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Research Article

Phenolic Constituents and Biological Activities of *Acalypha wilkesiana* F. Tricolor Müll. Arg.Seeds

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ABSTRACT
Acalypha wilkesiana F. tricolor is an ornamental plant widely distributed in Egypt. The seeds of *A.wilkesiana* were extracted by 70% MeOH, and then subjected to solvent-solvent extraction. The total extract and ethyl acetate fraction were subjected to both biological and chemical investigations. Antimicrobial activities of these extracts were carried out using disc diffusion test on six species of pathogenic bacteria and seven fungal species. Cytotoxic activities were evaluated using acid phosphatase assay on HepG2 hepatocellular and MCF7 breast cancer cell lines and antioxidant activity was performed using DPPH assay. The ethyl acetate fraction exhibited moderate antimicrobial activity, whereas, it exhibited potent antifungal activity especially towards *A. flavus, A. westerdijikia, F. verticelloides and F. proleferatum*. Fortunately, combination of conventional drug antibiotic (tetracycline) and *A. wilkesiana* seeds extract in portions (1:1 and 1:2) respectively, showed a distinct increase in the antibiotic activity compared to tetracycline itself, whereas the same combination with conventional antifungal drug(nystatin) did not show any increase in the antifungal activity. *A. wilkisiana* seeds showed also a distinct cytotoxic and antioxidant activities. Chemical investigation of ethyl acetate fraction extract led to isolation and identification of twelve polyphenolic compounds; among them the new natural product; brevifolin carboxylic propyl ester.

Keywords: *Acalypha wilkesiana*, Antibacterial, Antifungal, Antioxidant, Anticancer, Plant Phenolics, Ellagitannins and Propyl brevifolin carboxylate.

INTRODUCTION

Acalypha is the fourth largest genus of Euphorbiaceae family. Some citations reported that it comprises of about 450 species[1]. others reported that it comprises of about 570 species[2-4]. A large proportion of species of this genus are wild and can be found growing everywhere, while the other ornamental species have been introduced into West Africa and are cultivated as foliage plants in gardens and greenhouses. They are evergreen shrubs, trees and annuals, which native to the Tropical and Subtropical regions, mainly in the tropics of Africa, America and Asia[5]. *A. wilkesiana* is commonly known as copper leaf[6]. It is found all over the world most especially in the tropics of Africa, America and Asia. It is native to Fiji and nearby islands in the South Pacific and its common in Mauritius. It is a popular outdoor plant that provides color throughout the year, although it is also grown indoors as a container plant. *A. wilkesiana* leaves has antimicrobial properties[7-9]. as it used for treatment of fungal skin infections such as *Tinea versicolor*, *Pityriasis versicolar*, *Candida intetrigo*, *Impetigo contagiosa*[10]. Previous studies on *A. wilkesiana* leaves reported the presence of monoterpenes, sesquiterpenes, triterpenes, saponins, flavonoids, tannins, anthraquinone and glycoside[7-9,11,12]. Although *A. wilkesiana* seeds were used traditionally and reported to have anticancer and anti-inflammatory activities[13]. the seeds of this plant was not subjected to any chemical investigation. Therefore, the aim of this study is to investigate the chemical constituents, biological activities and to prove anticancer use of the seeds of *A. wilkesiana* which is widely distributed in Egypt as ornamental plant.

Experimental

General

^{1}H-NMR spectra were measured with a Bruker avance II 400 JHz NMR spectrometer, ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C-NMR chemical shifts to DMSO-d_6 and converted to the TMS scale by adding 39.5. HR-MS were measured by Bruker Daltonics - micrOTOF ESI ionization, UV recordings were made on a Shimadzu UV-Visible-1601 spectrophotometer. Paper chromatographic analysis (PC) and preparative paper chromatographic separation were carried out on Whatman No.1 and 3 MM papers respectively, using solvent systems 15% HOAc and BAW (n-BuOH-HOAc-H_2O, 4:1:5, upper layer). Vacuum Liquid Column (VLC) was performed using polyamide 11 as adsorbent.

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Plant material

The seeds of *A. wilkesiana* F. were collected from the garden of National Research Centre, Cairo, Egypt, in June 2013, and identified by Dr. Mohamed Al-Gebaly, former researcher of Botany, National Research Centre.

