Supplementary File

for

Phytochemical Investigation of the Bioactive Extracts of the Leaves of *Ficus cyathistipula* Warb.

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Experimental procedures

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S-A: Preparation of Mucilage and its hyrolysate

Mucilage was prepared by boiling 500 g of the air-dried powdered leaves with distilled water for 30 min.; the residue left after evaporation of solvent, was dissolved in the least amount of water, four volumes of absolute alcohol were added slowly with stirring in order to precipitate mucilage. The solution was passed through muslin and the hydro-alcoholic filtrate was concentrated under vacuum. The precipitation was repeated to separate all mucilage content. The total mucilage was washed with acetone and dried in desiccator till constant weight [12]. Fifteen mg of the prepared mucilage were kept under reflux in a boiling water bath with 30 ml of 2N sulphuric acid for 24 hours. At the end of hydrolysis, solution was separated and freed from sulphate by precipitation with barium carbonate. The precipitate was removed by filtration and washed repeatedly with water. The hydrolysate of the mucilage was evaporated under vacuum, dried and extracted with hot, distilled pyridine. The pyridine extract was then dried and reserved for study [13].

S-B: Material and methods for *in-vitro* biological study

Investigation of cytotoxic activity

The Sulphorhodamine B assay [19] was performed to evaluate the cytotoxicity of the ethanolic, aqueous leaf extracts and the four fractions of ethanolic extract: (petroleum ether, methylene chloride, ethyl acetate and *n*-butanol) on hepatocellular (HepG2), breast (MCF7) and cervical (HeLa) human carcinoma cell lines. The cells were grown expotentially in a 96-multi well plates for 24 hours before treatment with the tested extracts then incubated for 48 hours with different concentrations of each extract (0.5-50 µg/ml) in triplicates. The temperature was set at 37ºC in an atmosphere of 5% CO_2. The survival curve of each tumor cell line for a specified tested sample was fitted using linear equation and the dose of the sample which reduced survival to 50% (IC_{50}) was calculated. Cell lines used in the study were obtained from National Cancer Institute, Cairo, Egypt and doxorubicin (Sigma-Aldrich, Germany) served as reference drug.

Investigation of antimicrobial activity

The ethanolic, aqueous extracts and the four fractions of ethanolic were screened for their antimicrobial activity using the agar well diffusion assay [20]. 50 µl of each tested sample dissolved in dimethyl sulphoxide (E-Merck) at a concentration of 10 mg/ml were separately, transferred into sterile discs (5mm d). Dimethyl sulfoxide (10 µl) was used as negative control. Positive control of standard discs of gentamicin, ampicillin and amphotericin B (Sigma-Aldrich, Germany) were used for the Gram-negative, Gram-positive bacteria and fungi, respectively. The discs were placed onto the surface of the plates containing the solid bacterial medium (Mueller-Hinton agar) or the fungal medium (Dox's medium) heavily seeded with the spore suspension of the tested microorganisms. Inhibition zones were recorded in mm, after 25 hours of incubation period at 37ºC for bacteria and 48 hours of incubation period at 25ºC for fungi. The tested bacteria and fungi were supplied from the Regional Center for Mycology and Biotechnology, Anti-microbial Unit of the tested organisms: *Streptococcus pneumoniae* (RCMB 05922), *Bacillus subtilis* (RCMB 010067), *Staphylococcus aureus* (RCMB 010028), *Enterococcus faecalis* (RCMB 010068), *Pseudomonas aeruginosa* (RCMB 010043), *Escherichia coli* (RCMB 010052), *Klebsiella pneumonia* (RCMB 0010093), *Shigella flexneri* (RCMB 00100542), *Salmonella typhimurium* (RCMB 010072), *Aspergillus fumigatus* (RCMB 02568), *Syncephalastrum racemosum* (RCMB 05922) and *Candida albicans* (RCMB 05036).

Estimation of the minimum inhibitory concentration (MIC) was carried out using broth dilution method for each of the tested organisms in triplicates [21]. The samples at a concentration 10 mg/ml were separately, added to the nutrient broth and then a loopful of the test organism previously diluted to 0.5 McFarland turbidity standard was introduced to the tubes. Broth media only seeded with the tested organisms was served as control. Observation of Turbidity was observed in the tubes after 24 hours incubation at 37°C.

