Chemical composition and antimicrobial and cytotoxic activities of *Antidesm abunius* L.

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Abstract: It was deemed of interest to investigate *Antidesm bunius* aerial parts from phytochemical and biological points of view due to limited previous studies. Isolation and identification of phenolic compounds and evaluation of the potential antimicrobial and cytotoxic effects of *A. bunius* aerial parts was investigated. The petroleum ether (PEE) and 80% EtOH extracts (EE), as well as, n-hexane (HF), $CHCl_3$ (CF), EtOAc (EAF), *n*-BuOH (BF) and H_2O soluble fractions (WF) of the latter were prepared. Phytochemical study has been performed for isolation and identification of the major polyphenols. Antimicrobial activity, using diffusion agar technique, and potential cytotoxic effect against HepG2, MCF7 and HCT cell lines were evaluated. Malic acid (I), caffeic acid (II), methyl benzoate (III), (+)-catechin (IV), (-)-epicatechin (V), epicatechin-(4β→8)-catechin (procynidin B1, VI) and epicatechin-(4β→8)-epicatechin (procyanidin B2, VII) were isolated. Compounds I-VII showed strong to moderate antimicrobial activity, with MIC values in the range of 1.95-125µg/ml except for compounds 1 and IV, which did not show any effect. All tested samples showed dose dependent cytotoxic effect against all three tested cell lines. PEE showed strong cytotoxic activity (IC_{50}=23.7–38.2µg/ml). Furthermore, compound VI showed potent cytotoxicity against HepG2, MCF7 and HCT cell lines (IC_{50}=24.7, 16.5 and 18.0µg/ml) respectively. The strong to moderate antimicrobial activity and cytotoxic effect of the plant could be attributed to its content of phenolic acids, flavan-3-ols and/or proanthocyanidins. These findings were confirmed by results obtained for the isolated compounds.

Keywords: *Antidesm abunius,* antimicrobial, cytotoxicity, Flavan-3-ols, proanthocyanidins.

INTRODUCTION

*Antidesma*is a genus of tropical plants belonging to the family Phyllanthaceae, which comprises about 2000 species in 59 genera (Kathriarachchi *et al*., 2005).The plant was supposed to protect and be useful against snake bites. It was reported that the name of the genus indicates that the bark can be used for cordage (Quattrocchi, 2000). Several *Antidesma* species are used wide world in traditional medicine for treatment of infection caused by bacteria, fungi and viruses (Gitu, 2009); plant leaves are chewed and swallowed for vomiting spells; the bark is mixed with other plants and used as a wash for ulcers and scrofulous sores (Kaaiakamanu and Akina, 2003).

Many phytochemical studies conducted on *Antidesma* species reported the presence of triterpenoids, steroids, phenolic compounds/acids, megastigmanes, lignans, flavonoids, cyclopeptides, in addition to bicyclic and quinolide-type alkaloids (Buske *et al*., 2002; Rizvi *et al*., 1980; Buske *et al*., 1997; Bringmann *et al*., 2000; Arbain and Taylor, 1993). Furthermore, studies investigated different biological activities of *Antidesma* species including antimicrobial (Rangasamy *et al*., 2007), antioxidant (Butkhup and Samappito, 2011; Nuengchamnong and Ingkaninan, 2010), antifungal (Goun *et al*., 2003; Buske *et al*., 2002), diuretic (Rizvi *et al*., 1980), cytotoxic (Jose Rene *et al*., 2005), and antimalarial effects (Chhabra *et al*., 1993), in addition to its use in treatment of epilepsy (Moshi *et al*., 2005).

Previous research focused on the chemical constituents of *A. bunius*was very limited. The ripe fruits of *A. bunius* were reported to contain two groups of organic acids. The major group of organic acids includes tartaric, ascorbic, citric, and benzoic acids; while malic, lactic, oxalic and acetic acids were found in minor groups (Samappito and Butkhup, 2008). In addition, three flavan-3-ols i.e. catechin, procyanidin B1 and procyanidin B2 were isolated from the ripe fruits of *A. Bunius* (Butkhup and Samappito, 2008). Thus, this study represents the first chemical and biological investigation report of *A. buinis* aerial parts, aiming to study their phenolic constituents and evaluate their antimicrobial and cytotoxic activities.

MATERIALS AND METHODS

Plant material

The aerial parts of *Antidesma bunius* (L.) Spreng were collected from Al-Orman Botanical Garden, Giza, Egypt, in April 2010. The plant was kindly identified by Mrs. Therese Labib, Senior Manager and Specialist of Plant Taxonomy in Al-Orman Botanical Garden, Giza, Egypt. A voucher specimen was deposited in the Herbarium of

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the Pharmacogonsy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

Apparatus

A Büchi melting point apparatus was used for recording melting points (uncorrected), while a JASCO 320-A spectrometers was used for recording IR spectra. CD data were obtained in MeOH on a Jasco J-815 spectrophotometer. Optical activity was measured with a Perkin-Elmer 241 spectropolarimeter. The ESI-MS/EI-MS measurements were recorded on Quattro LCZ mass spectrometer from Waters in the positive and negative ion modes. Elemental analytical data was recorded on Carlo Erba, Model 1106 elemental analyzer. 1&2 D-NMR experiments were applied by using $CDCl_3$, DMSO-d_6 and $CDOD_3$ on a Varian Mercury 300BB (300MHz) and Varian Inova (500MHz) at ambient temperature with TMS as the internal standard. The δ-values are recorded as ppm, while coupling constants (*J*) are reported in Hz. Column chromatography (CC) was carried out using silica gel (230-400mesh). All the solvents are used in experiments were distilled prior to use. Other solvents were commercial grade without purification. Compounds were revealed by spraying with vanillin/boric acid and 1% ethanolic $FeCl_3$ reagents on TLC. Sephadex LH-20 was carried out in isolation of compounds by using methanol-water (v/v). Preparative TLC was performed on silica-gel plates (Kieselgel $60F_{254}$, 0.5mm, Merck) using toluene-acetone (60:40v/v).

Extraction and Isolation

The powder of shade-dried aerial parts of *A. bunius* (1kg) was defatted with petroleum ether (3x4Lx24hrs) at room temperature to get residue (PEE, 12.5g). Defatted plant material was further extracted at room temperature with 80% EtOH (EE), three times for 48hr (4L) at room temperature. The combined EtOH extract was evaporated to dryness at 50±5ºC using rotary evaporator to afford EtOH residue (EE; 200.2g). This residue was suspended in a mixture of MeOH-water (1:9), followed by successive solvent partitioning to give *n*-hexane (HF;35.5g), $CHCl_3$ (CF; 70.6g), EtOAc (EAF; 23.3g), *n*-BuOH (BF; 10.9g) and aqueous (WF) fractions.

