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Research Article

Cytotoxic Activity Assesment of Secondary Metabolites from *Tecomaria capensis* v. *aurea*

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ABSTRACT
Plants have been used for medical goal since the beginning of human being history; they are the basis of recent medicine. This study was conducted to determine the potential anticancer activity of bioactive compounds from *Tecomaria capensis* (Thunb.) Spach var. *aurea* [family Bignoniaceae] leaves against brine shrimp and human colon cancer cells (HCT-116) *in vitro*. Eight compounds were isolated from 85% methanol leaves extract and their structures were elucidated using ^{1}HNMR, ^{13}CNMR, mass spectroscopy and chemical methods. The isolated compounds were: quercetin 4'-*O*-methyl ether (**1**), rutin (**2**), myricetin (**3**), kaempferol 4'-*O*-methyl ether (**4**), luteolin (**5**), luteolin-7-*O*-*β*-D-glucuronopyranoside (**6**), apigenin (**7**) and apigenin-7-*O*-*β*-D-glucuronopyranoside (**8**). Moreover these compounds were isolated from *T. capensis* var. *aurea* leaves for the first time. *In vitro* biological investigations of the extracts and pure compounds indicated that; the investigated methanol extract, **1**, **2**, **3** and **7** possessed a strong activity among the cytotoxic parameters against brine shrimp; whereas compounds **1**, **3**, **7** and **8** showed potent inhibitory activity against colon cancer cells with LC_{50} values of 9.5, 6.5, 14 and 14.5 µg/ml, respectively. The results thus indicate efficacy for anticancer activity of the main constituents of the crude methanol extract of the chosen plant.

Keywords: brine shrimp, anticancer, methanol extract, flavonoids, myricetin, Bignoniaceae

INTRODUCTION

Cancer and tumor are the dangerous disease these days; moreover cancer is the uncontrolled cells growth followed by cells contravention into the surrounding cells and metastasized all over the body parts. Nearly chemotherapeutic drugs for cancer treatment are molecules specified and isolated from plants or their synthetic derivatives. Plants have been used as the essential element of traditional medicine systems to serve people all over the world for thousands of years[1]. Globally, at least 119 compounds derived from 90 plant species can be believed as important drugs. 74% of these substances were found by the chemical studies through the isolation of the bioactive constituents from plants used in traditional medicine[2]. An assessment of the World Health Organization validated that these ethical medical systems hold to play an important role in the primary health care of about 80% of the world's residents[3]. Chemotherapy provides some repose to cancer patients, but they are highly toxic, and minimal quantity of injected drug only can reach the cancerous tissue and may damage the normal system mostly bone marrow, epithelial tissue, reticuloendothelial system and gonads[4]. Plant derived compounds have played an important role in the evolution of several clinical useful anticancer agents[5-7].

Novel methodologies are required for its control and treatment and it is the leading health problems in all over the world[8]. 60 % of FDA approved anticancer drugs are from the plant origin. Recent times plants and plant derived compounds showed promising anticancer efficacy against various cancer cell lines and experimental samples[9]. The family Bignoniaceae includes 120 genera with 800 species mainly distributed within tropical and neotropical regions of America, Asia and Africa; however, some species are also used worldwide as ornamentals[10]. Particular groups of natural products from Bignoniaceae have been shown to have potential healing uses, such as antimicrobial activity (anthraquinones, flavonoids, phenylpropanoid glycolsides isolated from members of the genera *Tabebuia* and *Arrabaidea*)[11-15], and antiparasitic activity (antimalarial naphthoquinones isolated from the barks of *Sterespermum kunthianum*[16] and *Tabebuia incana*)[17]. The ethnobotanical uses of both complete extracts and isolated secondary metabolites comprise from applications as insect repellents (*Mansoa* sp. extracts) to systemic utilization (*Tecoma stans* infusions used as antidiabetic)[18,19]. *Tecomaria capensis* (Bignonaceae) is known as Capehoney suckles; it is a climber common to the tropical zone[20]. It is grown as an ornamental plant in gardens. Traditionally the leaves and flowers were used to treat pneumonia, enteritis, diarrhoea, fragrance and tonic[21]. It was reported to show antimicrobial[21], antifungal[21], antipyretic[20] and antioxidant activity[22]. It seems that one of the most promising antioxidant compounds are flavonoids; while flavonoids are considered very efficient radical scavengers and found in almost every plant.

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Antioxidant properties of flavonoids are probably related to their polyphenolic structure[23]. Flavonoids can be found as aglycon or as glycosides, more than 8000 of these polyphenolic compounds are identified by the presence of three rings in the molecule: two aromatic and one heterocyclic; so it can potentially bind to double stranded DNA (dsDNA); depending on reaction conditions antioxidative as well as pro-oxidative activities of flavonoids have been found in various systems[24,26]. *Tecomaria capensis* dried and powdered bark infusions are taken for sleeplessness[27]. Also *T. capensis* is included in the list of African plants estimated for *in vitro* antiplasmodial activity against *Plasmodium falciparum*[28]. Previously methanol extract of *T. capensis* leaves reported as antimicrobial[29] and antioxidant[30]; and its bark is indicated as antidiarrheal and anti-inflammatory agent[31]. Hence in the preset study a common Bignoniaceae member botanically identified as *Tecomaria capensis* v. *aurea* was selected with its methanol extract and pure compounds for anticancer evaluation against brine shrimp and HCT-116 cell line employing *in vitro* protocol.

MATERIALS AND METHODS

Collection of plant materials: The leaves of *Tecomaria capensis* v. *aurea* an ornamental plant were collected from Helwan University Garden, Ain-Helwan, Egypt. The plant was taxonomically identified by Dr Wafaa M. Amer, Professor of Plant Taxonomy, Faculty of Science, Cairo University. Voucher specimens (Reg. No. T-1) of the plant was deposited at the herbarium, Medicinal Chemistry Department, Theodor Bilharz Research Institute, Giza, Egypt.