Extraction and isolation

The freshly collected seeds of *A. wilkesiana* (1 kg) were extracted with 70% MeOH three times at room temperature. The filtrates were collected and evaporated under reduced pressure to dryness to give 100 gm of the concentrated extract. The extract was suspended into water and successively defatted with pet. ether and CH_2Cl_2, then the residue was extracted with ethyl acetate (EtOAc). The EtOAc extract (5 gm) was subjected to polyamide 11 VLC column chromatography, which eluted firstly with water then water-MeOH step gradient. Four major fractions were collected and tested by two dimension paper chromatography (2D PC), using two solvent systems (15% HOAc & BAW). The four fractions were further fractionated using Sephadex LH-20 column and Whatman 3MM paper for preparative fractionation to give Ac1-Ac12. All compounds were isolated using repeated Sephadex LH-20 CC and PPC using BAW and 15% acetic acid as eluents and finally purified on Sephadex LH-20 column by using methanol HPLC as eluent to yield pure isolated compounds.

Evaluation of antimicrobial activity

Test microorganisms

The inhibitory effects of extracts were carried out on six species of pathogenic bacteria. Two Gram-positive bacteria *Bacillus cereus* EMCC 1080, *Staphylococcus aureus* ATCC 13565 and four Gram-negative bacteria *Salmonella typhi* ATCC 25566, *Escherichia coli* 0157 H7 ATCC 51659, *Pseudomonas aeruginosa* NRRL B-272and *Klebsiella pneumoniae* LMD 7726 were obtained from the Holding Company for Biological Products and Vaccines (VACSERA), Egypt.The stock cultures were grown on nutrient agar slant at 37˚C for 24 h and then kept in refrigerator till use. Seven fungal species were used for antifungal assay *Aspergillus flavus* NRRL 3357, *A. parasiticus* SSWT 2999, *A. westerdijikia* CCT 6795, *A. ochraceus* ITAL 14, *A. carbonarus* ITAL 204, *Fusarium verticelloides* ITEM 10027 and *F. proleferatum* MPVP 328. The fungal isolates were obtained from Applied Mycology Dept., Cranfield Unvi., UK. The stock cultures were grown on potato dextrose agar slant at 25˚C for 5 days and then kept in refrigerator till use.

Media used for antimicrobial assay

Nutrient agar medium (Fluka, BioChemika, Spain) composed of meat extract 1g, yeast extract 2g, peptone 5g, sodium chloride 5g and agar 15g in 1000 ml distilled water. The pH was adjusted to 7.4±0.2 at 37˚C (ATCC, 1984),for bacterial disc diffusion test. Tryptic soya broth, TSB (BD, Sparks, USA) composed of pancreatic digest of casein 17g, papaic digest of soybean 3g, dextrose 2.5g, sodium chloride 5g and dipotassium phosphate 2.5g in 1000 ml distilled water. The pH was adjusted to 7.3±0.2 at 25˚C, for determination of minimum inhibitory concentration**.** Yeast extract sucrose medium (YES) composed of 20gyeast extract, 150gsucrose and 20g agar in 1000 ml distilled water[14]. for fungal disc diffusion test.