PEE (12.5g) was dissolved in petroleum ether (40-60^{o}C) and loaded on a silica gel column. Elution was successively carried out with *n*-hexane, followed by gradual addition of $CHCl_3$ then EtOAc, in increasing order of polarity, up to (*n*-hexane-$CHCl_3$-EtOAc, 12:1:1v/v/v), leading to four major sub-fractions I-IV. Fraction I, eluted with *n*-hexane-$CHCl_3$ (8:3v/v), showed three major spots on TLC. It was further subjected to column chromatography using *n*-hexane-$CHCl_3$ mixtures to afford compound I (25mg) and compound II (10mg) at 9:1v/v and 8:2v/v, respectively. The fraction III obtained from *n*-hexane-$CHCl_3$ (2:8v/v) was subjected to further purification using CC eluting with $CHCl_3$-MeOH (9:1v/v) to afford compound III (6.8mg).

A part of the CF (54.5g) was loaded on a silica gel column and the elution was successively carried out with $CHCl_3$-EtOAC-MeOH (10:1:1v/v/v) mixture to give eight major fractions (I-VIII). The major fraction I was chromatographed on Silica gel column using $CHCl_3$-MeOH (9:1v/v) as eluent to give compound IV (20mg). The major fraction IV was loaded on a Silica gel column eluted with $CHCl_3$-EtOAc (40:1v/v) to give five subfractions (C11-C15). Subfraction C11 was purified with EtOAc-MeOH-H_2O (8:1:0.1v/v/v), using Silica gel column to give compound V (15mg).The subfraction C15 was applied onto a Sephadex LH-20 column and eluted with MeOH-H_2O (6:4v/v) to give compound VI(15mg) and gradually increment the percentage of H_2O in MeOH (4:6v/v) to give compound VII (8mg).

Compound I (fig. 1): White amorphous solid; m.p.130–134°C; Microanalysis: C,35.83; H,4.51; O,59.66; (calc. for $C_4H_6O_5$); found: C,35.83, H,4.31 O,59.68; IR (ν_{max}^{KBR}), cm^{-1}:2837-2573, 1072 (-OH), 815, 684 (-C-O) and 1279 (-COH) very intensive stretching vibrations. Compared with the data given in literature (Cammas *et al*., 1993), it was identified as malic acid.

Compound II (fig. 1): Yellow amorphous solid; m.p. 223-225°C; Microanalysis: C,66.0; H,4.48; O,35.52; (Calc. for $C_9H_8O_4$); Found: C, 66.07; H, 4.44; O, 35.55; EI-MS: *m/z*180 $[M]^+$, 163, 136, 89, 77, 44. (IRν_{max}^{KBR}), cm^{-1}:1690 (C=O), 1639 (C=C), 1617, 1521 and 1447 (aromatic ring, C=C), 1247 and 1294 (carboxylic, C-O), 1189 (carboxylic, O-H). ^{1}H NMR: (500MHz, DMSO-d_6): δ ppm 12.12 (1H, *s*, COOH), 7.52 (1H, *d*, *J*=15.4 Hz, H-7), 7.01 (1H, *s*, H-2), 6.96 (1H, *d*, *J*=8.4 Hz, H-6), 6.75 (1H, *d*, *J*=8.4 Hz, H-5), 6.17 (1H, *d*, *J*=15.4 Hz, H-8). ^{13}C NMR (125MHz, DMSO-d_6): δ ppm 167.9 (C-9), 148.1 (C-4), 145.7 (C-3), 142.1 (C-7), 125.7 (C-1), 121.2 (C-6), 115.8 (C-5), 115.2 (C-2), 114.7 (C-8). Compared with the data given in reference (Ramaiah *et al*. 1984), it was identified as caffeic acid.

Compound III (fig. 1): Microanalysis; C,62.07; H,4.86; O,33.07; (Calc. for $C_8H_8O_2$); Found: C,62.07; H,4.84; O,33.10; ESI-MS:*m/z* 159.15 $[M+Na]^+$, 135.2 $[M-H^+]^-$,(-ve).(IRν_{max}^{KBR}), cm^{-1}:1720, 1615 and 1450 (carbonyl and aromatic). ^{1}H NMR: (500MHz, $CDCl_3$): δ ppm 8.10 (2H, br *d*, *J*=8.6 Hz, H-2, 6), 7.65 (1H, br , *J*=8.6 Hz, H-4), 7.41 (2H, *J*=8.6 Hz, H-3, 5), 3.56 (3H, *s*, O-CH_3). ^{13}C NMR (125MHz, $CDCl_3$): δ ppm 166.7 (C-7), 134.5 (C-4), 131.4 (C-1), 131.0 (C-2/6), 129.7 (C-3/5), 50.8 (-OCH_3) (Aldrich Library of ^{13}C and ^{1}H FT NMR Spectra; 1992), it was identified as methyl benzoate.

Compound IV (fig. 1): Yellow amorphous powder; m.p. 175–177^{o}C; Micoanalysis; C,62.07; H,4.86; O,33.07; (Calc. for $C_{15}H_{14}O_6$); Found: C,62.07; H,4.84; O,33.10. ESI-MS:*m/z* 313.1 $[M+Na]^+$, (+ve); 289.1 $[M-H]^-$, (-ve). (IRν_{max}^{KBR}), cm^{-1}:3400–2600, (C-H,O-H), 1620, (C=C

stretching); $[\alpha]_D^{20}$ +17.5° (c0.02, MeOH). ^{1}H NMR (500MHz, CD_3OD): δ ppm 6.95 (1H, brs, H-2'), 6.75 (1H, *d*, *J*=8.1 Hz, H-5'), 6.70 (1H, *d*, *J*=8.1 Hz, H-6'), 5.97 (1H, *d*, *J*=2.3 Hz, H-6), 5.94 (1H, *d*, *J*=2.3 Hz, H-8), 5.00 (1H, br *s*, H-2), 4.22 (1H, *m*, H-3), 2.75 (1H, *dd*, *J*=16.6, 8.6 Hz, H-4α). 2.90 (1H, *dd*, *J*=16.6, 2.7 Hz, H-4β), ^{13}C NMR (125MHz, CD_3OD): δ ppm 156.6 (C-5), 156.3 (C-7), 155.8 (C-9), 147.0 (C-3'), 145.8 (C-4'), 132.2 (C-1'), 117.7 (C-6'), 113.7 (C-5'), 110.9 (C-2'), 98.6 (C-10), 94.4 (C-6), 94.3 (C-8), 78.3 (C-2), 66.0 (C-3), 27.8 (C-4). The ^{1}H and ^{13}C NMR spectroscopic data were compared with those in previous studies and revealed that compound IV was (+)-Catechin (Vuong QV *et al*., 20 10; Hye MA 2009).

