European Journal of Medicinal Plants
2(2): 93-112, 2012

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Phytochemical Studies and Evaluation of Antioxidant, Anticancer and Antimicrobial Properties of *Conocarpus erectus* L. Growing in Taif, Saudi Arabia

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Research Article

Received 24th December 2011
Accepted 11th January 2012
Online Ready 6th February 2012

ABSTRACT

The use of plants and their derived substances increases day by day for the discovery of new therapeutic agents owing to their versatile applications. *Conocarpus erectus* L. (family Combretaceae) is one of two species in the genus *Conocarpus* widely spread in Taif and other parts of the Kingdom of Saudi Arabia. It is used in some countries as folk remedy for anemia, catarrh, conjunctivitis, diabetes, diarrhea and fever. To our knowledge from literatures there is little work about phytochemical contents and biological activities of *C. erectus* which encouraged the authors to carry out this study. The four defatted methanol extracts of *C. erectus* different parts (leaves, stems, fruits and flowers) showed high free radical scavenging activity toward DPPH radical with SC_{50} between 6.47-9.4 µg/ml. The *n*-BuOH fractions obtained from successive fractionation of the four defatted methanol extracts had radical scavenging activity with SC_{50} between 4.43-5.89 µg/ml higher than the EtOAc fractions (7.04-10.71 µg/ml). Due to the high antioxidant activity of the EtOAc and *n*-BuOH fractions, it was *in vitro* assayed toward two human cancer cell lines; HepG2 & MCF-7; using Sulphorhodamine-B assay method. The results showed that most fractions had cytotoxic activity with IC_{50}< 20µg/ml which fall within the American Cancer Institute criteria. A preliminary antibacterial screening of the four defatted methanol extracts of the different parts toward five bacterial strains were done. Phytochemical investigation of the

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EtOAc and *n*-BuOH fractions showed that phenolic compounds constitute the major components of it. In conclusion this is the first report on the phytochemical and biological properties of *Conocarpus erectus* growing in Taif governorate, KSA. More *in vivo* and *in vitro* studies along with detailed phytochemical investigation are needed in the hope to can use this plant (crude extract, fractions, sub-fractions or pure compounds) in the prevention and therapies of some diseases.

Keywords: Conocarpus erectus; ethnomedicine; antioxidant; anticancer; antibacterial; phytochemical characterization; phenolic compounds.

1. INTRODUCTION

From ancient time till now and tomorrow, the use of natural resources especially plants increases day by day for the discovery of new therapeutic agents. Natural products from some of these natural resources continue to be used in pharmaceutical preparations either as crude extracts, fractions, pure compounds or analogous compounds from highly active isolated compounds.

Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional medicines, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube et al., 2008; Nirmala et al., 2011). In spite of the recent domination of the synthetic chemistry as a method to discover and produce drugs, the potential of bioactive plants or their extracts to provide new and novel products for disease treatment and prevention is still enormous (Kviecinski et al., 2008; Newman et al., 2003; Verpoorte, 2000). Several active compounds have been discovered from plants and used directly as patented drugs like taxol, artemisinin and maprouneacin (Goodman and Walsh 2001; Klayman, 1993; Carney et al., 1999). Due to the multitargeting effect, inexpensive and safety of plant-based products compared to synthetic agents, there is a need for more and more searching and discovering of new drugs from plants.

Conocarpus erectus L. is one of two species in the genus *Conocarpus*, in the family Combretaceae growing on shorelines in tropical and subtropical regions around the world. *Conocarpus erectus*; known in English as buttonwood or button mangrove; is an evergreen tree 6 m tall with spreading crown, grey or brown bark, glaucous medium-green leaves and greenish flowers in dense cone-like heads in terminal panicles (Bailey, 1976). It is used in some countries as folk remedy for anemia, catarrh, conjunctivitis, diabetes, diarrhea and fever (Irvin, 1961). *C. erectus* is widely spread in Taif governorate and other parts of the Kingdom of Saudi Arabia. To our knowledge from the literatures, little is known about the phytochemical contents and biological activities of *C. erectus* (Nahla, 2010; Jagessar and Cox, 2010). In this work, a phytochemical study of different parts of *C. erectus* and their biological activities as antioxidant, anticancer and antimicrobial were done.

2. MATERIALS AND METHODS

2.1 Materials and Chemicals

All solvents and reagents used were of analytical grade. 1, 1-diphenyl picrylhydrazyl (DPPH) free radical and Folin–Ciocalteu's reagent (FCR) from Fluka Chemicals. Aluminum chloride, sodium carbonate, sodium phosphate, ammonium molybedate, rutin, ascorbic acid and gallic acid, all solvents and acids [petroleum ether, chloroform, ethyl acetate, *n*-butanol, ethanol, acetic acid, trichloroacetic acid (TCA) and sulphuric acid), dimethylsulphoxide (DMSO), RPMI-1640 medium, trypan blue, fetal bovine serum (FBS), penicillin/streptomycin, trypsin, sulphorhodamine-B (SRB)] from Sigma-Aldrich chemicals. Paper chromatography (PC) was done on Whatmann No. 1 (57 x 46 cm) while thin layer chromatography (TLC) was performed over pre-coated silica plates (GF_{254}, Merck). The absorbance measurements for antioxidant activity were recorded using the UV–Vis spectrophotometer Jenway 6405.

2.2 Collection and Preparation of the Plant Materials

The different parts (leaves, fruits, stems and flowers) of the plant under investigation were collected from Taif governorate, Kingdom of Saudi Arabia. The plant was kindly identified by Mrs. Traes Labib, general manager and head of specialists of Plant Taxonomy in El-Orman botanical garden, Giza, Egypt. Voucher specimen (given number CE1) was deposited at Natural products analysis laboratory, faculty of science, Taif University, Taif, KSA. The different parts were cutted and shade dried then finely powdered by electric mill and become ready for extraction process.