General

Experimental Procedures

^{13}CNMR (125 MHZ, DMSO-d_6) and ^{1}HNMR (500 MHZ, DMSO-d_6) spectra were recorded on JEOL-GX-spectrometer for all compounds except compound 3; its ^{1}HNMR (300 MHz) and ^{13}CNMR (75 MHz) data were recorded on JEOL-GLM spectrometers in DMSO-d_6. The chemical shifts were expressed in δ (ppm) with reference TMS and coupling constant (*J*) in Hertz. GC–MS spectral data were determined on an ESI–MS was measured on a Finnigan TSQ 700 GC/MS coupled with a Finnigan electro spray source. Melting points were determined on an electrothermal apparatus. The absorbance measurements for flavonoid compounds were recorded using the UV-Vis spectrophotometer Spectronic 601(Milton Roy, USA). Paper chromatography was carried out on Whatman No. 1 paper sheets (Whatman Ltd., England). Cellulose C (Merck), Sephadex LH 20 (Pharmacia, Uppsala, Sweden), polyamide 6S (Riedel de Darmstadt, Germany) and silica gel (70-230 mesh, Merck) were used for column chromatography. Silica gel 60 GF_{254} (Merck, Germany) were used for TLC. The chromatograms and TLC plates were visualized under UV lamp Vilber Lourmat (VL-6LC France) at 365 and 254 nm, and the spots were appears by absorption of UV radiation and spraying with 1% $FeCl_3$ in methanol, 10% H_2SO_4, 5% $AlCl_3$ and Naturstoff reagent (NA).

Material and Chemicals

All solvents and reagents used were of analytical grade. Aluminum chloride, ferric chloride, Naturstoff, DMSO (dimethyl sulfoxide), potassium dichromate, α-naphthol, acetic anhydride, ammonium hydroxide, magnesium metal were purchased from (Merck Chemical Co.), all solvents and acids [petroleum ether, chloroform, methylene chloride, ethyl acetate, n-butanol, acetone, methanol, ethanol, acetic acid, hydrochloric acid and sulphuric acid], were purchased from (Sigma-Aldrich Co.). Phosphate Buffered saline (Sigma).

Extraction, fractionation and primary pharmacological screening

About 2 Kg air dried powdered leaves extracted with 8 liter of 85% methanol and filtered off. The filtrate was evaporated to dryness in vacuum. The consecutive partition fraction of methanol crude extract of leaves was then made by using different solvent systems [petroleum ether (15 gm), chloroform (32 gm) and ethyl acetate (24 gm)]. Because of it was bioactivity-guided investigation; our main aim to resemble which crude extract was bioactive, before going to fractionation of the crude extract. In this manner, the cytotoxic activity described below of four crude extract were done by using brine shrimp lethality bioassay. From this, we found that; methanol crude extract of leaves showed a significant cytotoxic activity. So it draws our attention to do the successive fractionation of methanol crude extract of leaves in this consideration.

Chromatographic Isolation of the compounds

Chromatographic isolation of methanol fractions

The defatted crude methanol extract (150 gm) was subjected to column chromatography (CC) packed on polyamide S column (Φ 7 x 125 cm) and eluted with H_2O followed by gradual increase of MeOH portions to afforded seven collective fractions I-VII, according to their chromatographic properties (fluorescence-UV light, and responses towards different spray reagents on PC). The fractions (500 ml each) were collected and monitored via Paper chromatography technique (PC) and thin layer chromatography (TLC), in solvent systems; 15% AcOH (S1) (PC), BAW (n-BuOH: AcOH: H_2O; 4:1:5 top layer) (S2) (PC), $CHCl_3$:MeOH; (7.5:2.5) (S3) (TLC) and $CHCl_3$:MeOH:H_2O; (7:3:0.1) (S4) (TLC). TLC chromatograms were examined under UV light followed by spray with H_2SO_4:MeOH (10:90 v/v); while PC chromatograms were examined under UV light followed by spray with $AlCl_3$ and/or Naturstoff reagent and/or $FeCl_3$ reagent; then similar fractions were collected together.

Firstly fraction I was found to be a free sugars and inorganic salts with no phenolic properties. Fraction II was eluted with 95:5 (H_2O:MeOH), fraction III was eluted via 85:15 (H_2O:MeOH), fraction IV was eluted with 70:30 (H_2O:MeOH), fraction V was eluted with 60:40 (H_2O:MeOH), fraction VI was eluted via 50:50 (H_2O:MeOH) and fraction VII was eluted with 100% MeOH to afford compounds (1-8). Fraction II (9 g) was subjected to repeated 3 exclusive column chromatography (CC) on Sephadex LH-20 and eluted with 95:5 (H_2O:MeOH) to give pure **2** (70 mg). Fraction III (10 g) was fractionated on cellulose C with 85:15 (H_2O:MeOH)

as an eluent, followed by a Sephadex LH-20 column (2×60 cm) using BIW (*n*-BuOH:2-propanol:H_2O, 4:1:5 v/v/v, organic layer) to afford pure **6** (36 mg) and **8** (31 mg). Fraction IV (13 g) concentrated and subjected to successive re-chromatography on a Sephadex LH-20 column with methanol as the eluting solvent to obtain compounds **1** (39 mg) and **3** (52 mg). Fraction V (9 g) was concentrated and submitted to further purification on sub column silica gel (2×60 cm) with gradient mobile phase $CHCl_3$ and $CHCl_3$:MeOH then purified with preparative TLC using solvent system $CHCl_3$:MeOH (7.5:2.5) to give compounds **4** (27 mg) and **5** (30 mg). Fraction VI (11 g) was eluted with $CHCl_3$:MeOH (6.5:3.5) and packed with silica gel (column grade) to afford compound **7** (42 mg). These isolated compounds were then employed in the brine shrimp lethality bioassay and colon cancer cell line (HCT-116).