Compound V (fig. 1): Off white amorphous powder;m.p. 170–172°C; Microanalysis; C,62.07; H,4.86; O,33.07; (Calc. for $C_{15}H_{14}O_6$); Found: C,62.07; H,4.84; O,33.10. ESI-MS:*m/z* 313.2 $[M+Na]^+$ (+ve); ($IR\nu_{max}$ ^{KBR}), cm^{-1}:2400-3200 (C-H,O-H), 1620 (C=C stretching) 1287 (-COH), 864, 795 (-C-O); $[\alpha]_D^{20}$ −42º (c 0.004, MeOH). ^{1}H NMR (500 MHz, CD_3OD): δ ppm7.02 (1H, *d*, *J*=1.5 Hz, H-2'), 6.82 (1H, *dd*, *J*=8.1, 1.5 Hz, H-6'), 6.76 (1H, *d*, *J*=8.1 Hz, H-5'), 6.02 (1H, *d*, *J*=2.2 Hz, H-6), 5.82 (1H, *d*, *J*=2.2 Hz, H-8), 4.88 (1H, *s*, H-2), 4.20 (1H, br *s*, H-3), 2.74 (1H, *dd*, *J*=16.6 Hz, 2.5 Hz, H-4α), 2.87 (1H, *dd*, *J*=16.9 Hz,4.5 Hz, H-4β). ^{13}C NMR (125 MHz, CD_3OD): δ ppm157.3 (C-9), 156.2 (C-5/7), 145.86 (C-3'), 145.74 (C-4'), 132.2 (C-1'), 119.46 (C-6'), 115.9 (C-5'), 115.43 (C-2'), 100.1 (C-10), 96.8 (C-8), 96.2 (C-6), 76.7 (C-2), 66.7 (C-3), 28.2 (C-4), compound V was identify as *Cis-3,3',4',5,7-pentahydroxyflavan[(-)-Epicatecin]* (Agrawal PK, 1989; Pandya DJ and Anand IS, 2013).

Compound VI (fig. 1): Colourless needles; m.p. 231-232°C; Microanalysis; C,68.70; H,6.75; O,24.55; (Calc. for $C_{30}H_{26}O_{12}$); Found: C,68.67; H,6.56; O,24.51. ESI-MS:*m/z* 601.5 $[M+Na]^+$ (+ve); 577.5 $[M-H]^-$ (-ve). ($IR\nu_{max}$ ^{KBR}), cm^{-1}:2400–3200 (aromatic C-H, Phenolic and O-H), 1620 (aromatic C=C stretching), $[\alpha]_D^{20}$ +2.2° (c 0.002, MeOH). A dimer compound with two rings system (A and B). On the ring A, ^{1}H NMR (500MHz, $CDCl_3$): δ ppm7.25 (1H, *dd*, *J*=8.4, 1.8 Hz, H-6'), 7.16 (1H, *d*, *J*=8.4, H-5'), 6.88 (1H, *d*, *J*=1.8, H-2'), 6.00 (1H, *d*, *J*=1.5 Hz, H-8), 5.94 (1H, *d*, *J*=1.5 Hz, H-6), 5.15 (1H, *m*, H-3), 5.45 (1H, *s*, H-2), 4.70 (1H, *s*, H-4). On the ring B, ^{1}H NMR (500MHz, $CDCl_3$): δppm6.89 (1H, *d*, *J*=1.0, H-2'), 6.85 (1H, *dd*, *J*=8.4, 1.0 Hz H-6'), 6.68 (1H, *d*, *J*=8.3 Hz, H-5'), 6.68 (1H, *s*, H-6), 4.53 (1H, *d*, *J*=9.9 Hz, H-2), 5.05 (1H, *ddd*, *J*=9.9, 9.3, 6.6 Hz, H-3), 2.64 (1H, *dd*, *J*=16.4, 9.0 Hz, H-4α), 2.58 (1H, *dd*, *J*=16.3, 6.3 Hz, H-4β). This compound was identify as *Epicatechin-(4β→8)-catechin (Procyanidin B1)* (Nonaka G-I, 1981).

Compound VII (fig. 1): Yellow amorphous powder; m.p.196–198°C; Microanalysis; C,66.84; H,6.45; O,26.71; (Calc. for $C_{30}H_{26}O_{12}$) Found: C,66.76; H,6.42; O,26.69. ESI-MS:*m/z* 601.5 $[M+Na]^+$ (+ve); 577.5 $[M-H]^-$ (-ve). ($IR\nu_{max}$ ^{KBR}) cm^{-1}:2400-3200 (aromatic C-H, Phenolic and O-H), 1620 (aromatic C=C stretching), $[\alpha]_D^{20}$ + 4.5° (c 0.002, MeOH). A dimer compound with two rings systems (A and B). On the ring A, ^{1}H NMR (500MHz, $CDOD_3$): δ ppm6.89 (1H, *s*, H-2'), 6.73 (1H, *d*, *J*=8.1 Hz, H-5'), 6.70 (1H, *d*, *J*=8.1 Hz, H-6'), 5.99 (1H, *s*, H-6), 5.97 (1H, *s*, H-8), 5.09 (1H, *s*, H-2), 4.72 (1H, *s*, H-4), 3.89 (1H, *d*, *J*=1.0, H-3). ^{13}C NMR (125MHz, $CDOD_3$): δ ppm157.0 (C-7), 156.6 (C-9), 156.2 (C-5), 144.6 (C-2'), 144.1 (C-3'), 124.2 (C-6'), 122.0 (C-5'), 115.4 (C-1'), 115.1 (C-4'), 100.1 (C-10), 96.3 (C-8), 95.6 (C-6), 73.6 (C-2), 71.1 (C-3), 34.0 (C-4). On the ring B, ^{1}H NMR (500MHz, $CDOD_3$): δ ppm6.92 (1H, *s*, H-2'), 6.72 (1H, *d*, *J*=8.1Hz, H-5'), 6.70 (1H, *d*, *J*=8.1Hz, H-6'), 5.95 (1H, *s*, H-6), 4.27 (1H, *s*, H-2), 4.12 (1H, *m*, H-3), 2.70 (1H, *dd*, *J*=16.4, 4.6 Hz, H-4α), 2.54 (1H, *dd*, *J*=16.4, 3.8 Hz, H-4β). ^{13}C NMR (125 MHz, $CDOD_3$): δ ppm157.4 (C-7), 155.1 (C-5), 152.9 (C-9), 144.8 (C-2'), 141.2 (C-3'), 124.1 (C-6'), 122.0 (C-5'), 115.6 (C-1'), 115.1 (C-4'), 106.4 (C-8), 102.4 (C-10), 96.8 (C-6), 79.2 (C-2), 66.8 (C-3), 27.8 (C-4). Consistent with the reported spectral data of Epicatechin-(4β→8)-epicatechin (Procyanidin B2), in literature (Foo LY and Karchesy JJ, 1989).