2.3 Preparation of Defatted Methanol Extracts and its Successive Fractions

The plant powder (300 g) of each part was soaked in 1500 ml methanol for one week at room temperature with shaking day by day followed by filtration and again extraction for four times. The organic solvent was removed in vacuo using rotatory evaporator affording known weight of each crude methanol extract. The methanolic crude extracts were defatted by washing several times with petroleum ether (60-80 ℃). The defatted crude methanol extracts were ready for bioassay.

Twenty grams of the defatted methanol extracts were dissolved in 80 ml distilled water and then successively partitioned with chloroform, ethyl acetate and finally with *n*-butanol (3 x 100 ml solvent) affording known weight of each respective fractions.

2.4 Biological Studies

Crude defatted methanolic extracts and their successive fractions obtained from different parts of the plant under investigation were *in vitro* tested for their antioxidant, anticancer and antimicrobial activities.

2.4.1 Antioxidant activity

It is important to select and employ a stable and rapid method to assay antioxidant activity, because the determination of many samples is time-consuming. In this work, three different chemical methods were used for the evaluation of antioxidant activity of crude methanol extracts and its successive fractions; 1, 1-diphenyl picrylhydrazyl scavenging activity for

estimation the free radical scavenging properties, phosphomolybdenum method used for measurement the total antioxidant capacity and reducing power assay.

2.4.1.1 Scavenging ability towards 1, 1-Diphenyl Picrylhydrazyl (DPPH) radical

The (DPPH) assay was performed as described by Shirwaikar et al. (2006). This method depends on the reduction of purple DPPH radicals to a yellow coloured diphenyl-picrylhydrazine and the remaining DPPH radicals which show maximum absorption at 517 nm will be measured. Two ml of various concentrations of each sample were added to 2 ml solution of 0.1 mM DPPH. An equal amount of methanol and DPPH served as control. After 20 min of incubation at 37℃ in the dark, the absorbance was recorded at 517 nm. The experiment was performed in triplicates. The DPPH radical scavenging activity was calculated according to the following equation:

% DPPH radical scavenging activity = 1 - [$A_{sample}/A_{control}$] x 100,

where A_{sample} and $A_{control}$ are absorbance of sample and control. The SC_{50} (concentration of sample required to scavenge 50% of DPPH radicals) values were determined. The decrease of absorbance of DPPH solution indicates an increase of the DPPH radical scavenging activity.

2.4.1.2 Determination of the total antioxidant capacity by Phosphomolybdenum method

The total antioxidant capacities of ethyl acetate and n-butanol fractions were evaluated by phosphomolybdenum method as described by Prieto et al. (1999). The assay is based on the reduction of Mo (VI) to Mo (V) by the antioxidants and subsequent formation of a green phosphate/Mo (V) complex at acid pH 300 µml of each sample solution and ascorbic acid (100µg/ml) were combined with 3 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). A typical blank solution containing 3 ml of reagent solution and an appropriate volume of the same solvent was used for the sample. All tubes were capped and incubated in a boiling water bath at 95℃ for 90 min. After the samples have been cooled down to room temperature, the absorbance of the solution of each sample was measured at 695 nm against the blank using a UV/Vis spectrophotometer. The experiment was performed in triplicates. The antioxidant activity was expressed as the number of equivalents of ascorbic acid.

2.4.1.3 Reducing power assay

The reducing power of the samples was determined according to the method of Oyaizu, 1986. Two ml of each sample and ascorbic acid in methanol (200 µg/ml) were mixed with 2 ml of sodium phosphate buffer (0.2M, pH 6.6) and 2 ml of 1% $K_3Fe(CN)_6$ were incubated at 50℃ for 20min. After adding 2 ml of trichloro acetic acid, the mixture was centrifuged at 3000 rpm for 10min. The supernatant solution (2 ml) was taken out and immediately mixed with 2 ml of methanol and 0.5 ml of 0.1 % ferric chloride. After incubation for 10 min, the absorbance against blank was determined at 700 nm. Three replicates were made for each tested sample and ascorbic acid. The increase in absorbance of the reaction mixture indicates an increased reduction power. The reducing power activity was expressed as the number of equivalents of ascorbic acid.

2.4.2 Anticancer activity

Fractions (EtOAc and *n*-BuOH) of different parts were investigated *in vitro* towards two kinds of human cancer cell lines; liver carcinoma cell line (HepG2) and breast carcinoma cell line (MCF-7); using the method of Skehan et al. (1990), at the National Cancer Institute in Egypt. This is a colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye Sulphorhodamine-B (SRB). This dye is a bright pink aminoxanthrene dye with two sulphonic groups. It is a protein stain that binds to the amino groups of intracellular proteins under mildly acidic conditions to provide a sensitive index of cellular protein content. Cells were seeded in 96-well microtiter plates at a concentration of $5x10^4$-10^5 cell/well in a fresh medium and left to attach to the plates for 24 hrs. Wells were prepared for each individual sample and incubated for 48 h at 37℃ in 5% CO_2. After 24 hrs, cells were incubated with the appropriate different concentrations of the fraction under test (0, 5, 12.5, 25 and 50 µg/ml), completed to total of 200 µl volume/well using fresh medium and incubation was continued for 24, 48 and 72 hrs. Following 24, 48 and 72 hrs treatment, the cells were fixed with 50 µl cold 50 % trichloroacetic acid for 1 hr at 4℃. Wells were washed 5 times with distilled water and stained for 30 min at room temperature with 50 µl 0.4 % SRB dissolved in 1 % acetic acid. The wells were then washed 4 times with 1 % acetic acid. The plates were air-dried and the dye was solubilized with 100 µl/well of 10 mM tris base (pH 10.5) for 5 min on a shaker (Orbital shaker OS 20, Boeco, Germany) at 1600 rpm. The optical density (O.D.) of each well was measured spectrophotometrically at 564nm with an ELIZA microplate reader (Meter tech. Σ960, U.S.A.). The mean background absorbance was automatically subtracted and mean values of each drug concentration was calculated. The experiment was repeated 3 times for each cell line. The percentage of cell survival was calculated according to the following equation:

*% Survival fraction = O.D. of treated cell/O.D. of control cells * 100*

According to the National Cancer Institute guideline a natural product extract with IC_{50} values < 20µg/ml is considered active (Boik, 2001).