Disc diffusion technique

From the 24 h incubated nutrient agar slant of each bacterial species a loop full of the microorganism was inoculated in a tube containing 4 to 5 ml of tryptic soy broth. The broth culture is incubated at 35^0C for 2 - 6 h until it achieves the turbidity of 0.5 McFarland $BaSO_4$ standard. Barium sulfate 0.5 McFarland $BaSO_4$ standard was prepared by adding 0.5 ml of 1.175% (w/v) barium chloride dehydrate ($BaCl_2$. $2H_2O$) solution to 99.5 ml of 1% (v/v) sulfuric acid with constant stirring to maintain suspension. The density of turbidity standard was determined by using a spectrophotometer at 625 nm. The absorbance at 625nm should be (0.008 to 0.1) for the 0.5 McFarland standard. $BaSO_4$ turbidity standard should be vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance. The sensitivity test of microalgae crude extracts and fractions were determined with different bacterial cultures using disc diffusion method by Kirby-Bauer technique[15]. Petri dishes were prepared with 20 ml nutrient agar and the bacterial cultures were uniformly from tryptic soy broth using cotton swabs. Each extract and fraction was dissolved in 1 ml of dimethyl sulfoxide (DMSO) to give 10 mg ml^{-1} and 1 $mgml^{-1}$ for extract and fraction, respectively. Sterilized discs (6 mm) from Whatman No. 1 filter paper were loaded by extracts and dried completely under sterile conditions. The discs were placed on the seeded plates by using a sterile forceps. DMSO represented as negative control and tetracycline (500 µg ml^{-1}) was used as positive control. After that, inoculated plates were incubated at 37°C for 24h. At the end of the incubation period, inhibition zones were measured and expressed as the diameter of clear zone including the diameter of the paper disc. The fungal strains were plated onto potato dextrose agar (PDA) and incubated for 5 days at 25°C. The spore suspension of each fungus was prepared in 0.01% Tween 80 solution. The fungal suspension was compared with the 0.5 McFarland standard, the turbidity of the inoculum suspension represented approximately 2 x 10^8 cfu ml^{-1}. Similar to antibacterial test, sterilized filter paper discs (6 mm) were loaded with the extracts and dried completely under sterile conditions. Petri dishes of YES medium were inoculated with 50 µl of each fungal culture and uniformly spread using sterile L- glass rod. The extract loaded discs were placed on the seeded plates by using a sterile forceps. Negative control was prepared by using DMSO and the commercial fungicide Nystatin (1000 Unit ml^{-1}) was used as a positive control. The inoculated plates were incubated at 25°C for 24 - 48 h. At the end of the period, antifungal activity was evaluated by measuring the zone of inhibition (mm) against the tested fungus[16]. All treatments consisted of three replicates and the averages of the experimental results determined.

Determination of minimum inhibitory concentration (MIC)

The determination of MIC was conducted using tube dilution method[17]. A 24 h culture of the tested bacterial species was diluted in 10 ml of tryptic soy broth (TSB)

Table 1: Antibacterial activity

Bacteria	Inhibition zone mm (Mean±*S.E)					
	-ve Control	+ve Control	1	2	3	4
B. cereus	0	15.3±0.76^{B}	8.0±1.32^{D}	8.8±0.76^{C}	16.3±1.04^{B}	18.7±1.04^{A}
Staph. Aureus	0	14.7±0.76^{B}	9.2±0.58^{C}	8.2±0.76^{D}	17.2±1.89^{A}	17.8±1.04^{A}
E. coli	0	15.5±1.5^{B}	9.3±1.25^{C}	7.7±1.15^{D}	20.0±1.00^{A}	19.5±1.00^{A}
P. aeruginosa	0	19.2±1.26^{B}	9.0±1.00^{C}	8.2±1.04^{C}	23.3±1.26^{A}	22.7±0.58^{A}
S. typhi	0	16.7±0.76^{B}	7.5±0.28^{C}	7.5±0.86^{C}	19.5±1.32^{A}	18.5±1.00^{AB}
K. pneumonia	0	18.2±0.28^{A}	10.2±1.25^{D}	0	16.0±1.50^{C}	17.2±1.04^{B}

n=3, P<0.05, *S.E: standard error,negative control: DMSO, positive control: tetracycline, 1: ACE (EtOAc extract), 2: ACM(aq. MeOH extract),3: ACE: tetracycline (1:1), 4: ACM: tetracycline (1:1).

Antimicrobial activity:

Table 2: MIC values (mg ml^{-1})

Bacteria	MIC values mg ml^{-1}(Mean±S.E)					
	1	2	3	4	5	6
B. cereus	0.535±0.14	0.220±0.08	0.125±0.04	0.127±0.08	0. 25±0.14	0.20±0.08
S. aureus	0.415±0.11	0.550±0.21	0.225±0.04	0.225±0.05	0.35±0.05	0.30±0.06
E. coli	0.425±0.14	0.375±0.11	0.205±0.05	0.120±0.04	0.30±0.06	0.25±0.05
S. typhi	0.272±0.11	0.510±0.28	0.125±0.04	0.130±0.08	0. 22±0.04	0.230±0.08
P. aeruginosa	0.550±0.21	0.530±0.11	0.215±0.04	0.125±0.04	0.415±0.11	0.35±0.14
K. pneumonia	0.360±0.11	0.280±0.08	0.175±0.06	0.220±0.04	0.255±0.08	0.310±0.08

n=3, *S.E: standard error, negative control: DMSO, positive control: tetracycline, 1: ACE extract, 2: ACM extract, 3: ACE: tetracycline (2:1), 4: ACM: tetracycline (2:1), 5: ACE: tetracycline (1:1), 6: ACM: tetracycline (1:1).