Screening for antimicrobial activity

Antimicrobial activity of the obtained compounds I-VII, was carried out according to the National Committee of Clinical Laboratory Standards (NCCLS) (Farraro *et al*., 2000) using an American Type Culture Collection (ATCC) standard for four bacterial (two Gram positive, +ve and two Gram negative, -ve) and four fungal strains, *Bacillus subtilis*, *Streptococcus pneumonia*e, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus fumigatus*, *Candida albicans*, *Geotricum candidum* and *Syncephalastrum racemosum*, respectively.

Bacterial and fungal strains from stock cultures were grown in 10ml nutrient broth (Merck 5443, Darmstadt, Germany) or malt extract broth (Merck 5397, Darmstadt, Germany) cultures for 24h, respectively. Ten ml of broth culture were prepared overnight in nutrient or malt extract broths for 24h. Test fungi were grown in this culture at 25°C for 18h. The other microorganisms (MOs) were grown in nutrient broth at 35°C for 18h. All tested MOs were quantified in nutrient broth or malt extract broth by the serial dilution method. Their eventual cell concentrations were 106-107CFU/ml. The disc diffusion method (Bauer *et al*., 1996) was used to assess antimicrobial activity. The bacterial cell suspension was prepared from a 24h culture and adjusted to an inoculation concentration of $1x10^6$CFU/ml. Sterile nutrient agar (Immunpräparate, Berlin, D, 26g agar/L. distilled water) was inoculated with bacterial cells (100µl of bacterial cell suspension in 25ml medium) and poured into dishes to give a solid plate. Fungi ($1x10^6$ colony

forming units per mL) were inoculated into sterile Mueller-Hinton agar (Becton Dickinson, Heidelberg), according to DIN E 58940-3 for the agar disc diffusion assay (Al-Fatimi M *et al*., 2007). The screened compounds were dissolved in dimethylsulfoxide (DMSO, Merck, Germany) and loaded on a sterile discs (6mm diameter). Tested isolated compounds I-VII, at a dose of 5mg/ml, were used for the study. Different drugs were used as positive control, including Penicillin G for +ve bacteria, Streptomycin for -ve bacteria and Amphotericin B for fungi. DMSO was used as blank. The discs were stabilized on the surface of inoculated agar plates. The plates were preserved for 1h in the refrigerator to enhance pre-diffusion of compounds into the agar. The plates were incubated for 24h at 37°C, and 48h at 30°C with bacteria and fungi respectively. At the end of the incubation period, the inhibition zone diameters for each disc were measured. A mean zone of inhibition was calculated for the three replicates.

Determination of minimum inhibitory concentration

The MIC-value was determined for all isolated compounds I-VII using the agar diffusion method (Rajbhandari M and Schöpke T, 1999). Many concentrations of the tested samples were prepared as: 1, 0.50, 0.45, 0.40, 0.35, 0.30, 0.25, 0.20, 0.15, 0.10 and 0.05mg/ml, (Each assay was replicated three times). Hundred µl of each concentration was soaked in the disc. Then, the discs were loaded on the Petri dishes containing the MOs to be tested. The plates were incubated for 24h at 37°C and 48h at 30°C for bacteria and fungi respectively. After incubation, colonies numbers in each plate was counted.

Evaluation of cytotoxic activity

The potential cytotoxicity of different *A. bunius* extracts and isolated compounds was tested *in vitro* against three human cell lines i.e. the liver cancer cell line HepG2, the breast cancer cell line MCF7 and the colon cancer cell line HCT. The cytotoxic activity was estimated using the sulphurhodamine B assay (SRB) (Skehan P *et al*., 1990), in which the cells were placed in a 96-well plate (10^4cells/well) for 24h prior treatment with the extracts/compounds to permit attachment of cells to the wall of the plate. Various concentrations of tested samples in DMSO (1.56, 3.125, 6.25, 12.5, 25 and 50µg/ml) were added to the cell monolayer. DMSO and Doxorubicin (Sigma-Aldrich Co.) were used as blank and positive control respectively. Six concentrations of doxorubicin from 2to64µg/ml using two fold serial dilutions in DMSO were prepared. Triplicate wells were incubated with the samples, blank and positive controls, for 48h at 37ºC in an atmosphere of 5% CO_2. The cells were then fixed, washed and stained with SRB. Excess stain was cleaned with 1% glacial acetic acid and adhesive stain was recovered with Tris-EDTA buffer. The colour intensity was measured in an ELISA reader at 564nm. The percent cell survival was calculated compared to the untreated cell (negative control), that was assumed as 100% viable. The relationship between the surviving fraction and the drug concentration is plotted to assess the survival curve for each cancer cell line, after treatment with the specified test sample and positive control. The curves were fitted using a linear equation and the IC_{50} was calculated.

RESULTS

Chemical results

The procyanidin constitution of CF was proved by TLC and by spraying the TLC by using vanillin/boric acid reagent, which afforded cyanidin as the principle pigment. Structures of compounds I-VII are presented in s. 1.