2.4.3 Antimicrobial activity

2.4.3.1 Microorganisms

Clinical isolates of *Escherichia coli* (HB 101, K 12), *Pseudomonas aeruginosa* (PAO1), *Salmonella typhimurium* (clinical isolate), *Klebsiella pneumoniae* (clinical isolate), *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (168) were used in this study.

2.4.3.2 Assessment of antibacterial activity of extracts (NCCLS 1999)

Bacteria suspension of each tested bacteria (107 CFU/ml) was spread onto the surface of Muller-Hinton agar plates. Eight mm cork borer was used to punch wells into the plates and 100 µl of each extract dissolved in DMSO (10 mg/ml) as well as were applied to each well. The plates were incubated for 18 h at 37℃. The inhibition zones diameter for each extract was measured and the phenol coefficient was calculated.

2.4.3.3 Phenol equivalence assay for antibacterial activity (Allen et al., 1991)

Aliquots of 100 µl of phenol standard solutions (2%, 3%, 4%, 5%, 6%, and 7%) were placed, simultaneously, with extracts in wells of the assay plates. Negative controls of sterile

deionized water were included. The diameter of each zone of inhibition was measured using calipers. The mean diameter was squared, and a standard curve was generated of phenol concentration against the mean squared diameter of the zone of inhibition. The activity of each extract was calculated using the standard curve of phenol and was expressed as the equivalent phenol concentration (% w/v).

2.5 Phytochemical Analysis

The EtOAc and *n*-BuOH fractions obtained from successive fractionation of the defatted methanol extracts of the leaves, stems, flowers and fruits of *C. erectus* were selected for phytochemical analysis to explore the major classes of natural products, which may be responsible for activity as follows:

2.5.1 Phytochemical tests

Tests for sterols/triterpenes, phenolic compounds, flavonoids, tannins, carbohydrates/ glycosides, saponins and alkaloids were carried out according to the previously reported methods (Harborne, 1973).

2.5.2 Thin layer and paper chromatography

Phytochemical screening for the presence of secondary metabolites was performed using TLC analyses (pre-coated aluminum silica gel plates, GF254, Merck) with different eluting systems. The solvent systems were (CH_2Cl_2: MeOH, 19:1), $CHCl_3$: EtOAc:MeOH; 2:2:1), ($CHCl_3$: MeOH, 9:1 and 8:2), ($CHCl_3$: MeOH: H_2O; 65: 35: 5), (*n*-BuOH: MeOH: H_2O, 5:1:1). Spray reagents used in order to develop the spots were: 1% ferric chloride (tannins), 2% aluminium chloride in ethanol (flavonoids), 40% sulphuric acid/methanol (saponins) and Dragendorff reagent (alkaloids) (Wagner et al., 1984). Also, one dimensional PC was performed on Whatman No. 1 (57 x 46 cm) using BAW solvent systems (*n*-BuOH:AcOH: H_2O, 4:1:5 organic layer) and 15% AcOH/H_2O. The change of spot colours on the chromatograms was detected by exposing to ammonia vapour or spraying with 1% methanol $AlCl_3$ or $FeCl_3$ (Mabry et al., 1970).

2.5.3 Estimation the total amount of phenolic, flavonoid and tannin compounds

The total phenolic, flavonoid, and tannin contents of each active fraction was measured according to the methods described by Kumaran and Karunakaran (2006) for total phenolics and flavonoids and by Grubesic et al. (2005) for total tannins. The total phenolic content of plant extracts was determined using Folin-Ciocalteu's reagent (FCR). This method depends on the reduction of FCR by phenols to a mixture of blue oxides which have a maximal absorption in the region of 750 nm. About 100 µl of plant extracts (100 µg/ml) and also 100 µl of gallic acid (100 µg/ml) were mixed with 500 µl of the FCR and 1.5 ml of 20% sodium carbonate. The mixture was shaken thoroughly and made up to 10 ml using distilled water. The mixture was allowed to stand for 2 h. Then the absorbance at 765 nm was determined against a blank which contain all reagents without the samples or the gallic acid at the same conditions. All determinations were carried out in triplicates. The total phenolic content is expressed as the number of equivalents of gallic acid (GAE).

The flavonoids content was determined by aluminium chloride method using rutin as a reference compound. This method based on the formation of a complex flavonoid-aluminum having the absorptivity maximum at 415 nm. About 100 µl of plant extracts in methanol (10

mg/ml) was mixed with 100 µl of 2% aluminium trichloride in ethanol and a drop of acetic acid, and then diluted with methanol to 5 ml. The absorption at 415 nm was read after 40 min. Blank were prepared from all reagents without the samples. The absorption of standard rutin solution (0.5 mg/ml) in methanol was measured under the same conditions. All determinations were carried out in triplicates. The amount of flavonoids in plant extracts in rutin equivalents (RE) was calculated by the following formula:

$X = (A - m_o)/(A_o - m)$.

where X is the flavonoid content, mg/mg plant extract in RE, A is the absorption of plant extract solution, A_o is the absorption of standard rutin solution, m is the weight of plant extract, mg and mo is the weight of rutin in the solution, mg.