with reference to the 0.5 McFarland standard to achieve inocula of 10^8 cfu ml^{-1}. In culture tube containing nine different concentrations of each crude extract and fraction (4.0, 2.0, 1.75, 1.5, 1.0, 0.75, 0.50, 0.25, 0.1 mg ml^{-1} in DMSO) were prepared. Each tube was inoculated with 100µl of bacterial cell suspension and incubated at 37^{0}C for 24h. The growth of the inoculum in broth is indicated by turbidity of the broth and the lowest concentration of the extract which inhibited the growth of the test organism was taken as the minimum inhibitory concentration (MIC). MIC against fungi was performed by using the technique of Perrucci*et al*. 2004. Crude extracts at different concentrations were separately dissolved in 0.5 ml of 0.1% Tween 80 (Merck, Darmstadt, Germany), then mixed with 9.5 ml of melting, 45^{0}C, PDA and poured into Petri dish (6 cm). The prepared plates were centrally inoculated with 3µl of fungal suspension (10^8 cfu ml^{-1}; 0.5 McFarland standard). The plates were incubated at 25°C for 24-48h. At the end of the incubation period, mycelial growth was monitored and MIC was determined[18].

Evaluation of anticancer activity:

Cell culture

HepG2 hepatocellular carcinoma and MCF7 breast carcinoma human tumor cell lines were cultured in 95% humidity, 5% CO2 and 37°C. HepG2 and MCF7 cell lines were maintained in RPMI-1640, supplemented with 10% foetal bovine serum.

Cytotoxicity assay

The acid phosphatase assay[19] was used to assess cytotoxicity. Five thousand cells were seeded per well in 96 well plates, left to attach overnight, and then treated with samples for three days. For one plate, a substrate solution was prepared where 20 mg tablet of pNPP (Sigma; cat. no. N2765) was dissolved in 10 ml buffer solution (0.1 M sodium acetate, 0.1% triton X-100, pH=5). Cell monolayers were washed twice with 250 µl PBS. 100 µl of pNPP substrate solution were added per well, then plates were incubated for 4 hours at 37°C. 10 µl of 1N sodium hydroxide stop solution were added per well. Absorbance was measured directly at wavelength 405 nm. All samples were tested in triplicates, and 1 µl DMSO was used as negative control and 1µMstaurosporine was used as positive control. Plant samples were tested at 100 µg/ml. Percent cytotoxicity was calculated by the formula:

[1-(D/S)] × 100, where D and S denote the optical density of drug and solvent treated wells, respectively.

Antioxidant activity

The free radical scavenging activity of plant samples was evaluated by using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay[20]. Plant samples were screened at final concentrations as follows: 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200 µg/ml using 0.1mM DPPH dissolved in methanol. After incubation for 30 min in dark at room temperature, the absorbance was measured at 517 nm and a reference wavelength of 690 nm. Ascorbic acid (vitamin C) was used as positive control. The DPPH / methanol mixture was used as a negative control. The DPPH scavenging activity of plant samples was calculated according to the following equation:

Percentage reduction = (1-(X/ (av(NC)))*100

Where x indicates the absorbance of sample and av (NC) indicates the average absorbance of the negative control. EC_{50} values were calculated using probit analysis utilizing the SPSS computer program (SPSS for windows, statistical analysis software package / version 9 / 1989 SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSIONS

