New ellagic acid derivative from the fruits of heat-tolerant plant *Conocarpus lancifolius* Engl. and their anti-inflammatory, cytotoxic, PPAR agonistic activities

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Abstract: *Conocarpus lancifolius* (Combretaceae), "دمس سنائي" in Arabic, is distributed in Riyadh, Saudi Arabia. It is a heat-tolerant Saudi medicinal plant, demonstrates prominent antidiabetic potential and the fruit extract fruits showed cytotoxicity against MRC-5 cancer cell line, as well as prominent antiprotozoal and antibacterial activities. The objective of this study is to isolate the compounds and evaluate the anti-inflammatory, cytotoxic, PPAR agonistic and antioxidant activities of extracts and pure constituents from *C. lancifolius*. A new trimethoxyellagic acid derivative 1 and two compounds, kaempferol 3-*O*-rutinoside 2 and β-sitosterol glucoside 3 were isolated from the fruits of *Conocarpus lancifolius*, a heat-tolerant plant. Compound 2 showed strong dual activation for PPARα and PPARγ with 2.6 fold increment in PPARα activity, while 2.2 fold increment in PPARγ at 25µg/mL. This is first report on isolation and screening of different biological activities of extract and pure constituents from fruits of *C. lancifolius*.

Keywords: Combretaceae; *Conocarpus lancifolius;* anti-inflammatory activity; cytotoxic activity; PPAR agonistic activity.

INTRODUCTION

Conocarpus lancifolius Engl. is an ornamental tree from the Combretaceae family with a high tolerance to heat, growing all over in Saudi Arabia (Al-Surrayai *et al.*, 2009). *C. lancifolius*, one of the two species in the genus *Conocarpus* is naive to coastal and river areas of Somalia, Djibouti, and Yemen (Abdel-Hameed *et al.*, 2012). It is also found throughout East Africa, the Arabian Peninsula, and South Asia (Redha *et al.*, 2011). The other species *C. erectus* is used as a traditional medicine for fever, catarrhal inflammation, diabetes, conjunctivitis, anemia and diarrhoea (Ayoub, 2010). According to the literature, methanol soluble extract of aerial part of *C. lancifolius* gave prominent antidiabetic potential due to gluconeogenesis suppression in alloxan induced diabetic rabbits (Saadullah *et al.*, 2014). The methanol soluble extract of the fruits of *C. lancifolius* showed significant cytotoxicity against MRC-5 Cells, as well as antiprotozoal activity (Al-Musayeib *et al.*, 2012). The alkaloidal extract of *C. lancifolius* leaves exhibited antibacterial activity (Hayssam et al. 2013). Total methanol soluble extract of *C. lancifolius* showed moderate antibacterial and low antifungal activities (Saad *et al.*, 2014).

Considering the fact that biological activities and phytochemical studies of *C. lancifolius* have not been fully investigated and their therapeutic potency has not yet been fully summarized, so, herein we aimed to isolate and investigate the anti-inflammatory, cytotoxic, PPAR agonistic and antioxidant activities of extract and its constituents.

MATERIALS AND METHODS

General experimental procedures

The experimental procedures are same as published before (Al-Taweel *et al.*, 2015).

Plant material

Fresh fruits of *C. lancifolius* (4.0 kg) were collected from Riyadh (Saudi Arabia) during October 2013 and taxonomist of Pharmacognosy Department from King Saud University identified it, while a voucher (15103) has been deposited.

Extraction and isolation

The extraction and isolation scheme of extract and isolates has given as supplementary material.

Compound 1

White amorphous powder; $[\alpha]^{D}_{27}$= -35 (*c*=0.4 in CH_3OH); ESI-MS (neg ion mode): *m/z* 425 $[M-H]^-$, 342 [M-cyclopentanone ring]$^-$; ^1H and ^{13}C NMR data (table 1).

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STATISTICAL ANALYSIS

All of the experiments were repeated thrice and the results expressed as means ± standard deviation (SD) of three experiments. Significant differences were analyzed by ANOVA statistics.