Cytotoxic assay

HCT-116 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 µg/ml gentamycin. The cells were maintained at 37 ℃ in a humidified atmosphere with 5% CO_2 and were sub cultured two to three times a week. While eggs of *Artemia salina* are from (Artemia Inc., California) and saline artificial sea (Instant Oceanic, Marine land Labs, USA).

Pharmacological screening

Brine shrimp lethality bioassay method was utilized for pharmacological screening[32]. The eggs of brine shrimp, artemia salina leaches were taken in a conical flask and seawater (prepared by dissolving 38 gm of NaCl in one liter of distilled water) was added to it. Two days were allowed to hatch the shrimp and to become matured as nauplii. Measured toxicity of each sample was tested at 5, 10, 20, 100, 200, 400 and 600 ppm in 10 ml seawater with 1% DMSO (v/v). From each of these test solutions 100 µl were added to the prepared numbered glass test tubes containing 5 ml of sea water and 10 shrimp (larvae) nauplii and by using a Pasteur pipette take 10 living nauplii then were put to each of the tubes. Three replicates were used for each concentration and then a parallel series of tests with the standard potassium dichromate solution and the blank control one test tube containing the same volume of DMSO plus seawater and brine shrimp nauplii were used. After 24 hours, the test tubes were observed with a magnifying glass and number of survived nauplii in each test tube was counted. From this data, the percent of the lethality mortality of brine shrimp nauplii was calculated for each concentration in µg/ml. finally the data were analyzed and LC_{50} values calculated according to Reed-Muench method[33].

In vitro cytotoxicity testing

The antitumor activity was evaluated on Human colon carcinoma (HCT-116) cells. The cells were grown as monolayers in growth RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50µg/ml gentamycin. The monolayers of 10.000 cells adhered at the bottom of the wells in a 96-well microtiter plate incubated for 24 h at 37 ℃ in a humidified incubator with 5% CO_2. The monolayers were then washed with sterile phosphate buffered saline and simultaneously the cells were treated with 100 µ1 from different dilutions of tested sample in fresh maintenance medium and incubated at 37 ℃. A control of untreated cells was made in the absence of tested sample. A positive control containing Doxroubicin drug was also tested as reference drug for comparison. Six wells were used for each concentration of the test sample. Every 24 h the observation under the inverted microscope was made. The number of the surviving cells was determined under the inverted microscope was made. The number of the surviving cells was determined by staining the cell lysing using 33% glacial acetic acid and read the absorbance at 590 nm using ELISA reader (SunRise, TECAN, Inc, USA) after wall mixing. The absorbance values from untreated cells were considered as 100% proliferation. The number of viable cells was determined using ELISA reader as previously (ODt/ODc) x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots[34].

Preliminary phytochemical screening

Different solvent extracts of *Tecomaria capensis* v. *aurea* leaves extracts were subjected separately to the following phytochemical tests and the results were recorded in table **(**1**)**.

Test of saponins

About 10 gm of each extract was shaken with distilled water and filtered. This filtrate was shaken strongly and allowed to stand for about five minutes. The presence of voluminous froth indicates the presence of saponins[35].

Test for carbohydrates and glycosides

One gm of each extract was mixed with 10 ml of 50 % aqueous ethanol. 5 ml of each extract in ethanol were mixed with 0.5 ml of ethanolic α-naphthol solution followed by one ml of H_2SO_4 poured carefully on the wall of the test tube. The appearance of a violet ring between the two layers indicates the presence of carbohydrates and /or glycosides[36].

Test for sterols and/or triterpenes

About 10 ml of each extract in ethanol were evaporated to dryness. The residue was dissolved in 20 ml of chloroform solution and filtered. The filtrate was subjected to the following tests.

Salkowski test

To about 5 ml of the chloroform solution, an equal volume of sulphuric acid were added carefully. Formation of a red colour mentions the presence of sterols and /or triterpenes.

Liebermann-Burchard test

Evaporate about 5 ml of the chloroform solution of each sample to a small volume, about 1ml of acetic anhydride was added followed by 2 ml of H_2SO_4 poured carefully on the wall of the test tube and allow forming a lower layer. A reddish brown colour appeared at the junction between the two layers mention the presence of unsaturated sterols and/or triterpenes[37].

Test for alkaloids and/or nitrogenous bases

10 gm of each extract was mixed with 100 ml of dilute hydrochloric acid. The acidic extract was filtered, and converted to alkaline with ammonium hydroxide solution, followed by extraction with chloroform. The chloroform extract was evaporated till dryness and the extract was dissolved in 2 ml of HCl solution. The formation of very faint brown precipitate with Wagner's reagent and very slight precipitate with Mayer's reagent confirming the presence of nitrogen bases[38].

Test for tannins

About 2 gm of each extract was added to 20 ml of 50 % aqueous ethanol and filtered. Add few drops of ferric chloride solution; if a green colour obtained confirming the probability of existence of tannins[39].

Test for flavonoids

5 gm of each each extract sample was soaked overnight with 150 ml of 1% hydrochloric acid solution and filtered. The filtrate was subjected to flavonoid compounds tests as follows. About 10 ml of the filtrate was converted to alkaline with NaOH solution. The appearance of a yellow color denotes that the presence of flavonoids. About 5 ml of the filtrate was mixed with 5 ml of HCl acid solution and few pieces of magnesium metal were added. The formation of a red colour indicates the presence of flavonoids[40].

Acid hydrolysis of compounds 2, 6 and 8

5 mg of each isolated compound was reflux with 10 % HCl at 90 ^{o}C in aqueous methanol for about two hours; then the reaction solution was concentrated and extracted with chloroform solution. Tell chloroform extract to evaporate to dryness. Aglycons were identified by direct comparison with an authentic sample by Co-TLC which using solvent system ($CHCl_3$:MeOH; 9:1). Whereas the aqueous layer was neutralized with 2N KOH solution, filtered and concentrated and the sugar moieties were identified with direct comparison with authentic sugars by PC using solvent system, (iso-PrOH:*n*-BuOH:H_2O; 7:1:2) and silica gel TLC using solvent system, ($CHCl_3$:MeOH:H_2O, 30:12:4) and by; detection with aniline hydrogen phthalate, which showed that the sugars of 2 to be rhamnose and glucose; whereas for compounds 6 and 8 the sugar was glucuronic acid.