Table 3: Antifungal activity

Fungi	Inhibition zone mm (Mean±*S.E)					
	-ve control	+ve control	1	2	3	4
A. flavus	0	13.5 ± 0.50^{C}	14.3 ± 1.61^{B}	13.3 ± 0.58^{C}	14.2 ± 0.58^{B}	15.5 ± 1.32^{A}
A. ochraceus	0	15.8 ± 0.76^{A}	14.5 ± 0.86^{B}	11.3 ± 1.04^{C}	13.5 ± 1.50^{BC}	15.8 ± 0.28^{A}
A. parasiticus	0	16.3 ± 0.29^{A}	9.5 ± 1.00^{D}	7.2 ± 0.28^{E}	14.2 ± 1.61^{C}	15.7 ± 0.76^{B}
A. westerdijikia	0	15.5 ± 0.87^{A}	13.7 ± 2.08^{C}	12.5 ± 1.80^{D}	14.7 ± 1.61^{B}	14.2 ± 1.51^{BC}
A. carbonarus	0	16.0 ± 1.00^{B}	16.7 ± 0.76^{A}	12.8 ± 0.76^{D}	15.0 ± 1.50^{C}	15.2 ± 1.04^{C}
F. verticelloides	0	13.8 ± 0.28^{C}	15.0 ± 1.32^{A}	11.2 ± 1.26^{D}	13.5 ± 1.80^{C}	14.3 ± 0.28^{B}
F. proleferatum	0	11.5 ± 0.86^{C}	15.2 ± 0.28^{A}	13.0 ± 1.32^{B}	11.7 ± 0.76^{C}	10.8 ± 1.04^{D}

n=3, P<0.05,*S.E: standard errornegative control: DMSO, positive control:Nystatin, 1: ACE extract, 2: ACM extract,3: ACE: Nystatin (2:1), 4: ACM: Nystatin (2:1).

Application of *A.wilkisiana* seeds EtOAc extract to polyamide 11 column chromatography on VLC system using H_2O/MeOH mixtures as eluents led to isolation and identification of twelve phenolic compounds (Ac1-Ac12). These compounds were identified as follow; Gallic acid (Ac1), methyl gallate (Ac2), ellagic acid (Ac11) and monomethoxy ellagic acid (Ac12) were identified by authentic samples obtained from the Department of phytochemistry and plant systematic. All other compounds were identified by high resolution mass spectrometry, HR/MS NMR and 2D COSY and compared with those published data as follow; 3, 6-(R)-hexahydroxydiphenoyl(α/β)-$^{1}C_4$-glucopyranose[21], Punicalin[22](Ac4) Corilagin[21] (1-*O*-galloyl-3,6-hexahydroxy diphenoyl-B1,4-β-D-glucopyranoside) (Ac5), Geraniin[23] (Ac6), Kaempferol-3-*O*-α-L-rahmnoside[24] (Ac7), Brevifolin carboxylic acid[21] (Ac_8), Methyl brevifolin carboxylate[21] (Ac9).

Structure elucidation of the new natural product Ac 10

Ac 10 was Isolated as yellow amorphous powder, changed to blue color with ferric chloride indicating its phenolic nature, UV spectrum very similar to that of brevifolin carboxylic acid[21]. moreover, it shows yellow spot under Ultraviolet light lamp, it also show rate of flow in BAW (Butanol:Acetic acid: Water) more than brevifolin carboxylic acid and less than it in 15% acetic acid. Ac 10 exhibited a molecular weight of 334 derived from positive high resolution ESI-MS analysis spectrum molecular ion 334.8977 $C_{16}H_{14}O_8$ suggesting the structure to be propyl brevifolin carboxylate. For more confirmation, ^{1}H& ^{13}C-NMR and ^{1}H -^{1}H COSY experiments were performed. The ^{1}H- NMR spectrum (DMSO –d_6) of Ac 10 confirmed the supposed structure and it showed the brevifolin moiety signal pattern as follow[(21)]: δ 7.20 (1H, s, H-7), δ 4.42 (1H, d, *J*= 8, H-2), δ 2.96 (1H, dd, *J*=20&8, H-3a), δ 2.45 (1H, d, *J*=20, H-3b). It also confirmed the presence of propyl moiety through signal at δ ppm 3.37(2H, d, *J*=6.4,α CH_2), δ1.30 (2H, m, β CH_2), 0.85(3H, t, *J*=7.2, γ CH_3). ^{13}C-NMR spectra finally prove the proposed structure of propyl moiety to brevifolin carboxylic acid through upfield shift of carboxyl δ 172.56 of Ac 10 compared to δ ppm 173.02 in case of brevifolin carboxylic acid[21,25-26]. It also showed propyl moiety signals at δ ppm 60.82(α CH_2), δ 19.11 (β CH_2), δ 14.34 (γ CH_3)[25-26]. Therefore, the structure of Ac 10 is the new natural; product propyl brevifolin carboxylate.