Determination of anti-oxidant activity using DPPH assay

The ethanolic, aqueous extracts and the four fractions of ethanolic extract were screened for their anti-oxidant activity using DPPH assay [22]. Tested samples were separately dissolved in 70% methanol (50 - 1000 µg/ml) where 0.3 ml of each test solution was added to 2 ml DPPH solution. The absorbance was determined at 492 nm against blank of methanol, after incubation at room temperature for 30 minutes. Ascorbic acid (Misr Company for Pharmaceutical Industry, Mataria, Cairo, Egypt) was used as a positive control (1- 50 µg/ml). IC_{50} (the concentration inhibiting DPPH formation by 50% relative to methanol) was calculated as the average of three determinations and used to express the anti-oxidant activity of each sample. Percentage Inhibition of the DPPH free radical (I%) was calculated according to the equation:

I%= [(Absorbance of the blank-Absorbance of the sample) / Absorbance of the blank] x 100

S-C: Investigation of secondary metabolites

Preparation of extract

Dried leaves were ground in presence of liquid nitrogen then the powder (30 mg) was homogenized five times, each for 20 seconds, with 2.5 ml methanol containing 5 µg/ml umbelliferone (internal standard for relative quantification) using Turrax mixer (11000 RPM). Mixing periods were separated by 1 minute to avoid heating. Plant debris were removed by vigorous vortex and centrifugation of the extract at 3000 g for 30 min. An aliquot of 500 µl was put on C18 cartridge (500 mg) preconditioned with methanol and water then eluted using 70% and 100% methanol (3 ml, each). The solvent in the eluent was removed by evaporation under nitrogen and the residue is resuspended in methanol (500 µl) and kept for analysis [26].

High-resolution UPLC-PDA-qTOF-MS analysis

Analysis of methanol-soluble constituents was carried out on HSS T3 column (100 x 1.0 mm, particle size 1.8 μm; Waters). The mobile phase, at a flow rate of 150 µl/ min, was set: 0 to 1 min, isocratic 95% A, 5% B; 1 to 16 min, linear from 5 to 95% B; 16 to 18 min, isocratic 95% B; 18 to 20 min, isocratic 5% B; A and B being 99.9% water/formic acid and 99.9% acetonitrile/formic acid, respectively. The injection volume was 3.1 µl (full loop injection). Detection of the eluted compounds was performed from m/z 100 to 1000 in negative ion mode using the following instrument settings: nebulizer gas, nitrogen, 1.6 bar; dry gas, nitrogen, 6 liters/min, 190ºC; capillary, -5500 V (+4000V); end plate offset, -500V; funnel 1 RF, 200 Vpp; funnel 2 RF, 200 Vpp; in-source CID energy, 0 V; hexapole RF, 100 Vpp; quadrapole ion energy, 5 eV; collision gas, argon; collision energy, 10 eV; collision RF 200/400 Vpp (timing 50/50); transfer time, 70 µs; pulser frequency, 10 KHz; spectra rate, 3Hz. Internal mass calibration was done by infusion of 20 µl 10 mM lithium formate in isopropanol:water, 1:1 (v/v), at a gradient time of 18 min using a diverter valve [26]. Metabolic assignments were made by comparing retention time, UV/Vis spectra and MS data (accurate mass, isotropic distribution and fragmentation pattern in negative ion mode) of the compounds detected with reference literature and searching in the existing phytochemical dictionary of natural products database (compact reinforced composite (CRC), Wiley).

S-D: Study of lipoidal content

Preparation of unsaponifiable and saponifiable matters

One gram of the petroleum ether fraction of the leaves was treated for an overnight with 20% ethanolic potassium hydroxide at room temperature**.** The saponified solution was then concentrated under reduced pressure and suspended in 100 ml distilled water followed by extraction with ether (50 ml × 3). The combined ethereal extracts washed with distilled water till free from alkalinity, dried over anhydrous sodium sulphate and evaporated to dryness [28]. The obtained residue represented the unsaponifiable matters (USM) and saved for gas chromatography. The aqueous solution left after separation of USM was acidified with dilute HCl (10%) to liberate the free fatty acids and extracted with ether till exhaustion. The combined extracts were washed thrice with distilled water, dried over anhydrous sodium sulphate and evaporated to dryness. The fatty acid mixture was dissolved in small amounts of anhydrous methanol and methylated by dropwise addition of an ethereal solution of diazomethane, until gas evolution ceased and the mixture acquired a pale yellow color. The mixture was freed from the solvent by evaporation under a stream of nitrogen and the obtained fatty acids methyl esters (FAMEs) were saved for GC analysis [30].