RESULTS AND DISCUSION

T. capensis v. *aurea* leaves dry powder was extracted with methanol, petroleum ether, chloroform and ethyl acetate and phytochemical screening was then conducted. The phytochemical screening tests indicated the presence of alkaloids, saponins, tannins, terpenoids, glycosides, carbohydrates, steroids, sterols and flavonoids in the methanol extract (Table 1). The chloroform, petroleum ether and ethyl acetate extracts constituents are found in (Table 1). Repeated chromatographic isolation and purification of 85% methanolic extract of *T. capensis* v. *aurea* leaves gives eight compounds; their structures were elucidated using some spectroscopic and chemical methods. The structure of the isolated compounds was formulated as quercetin 4'-*O*-methyl ether (1), rutin (2), myricetin (3), kaempferol 4'-*O*-methyl ether (4), luteolin (5), luteolin-7-*O*-*β*-D-glucuronopyranoside (6), apigenin (7) and apigenin-7-*O*-*β*-D-glucuronopyranoside (8).

Characterization of the target compounds

The chemical structure of each isolated compound was identified according to its ESI-MS, ^{1}H-NMR and ^{13}C-NMR data; these data are provided below.

*Characterization of Compound **1***

Depends on chromatographic properties and UV spectral data; compound 1 was expected to be quercetin derivatives like structure[40,41]. It is a yellow needels, m.p. 265 ^{o}C, R_f: 0.59 [S1], 0.64 [S2], it showed two major absorption bands in methanol, band I at 371 nm and band II at 255, 270 (sh) nm; also ESI-MS give a molecular ion peak at m/z 317 $[M+H]^+$. Based on spin coupling systems; two trademark were distinguish in its ^{1}HNMR spectrum the first one was recorded as a meta doublet at δ 7.64, a doublet of doublet at δ 7.61 and an *ortho* doublet of one proton at δ 7.04 which were characteristic to H-2', H-6' and H-5', respectively of 3',4'-disubistituted B-ring. The second coupling one was described as two *meta*- coupled doublets at δ 6.13 and δ 6.39 for H-6 and H-8, respectively of 5,7-dihydroxy group at A-ring. The downfield location of H-5' (about +0.2) demonstrated substitution at 4'-position. A singlet at δ 3.81 was assignable for OCH_3 group in 4'-OH. The ^{13}CNMR spectrum of 1 showed the typical 16-carbon of ^{13}CNMR for quercetin 4'-*O*-methyl ether moiety. The downfield shift of C-1', C-4' at 123.4 and 149.3, respectively affected by the methoxy group which appear at 55.6. The other carbons have been assigned throughout

Table 1: Preliminary phytochemical studies of *Tecomaria capensis* v. *aurea* leaves dry powder extracts

Solvents					
Phytoconstituent	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Aqueous
Saponins	+	-	-	+	+
Glycosides	+	-	-	+	-
Steroids	-	-	+	+	-
Carbohydrates	-	+	+	+	+
Alkaloid	-	+	+	+	+
Sterols	+	-	-	+	+
Teriterpens	+	+	+	+	+
Tannins	-	-	+	+	-
Flavonoids	+	+	+	+	-

+ Signified the presence of the constituents

- Signified the absence of the constituents

Table 2: ^{13}CNMR spectral data of 1, 2, 4, 6 and 8 (500, 125 MHz, DMSO d_6)

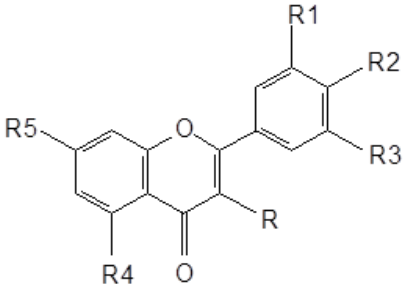
C.	1		2		4		6		8	
NO.	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
2	146.2	-	157.1	-	146.1	-	164.5	-	164.7	-
3	136.1	-	133.5	-	136.0	-	103.8	6.74 *s*	103.7	6.8 *s*
4	175.8	-	177.6	-	176.1	-	182.5	-	182.5	-
5	160.7	-	161.6	-	160.7	-	161.8	-	161.7	-
6	98.2	6.13 *d* (1.8)	99.1	6.21 *d* (2.5)	97.9	6.16 *d* (1.8)	100.2	6.93 *d* (1.9)	100.1	6.46 *d* (1.9)
7	163.9	-	164.1	-	164.01	-	163.4	-	163.3	-
8	93.4	6.39 *d* (1.8)	93.6	6.42 *d* (2.5)	93.4	6.43 *d* (1.8)	95.4	6.80 *d* (1.9)	95.3	6.83 *d* (1.5)
9	156.2	-	157.1	-	157	-	157.6	-	157.5	-
10	103.30	-	104.0	-	104.1	-	106.1	-	105.9	-
1'	123.4	-	121.4	-	123.4	-	121.7	-	121.3	-
2'	111.8	7.64 *d* (2.1)	115.6	-		-	114.1	-		-
2'/6'		-		7.52 *m*	130.1	8.11 *d* (8.4)		7.45 *m*	129.1	7.9 *d* (8.6)
3'	146.2	-	145.2	-			146.4	-		-
3'/5'		-		-	114.6	7.07 *d* (8.4)		-	116.7	6.9 *d* (8.6)
4'	149.3	-	148.7	-	160.7	-	150.5	-	162.3	-
5'	114.6	7.04 *d* (8.4)	116.7	6.85 *d* (7.5)		-	116.5	6.59 *d* (8.4)		-
6'	119.7	7.61 *dd* (8.4, 2.1)	121.7	-		-	119.7	-		-
OCH_3	55.6	3.81 *s*	-	-	55.6	3.77 *s*	-	-	-	-
1"		-	101.3	5.32 *d* (7.5)			100.2	5.2 *d* (7)	100.1	5.2 *d* (7)
2"		-	74.5				73.5		73.6	
3"		-	75.7				75.7		75.54	
4"		-	70.1				72.0		71.3	
5"		-	77				76.7		76.7	
6"		-	66.9	3.7			172.1		172.1	
1"'		-	101.1	4.4 *brs*						
2"'		-	70.7							
3"'		-	71.1							
4"'		-	72.5							
5"'		-	69.1							
6"'		-	18.5							