Identification of Ac10

Isolated as yellow amorphous powder, blue color with ferric chloride indicating its phenolic nature, ^{1}H NMR (DMSO-d_6, 400 JHz): δ 7.20 (1H, s, H-7), δ 4.42 (1H, d, *J*= 8, H-2), δ 2.96 (1H, dd, *J*=20&8, H-3a), δ 2.45 (1H, d, *J*=20, H-3b), Propyl moiety: δ 3.37(2H, d, *J*=6.4,α CH_2), δ1.30 (2H, m, β CH_2), 0.85(3H, t, *J*=7.2, γ CH_3). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 193.30(C-4)**,** δ 172.56(C-1), δ 160.96(C-6), δ 150.70(C-4a), δ 145.70(C-10), δ 145.13(C-8), δ 142.77(C-9), δ 139.53(C-10a), δ 114.53(C-4b), δ 110.68(C-6a), δ 108.00(C-7), δ 41.10 (C-2), δ 37.48(C-3), Propyl moiety; δ 60.82(α CH_2), δ 19.11 (β CH_2), δ 14.34 (γ CH_3).

Identification of known compounds

Gallic acid (Ac1), methyl gallate (Ac2), ellagic acid (Ac11) and monomethoxy ellagic acid (Ac12) were identified by authentic samples; Department of phytochemistry and plant systematic. All other compounds were identified by high resolution mass spectrometry, NMR and 2D COSY and compared with those published data.

3,6-(R)-hexahydroxydiphenoyl(α/ β)-$^{1}C_4$-glucopyranose,(Ac3) . Obtained as yellow powder, ^{1}H NMR (DMSO-d_6, 400 JHz): α form δ 5.03 (1H, brs, H-1), δ 3.97 (1H, brs, H-2), δ 5.05 (1H, d, J=4, H-3), 4.13 (1H, m, H-4), 3.93 (1H, brs, H-5), 4.07 (1H, m, H-6,6`), β form: δ 5.33 (1H, d, J=4, H-1), δ 3.93 (1H, d, J=4, H-2), δ 5.22 (1H, d, J=4, H-3), 4.20 (1H, m, H-4), 3.88 (1H, brs, H-5), 4.04 (1H, m, H-6,6`), HHDP of both forms: α form 6.50, 6.52 and β form: 6.39, 6.45. ^{13}C NMR: α glucose: δ 102.22 (C-1), δ 79.53 (C-2), δ 77.22 (C-3),δ 76.71(C-4), δ 70.34(C-5), δ 65.56 (C-6)**.** β glucose**:** δ 95.68(C-1), δ 79.34 (C-2), δ 76.70 (C-3),δ 73.59 (C-4), δ 70.47 (C-5), δ 65.62 (C-6)**.** HHDP of both forms: δ 123.04,123.57, 123.92, 124.73(C-1,1'), δ 106.30, 106.33, 106.82, 106.88 (C-2,2'), δ 143.69, 143.75, 145.77, 145.82 (C-3,3'& C-5,5'), δ 135.47, 135.67, 135.75, 135.88 (C-4,4'), δ115.76, 116.03, 116.16, 116.20 (C-6,6'), 166.51, 166.68, 168.47, 168.79 (C=O).

Punicalin, (Ac4): Obtained as yellow powder**,** ^{1}H NMR (DMSO-d_6, 400 JHz): sugar moiety: δ 4.87 (1H, d, J=3.6, α-1H), δ 4.24 (1H, d, J= 8, β-1H), δ 2.81- 4.11(the rest of sugar protons, hidden by water signal). Gallagyl moiety: δ 6.54 (1H, s, α H_D), δ 6.96 (1H, s, α H_D), δ 6.57 (1H, s, β H_D), δ 6.98 (1H, s, β H_D). ^{13}C NMR: α glucose: δ 91.40(C-1), δ 72.6 (C-2), δ 72.40 (C-3),δ 74.99(C-4), δ 70.02(C-5),