The petroleum ether (PEE) and the 80% aqueous EtOH (EE) extracts of *Antidesmabunius* (L.) Spreng.aerial parts, as well as, the *n*-hexane (HF), $CHCl_3$ (CF), EtOAc (EAF), *n*-BuOH (BF) and H_2O (WF) soluble fractions of the latter were prepared. Phytochemical study performed on *A. bunius* aerial parts led to the isolation of malic acid (I), caffeic acid (II), methyl benzoate (III) from the PEE, while trans-3,3',4',5,7-pentahydroxyflavan [(+)-Catechin] (IV), cis-3,3',4',5,7-pentahydroxyflavan [(-)-Epi-catechin] (V), Epicatechin-(4β→8)-catechin (Procyanidin B1) (VI) and epicatechin-(4β→8)-epicatechin (PB2) (VII) were isolated from the CF.

Antimicrobial activity and MIC

All tested compounds I-VII showed strong to moderate antimicrobial activity, except for compounds I and IV, which did not show any effect on all tested microorganisms. The most active compound was found to be V, with zones of inhibition ranging from 25.6-17.3mm followed by compounds VI (19.8-15.2mm), II (18.9-16.0mm), VII (15.6-13.2mm) and then III (14.5-10.0mm). All tested compounds showed no antimicrobial activity against the Gram negative bacteria *Pseudomonas aeruginosa* and the fungi *Candida albicans*. MIC of all tested compounds were in the range of 0.03-125µg/ml. Compound VII showed the lowest range of MIC (0.03–15.63µg/ml), followed by V (1.95-62.5µg/ml), II (3.9-62.5µg/ml), VI (31.25–125µg/ml) and finally III (62.5-125µg/ml). Antimicrobial activities and MIC of compounds I-VII and reference drugs are compiled in table 1.

Cytotoxic activity

The potential cytotoxicity of different *A. bunius* extracts, fractions (PEE, EE, CF and WF) and the isolated compounds (I-VII) were tested *in vitro* against the three human cell lines HepG2, MCF7 and HCT. All tested extracts, fractions and compounds showed dose dependent cytotoxic effect against all three cell lines. The tested extracts and fractions showed cytotoxic activities against HepG2 cells, with PEE being the most active followed by WF, CF and then EE. In addition, the IC_{50} of the PEE and WF were 23.7µg and 39.7µg, respectively.

Table 1: Antimicrobial activity and MIC of compounds I–V11 isolated from *Antidesma bunius* using agar diffusion technique.

Microorganism	Cpd. I		Cpd. II		Cpd. III		Cpd. IV		Cpd. V		Cpd. VI		Cpd. VII		penicillin G		Streptomycin		Amphotericin B	
	M ±S.D[a]	MIC	M ±S.D[a]	MIC	M ± S.D[a]	MIC	M ± S.D.[a]	MIC	M ±S.D[a]	MIC	M ±S.D[a]	MIC	M ±S.D[a]	MIC	M ±S.D[a]	MIC	M ± S.D[a]	MIC	M ±S.D[a]	MIC
Bacillus subtilis	NA	NA	18.3 ± 0.25	7.81	14.5 ± 0.67	62.5	NA	NT	23.5 ±0.54	1.95	19.8 ± 0.19	31.25	15.6 ± 0.16	0.12	26.4 ± 0.4	0.06	NT	NT	NT	NT
Streptococcus pneumonia	NA	NA	16.0 ± 0.44	31.25	12.4 ± 0.44	125	NA	NT	20.3 ±0.33	7.81	17.5 ± 0.25	125	13.5 ± 0.34	1.95	25.7 ± 0.2	0.12	NT	NT	NT	NT
Escherichia coli	NA	NA	NT	62.5	10.0 ± 0.46	125	NA	NT	17.6 ±0.28	15.63	16.8 ± 0.19	125	NT	7.81	NT	NT	26.7 ± 0.9	0.06	NT	NT
Pseudomonas aeruginosa	NA	NA	NA	NA	NA	NA	NA	NT	NA	NA	NA	NA	NA	NA	NT	NT	20.4 ± 0.3	3.9	NT	NT
Aspergillus fumigatus	NA	NA	18.6 ± 0.25	3.9	NA	NA	NA	NT	21.7 ±0.28	1.95	19.8 ± 0.25	31.25	15.3 ±0.39	0.49	NT	NT	NT	NT	23.7 ± 0.1	0.12
Candida albicans	NA	NA	NA	NA	NA	NA	NA	NT	NA	NA	NA	NA	NA	NA	NT	NT	NT	NT	25.4 ± 0.1	0.06
Geotricum candidum	NA	NA	18.9 ± 0.58	3.9	NA	NA	NA	NT	25.6 ±0.29	1.95	19.6 ± 0.58	125	14.3 ±0.25	0.03	NT	NT	NT	NT	28.7 ± 0.2	0.015
Syncephalastrum racemosum	NA	NA	17.2 ± 0.34	15.63	NA	NA	NA	NT	17.3 ±0.27	62.5	15.2 ± 0.25	125	13.2 ±0.19	15.63	NT	NT	NT	NT	19.7 ± 0.2	1.95

Samples were tested at a concentration of 5mg/ml; M±S.D.: mean ± standard deviation; Diameter of the inhibition zone (mm) beyond the well diameter of 6 mm; NT: not tested; NA: no activity; MIC expressed in µg/ml.

Table 2: Inhibitory activity of different *A. bunius* extracts/isolated compounds against *Hepatocellular carcinoma* cells (HepG2).

Make a cell for	Cell viability %							
Sample conc. (µg)	0	1.56	3.125	6.25	12.5	25.0	50.0	IC_{50} (µg)
EE	100	100	98.96	96.24	89.92	73.46	60.12	-
PEE	100	92.62	80.18	71.39	59.76	47.84	33.12	23.7
CF	100	100	100	98.91	89.12	76.54	57.28	-
WF	100	98.24	94.17	90.98	79.02	63.38	40.59	39.7
I	100	100	100	100	97.84	82.48	37.32	42.9
II	100	100	97.50	93.44	81.98	68.14	21.86	34.8
III	100	100	100	97.32	86.54	70.62	34.36	32.7
IV	100	100	100	100	96.18	80.62	35.44	41.9
V	100	100	100	97.98	88.14	69.86	19.58	34.9
VI	100	100	96.92	91.26	73.87	49.32	26.47	24.7
VII	100	100	100	95.14	83.78	67.29	18.72	33.9

(-) indicates weak inhibitory activity under these experimental conditions.

Table 3: Inhibitory activity of different *A. bunius* extracts/compounds against breast carcinoma cells (MCF-7).