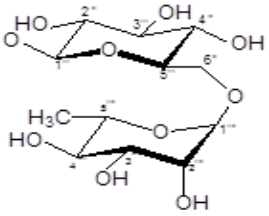
Δ δ in ppm and *J* values (Hz),were given in parentheses

comparison with the literature data[42,43]. Therefore 1 was recognized as quercetin 4'-*O*-methyl ether (Tamarixetin)[44].

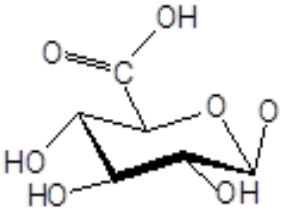
Characterization of Compound ***2***

The structure of 2 was predictable to be quercetin 3-*O*-rhamnosylglucoside depending upon the basis of its chromatographic data, UV spectral data in methanol, band I at 360 nm and band II at 232, 267 (sh) nm and complete acid hydrolysis. It is a pale yellow powder, m.p. 195 ^{o}C, R_f: 0.50 [S1], 0.54 [S2]. ESI-MS data give a molecular ion peak for the dimeric adduct at m⁄z 1221 $[2M+H]^+$ and the molecular ion peak at m⁄z 611.1 $[M+H]^+$ in its positive ESI-MS spectrum. Also the aglycone was proved to be quercetin on the basis of its fragment ion peak at m⁄z 449 $[M+H-162]^+$ and m⁄z 303 $[M+H-162-146]^+$ = $[aglycone+H]^+$. ^{1}HNMR exhibited an anomeric proton doublet signal of inner glucosyl moiety at δ 5.32 and H-6"' CH_3 as a doublet at 1.1 (J = 6.5 Hz) of a terminal α-rhamnosyl moiety; and the remaining glucosyl protons appears at δ 3.30-3.49 (4H, m, H-2", H-3", H-4", H-5"). The H-1"' (*brs*) signal at δ 4.4 together with the downfield appearance of H-6" at δ 3.7 was a confirmative proof for (1"'→6") rhamnosyl glucoside linkage[45]. Like previous quercetin 4'-*O*-methyl ether (Tamarixetin), the ABX (H-2⁄5⁄6', B-ring) and AM (H-8⁄6, A-ring) spin coupling systems were confirmative documents in their δ and J values for quercetin aglycone[46]. ^{13}CNMR spectrum, exhibited typical 15-carbon of ^{13}C-resonances for a 3-*O*-subistituted quercetin[43]. δ values of the 12 carbon sugars





O-rutinosyl



Glucuronic acid

	R	R1	R2	R3	R4	R5
1:	OH	OH	OCH_3	H	OH	OH
2:	O-rutinosyl	H	OH	OH	OH	OH
3:	OH	OH	OH	OH	OH	OH
4:	OH	H	OCH_3	H	OH	OH
5:	H	OH	OH	H	OH	OH
6:	H	OH	OH	H	OH	Glu
7:	H	H	OH	H	OH	OH
8:	H	H	OH	H	OH	Glu

Skeletal structure of compounds 1-8

^{13}C-resonances were in complete agreement with those of a rutinoside moiety, particularly the downfield shift of C-6" (about +7ppm) and upfield of C-5" (about 1ppm) due to 1'"→6" *O*-glycosedation[42,43,47]. Therefore 2 was identified as quercetin 3-*O*-α-L-rhamnopyranosyl-(1'"→6")-*β*-D-glucopyranoside (Rutin)[48].

*Characterization of Compound **3***

As shown from the spectral data, compound 3 appears to be myricetin; it is a yellow needels, m.p. 356 °C, R_f, 0.17 [S1], 0.30 [S2], its ESI-MS spectrum gives a molecular ion peak at *m/z* 319 $[M+H]^+$. Its chromatographic properties and UV spectral data in methanol; band I at 377 nm and band II at 254, 272 (sh) nm. ^{1}HNMR spectrum showed in its aromatic region a spin coupling system appeared as two *meta* doublets coupled protons, which were characteristic to H-6 and H-8 of 5,7-dihydroxy A-ring at *δ* 6.19 and 6.38, respectively. Beside a singlet signal attributed to H-2′⁄6' at *δ* 7.33. The ^{13}CNMR spectrum of 3 showed the typical 14 ^{13}CNMR signals for myricetin moiety. ^{13}CNMR spectrum showed a signals at *δ* 146.73 (C-2), 135.98 (C-3), 175.61 (C-4), 160.71 (C-5), 98.40 (C-6), 164.06 (C-7), 93.21 (C-8), 156.08 (C-9), 120.73 (C-1′), 107.41 (C-2′, 6′), 145.69 (C-3′, 5′), 135.87 (C-4′), 102.96 (C-10). ^{1}HNMR and ^{13}CNMR spectral data were in agreement with literature data[40].