δ 64.75(C-6)**.** β glucose**:** δ 97.20(C-1), δ 75.99 (C-2), δ 76.90 (C-3),δ 74.52 (C-4), δ 73.29 (C-5), δ 65.23 (C-6). Gallagyl moiety: α-form: δ 170.60 (C-7), δ 171.20 (C-7`), lactone 160.75& 161.88. β –form: δ 171.50 (C-7), δ 169.60 (C-7`), lactone 158.54& 159.90. Corilagin(1-*O*-galloyl-3,6- hexahydroxy diphenoyl-B1,4-β-D-glucopyranoside), (Ac5), It was obtained as a yellow amorphous powder, ^{1}H NMR (DMSO-d_6, 400 JHz): δ 7.02 (2H, s, galloyl protons), δ 6.56, 6.49 (2H, s, protons of two HHDP), δ 6.21 (1H, d, J=7.2, anomeric sugar proton), δ 3.87-4.60(the rest of sugar protons). DEPT NMR (DMSO-d_6, 100 MHz): glucose moiety δ 92.62(C-1), δ 72.12(C-2), δ 78.06(C-3),δ 62.62(C-4), δ 76.83(C-5), δ 64.42(C-6), δ 109.43(C-1,6 galloyl), δ 106.45 (C-5 of HHDP), 107.39(C-5` of HHDP). Geraniin (Ac6), Was obtained as a yellow amorphous powder, HR-ESI-MS *m/z*: 975.0695 $[M+Na]^+$, ^{1}H NMR (DMSO-d_6, 400 JHz): glucose moiety: δ 6.36 (1H, brs, H-1), δ 5.33 (1H, brs, H-2), δ 5.41 (1H, brs, H-3), δ 5.22 (1H, brs, H-4), δ 4,72 (1H, t, H-5), δ 4.38 (2H, m, H-6), Galloyl moiety: δ 7.04 (2H, s, H-2,6), HDDP moiety: δ 6.46 (1H, s, H-3), 6.79 (1H, s, H-3`), DHHDP moiety: δ 7.06 (1H, s, H-3), δ 4.89 (1H, s, H-1`), δ 6.39 (1H, s, H-3`). $^{13}$C NMR (DMSO-$d_6$, 100 MHz): glucose moiety: δ 91.40 (C-1), δ 70.52 (C-2), δ 65.44 (C-3), δ 63.48 (C-4), δ 73.78 (C-5), δ 63.48 (C-6), Galloyl moiety: δ 118.02(C-1), δ 109.28 (C-2,6), δ 145.51(C-3,5), δ 143.97 (C-4), δ 165.32(C-7), HDDP moiety: δ 115.0 (C-1), δ 116.2 (C-1`), δ 122.5 (C-2), 123.1 (C-2`), δ 106.3 (C-3), 107.6 (C-3`), δ 144.7 (C-4), 144.8 (C-4`), δ 135.3 (C-5), δ 136.2 (C-5`), δ 145.6 (C-6), δ 145.6 (C-6`), DHHDP Ring D: δ 117.4 (C-1), δ 114.8 (C-2), δ 111.7 (C-3), δ 142.9 (C-4), δ 138.4 (C-5), δ 144.2 (C-6), δ 164.4 (C-7). DHHDP Ring E: δ 45.0 (C-1`), δ 152.3 (C-2`), δ 128.0 (C-3`), δ 191.3 (C-4`), δ 90.9 (C-5`), δ 95.7 (C-6`), δ 164.1 (C-7`). Kaempferol-3-*O*-α-L-rahmnoside, (Ac7), Obtained as yellow powder, ^{1}H NMR (DMSO-d_6, 400 JHz): δ 6.17 (1H, brs, H-6), δ 6.40 (1H, brs, H-8), δ 6.92 (2H, d, J=8.4, H3`,5`), δ 7.92 (2H, d, J=8.4, H2`,6`), δ 5.43 (1H, brs, H-1``), δ 0.98 (3H, d, J=6.04, H-6``).^{13}C NMR (DMSO-d_6, 100 MHz): δ 156.62(C-2), δ 133.94(C-3), δ 177.89(C-4), δ 161.56 (C-5), δ 99.12(C-6), δ 164.58(C-7), δ 94.50 (C-8), δ 157.23 (C-9), δ 104.32 (C-10), δ 120.66 (C-1`), δ 131.2 6(C-2`&6`), δ 116.26 (C-3`&5`), δ 160.44 (C-4`), δ 101.45 (C-1``), δ 70.78 (C-2``), δ 70.84 (C-3``), δ 71.12(C-4``), δ 69.90(C-5``), δ 18.39 (C-6``).