The total content of tannins adsorbed by casein was determined using FCR. About 10 ml (100µg/ml) of each fraction (solution 1, S1) was mixed with 100 mg of casein with shaking for two hours (adsorption of tannins) and then filtered (solution 2, S2). The total phenolic contents for both solutions S1 and S2 using Folin–Ciocalteu's method as described before. The difference between absorbance of S1 and S2 correspond to concentration casein-adsorbed tannins in sample. All determinations were carried out in triplicates. The total casein-adsorbed tannins are expressed as the number of equivalents of gallic acid (GAE).

2.5.4 RP-HPLC-UV analysis of some phenolics

2.5.4.1 Apparatus and chromatographic conditions

Reversed phase HPLC analysis was done using a system consists of two pump (Waters 515), UV detector (Waters 486 at 280 nm), Rheodyne injector, CBM-20A communication bus module, Waters automated gradient controller, reversed phase Symmetry C18 column (5µm, 4.6×150mm) from Waters and Empower software (build number 1154). After several trails, chromatographic separation was carried out at room temperature with flow rate 1.0 ml/ min of gradient elution using two solvent; A (0.1% formic acid (FA) in water) and solvent B (0.1% FA in acetonitrile) with a linear gradient elution: 95% A (5 min), 95–90% A (15 min), 90–50% A (50 min), 50–95% A (60 min), 95% A (65 min).

2.5.4.2 Preparation of standard and sample solutions

Standard stock solution of gallic acid (500 µg/ml), catechin (400 µg/ml), quercetin-3-glc-6-gallic acid (200 µg/ml), rutin (500 µg/ml), quercetin-3-glc (200 µg/ml), kaempferol-3-glc (200 µg/ml), quercetin (500 µg/ml) and apigenin (200 µg/ml) were prepared in HPLC grade of 50% acetonitrile/water and filtered using membrane disc filter (0.45 µm). Firstly, each compound was chromatography using the previous analytical condition. For each compound the retention time was determined. From each individual standard stock solution, a mixed stock solution containing eight analytes were prepared and diluted to appropriate different concentrations for establishing calibration curves. For samples, stock solution (2.5 mg/ml) of the EtOAc and *n*-BuOH fractions from different parts of *C. erectus* were prepared in HPLC grade of 50% acetonitrile /water and filtered using membrane disc filter (0.45 µm).

2.5.4.3 Calibration curve and sample analysis

For quantitative analysis, six different concentrations of a mixed stock solution containing eight analytes (3.125, 6.250, 12.50, 25.00, 37.50 and 50.00 µg/ml for gallic acid, rutin, and

quercetin; 2.50, 5.00, 10.00, 20.00, 30.00 and 40.00 µg/ml for catechin; 1.25, 2.50, 5.00, 10.00, 15.00 and 20.00 µg/ml for quercetin-3-glc-6-gallic acid, quercetin-3-glc, kaempferol-3-glc and apigenin) were injected. By Empower software a calibration curve was obtained by plotting the peak areas versus the concentration of each analyte. Chromatograms of samples obtained were analyzed using the Empower software based on retention times comparing of sample with those of the standards for qualitative analysis and calibration curve for quantitative analysis.

2.6 Statistical Analysis

All determinations for bioassay were carried out triplicates and the values in tables are mean ± standard deviation. The statistical analyses were carried out using SPSS 13.0 and Microsoft Excel programs.

3. RESULTS AND DISCUSSION

It was noticed by authors that the tree of *Conocarpus erectus* is widely spread in Taif governorate, Kingdom of Saudi Arabia and many places of the Kingdom. Also, the tree is evergreen with plenty of flowers and fruits. Due to this and also the lake of phytochemical and biological work of this plant (Nahla, 2010; Jagessar and Cox, 2010), the authors encouraged to investigate the different parts of this plant as antioxidant, anticancer and antibacterial agents. As known the general strategy of natural products drug discovery from natural resources (plant, microbes, fungi, etc.) started with preliminary bioassay screening (antioxidant, cytotoxic, antibacterial, etc.) of its crude extracts followed by bioassay guided fractionation, isolation and structure elucidation of the bioactive compounds (Abdel-Hameed, 2009). In this work the defatted methanol extract of different parts (leaves, stems, fruits and flowers) of *C. erectus* were bioassayed for their antioxidant properties followed by successive fractionation of the defatted methanol with some organic solvents of different polarities. The most active fractions were bioassayed as antioxidant agent using three *in vitro* methods and as anticancer toward two cancer cell lines (HepG2 and MCF-7). The fractions were phytochemically investigated to know the natural product classes which may be responsible for the activity. Also, the defatted methanol extract of *Conocarpus erectus* different parts were preliminary investigated as antimicrobial agents.

3.1 As Antioxidant Agents

Free radicals are highly reactive oxygen species produced through oxidative process within the mammalian body. In normal conditions, the human body possesses many defense mechanisms against oxidative stress, including antioxidant enzymes and non-enzymatic compounds but under some circumstances including exposure to some environmental pollutants, e.g., cigarette smoke, pesticides, smog, UV radiation, etc. the natural antioxidant mammalian mechanism become insufficient and then the excess of free radicals can damage both the structure and function of a cell membrane in a chain reaction leading to many degenerative diseases (Atta-ur-Rahman and Choudhary, 2001). Many antioxidant-based drug formulations are used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer (Mosquera et al., 2007). Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods, cosmetics or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity (Sasaki et al., 2002).

The defatted methanol extracts of *Conocarpus erectus* different parts and its successive fraction were investigated for their antioxidant properties using three rapid and stable *in vitro* methods.