RESULTS

The methanol extract of the *C. lancifolius* fruits was divided into fractions soluble in $CHCl_3$, *n*-BuOH and H_2O. After a chromatographic separations of chloroform and *n*-butanol fractions resulted the isolation of one new compound (1) (fig. 1) and two known constituents, namely kaempferol 3-*O*-rutinose (2) (Shyaula *et al.*, 2012) and β-sitosterol 3-*O*-glucoside (3) (Rahman *et al.*, 2009). Structures of compounds were assigned from ^1H, ^{13}C NMR and ESIMS experiments and comparison with published data.

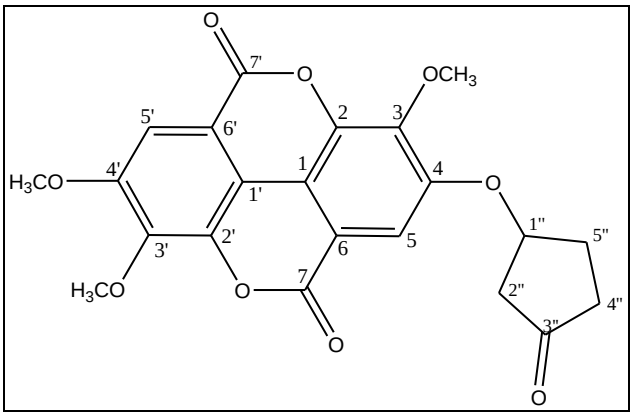

Fig. 1: Structure of compound 1.

Compound (1) obtained as white solid having molecular formula $C_{22}H_{18}O_9$ determined by the ESI-MS gave $[M-H]^-$ signal at *m/z* 425. This MF $C_{22}H_{18}O_9$ was further confirmed by the help of ^{13}C-NMR spectra (table 1), which showed fifteen peaks comprising on three methoxy (OCH_3), three methylene (CH_2), three methine (CH) and thirteen (C) quaternary carbons. Fragmented peak at *m/z* 342 $[M-H-cyclopentanone]^-$ in the ESI-MS spectrum showing the presence of one cyclopentanone ring, which confirmed by presence of quaternary carbon at *d*(C) 205.0 in ^{13}C-NMR spectrum. The ^1H-NMR spectrum showed prominent peaks in aromatic region at δ 7.46 (s, 1H) and 8.16 (s, 1H) characteristic to the ellagic acid skeleton (Ayoub, 2010) together with three methoxy protons at δ 3.92, 4.02 and 4.12 (s, 9H) indicating a trimethoxy ellagic acid. The ^1H NMR spectrum showed few more signals in the high field region for the three sets of methylene (CH_2) protons at δ 1.80, 1.98 (m, 2H), 1.92, 2.06 (m, 2H) and δ 3.22, 3.42 (m, 2H) and one oxymethine (O-CH) proton at δ 5.33 (m, 1H) (table 1) which confirm the presence of pentacyclic ring of compound 1. In the ^{13}C-NMR spectrum, fourteen carbon signals were assigned to ellagic acid, five carbon signals to cyclopentanone moiety and the remaining three carbon signals were assigned to methoxyl groups (table 1).

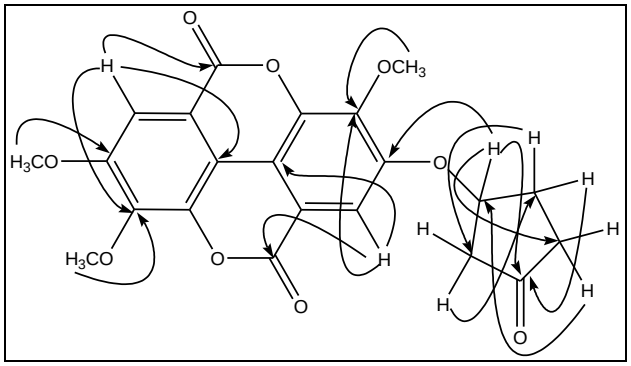

Fig. 2: Important HMBC correlations of compound 1.