Brevifolin carboxylic acid, (Ac_8) Isolated as yellow powder, blue color with ferric chloride indicating its phenolic nature, ^{1}H NMR (DMSO-d_6, 400 JHz): δ 7.15 (1H, s, H-7), δ 4.35 (1H, d, *J*= 6.4, H-2), δ 2.99 (1H, d, *J*=17.3, H-3a), δ 2.62 (1H, d, *J*=17.3, H-3b).^{13}C NMR (DMSO-d_6, 100 MHz): δ 195.33(C-4) **,** δ 173.02(C-1), δ 161.38(C-6), δ 148.90(C-4a), δ 145.95(C-10), δ 143.13(C-8), δ 142.57(C-9), δ 141.39(C-10a), δ 116.01(C-4b), δ 113.10(C-6a), δ 108.50(C-7), δ 42.72(C-2), δ 37.82(C-3).

Brevifolin carboxylic acid methyl ester (Ac 9) Isolated as pale yellow powder, blue color with ferric chloride indicating its phenolic nature, ^{1}H NMR (DMSO-d_6, 400 JHz): δ 7.19 (1H, s, H-7), δ 4.41 (1H, d, *J*= 6.4, H-2), δ 2.98 (1H, d, *J*=17.3, H-3a), δ 3.40(3H, s, OMe), δ 2.51 (1H, d, *J*=17.3, H-3b).^{13}C NMR (DMSO-d_6, 100 MHz): δ 193.28(C-4)**,** δ 173.10(C-1), δ 160.95(C-6), δ 150.71(C-4a), δ 145.97(C-10), δ 145.39(C-8), δ 142.53(C-9), δ 139.79(C-10a), δ 114.53(C-4b), δ 110.69(C-6a), δ 107.97(C-7), δ 41.06(C-2), δ 37.47(C-3), δ 52.45 (OMe).

Evaluation of the biological activities;

Evaluation of antibacterial activity

The antimicrobial and MIC assays (Tables 1-2) were performed by the agar disc diffusion method. The results obtained by measuring the zone of inhibition diameter (mm). The first concentration not to produce visible inhibition zone after incubation was considered to be the MIC.

Antibacterial effects

Both 70%(ACM)and EtOAc (ACE) of *A. Wilkesiana* seeds extracts demonstrated moderate to weak antibacterial effects against Gm-ve and Gm+ve strains when compared to tetracycline with MIC values ranging from 0.22 to 0.55 mg/ml for the tested organisms.

Whereas, combinations of ACE and ACM extracts with tetracycline showed an interested increase in Tetracycline antibacterial activity as shown in tables (1-2). These results is due to the highly content of ellagitannins which are reported for their synergistic effect in combination with conventional antibiotics[27]. Therefore, we can conclude that ACM and ACE decrease the resistance of the above mentioned micro-organisms towards tetracycline (β-lactams antibiotics).

Evaluation of antifungal activity

Antifungal effects

The EtOAc (ACE) extract showed equipotent antifungal effects to that of nystatin against A. oc, A. car and F. vericell. Both ACM and ACE extracts displayed higher antifungal effects than the positive control when tested against *F. pro* and showed strong potency towards *A. flavus* (Table 3). The potent antifungal activity of the *A. wilkisiana* seeds extracts is due to presence of ellagitannins especially corilagin and geraniin which are reported for their potency as antifungal agents[23,28]

Evaluation of anticancer activity

Wilkisiana seeds extract were reported in traditional use in Nigeria for healing breast cancer[13,29]. Our investigation of ACE (100 ug/ml) on HepG2 hepatocellular carcinoma and MCF7 breast carcinoma human tumor cell lines showed distinctive activity 75.8% and 87.1% inhibition, respectively. On the other hand ACM showed less activity 66.4% and 74.1%, respectively**.** The distinctive cytotoxic activity of ACE is result of richness of ellagitannins and presence of antitumor agents like geraniin, corilagin, punicalin and brevifolin caroboxylic acid derivatives[30-32].

Evaluation of antioxidant activity:

ACM and ACE showed potent antioxidant activity of EC_{50} 3.2 and 9.1, respectively. The potential antioxidant activity of both extracts is due to their polyphenolic components[33-34].

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