*Characterization of Compound **4***

Depending on its chromatographic properties UV spectral data; compound 4 was expected to be kaempferol 4'-*O*-methyl ether[40,41]. It is a yellow powder, m.p. 226 °C, R_f: 0.30 [S3], 0.68 [S4], UV spectra in methanol showed band I at 369 nm and band II at 268, 325 (sh) nm; ESI-MS give a molecular ion peak at m/z 301 $[M+H]^+$. This evidence was supported by ^{1}HNMR spectrum, showed two characteristic spin coupling systems, each of two doublets in its aromatic region, for 4'-methoxy B-ring and 5,7-dihydroxy A-ring of a kaempferol moiety. The 1st system was described as AX of two *ortho* doublets at *δ* 8.11 and 7.07 for H-2′⁄6' and H-3′⁄5', respectively, while the 2nd system was of two *meta* doublets at *δ* 6.43 and 6.16 of H-8 and H-6, respectively. A singlet at δ 3.77 ppm was characteristic for OCH_3 group in 4'-OH. The 4'-*O*-subistituted kaempferol moiety was confirmed from its 14 resonances in the ^{13}CNMR spectrum as it was interpretated above, specially signals at 130.1 and 114.6 of C-2′⁄6' and 3′⁄5' substanial for 1',4'-disubstituted B-ring of kaempferol aglycone. The methoxy group at 55.6 reflected on a downfield shift of C-4'. Accordingly 4 was identified as kaempferol 4'-*O*-methyl ether (kaempferide)[49].

*Characterization of Compound **5***

On the bases of its chromatographic properties, compound 5 was expected to be luteolin-like structure. It is a yellow needles, m.p. 329 °C, R_f: 0.41 [S3], 0.58 [S4], UV spectra in methanol showed band I at 350 nm and band II at 290 (sh) nm and ESI-MS give a molecular ion peak at m/z 287 $[M+H]^+$. Two characteristic spin coupling systems were recognized in its ^{1}HNMR spectrum. The first system was appeared as a *meta* doublet at *δ* 7.40 a doublet of doublet at 7.37 and an *ortho* doublet of one proton at *δ* 6.85, which were assignable to H-2', 6' and 5' of 3', 4'-dihydroxy B-ring. The second coupling system was recorded as two *meta* coupled doublets at *δ* 6.21 and 6.42 for H-6 and H-8, respectively of 5,7-dihydroxy A-ring. Also a singlet signal corresponding to H-3 at *δ* 6.64. From all these findings compound 5 was identified as 3',4',5,7-tetrahydroxy flavone (luteolin)[42].

*Characterization of Compound **6***

Compound 6 indicated fundamentally the same chromatographic conduct, chemical and ^{1}HNMR spectral data to **5** except for the ^{1}HNMR spectroscopic data assignable to the glucuronyl sugar moiety. It is a yellow amorphous powder, m.p. 244 °C, R_f: 0.35 [S1], 0.40 [S2], UV spectral data in methanol showed band I at 347 nm and band II at 268 (sh) nm, its ESI-MS give a molecular ion peak at m/z 463 $[M+H]^+$ and at m/z 287 $[M+H-176]^+$ indicating the loss of glucuronic acid from the last fragment = $[aglycone+H]^+$. Acid hydrolysis of 6 afforded glucuronic acid in the aqueous phase along with luteolin which showed in the organic phase. The 7-*O*-glycosedation of 6 was detected from the downfield shift of both H-6 and H-8 (about + 0.2 ppm with respect to the reported unsubstituted A-ring)[40]. And also the *β*-anomeric at *δ* 5.2 (*d*, *J* = 7 Hz) corresponding to the adjustment of $^4C_1$-pyranose structure of sugar moiety. In ^{13}CNMR spectrum slight upfield shift of C-7 at 163.4 ppm and downfield shift of C-6 and C-8, was demonstrative to the glycosedation at 7-OH[43]. Sugar moiety was confirmed as

glucuronic acid due to the resonance of C-6" at 172.1 ppm. The sugar moiety assumes $^4C_1$-pyranose adaptation on the basis of the typical *J*- and *δ*-values in its ^{1}H and ^{13}CNMR spectral data[50]. From all above information compound 6 was distinguished as luteolin 7-*O*-*β*-D-$^4C_1$-glucuronopyranoside[51].

*Characterization of Compound **7***

Based on its chromatographic properties, 7 was expected to be apigenin; it is a yellow powder, m.p. 346 ºC, R_f: 0.64 [S3], 0.76 [S4], UV spectra in methanol showed band I at 335 nm and band II at 266, 295 (sh) nm, ESI-MS give a molecular ion at m/z 271 $[M+H]^+$. ^{1}HNMR displayed an AX coupling system in its aromatic region of two *ortho* doublets each integrated to two protons of 1',4'-disubistituted B-ring at *δ* 7.45 and 6.90 assignable to H-2'/6' and H-3'/5', respectively. Beside a 5,7-dihydroxy A-ring was concluded from 1 proton of the two meta signals at *δ* 6.93 and 6.80, which were assignable to H-6 and H-8, respectively. As well as, a singlet signal attributed to H-3 at *δ* 6.74. Based on the above described data compound 7 was identified as 5,7,4'-trihydroxy flavones (apigenin)[46].

*Characterization of Compound **8***

It indicated fundamentally the same chromatographic conduct, chemical and ^{1}HNMR spectral data to 7 except for the ^{1}HNMR spectroscopic data assignable to the sugar moiety. It is a yellow crystals, m.p. 265 ºC, R_f: 0.34 [S1], 0.63 [S2], UV spectra in methanol showed band I at 333 nm and band II at 270 (sh) nm, ESI-MS give a molecular ion peak at m/z 447 $[M+H]^+$ and at m/z 271 $[M+H-176]^+$ indicating the loss of glucuronic acid = $[aglycone+H]^+$. Acid hydrolysis of 8 afforded glucuronic acid in the aqueous phase along with apigenin in the organic phase. The glycosedation at 7-OH was shown from the downfield shift of both H-6 and H-8 (about +0.2 ppm) and the *β*-anomeric proton signal at *δ* 5.2 (*d*, *J* = 7 Hz). In ^{13}CNMR spectral data, glycosedation at 7-OH was detected from slight upfield shift of C-7 and downfield shift of both C-6 and C-8[42]. Moreover, the sugar moiety was concluded as glucuronic acid, from the resonance of C-6" at 172.1 ppm. The sugar moiety was found to have a $^4C_1$ pyranose conformation based on the typical *J* and *δ* values in its ^{1}HNMR and ^{13}CNMR spectral data[50]. Finally compound 8 was identified as apigenin 7-*O*-*β*-D-$^4C_1$-glucuronopyranoside[51].