3.1.1 Free Radical scavenging activity by DPPH$^{\cdot}$

The radical scavenging activity of the four defatted methanol extracts of *C. erectus* different parts on DPPH$^{\cdot}$ (see supplementary data) showed high activity with SC_{50}<10 µg/ml. The activities of the four extracts were in order: fruits > stems> flowers > leaves with SC_{50} = 6.47, 7.24, 7.65 and 9.40 µg/ml respectively. The ethyl acetate and n-butanol fractions obtained from successive fractionation of the defatted four methanol extracts showed antiradical activity toward DPPH at SC_{50}<11 µg/ml (table 1) whereas the chloroform fractions showed weak activity with SC_{50} > 100 µg/ml. The ethyl acetate fraction of flowers showed the strongest activity with SC_{50} = 7.04 µg/ml followed by fruits (7.12 µg/ml), stems (8.96 µg/ml) and leaves (10.71 µg/ml). On the other hand, the *n*-butanol fraction of fruits showed the highest antiradical activity with SC_{50} = 4.43 µg/ml followed by stems (4.87 µg/ml), flowers (5.89 µg/ml) and leaves (5.89 µg/ml).

The model of scavenging of the stable DPPH radicals is a widely method to evaluate the antioxidant activity of the investigated sample in relatively short time compared with other methods**.** The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Gulcin et al., 2004). The addition of the investigated extracts to the DPPH solution caused a decrease in the optical density at 517 nm (the maximum absorption of a stable DPPH radical at 517 nm). The decrease in absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecules and radical which results in the scavenging of the radical by hydrogen donation. This is visualized as a discoloration from purple to yellow.

3.1.2 Antioxidant capacity by phosphomolybdenum method

Table (1) showed that the ethyl acetate fraction of the flower has the higher antioxidant capacity (358.81 mg equivalent to ascorbic acid /g extract) than the other three ethyl acetate fractions; fruits, stems and leaves with 334.96, 333.9 and 326.8 mg equivalent to ascorbic acid /g extract respectively. Also, the four *n*-butanol fractions showed high antioxidant capicity in order: stem>fruits>flower>leaves with 575.1, 542.0, 430.4 and 423.85 mg equivalent to ascorbic acid /g extract.

This method was based on the reduction of Mo(VI) to Mo(V) by the antioxidant agent and the formation of a green phosphate/ Mo(V) complex with a maximal absorption at 695 nm. It has been reported that phosphomolybdenum method is successfully used to measurement of total antioxidant capacities of many plant extracts (Kumaran and Karunakaran 2005; Abdel-Hameed, 2009).

3.1.3 Reducing power activity

The data of reducing power activity of the four ethyl acetate fractions in table 1, showed that the ethyl acetate fraction of the flowers has the highest activity (610.2 mg equivalent to ascorbic acid /g extract) followed by the ethyl acetate fractions of fruits, stems and leaves with 546.4, 437.8 and 410.0 mg equivalent to ascorbic acid /g extract respectively.

Table 1. Yield, DPPH free radical scavenging activity, total antioxidant capacity and reducing power activity of ethyl acetate and *n*-butanol fractions from successive fractionation of defatted methanol extract of *Conocarpus erectus* different parts

Plant part	Yield (% crude defatted MeOH) and DPPH free radical scavenging activity SC_{50} [µg/ml]				Total antioxidant capacity [mg equivalent to ascorbic acid /g extract][a]		Reducing power activity [mg equivalent to ascorbic acid /g extract][b]	
	Ethyl acetate		*n*-butanol		Ethyl acetate	*n*-butanol	Ethyl acetate	*n*-butanol
	Yield (%)	SC_{50} [µg/ml]	Yield (%)	SC_{50} [µg/ml]				
Leaves	8.0	10.71±0.06	8.2	5.89±0.04	326.8±4.30	423.85±4.87	410.0±2.84	715.9±3.41
Stems	2.9	8.96±0.08	7.3	4.87±0.03	333.9±8.18	575.1±6.57	437.8±1.97	888.3±3.41
Flowers	5.6	7.04±0.07	13.2	5.89±0.06	358.81±6.57	430.4±4.09	610.2±3.94	676.5±6.65
Fruits	4.9	7.12±0.07	12.2	4.43±0.06	334.96±5.86	542.0±2.48	546.4±6.21	893.6±1.45

Values of SC_{50}, total antioxidant capacity and reducing power activity are expressed as mean of triplicate determinations ± standard deviation
[a]Antioxidant capacity monitored by the phosphomolybdenum method expressed by mg equivalent to ascorbic acid /g extract.
[b]Reducing power activity expressed by mg equivalent to ascorbic acid /g extract.

On the other hand the *n*-butanol fraction of fruits has the highest reducing power activity (893.6 mg equivalent to ascorbic acid /g extract) followed by stems, leaves and flowers with 888.3, 715.9 and 676.5 mg equivalent to ascorbic acid /g extract respectively.

Reducing power indicates compounds that are electron donors, which can act as primary and secondary antioxidants. Reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. Different studies have been indicated that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain through the donation of a hydrogen atom (Lin et al., 2010; Li and Lin, 2010).

3.2 As Anticancer Agents

Cancer is a major public health burden in both developed and developing countries. It was estimated 12.7 million new cancer cases and 7.6 million cancer deaths occur in 2008 (Ferlay et al., 2010). The environmental, chemical, physical, metabolic and genetic factors play a direct and/or indirect role in the induction and deterioration of cancers. The limited success of clinical therapies including radiation, chemotherapy, immunomodulation and surgery in treating cancer, as evident by the high morbidity and mortality rates, indicates that there is an imperative need of new cancer management (Dai and Mumper, 2010). Drug discovery from medicinal plants has played an important role in the treatment of cancer and, indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied towards combating cancer (Balunas and Kinghorn, 2005). The National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity. It was estimated that 14 cancer drugs of the top 35 drugs in year 2000 based on worldwide sales were natural products and natural product derivatives (Shoeb, 2006). Thus, it is urgent to find more and more safe new compounds that kill cancer cells.