The methine (CH) proton of cyclopentanone ring at δ 5.33 correlating to the C-4 (δ 147.9) in *heteronuclear multiple bond correlation* spectrum, pointing out the linkage of cyclopentanone unit at C-4. The cyclopentanone unit was further confirmed by the presence of enol olefin proton signal at δ 6.16 in ^1H-NMR spectrum, showing the keto-enol tautomerism of the carbonyl group. In a NOESY experiment, correlation was noticed between H-5′ and OMe-4′ and another correlation noticed between H-5 and H-2′′ of the cyclopentanone ring, although there was no correlation observed for H-5 protons to OMe-3. Results further confirm the linkage of the cyclopentanone ring at C-4 of the ellagic acid. The positions of three methoxy groups at C-3, C-3′ and C-4′ were decided through ^3J HMBC correlations (fig. 2). Accordingly, compound 1 was characterized as 3,3′,4′-trimethoxy 4-*O*-cyclopentanone ellagic acid. This is the first report on phytochemical investigation of *C. lancifolius* and on the best of our knowledge there is no compound has been isolated before from this plant. Kaempferol 3-*O*-rutinose (2) and β-sitosterol 3-*O*-glucoside (3) were the major constituents of *n*-butanol soluble fraction and their spectra completely matched with previous published literature (Shyaula *et al.*, 2012; Rahman *et al.*, 2009).

Assay for antioxidant activity

The assay was performed in human hepatoma cell line (HepG2) according to the method described earlier (Al-Taweel *et al.*, 2015).

Assay for iNOS inhibition

The assay for iNOS inhibition was also performed as described earlier (Al-Taweel *et al.*, 2015; Zhao *et al.*, 2014).

Assay for cytotoxicity

In vitro cytotoxic activities of the compounds were determined against four different human cancer cell lines (SK-MEL, KB, BT-549, SK-OV-3) and two noncancerous kidney cell lines (LLC-PK1 and VERO). These cell lines

obtained from American Type Culture Collection (ATCC, Rockville, MD) (Borenfreund *et al.,* 1990).

Table 1: ^{1}H- and ^{13}C-NMR data for Compound 1

1		
Position	d (H)	d (C)
1	-	114.3
2	-	141.2
3	-	143.8
4	-	147.9
5	8.16 (s)	118.1
6	-	111.6
7	-	155.0
1′	-	112.9
2′	-	141.0
3′	-	141.4
4′	-	154.6
5′	7.46 (s)	107.6
6′	-	112.8
7′	-	158.5
3-OCH_3	4.12 (s)	61.8
3′-OCH_3	4.02 (s)	61.6
4′-OCH_3	3.92 (s)	57.0
1′′	5.33 (m)	81.9
2′′	3.22 (m)	46.9
	3.42 (m)	
3′′	-	205.0
4′′	1.80 (m)	34.5
	1.98 (m)	
5′′	1.92 (m)	21.9
	2.06 (m)	

δ in *ppm* and *J* in Hz.
Recorded in DMSO-d_6 at 500 MHz

Assay for activation of PPARα and PPARγ
The assay was described earlier (Zhao *et al.,* 2014).

DISCUSSION

According to literature, the methanol extract showed strong antidiabetic activity in alloxan induced diabetic rabbits, so here in we aim to study the Peroxisome proliferation effects (PPARα and PPARγ) a phenomenon occurs when response of animals are exposed to the compounds named peroxisome proliferators. Peroxisome proliferation is usually regulated by nuclear receptor known as peroxisome proliferator-activated receptor (PPAR) (Cajaraville *et al.,* 2003). The agonistic effects of the extract and compounds on PPARs were determined, PPARα and PPARγ are depends on ligand transcription factors which regulate the carbohydrate and lipid metabolism as well as inflammatory process. These are considered significant targets related to metabolic disorder which is a combination of diabetes, cardiovascular disease and inflammation. The extract (50 µg/mL) did not show any agonistic effect on PPARα and PPARγ but compound 2 showed strong dual activation of both PPARα and PPARγ with 2.6 fold increment in PPARα activity, while 2.2 fold increment in PPARγ at 25 µg/mL. Compound 1 was less active with 1.4 time fold increment in PPARα activity and 1.5 fold increment in PPARγ activity at 50µg/mL. Compound 3 showed activity towards PPARγ only with a fold increment of 1.4 at 25µg/mL. The control drugs ciprofibrate (10µM, cardiovascular) and rosiglitazone (10µM, antidiabetic) showed a fold induction of 2.3 and 3.7 for PPARα and PPARγ, respectively (table 2).