Brine shrimp lethality activity

Brine shrimp lethality bioassay is a simple method conducted cytotoxic activity of bioactive compounds. It is a process established on the death ability of compounds on brine shrimp (*Artemia salina*)[52]. It's a preliminary toxicity test screening and followed by further experiments on cell line or mammalian animal models[53].

As shown in Fig. 1; methanol leaves extract exhibited the highest brine shrimp lethality with LC_{50} value of 30µg/ml followed by ethyl acetate, chloroform and petroleum ether fractions with LC_{50} values of 110, 435 and 515µg/ml, respectively. Myricetin showed potent significant cytotoxic activity with LC_{50} value of 5µg/ml followed by compounds 1, 2, 7 and 8 with LC_{50} values 10, 12, 14 and 18µg/ml, respectively; whereas compounds 4, 5 and 6 exhibited a lower cytotoxic activity with LC_{50} values of 210, 65 and 70µg/ml, respectively (Fig. 2 and 3).

Cytotoxicity against colon cancer HCT-116 cells

Fig. 4, shown the treatment of HCT-116 cells with leaves methanol extract depressed remarkably the cell growth with IC_{50} 21*µ*g/ml, but EtOAc extract has a low activity of IC_{50} 49*µ*g/ml. The findings of the present study showed differences between the cytotoxic activities of crude MeOH extract and extracts from partition; this indicates that the active principles might be more concentrated in methanol fraction. This suggests that ***T. capensis*** leaves contain several antitumor principles with different polarities as shown throw the phytochemical study herein. All the isolated compounds showed cytotoxic activities. Among the tested compounds, myricetin was the highest growth inhibitor of HCT-116 cells with IC_{50} 6.5*µ*g/ml followed by compound 1 which exhibited a strong cytotoxic effect against HCT-116 cells with IC_{50} 9.5*µ*g/ml, (Fig. 5). Compounds 7 and 8 were cytotoxic at IC_{50} 14 and 14.5*µ*g/ml, respectively (Fig. 6). The treatment with 2, 4, 5 and 6 had effect on the growth of HCT-116 cells with IC_{50} 28, 31, 25 and 29.5*µ*g/ml, respectively (Fig. 5 and 6).

Cytotoxicity assays and cell viability are used for cytotoxicity tests of plants chemical constituents. Flavonoids are reported to have a range of biological activities including antioxidant, anti-inflammatory, and anti-tumorigenic properties. In particular, flavonoids are active at different stages of cancer development by protecting DNA from oxidative damage, activating carcinogen metabolism and detoxification, preventing cellular proliferation, and/or inducing cellular cytotoxicity[53,54]. Furthermore, it is suggested that flavonoid compounds are incorporated into cells then, increases intracellular Reactive Oxygen Species (ROS) levels which are reactive molecules containing oxygen such as peroxides and superoxide and then exert cytotoxicity. Reported data suggest that quercetin 4'-*O*-methylated metabolite may provide protection against oxidatively induced cellular damage *in vivo*[55]. The flavonol glycoside of quercetin molecule is rutin (quercetin-3-*O*-rutinoside); it serves an important rules in the human being health such as its potent scavenging activities which acts on oxidizing species such as superoxide radical, hydroxyl radical and peroxyl radical[56]. Previous studies have demonstrated that myricetin and quercetin, showed inhibitory effect on mammalian TrxR and also that activity may be because of these flavonols are readily autooxidizable forming superoxide radicals[57]. In addition, kaempferol may possess protective effects against gastric cancer[58]. Luteolin is cytotoxic towards H4IIE cells inducing an apoptotic cell death accompanied by induction of oxidative stress measured as increase in malondialdehyde formation[59]. Apigenin-7-*O*-*β*-D-glucopyranoside showed a strong anticancer activity against breast MCF-7 cell line[60]. Apigenin is a potential chemopreventive and chemotherapeutic agent in leukemia due to the stimulation on signaling pathways that provoke inhibition of cell proliferation and cell cycle arrest of fast cycling cells[61]. These findings support the traditional use of this plant in the remediation of tumor diseases.

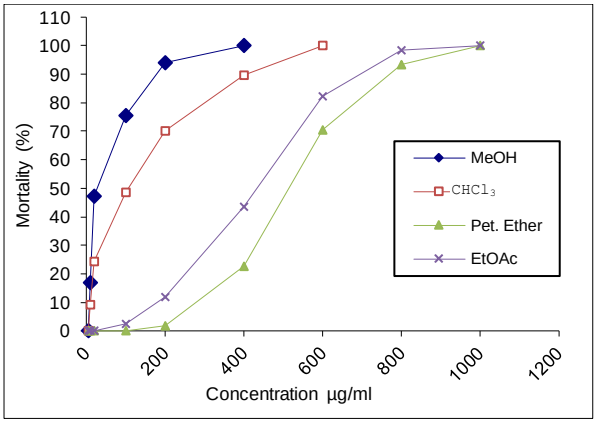


Figure 1: The cytotoxic activity of *T. capensis* methanol, chloroform, petroleum ether and ethyl acetate extracts against brine shrimp