Make a cell for	Cell viability %							
Sample conc. (µg)	0	1.56	3.125	6.25	12.5	25.0	50.0	IC_{50} (µg)
EE	100	100	100	97.38	90.12	79.54	68.36	-
PEE	100	100	98.18	87.96	74.25	61.84	40.12	38.2
CF	100	100	95.18	86.22	70.65	48.12	38.46	24.0
WF	100	100	96.98	91.14	76.72	63.46	47.38	45.9
I	100	100	96.92	89.46	79.24	67.18	38.92	40.2
II	100	97.58	93.17	82.38	69.44	53.90	32.56	29.6
III	100	100	97.24	89.72	77.15	60.84	34.86	35.4
IV	100	99.02	95.37	84.53	68.32	49.46	31.85	24.6
V	100	96.86	91.12	80.44	61.36	47.18	32.30	22.5
VI	100	93.80	86.14	73.31	57.16	34.98	22.32	16.5
VII	100	96.93	94.36	87.12	75.90	58.71	32.44	33.3

(-) indicates weak inhibitory activity under these experimental conditions.

Table 4: Inhibitory activity of different *A. bunius* extracts/compounds against colon carcinoma cells (HCT).

Make a cell for	Cell viability %							
Sample conc. (µg)	0	1.56	3.125	6.25	12.5	25.0	50.0	IC_{50} (µg)
EE	100	100	100	100	100	98.34	91.22	-
PEE	100	100	97.68	89.40	78.92	59.84	38.15	36.3
CF	100	100	97.48	90.85	82.52	63.46	51.94	-
WF	100	100	100	98.34	93.12	81.98	68.26	-
I	100	97.92	91.38	85.24	72.76	64.89	39.18	39.5
II	100	99.18	95.42	89.38	77.14	60.96	34.84	35.5
III	100	98.24	92.16	83.35	71.04	58.69	29.72	32.5
IV	100	100	96.50	88.16	80.94	62.33	37.68	37.5
V	100	97.18	93.24	84.51	69.26	48.32	31.14	24.0
VI	100	97.46	90.22	73.18	57.96	39.74	25.28	18.0
VII	100	95.88	89.15	80.94	69.39	38.18	21.34	20.3

(-) indicates weak inhibitory activity under these experimental conditions.

The cytotoxic activities of the tested extracts and fractions against MCF-7 cells were similar to those of HepG2, except for the CF being more active than the PEE. For CF, PEE and WF the IC_{50} values were 24.0, 38.2 and 45.9 µg/ml, respectively. Furthermore, the tested extracts and Fractions showed lower activity towards HCT cells than on both other cell lines. The IC_{50} could only be determined for the PEE, being 36.3µg/ml.

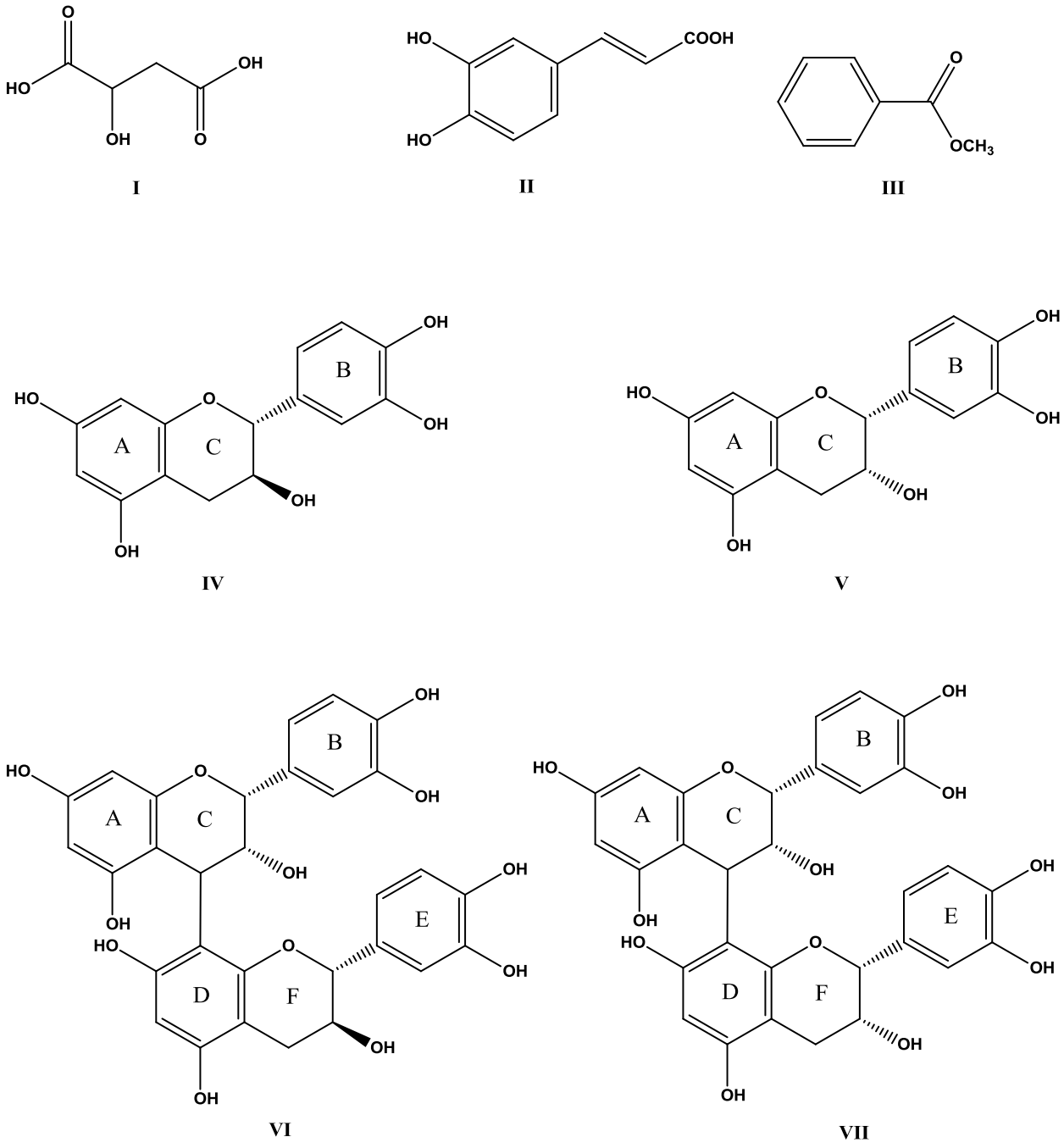

Fig. 1 Structures of compounds I-VII

All tested compounds I-VII showed dose dependent cytotoxic effect on all three cell lines. TheIC_{50} values of compounds I-VII were in the ranges of 24.7-42.2µg/ml, 16.5-40.2µg/ml, and 18.0-39.5µg/ml for HepG2, MCF-7 and HCT cell lines respectively. Furthermore, compound VI was found to possess potent cytotoxicity against HepG2, MCF7 and HCT cell lines, with IC_{50} of 24.7, 16.5 and 18.0µg/ml, respectively. Results of the *in vitro* cytotoxicity study are recorded in tables 2-4.