GLC analyses of unsaponifiable matter and fatty acids methyl esters

USM analysis was performed on a TR-5MS column (5 % phenyl polysil phenylene siloxane, 30 m x 0.25 mm I.D. x 0.25 µm film), injection temperature was set at 270°C and detector temperature at 280°C (FID). The flow rate of the carrier, N_2, was 30 ml/min. The column temperature was 70°C isothermally for 5 min then increased to 280°C by the rate of 4°C/min. Aliquots, 2 µL of 10% chloroformic solution of the USM and reference samples were co-chromotographed. FAMEs were analyzed on a DB-5 column (5 % phenyl methyl siloxane, 30 m x 0.25 mm I.D. x 0.25 µm film), injection temperature was set at 210°C and detector temperature at 220°C (FID). The flow arte of the carrier, N_2, was 30 ml/min. The column temperature was 140°C then increased to 200°C by the rate of 5°C/min. Aliquots, 2 µL of 10% chloroformic solution of the FAME and reference FAMEs were co-chromotographed. Identification of the hydrocarbons, sterols and fatty acid methyl esters was based on comparison of the retention times of their peaks with those of the available reference standards (Sigma Chemical Co., St. Louis, MO, USA) similarly analyzed. The amount of each component was calculated *via* peak area measurement using a computing integrator.

S-E: Spectrophotometric determination of total steroidal content

Spectrophotometric determination of total sterol content was based on measuring the intensity of the green color developed when a steroidal compound complexes with Libermann-Burchard's reagent [31]. The concentration of the total steroids was calculated as β-sitosterol equivalent (BSE/ 100g d.wt.). One gram of the 70% ethanolic extract of the leaves was dissolved in chloroform to 10 ml and further diluted to 10 times. From the extract, 0.5 ml was transferred to a test tube and mixed with 2 ml of Libermann-Burchard reagent (0.5 ml sulfuric acid dissolved in 10 ml acetic anhydride) then kept in dark and ice for 15 minutes. A standard calibration curve ($R^2 = 0.999$) was prepared using different aliquots of β-sitosterol solution (Sigma Chemical Co., St. Louis, MO, USA) equivalent to 100-1000 µg/ml. The absorbance of the resulting green color was measured at 640 nm using UV spectrophotometer. For each concentration, three replicates were carried out and the average of the obtained absorbances was plotted versus concentration.

S-F: Spectrophotometric determination of total phenolic content

The total phenolic content was performed using Folin-Ciocalteu reagent [32]. An aliquot (0.8 ml) of the ethanolic extract dissolved in 50% methanol in a concentration of 1 mg/ml was mixed with 0.4 ml of Folin-Ciocalteu reagent and 4 ml distilled water, then diluted to 10 ml with 290 g/L sodium carbonate solution. A standard calibration curve ($R^2 = 0.9987$) was prepared using different aliquots of standard gallic acid solution (Sigma Chemical Co., St. Louis, MO, USA) equivalent to 25-125 µg/ml. The absorbance of the resulting blue color was measured after 30 min at 760 nm using UV spectrophotometer. The results were expressed as mg gallic acid equivalent (GAE)/100 g d.wt. For each concentration, three replicates were carried out.

S-G: Spectrophotometric determination of flavonoid content

The total flavonoid content was estimated for the 70% ethanolic extract based on measuring the intensity of of the color developed when flavonoids were complexed with aluminium chloride reagent [33]. An aliquot (0.5 ml) of the extract dissolved in 95% ethanol in a concentration of 1 mg/ml was evaporated. The residue obtained was treated with 5ml of 0.1M aluminium chloride. A standard calibration curve ($R^2 = 0.9984$) was prepared using different aliquots of standard rutin solution (Sigma Chemical Co., St. Louis, MO, USA) equivalent to 20-160 µg/ml. The intensity of the developed yellow color was measured at 420 nm using a UV spectrophotometer expressed as rutin equivalent/ 100g d.wt. Results were the average of three determinations.

S-H: Gravimetric determination of tannin content

The tannin content was determined adopting the hide powder method [34]. The air dried powdered leaves (0.75 g) were boiled with 150 ml distilled water in a 250 ml Erlenmyer flask and then left on a boiling water bath for 30 minutes. The mixture was cooled, transferred to a 250 ml measuring flask. The volume was completed to 250 ml with distilled water, left to sediment for 1-2 hours and then filtered. The first 50 ml of the filtrate were rejected then 25 ml of the filtrate were transferred to a pre-weighed 50 ml beaker, allowed to evaporate and dried in a hot air oven at 70 ºC. After cooling, the beaker was kept in a desiccator till a constant weight was recorded. The residue in the beaker was then weighed and recorded (G_1). Another 50 ml of the filtrate were mixed with 0.5 g hide powder and left for one hour with continuous shaking. The mixture was filtered and 25 ml of it was transferred to a pre-weighed 50 ml beaker and treated as before. The residue in the beaker was weighed (G_2). A blank determination was carried out twice using distilled water. The residue left was weighed ($G_0 = 0$). The tannin percentage was calculated according to the following equation:

$$\text{Tannin } \% = \frac{G_1 - (G_2 - G_0) \times 1000 \times 100 \times 10}{W}$$

G_1 = weight of residue containing tannins in g; G_2 = weight of residue after removal of tannins in g; G_0 = Blank; W= weight of powdered drug in mg.
The result in each case was the average of three determinations.