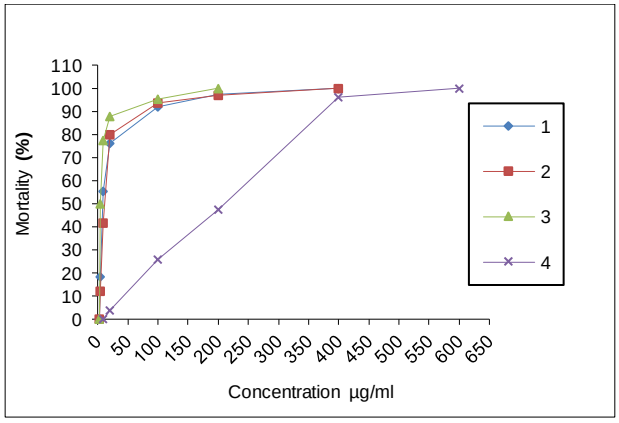


Figure 2: The cytotoxic activity of compounds 1,2, 3 and 4 against brine shrimp

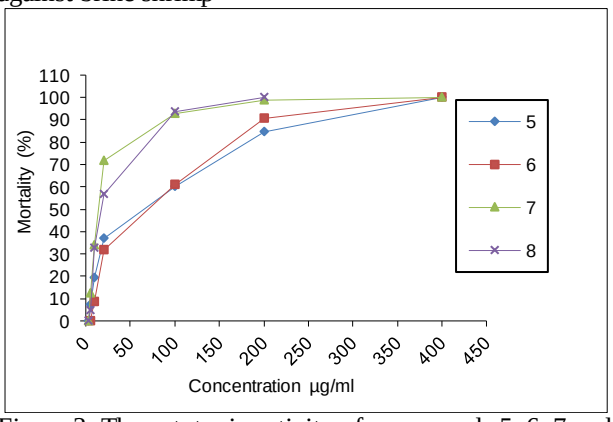


Figure 3: The cytotoxic activity of compounds 5, 6, 7 and 8 against brine shrimp

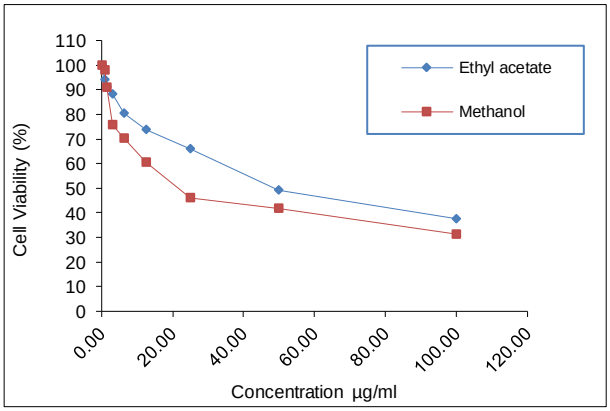


Figure 4: The cytotoxic activity of *T. capensis* methanol and ethyl acetate extracts against HCT-116 cells

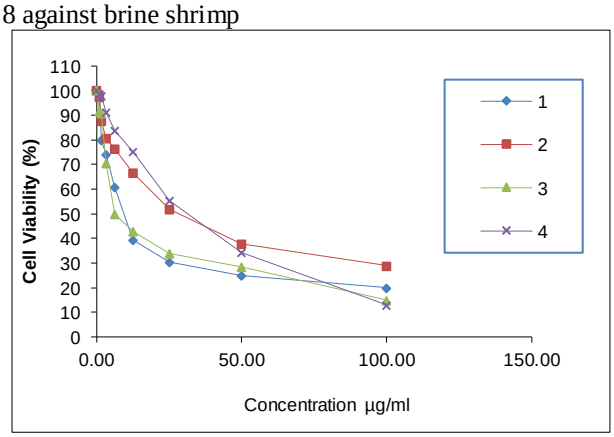


Figure 5: The cytotoxic activity of compounds 1, 2, 3 and 4 against HCT-116 cells

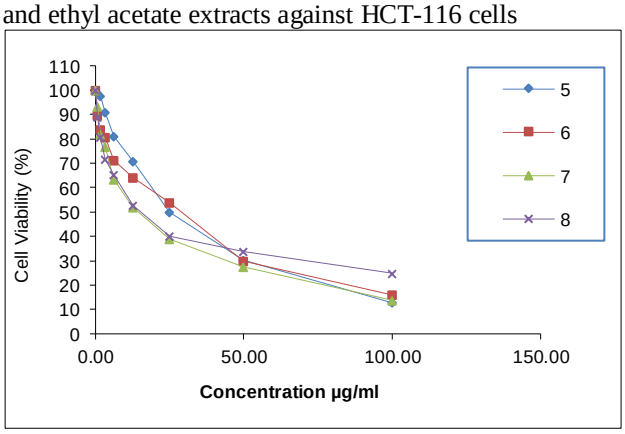


Figure 6: The cytotoxic activity of compounds 5, 6, 7 and 8 against HCT-116 cells

The structure-activity relationship (SAR)

Myricetin is the most cytotoxic compound among the substituted flavonols; while SAR performed in our study provided some new information on this molecule. From the view point, it seems that; a presence of free hydroxyl groups on the A- or B-ring is to be necessary for the cytotoxic activity of the compound. Also this result confirms the importance of a free 3-OH group in myricetin and quercetin is a corner stone in their activities; while the 3-OH substitution of quercetin as in rutin molecule decreases the activity.

CONCLUSION

Our study showed the cytotoxic activity of methanolic extract of *Tecomaria capensis* v. *aurea* leaves and its derived fractions as well as chromatographic isolation of active chemical constituents which confirmed by ^{1}HNMR, ^{13}CNMR and ESI-MS analysis. The outcomes uncovered among the isolated metabolites that; myricetin showed potent significant noteworthy cytotoxic activity against colon cancer cell line (HCT-116). Presented the beneficial effects of phenolic compounds, the antitumor activity of the plant may be attributed to its flavonoid contents. The above results supports that the natural sources concepts of

bioactive compounds can be solve the cancer disease problem.

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