The anti-inflammatory capability of extract and compounds was gained from their ability to inhibit transcriptional activity of *NF-kappa B*, iNOS activities and generation of ROS in cellular system exposed to proinflammatory stimuli. The extract of *C. lancifolius* fruits showed antioxidant activity with a 43% decrease in the oxidative stress in a cellular assay in comparison to a 74% decrease by Quercetin (at 50µM) as positive control. The other targets related to anti-inflammatory activity were not affected by the extract such as iNOS and NF-kB (table 3). Parthenolide inhibited iNOS and *NF-kappa B* activities with the IC_{50} values of 0.35µg/mL and 0.5 µg/mL, respectively. The extract was also not cytotoxic to any of the cell lines up to 100µg/mL in our assay, although the cytotoxicity of the extract has been reported earlier for different cell lines (Al-Musayeib *et al.,* 2012).

The isolated compounds did not show any decrease in oxidative stress up to 1000µg/mL but iNOS activity was inhibited by two of them (1 and 2). Compound 2 was more active than compound 1 in inhibiting iNOS activity (IC_{50} value 18 vs 50µg/mL). No significant NF-kB inhibition was observed by these isolates up to 50µg/mL. The iNOS inhibitory activity could be related to the cytotoxicity of compound 2 (table 2). The compounds were not cytotoxic on concentration of 25µg/mL to the four cancer cell lines included in this study (data not shown).

These results indicate that the previously reported anti-inflammatory and antidiabetic activity of the plant could be due to the presence of compounds like compounds 1 and 2. Anti-inflammatory potential of plant could be explained in terms of iNOS and inhibition of nitrite production. The agonistic effect of its constituents towards PPAR may also contribute towards the activity against metabolic disorder comprising of diabetes, cardiovascular disease and inflammation.

According to the literature data, different biological activities of ellagic acid and kaempferol 3-*O*-rutinoside have been explored. It has been found that ellagic acid

Table 2: PPAR agonistic activity of *C. lancifolius* fruit extract and isolated compounds

Sample name	Fold Induction	
	PPAR alpha	PPAR gamma
C. lancifolius fruit extract 50µg/mL	NA	NA
Compound 1, 50µg/mL	1.4± 0.15	1.5± 0.21
Compound 2, 25µg/mL	2.6± 0.31	2.2± 0.18
Compound 3, 25µg/mL	NA	1.4± 0.25
Ciprofibrate 10µM	2.2± 0.26	NT
Rosiglitazone 10µM	NT	3.7± 0.35

Table 3: Anti-inflammatory & cytotoxic activities of *C. lancifolius* fruit extract and isolated compounds

Sample Name	Antioxidant activity	iNOS inhibition	NF-kB inhibition	Cytotoxicity to
	% decrease in			kidney cell lines
	oxidative stress	IC_{50} in µg/mL	IC_{50} in µg/mL	IC_{50} in µg/mL
C. lancifolius fruit extract	43%	NA	NA	NA
Compound 1	NA	50± 0.21	NA	NA
Compound 2	NA	18± 0.12	NA	15± 0.17
Compound 3	NA	NA	NA	>25
Quercetin (50µM)	74± 0.18	NT	NT	NT
Parthenolide	NT	0.35± 0.22	0.5± 0.21	NT
Doxorubicin	NT	NT	NT	0.85± 0.32

NA: not active, NT : not tested

exhibited anti-cancer activities like apoptosis and induction of cell cycle (Narayanan *et al.*, 1999), along with inhibition of the formation of tumor and its growth in different animals (Stoner & Morse, 1997; Khanduja *et al.*, 1999). Kaempferol 3-*O*-rutinoside was proved to have potent antidiabetic effect (Tadera *et al.*, 2006), as well as antiglycation activity which is good for treatment of diabetes. It was also shown to possess non-cytotoxic nature (Shyaula *et al.*, 2012).

CONCLUSION

In conclusion, the investigation of chloroform and *n*-butanol fraction of the fruits of *C. lancifolius* growing in Saudi Arabia yielded one new compound 3,3′,4′ trimethoxy 4-*O*-cyclopentanone ellagic acid and two known compounds, kaempferol 3-*O*-rutinose (2) and β-sitosterol 3-*O*-glucoside (3). Their structures were established by using extensive spectroscopic studies. Compounds 1 and 2 showed strong anti-inflammatory activity and significant PPAR agonistic activity, which is in agreement with literature on antidiabetic effect done before. Phytochemical and pharmacological potential of *C. lancifolius* has not been investigated earlier, so the results obtained from our work could be contribute to understand their chemical constituents and its biological potential as reported in literature.

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