* produced better yield.

Phenolic contents from HPLC

Quantification of phenolic contents of methanol extracts of the aerial and root parts of *Conocarpus lancifolius* by HPLC has been done.

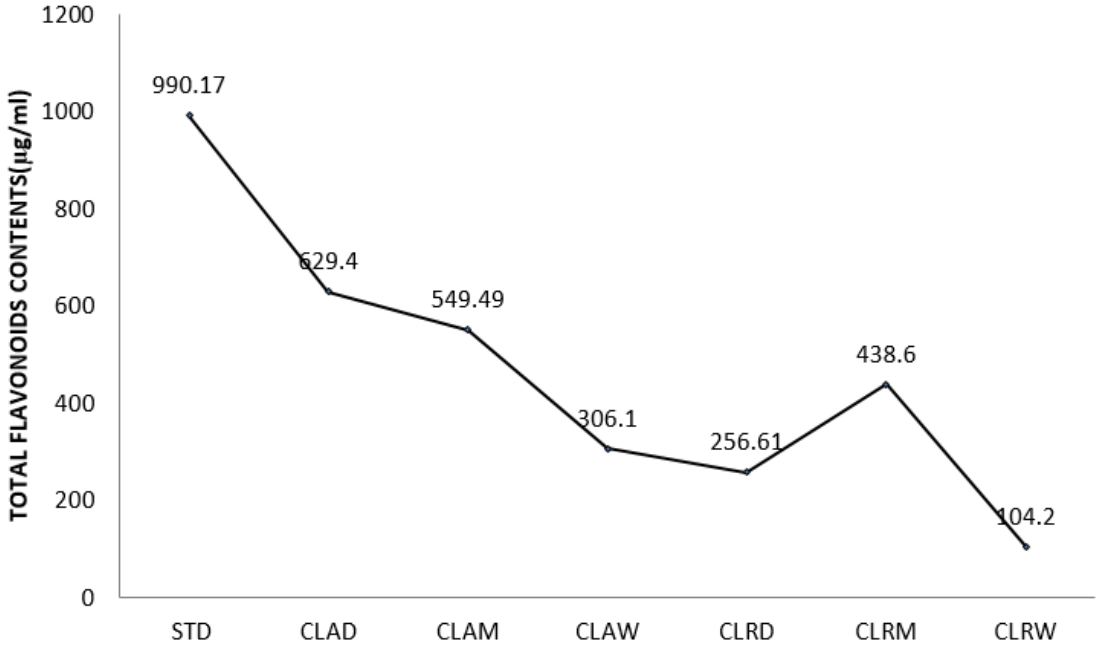

Figure 1: Flavonoid contents of *Conocarpus lancifolius*

Table 4: Phenolic contents derived from HPLC

Compound	*Conocarpus lancifolius*					
	Arial			Root		
	Retention time	***Area(mV.s)***	***Concentration (ppm)***	***Retention time(min)***	***Area(mV.s)***	***Concentration (ppm)***
Quercetin	2.753	497.586	45.772	2.742	297.586	15.772
Gallic acid	4.227	271.652	9.779	4.325	360.740	14.0149
Vitamin c	-	-	-	23.667	517.799	10.356
Chlorogenic acid	15.673	901.329	70.304	15.593	475.745	37.108
M.Coumeric acid	-	-	-	19.627	247.604	9.0325
Sinapicacid	-	-	-	26.580	601.639	7.82
Ferulic acid	22.587	1046.640	74.93	22.587	451.092	32.4786
4-OH 3-methoxy benzoic acid	14.387	545.298	57.80	-	-	-
Caffeic acid	-	-	-	12.993	333.265	15.330

The calculated phenolic contents of methanol extract of aerial part of *Conocarpus lancifolius* with reference to quercetin, gallic acid, chlorogenic acid, ferulic acid and 4-hydroxy-3-methoxy benzoic acid was with concentration 45.772 ,9.779 , 70.304 , 74.93 and 57.80 ppm respectively.

While the methanol root extract calculated with reference toquercetin, gallic acid, chlorogenic acid, coumeric acid, sinapic acid, ferulic acid and caffeic acid with concentration 15.772, 14.0149 , 10.356 , 37.108, 9.0325, 7.82, 32.4786 and 15.330 ppm, respectively.

DISCUSSION

Plant possessed significant phytotoxic and antioxidant activities. These properties may be due to the presence of flavonoids and phenols [18]. Currently, the synthetic chemical pesticides are being used for prevention of crop production. However, some serious flaws are associated with the use of these synthetic pesticides including pest resistance and negative impact on natural enemies in addition to environment and health related concerns [19]. These problems have resulted in the renewed interest in the development and use of botanical pesticides, which could be an appropriate and non-hazardous alternative to the currently used synthetic agrochemicals as the natural products [20]. The *Lemna* assay is a helpful tool in primary investigation or screening for herbicidal and weedicidal potential. The existing synthetic herbicidal and weedicidal are non-selective, expensive and hazardous to human health. So, that weedicides from the natural sources are better substitute to improve theses demerits of synthetic chemicals.

Plants are potential source of natural antioxidants such as ascorbic acid, tocopherol, cartenoids, flavonoids and phenolic acids [21]. The activities of antioxidants have been attributed to various mechanisms including prevention of chain initiation, decomposition of peroxides, radical scavenging and reducing capacity [22]. Consequently, these activities vary with assay methods and a single assay may be inadequate. Therefor antioxidant potential of dichloromethane, methanol and water extracts of root and aerial parts of *Conocarpus lancifolius* by using DPPH, NO scavenging and ferrous reducing power activity (FRAP) methods was evaluated. Phenols and flavonoids represent two phytochemicals whose relative abundance in plant extracts has been profusely linked to antioxidant activities. Phenols and flavonoids in extracts may explain their high antioxidant activities. DPPH radical scavenging assay provides an easy, rapid, and convenient method to evaluate antioxidants and radical scavengers [23]. It is based on the ability of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515nm and also for the visible deep purple colour.

Certain synthetic compounds have shown potentials as antiurease agents, e.g., hydroxyurea, flurofamide, and hydroxyamic acid, however, the *in vivo* use of some of these has been prohibited because of their toxicity or instability, for instance, acetohydroxyamic acid has been demonstrated to be teratogenic in rats. The discovery of potent and safe urease inhibitors has been a very important area of pharmaceutical research.

CONCLUSION

Antioxidant activity is highly dependent on phenolics, including flavonoids. The results of the present study indicate that the extracts exhibited varying but potent antioxidant, phytotoxic and anti-urease activities that should not be ignored.

The results support the traditional healers' claim on the therapeutic properties of *Conocarpus lancifolius*.

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