Table (2) showed the cytotoxic effects of the ethyl acetate and *n*-butanol fractions of the different parts of *C. erectus* against HepG2 and MCF-7 cell lines using the sulforhodamine B (SRB) method (Skehan et al., 1990). The SRB method, which was developed in 1990, remains one of the most widely used methods for *in vitro* cytotoxicity screening. It has been widely used for drug-toxicity testing against different types of cancerous and non-cancerous cell lines (Vichai and Kirtikara, 2006).

The ethyl acetate fraction of the stems and leaves showed high cytotoxic activity toward the HepG2 cell line with IC_{50} = 8.97 and 8.99 µg/ml respectively followed by the ethyl acetate fractions of fruits and flowers with IC_{50} = 10.12 and 11.29 µg/ml respectively. On the other hand the *n*-butanol fraction of leaves showed higher cytotoxic activity against HepG2 cell line with IC_{50} = 4.89 µg/ml followed by *n*-butanol fractions of flowers, fruits and stems with IC_{50} = 9.73, 20.68 and 23.9 µg/ml respectively. The cytotoxic activity of the ethyl acetate fractions toward MCF-7 showed that the ethyl acetate fraction of fruits has the high activity with IC_{50} = 10.82 µg/ml followed by the ethyl acetate fractions of stems, leaves and flowers with IC_{50} = 12.35, 15.84 and 20.86 µg/ml respectively. The *n*-butanol fraction of flowers showed the highest cytotoxic activity with IC_{50} = 7.60 µg/ml followed by leaves (15.04 µg/ml), stems (19.65 µg/ml) and fruits (24.01 µg/ml).

According to the American Cancer Institute (NCI), the criteria of cytotoxic activity for the crude extract is an IC_{50}< 20 µg/ml (Boik, 2001). Most of the fractions showed IC_{50} fall within the NCI criteria, thus these fractions are considered as promising anticancer potential.

Table 2. Cytotoxic activity (IC_{50}) of ethyl acetate and *n*-butanol fractions from successive fractionation of defatted methanol extract of *Conocarpus erectus* different parts on liver carcinoma cell line (HepG2) and breast carcinoma cell line (MCF-7)

Plant part	HepG2 (µg/ml)		MCF-7 (µg/ml)	
	Ethyl acetate	n-butanol	Ethyl acetate	n-butanol
Leaves	8.99±1.23	4.89±0.85	15.84±0.55	15.04±.60
Stems	8.97±0.72	23.9±1.17	12.35±0.48	19.65±1.34
Flowers	11.29±0.17	9.73±0.38	20.86±0.91	7.60±0.03
Fruits	10.12±1.03	20.68±0.70	10.82±1.06	24.01±1.25

3.3 As Antimicrobial Agents

Infectious disease caused by bacteria, viruses, fungi and parasites are still a major threat to public health, despite of the tremendous progress in human medicine Bacterial and fungal pathogens have evolved numerous defense mechanisms against antimicrobial agents, and resistance to old and newly produced drugs is on the rise (Buvaneswari, et al., 2011). The development of microbial resistance to available antibiotics due to random selection and possible side effects, have led some authors to investigate the antimicrobial activity of indigenous medicinal plants in many parts of the world (Zampini et al., 2009; Abdel-Hameed et al., 2008; Barbour et al., 2004).

In this study, the antibacterial activity of defatted methanolic extracts of leaf, stem, flower and fruit of C. erectus was assessed against two Gram-positive bacteria (*S. aureus* and *B. subtilis*), three Gram-negative bacteria (*E. coli, K. pneumonia* and *P. aeruginosa*) and an acid-fast bacterium (*M. phlei*). The largest detected inhibition zones for extracts were detected in Gram-positive bacteria followed by the acid fast bacterium, *M. pheli*, and then Gram-negative bacteria (Table 3).

Table 3. Zones of inhibition of defatted methanol extracts of different parts of *Conocarpus erectus* against different types of bacteria

Bacteria	Part used			
	Leaves	Stem	Flower	Fruit
	Inhibition zone			
Staphylococcus aureus	21.5 ± 0.31	21.0± 0.67	23.0± 0.47	22.0± 0.61
Bacillus subtilis	22.0 ± 0.87	21.0± 0.11	22.0± 0.72	21.5± 0.87
Mycobacterium pheli	15.5± 0.56	16.5± 0.40	17.5± 0.70	16.5± 0.42
Escherichia coli	11.5± 0.59	11.5± 0.61	12.5± 0.35	12.0± 0.42
Pseudomonas aeruginosa	12.0 ± 0.53	15.0± 0.11	18.0± 0.32	15.5± 0.59
Kelbsiella pneumoniae	10.5 ± 0.39	11.0± 0.62	13.0± 0.47	10.5± 0.63

The higher sensitivity of Gram-positive bacteria to extracts of natural plants has been documented (Dulger and Gonuz, 2004; Chaieb, et al., 2011). The phenol equivalent of extracts was calculated for each extract (Table 4). Extracts demonstrated antibacterial activity equivalent to phenol concentration ranging between 2.9% and 11.9 % (w/v) (Table 3). The highest phenol equivalent was found in *S. aureus* and ranged between 10 and 11.9 (Table 4). On the other hand the lowest phenol equivalent was found in the case of *K.*

pneumonia and ranged between 3.0 and 4.1 (Table 4). Flower and fruit extracts had relatively the highest antibacterial activity (Table 3, 4). More detailed antimicrobial studies are now ongoing on these extracts and their successive fractions.