DISCUSSION

Compounds I and II were identified as malic acid and caffeic acid, respectively as compared for their physical, spectral data and co-chromatography with those of reference samples (Samappito and Butkhup, 2008; Cymerman Craig and Roy, 1965).

IR spectrum of compound III disclosed absorption bands at 1720, 1615 and 1450cm^{-1}, owing to ester carbonyl and

aromatic functions. ESI-MS: *m/z* 159.15 $[M+Na]^+$, 135.2 $[M-H^+]^-$, corresponds to molecular formula $C_8H_8O_2$. The ^1H NMR spectrum of compound III disclosed three resonances in the aromatic region at δ8.10 (2H, br *d*, *J*=8.6 Hz), 7.65 (1H, br *t*, *J*=8.6 Hz) and 7.41 (2H, *t*, *J*=8.6 Hz), corresponding to H-2/6, H-4, H-3/5 andcarbomethoxy protons at 3.56 (3H, s). The ^{13}C NMR spectrum of compound III confirmed the presence of six carbon signals for one methyl (50.8ppm), five methine (in three carbon signals) and two quaternary carbons (C-1 & C-7). The downfield signal at 166.7 might be assigned to ester carbonyl, whereas the signals in the aromatic region at δ134.5, 131.4, 131.0 and 129.7 were assigned to the aromatic methines and quaternary carbon atoms. The physical and spectral data of III coincided with those represented in the literature for methyl benzoate (Aldrich Library of ^{13}C and ^1H FT NMR Spectra; 1992).

Compound IV was found as yellow amorphous powder. Positive ion ESI-MS gave molecular ion peak at *m/z* 313.1 $[M+Na]^+$ as an adduct with Na^+, consistent with the molecular formula of $C_{15}H_{14}O_6$ and Mwt. of 290.1(Calc. for $C_{15}H_{14}O_6$, 290.1). The IR spectrum of compound IV showed a broad band at 3400–2600 cm^{-1}, indicating the presence of aliphatic and aromatic C-H, phenolic and alcoholic O-H stretching, and a sharp band at 1620cm^{-1} suggesting the presence of aromatic C=C stretching. The ^1H and ^{13}C NMR spectra indicated that this compound belongs to flavonoids. ^1H NMR spectrum of compound IV showed two meta doublets at δ5.97 and 5.94, due to H-6 and H-8 protons, respectively. The ^{13}C NMR spectrum of IV exhibited 15 carbon signals for one methylene, eight methine and six quaternary carbons. It revealed signals at δ78.3, 66.0, 27.8,94.4, 94.3, 110.9, 113.7 and 117.7 assignable to C-2, C-3, C-4, C-6,C-8,C-2', C-5'andC-6', respectively, in addition to others aromatic carbons peaks at δ156.3, 156.6, 155.8, 98.6, 132.2, 147.0 and 145.8. Based on NMR spectral data, chemical analysis and a comparison of the data that present in the literature (Hye MA *et al*., 2009; Nikkon *et al*., 1995) compound IV was identified as (+)-catechin.

Compound V was obtained as an off white amorphous powder and gave positive reaction with vanillin/boric acid reagent. ESI-MS spectrum obtained in the positive ion mode showed signals at *m/z*313.2 interpretable for adduct ion with Na^+ and corresponding to Mwt. 290.1, presenting a monomer (catechin or epicatechin). The ^1H NMR spectrum of V displayed two singlet signals at δ4.88 and 4.20 corresponding to H-2 and H-3, respectively, in addition to two doublets of doublets at δ2.87 and 2.74 assigned to H-4 protons (coupled to each other with *J*=16.9 Hz and to H-3 with *J*=4.5 and 2.5 Hz). Aromatic signals appeared at 7.02 (*d*, *J*=1.5 Hz), 6.76 (*d*, *J*=8.1 Hz) and 6.82 (*dd*, *J*=8.1, 1.5 Hz) were indicative to H-2', H-5' and H-6' of 3,4-dihydroxy of ring B. In addition to a pair of meta-coupled doublets at δ 6.02 (*d*, *J*=2.2) and 5.82 (*d*, *J*=2.1) for H-6 and H-8 of 5, 7-dihydroxy ring A. Accordingly, ^1H NMR spectrum clearly suggested an epicatechin structure for compound V. This was further approved by the^{13}C NMR spectrum in which 15 carbon signals were observed, including three key aliphatic carbon signals at δ76.7 (C-2), 66.7 (C-3) and 28.2 (C-4), respectively (Porter LJ *et al*., 1982). Therefore, compound V was identified as (-)-epicatechin (Nikkon *et al*., 1995).

Compound VI was obtained as colourless needles. The ESI-MS spectrum (positive ion mode) of VI revealed an aduct ion at *m/z*601.5 $[M+Na]^+$ and $[M-H]^+$ *m/z* at 577.5 in negative ion mode, corresponding to the molecular formula $C_{30}H_{26}O_{12}$. In the ^1H NMR spectrum, heterocyclic coupling constant ($J_{2,3}$<2) confirmed relative 2,3-*cis* configuration of the 'upper' constituting flavan unit, whereas the 2,3-trans stereostructure of 'lower' unit was indicated by large value of $J_{2,3}$ (9.9Hz). The aromatic region showed two ABM- coupling systems for H-6', H-5' and H-2' (7.25, 7.16, 6.88ppm) & (6.85, 6.68, 6.89ppm) confirming the ortho-dihydroxylation of B and E rings. The assignment of H-6 (A), H-8 (A) and H-6 (D) at δ5.94, 6.00 and 6.68ppm, respectively, as well as H-2 (F) at 4.53 ppm, indicated (4β→8) linked dimeric proanthocyanidin (Mello JP *et al*., 1996), which was confirmed by downfield shift of C-8 (lower) at 107.0 (Δ≈+10ppm) and confirmed by HMBC. The spectral data of compound VI were consistent with Epicatechin-(4β→8)-catechin (procyanidin B1), also reported in literature (Lu Y and Foo LY, 1999).