Table 4. Phenol equivalent of different defatted methanol extracts *Conocarpus erectus* against different types of bacteria.

Bacteria	Part used			
	Leaves	Stem	Flower	Fruit
	% phenol coefficient			
Staphylococcus aureus	10.5 ± 0.61	10.0 ± 0.63	11.9 ± 0.59	11.0 ± 0.59
Bacillus subtilis	6.9 ± 0.55	6.4 ± 1.14	6.9 ± 0.32	6.4 ± 0.22
Mycobacterium pheli	8.6 ± 0.70	9.3 ± 0.40	10.0 ± 0.15	9.3 ± 0.31
Escherichia coli	5.8 ± 0.59	5.8 ± 0.42	6.4 ± 0.53	6.0 ± 0.61
Pseudomonas aeruginosa	2.9 ± 0.47	3.6 ± 0.61	4.9 ± 0.80	4.0 ± 0.87
Kelbsiella pneumoniae	3.0 ± 0.6	3.7 ± 0.4	4.1 ± 0.2	3.0 ± 0.2

3.4 Phytochemical Analysis

To get rapid idea about the major natural products classes present in the ethyl acetate and *n*-butanol fractions, some phytochemical tests with TLC and PC analysis were done. The results obtained showed that the phenolic compounds especially tannins and flavonoids are the major constituent in all fractions. In addition the *n*-butanol fractions contain moderate amount of saponins identified by froth formation test.

The result of phytochemical screening revealed that phenolic compounds constitute the major components of the most active fractions. Then the total amounts of phenolic, tannin and flavonoid components of EtOAc and n-BuOH fractions were chemically estimated. The content of total phenolics and tannins in the fractions were measured using the Folin-Ciocalteus assay and is expressed as gallic acid equivalent (GAE) while the total flavonoids were estimated using aluminum chloride method (expressed as rutin equivalent, RE). The Folin–Ciocalteu's assay is one of the oldest methods designed to determine the total contents of phenolics in foods or medicinal plants (Roginsky and Lissi, 2005). Table (5) showed that the ethyl acetate fraction of fruits and flowers contains high total phenolic contents equivalent to 303.45 and 301.15 mg/g GAE respectively whereas the ethyl acetate fractions of leaves and stems have the lowest total phenolic contents (186.21 and 181.61 mg/g GAE). On the other hand the *n*-butanol fractions of fruits and stems have the higher total phenolic contents equivalent to 418.39 and 316.09 mg/g GAE respectively. The ethyl acetate fraction of flowers and *n*-butanol fractions of leaves (table 5) have the highest total flavonoid contents equivalent to 64.17 and 51.36 mg/g RE respectively. The estimation of total casein-adsorbed tannin content of the ethyl acetate and *n*-butanol fractions by FCR method (table 5) revealed that the three *n*-butanol fractions of stems, fruits and flowers have the highest contents equivalent to 165.52, 163.22 and 163.22 mg/g GAE respectively whereas the remain fractions contain the lowest contents (16.09-75.86 mg/g GAE).

HPLC chromatogram of eight standard phenolic compounds mixture (see supplementary data); gallic acid, catechin, quercetin-3-glc-6-gallic acid, rutin, quercetin-3-glc, kaempferol-3-glc, quercetin and apigenin; was obtained after several trials to get good separation.

Calibration curve (see supplementary data) for each compound in the standard mixture was obtained with linearity range of 3.125-50 µg/ml for gallic acid, rutin, and quercetin, 2.5-40 µg/ml for catechin, 1.25-20 µg/ml for quercetin-3-glc-6-gallic acid, quercetin-3-glc, kaempferol-3-glc and apigenin. Regression equation and correlation coefficient (see supplementary data) revealed a good linearity response for method. The presence or absence of the eight standard phenolic compounds in the ethyl acetate and n-butanol fractions were done by comparing the HPLC chromatograms of them with the HPLC chromatogram of standard based on the retention time at the same conditions. The quantity of each identified peak in the fractions was estimated from the calibration curve for each component in the standard mixture (table 6). It was noticed that the gallic acid and rutin identified in all fractions with different percentage while the other identified compound may be present, traces or absent in the different fractions. The ethyl acetate fraction of fruits has the highest content of gallic acid (95.26 mg/g) followed by ethyl acetate fractions of leaves (42.77 mg/g), flowers (38.77 mg/g) and stems (31.99 mg/g). The rutin compound found with high content in both ethyl acetate fractions of flower (84.55 mg/g) and leaves (44.81 mg/g) whereas the other fraction found in rang (6.62-18.97 mg/g). Now, large scale extraction take place followed by chromatographic isolation and separation of the unknown compounds and identify it through spectroscopic and chemical analysis.

3.5 Relationship between Total Phenolic, Flavonoid and Tannin Contents with Antioxidant and Anticancer Activities

Data of correlation (see supplementary data) showed linear correlation appeared between the total phenolic contents of the ethyl acetate & n-butanol fractions with reducing power, free radical scavenging activity and total antioxidant capacity with excellent correlation coefficient (R^2 = 0.941 for reducing power, R^2 = 0.925 for free radical scavenging activity and R^2 = 0.779 total antioxidant capacity). On the other hand there is no correlation between the total flavonoids with reducing power, free radical scavenging activity and total antioxidant capacity. Positive correlation appeared between the total tannin contents of the different fractions with the total antioxidant (R^2 = 0.834), free radical scavenging activity (R^2 = 0.697) and reducing activity (R^2 = 0.797). The positive correlation between the total phenolic and tannin contents with the three antioxidant parameter revealed that the phenol and tannin compounds are the only or the major class responsible for the activity. The negative correlation between the total flavonoids content of the fractions with the three antioxidant parameter can be also attributed to the high content of other phenolic and tannin compounds which hide the effect of flavonoid compounds. The correlation between the total content of phenolics and the antioxidant capacity were studied by many authors. Several studies established a linear correlation between the total content of phenolics and the antioxidant capacity (Cai et al., 2004; Kumaran and Karunakaran, 2006) whereas some studies reported that there is no correlation (Yu et al., 2002).

Plant phenolics constitute one of the major groups of compounds acting as primary antioxidant or free radical terminators. Phenolic compounds such as flavonoids, phenolic acids and tannins are considered to be the major contributor to the antioxidant activity of vegetables, fruits or medicinal plants. The antioxidant activity of the phenolic compounds were attributed to its redox properties, which allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers and have also metal chelating properties (Rice-Evans et al., 1996). These compounds have been effective in many health related properties such as anticancer, antiviral, anti inflammatory activities, effects on capillary fragility and ability to inhibit human platelet aggregation (Cai et al., 2004; Gulcin et al., 2004).

Table 5. Total amount phenolic, flavonoid and tannin compounds of ethyl acetate and *n*-butanol fractions from successive fractionation of defatted methanol extract of *Conocarpus erectus* different parts

Plant part	Total phenols (mg gallic acid equivalents/g plant extract)[a]		Total flavonoids (mg rutin equivalents/g plant extract)[b]		Total tannins (mg gallic acid equivalents/g plant extract)[c]	
	Ethyl acetate	*n*-butanol	Ethyl acetate	*n*-butanol	Ethyl acetate	*n*-butanol
Leaves	186.21±6.89	328.74±10.53	35.31±0.25	51.36±0.74	16.09±3.98	75.86±6.89
Stems	181.61±3.98	416.09±14.35	14.78±0.36	8.47±0.32	18.39±3.98	165.52±13.79
Flowers	301.15±10.53	337.93±11.94	64.17±0.43	32.26±0.78	55.17±6.89	158.62±11.94
Fruits	303.45±6.89	418.39±7.96	35.14±0.64	29.50±0.45	18.39±3.98	163.22±14.35

[a]*Total phenols expressed by mg equivalent to gallic acid /g extract.*
[b]*Total flavonoids expressed by mg equivalent to rutin /g extract.*
[c]*Total tannins expressed by mg equivalent to gallic acid /g extract.*

Table 6. Quantity of some phenolic compounds (mg/g extract) identified in ethyl acetate and *n*-butanol fractions from successive fractionation of defatted methanol extract of *Conocarpus erectus* different parts

Plant part	Leaves		Stems		Flowers		Fruits	
	Ethyl acetate	*n*-butanol	Ethyl acetate	*n*-butanol	Ethyl acetate	*n*-butanol	Ethyl acetate	*n*-butanol
Phenolic compounds								
Gallic acid	42.77	10.46	31.99	6.77	38.77	4.86	95.26	25.62
Catechin	nd	7.48	t	8.18	t	7.70	t	t
Quercetin-3-glc-6-gallic acid	t	5.09	5.64	Unresolved peak	12.15	t	t	Unresolved peak
Rutin	44.81	18.97	14.90	Unresolved peak	84.55	15.81	16.57	6.62
Guercetin-3-glc	t	t	t	t	39.62	Unresolved peak	9.27	9.16
Kaempferol-3-glc	t	t	t	t	t	15.79	9.41	5.13
Quercetin	7.23	9.65	t	t	Unresolved peak	t	t	nd
Apigenin	23.06	12.23	t	nd	17.88	t	t	t

t = traces
nd = not detected

Only a moderate correlation appeared between the total phenolic and tannin contents of the fractions with the cytotoxic effect toward the cell line HepG2 (R^2 = 0.471 and 0.457 respectively) whereas there is no correlation with the total flavonoids. On the other hand there is no correlation between the total phenolic, flavonoids and tannin contents with the cytotoxicity toward the cell line MCF-7 with reducing power, free radical scavenging activity and total antioxidant capacity. This indicated that the phenolic components of these fractions play a weak role in cytotoxic activity and there is another natural product classes (may be saponin compound as detected by froth formation) play a part in cytotoxic activity.

From the above results in this paper, the phenolic compounds are only responsible for the antioxidant activity and not only responsible for anticancer properties but it can be play indirect part as cancer prevention by protects cells from the damage caused by free radicals. Many studies have revealed that intake of natural antioxidants is correlated with low incidence of cancer, heart disease, diabetes, and other diseases associated with ageing (Cai et al., 2004). Phenolics in vegetables, fruits, spices, and medicinal herbs might prevent cancer through antioxidant action and/or the modulation of several protein functions. Phenolics may inhibit carcinogenesis by affecting the molecular events in the initiation, promotion, and progression stages (Yang et al., 2001). On the other hand, some phenolics including phenolic acids, flavonoids quinones coumarins lignans stilbenes and tannins, etc. possessed potent antioxidant activity and also had anticancer/anticarcinogenic/ antimutagenic activities (Cai et al., 2004).

4. CONCLUSION

In conclusion, this is the first report on the phytochemical and biological properties of *Conocarpus erectus* growing in Taif governorate, KSA. The results of this study provide evidence that different parts; leaves, stems, fruits and flowers; have antioxidant, anticancer and antimicrobial properties. Also, phenolic compounds especially tannins are the major components of this species. More *in vivo* and *in vitro* studies along with detailed phytochemical investigation are needed in the hope to can use this plant (crude extract, fractions, sub-fractions or pure compounds) in the prevention and therapies of some diseases.

ACKNOWLEDGEMENTS

The authors are very grateful to Taif University, Kingdom of Saudi Arabia for supporting this work (Project no. 1-432-1214).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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