Compound VII was obtained as yellow amorphous powder; m.p.196-198^oC; $[\alpha]_D^{20}$+4.5^o. The ESI-MS spectrum (positive ion mode) of VII revealed an aduct ion at *m/z*601.5 $[M+Na]^+$ and $[M-H]^+$ *m/z* at 577.5 in negative ion mode, corresponding to the molecular formula $C_{30}H_{26}O_{12}$. Carbon signals at δ_C73.6, 79.2 (125MHz, $CDOD_3$) were consistent with C-2 resonances of epicatechin moieties.The observation of C-2 carbon signal at δ73.6 is convenient with a 2,3-cis-3,4-trans structure in epicatechin (Foo LYand Karchesy, 1989). The signals that are noticed at δ100.1-102.4, 96.3-106.4, 115.4-115.6 are owing to C-10, C-8 and C-1'. The aromatic CH resonances at 95.6-96.8, 144.6-144.8, 122.0-122.0 and 124.2-124.1 are assigned to C-6 and C-2', C-5' and C-6'. The high-amplitude negative cotton effects in the 240-300nm region of the circular diachroism confirmed the intraflavan linkage to be 4S. Furthermore, the chemical degradation suggested that both of the two units in the structure of VII are epicatechin. Hence the structure of compound VII was confirmed as epicatechin-(4β→8)-epicatechin (Procyanidin B2), which previously described in literature by (Antonelli-Ushirobira *et al*., 2007).

The antimicrobial activities of the isolated compounds I-VII were evaluated using diffusion agar technique. Compounds I-VII showed strong to moderate antimicrobial

activity, except for compounds I and IV, which did not show any effect on all tested micro organisms. Caffeic acid is reported to inhibit the growth of -ve bacteria including *Escherichia coli* and completely inhibited the growth of gram positive bacteria as *Bacillus cereus*. In addition, caffeic acid completely inhibited the growth and aflatoxin production of some *Aspergillus* species (Aziz *et al*., 1998). The -ve bacteria differ from +ve bacteria in having a thick liposaccharide coated cell wall, which might be impermeable to the polar phenolic acids (Bais *et al*., 2002; Chakraborty *et al*., 2007).Our study confirmed these findings, as compound II showed MIC values of 7.81 and 31.25µg/ml against the Gram positive bacteria *Bacillus subtilis* and *Streptococcus pneumonia*e, respectively, while the MIC value was 62.5µg/ml against the Gram negative bacteria *Escherichia coli* and compound II was not active against *Pseudomonas aeruginosa*. In addition, compound II showed MIC values of 3.9, 3.9 and 15.63µg/ml against the fungi *Aspergillus fumigatus, Geotricum candidum* and *Syncephalastrum racemosum*, respectively. Furthermore, benzoic acid esters showed good antimicrobial activity; this may be due to the presence of an ester group. The organic acid esters that are widely used as food preservatives are alkyl esters of p-hydroxyl benzoic acid, commonly referred to as parabens. Compound III possess a chemical structure similar to parabens and may therefore exert similar antimicrobial activity like parabens (Goldberg and Williams, 1991; Sathish *et al*., 2013). The antimicrobial activity of flavan-3-ols has been reported and the antimicrobial effect of the flavan-3-ols epigallocatechin gallate and epicatechin have been investigated (Ikigai *et al*., 1993). They found that Gram-positive bacteria were more susceptible than Gram-negative bacteria towards the effect of these catechins. They suggested that the mode of action of these flavan-3-ols was to cause damage to the bacterial cell membrane. In addition, they suggested that the outer membrane of -ve bacteria provides a protective barrier and prevents these flavan-3-ols from binding to the bacterial cell and are therefore less susceptible to the effect of this class of compounds. These results are in accordance with the effect of the investigated compounds, since compounds V, VI and VII showed MIC ranging from 0.12–125µg/ml against the tested Gram positive bacteria, while they were in the range of 7.81–125µg/ml against the tested Gram negative bacteria. In addition, compounds V-VII were not active against the Gram negative bacteria *Pseudomonas aeruginosa*. Furthermore, flavan-3-ol dimers and oligomers are reported to act as antifungal compounds to extend shelf life and to enhance quality preservation (Hébert C *et al*., 2002).The potential cytotoxic activities of PEE, EE, CF and WF and the isolated compounds I-VII were investigated *in vitro* against HepG2, MCF7 and HCT cell lines. All tested extracts, fractions and compounds showed dose dependent cytotoxic effect against all three cell lines examined. The PEE and CF were found to be the most active samples among all tested extracts and fractions on the cell lines MCF-7 and HCT, while WF was more active than CF in case of HepG2 cell line. The cytotoxic activity of PEE could be attributed to the presence of caffeic acid, since it is reported to cause the reduction in cell viability of HepG2 (Chung *et al*., 2004). In addition, aromatic aerobic biotransformation products of toluene - including benzaldehyde, methyl benzoate - showed toxicity on HeLa cells (Shen *et al*., 1989). The cytotoxic effect of CF may be attributed to the presence of flavan-3-ol (catechin) and/or proanthocyanidins. In 2002, a phytochemical study manifested that extracts of whole cranberry containing proanthocyanidins and other flavonoids inhibited *ornithine* decarboxylase (ODC) activity in mouse epithelial cells (ME-308). Characterization of an active subfraction confirmed the presence of dimers and oligomers of catechinepicatechin, monomeric catechins, and quercetin glycosides (Kandil FE *et al*., 2002). Furthermore, proanthocyanidins in grape seeds are confirmed to inhibit the growth of breast cancer cells both *in vitro* (Ye *et al*., 2002; Agarwal *et al*., 2000) and *in vivo* (Kim *et al*., 2004).

CONCLUSION

According to the findings of this study, antimicrobial and cytotoxic activities of *A. bunius* extracts, fractions and/or pure isolates could be attributed the plant's content of phenolic acids, flavan-3-ol and/or proanthocyanidin and possibly other classes of compounds. Based on the different classes of secondary metabolites reported in the other *Antidesma* species e.g. triterpenoids, steroids, megastigmanes, lignans, cyclopeptide and quinolide-type alkaloids, along with their diversity of biological activities including antioxidant, diuretic and antimalarial effects and their use in treatment of epilepsy; it is encouraging for further phytochemical and biological investigation of *A. bunius* aerial parts.

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