BIOACTIVITY OF *ANTIDESMA BUNIUS* LEAVES (EUPHORBIACEAE) AND THEIR MAJOR PHENOLIC CONSTITUENTS

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Abstract

Chromatographic fractionation of the methanol extract of *Antidesma bunius* leaves afforded six polyphenols, namely, corilagin (**1**), gallic (**2**), ferrulic (**3**) and ellagic (4) acids in addition to the flavone vicinin II (**5**) and the dimmer amentoflavone (**6**). Their structures were elucidated by NMR and HRESI analysis. This is the first reported occurrence of the biflavone (amentoflavone) and vicinin in this species. The total phenolic content was estimated at 90 mg/ml of gallic acid equivalent (GAE) per 100 g plant extract using the Folin-Ciocalteu method. Antioxidant biochemical assay of the plant methanol extract was carried out using culture medium of hepatocyte cell lines. An increase in glutathione reductase enzyme and a reduction of the nitric oxide level in the cell culture revealed that *A. bunius* leaf extract possesses significant antioxidant activity in comparing with quercetine as a reference. The hepatotoxicity and hepatoprotective activities of *A.bunius* leaf extract and the pure isolated compounds: **1** , **2** and **5** were evaluated by adopting the MTT colorimetric assay. Compounds **1** and **2** revealed a margin of safety on the monolayer hepatocyte with an IC_{50} > 1000 µg/ml while for **5**, IC_{50} was at 125 µg/ml. The hepatoprotective activities for *A.bunius* leaf extract, **1** and **2** in comparison to Silymarin (50 µg/ml) were at 6.5, <12.5 and 12.5 µg/ml respectively while **5** did not show hepatoprotective activity at the tested concentrations.

Keywords : *Antidesma bunius*, Euphorbiaceae, phenolics, amentoflavone, antioxidant effect, hepatotoxicicity, hepatoprotection

Introduction

Antidesma bunius (L.) Spreng (family Euphorbiaceae) is a medicinal plant found in many dipterocarp forested areas in Thailand, Philippines, and also throught the region of southest Asia (Roger, 2004). The plant is commonly known to Thai local people as Mao Luang while in Philippines as Bignay. It is a shrub species with wide-spreading branches forming a dense crown evergreen. The fruits when ripe are traditionally used as a medicinal plant for gastric intestinal problem, e.g. diabetes, dysentery, indigestion and constipation. Its leaves usually used as flavoring when combined with other vegetables while its fruits are processed into jam and jelly and its juices fermented into wine and brandy. Recently, bignay was used to treat different illnesses ranging from colds to cancer (Magsino, 2003).

Previous studies demonstrated cytotoxic activity against brine shrimp (Micor *et al.*, 2005**)** of *A. biunius* leaves and fruits .Various alkaloids specially the glycine-derived acetogenic quinoline alkaloid and antidesmone have been detected in some *Antidesma species* (Steenkamp *et al*, 2009; Bringmann *et al.* 2000; Watt and Breyer- Brandwijk, 1962). *Antidesma species* are used by the Vhavenda for the treatment of gynaecological complaints (Steenkamp *et al*, 2009). The root-bark is taken for dysentery and washed in to ease body pain (Palgrave, 2002; Gerstner, 1938). Fruits of *A.biunius* were analyzed by Butkhup and Samappito (2008) for the contents of catechins and procyanidins qualitatively and quantitatively. Furthermore, this crop plant has been considered to be good source of bioactive phenolics. To our best of knowledge, it could be possibly inferred that our report is the second one concerning isolation and characterization of *A. biunius* leaves phenolics. The previous phytochemical investigation of its phenolics lead to the isolation of corilagin, gallic and chebulic acid (Hui and Sung, 1968)**.** We aimed to isolate and characterize major phenolic constituents from the methanol extract of the leaves of *A. biunius* (MEABL) , to estimate their total content and evaluate the antioxidant activity of MEABL through biochemical level assays (Lactate dehydrogenase (LDH), nitric Oxide (NO) production) and glutathione reductase (GR) in culture medium of hepatocyte cell lines (Rec.GSCC (DGKC) 1970; Montgomery and Dymock, 1961; Goldberg and Spooner,1983) . In addition, we aimed to evaluate the *in vitro* hepatotoxicity and hepatoprotective activity of MEABL and isolated pure phenolics (Seglen, 1976; Kiso *et al*., 1983).

Experimental

Instruments

1D & 2D NMR experiments (^{1}H, ^{13}C and HMBC) were recorded using a Jeol EX-500 spectrometer: 500MHz (^{1}H NMR) 125 MHz (^{13}C NMR) DMSO-*d6* and a Brucker spectrometer: 400 MHz (^{1}H NMR), 100 MHz (^{13}C NMR) HMQC and HMBC in CD_3OD. The chemical shifts are expressed in

(ppm) and the coupling constants are in Hz. The mass spectra (EIMS) were measured on a Finnigan-Mat SSQ 7000 spectrometer; *m/z* (rel. %) while the high resolution mass spectra (HREIMS) were obtained on a VG Auto Spec M series sector (EBE) mass spectrometer. The UV spectra were recorded on UV spectrophotometer (Shimadzu UV-240). Column chromatography was carried out on Polyamide 6S (Riedel-De-Haen AG, Seelze Haen AG, Seelze Hanver, Germany) and Sephadex LH-20 (Pharmacia). The paper chromatography (Whatman No. 1 and 3 MM) was performed using solvent systems: 1) H_2O, 2) 15% HOAc (H_2O–HOAc 85:15), 3) BAW (*n*-BuOH–HOAc–H_2O 4:1:5, upper layer), 4) (C_6H_6–*n*-BuOH–H_2O–pyridine 1:5:3:3, upper layer). Solvents 3 and 4 were used for sugar analysis.

Chemicals and standards

Folin – Ciocalteu's phenol reagent and acetic acid were from Merck; gallic acid (3,4,5-trihydroxybenzoic) were from Sigma, hydrochloric acid (minimum 37%); sodium chloride, methanol and sodium sulphate were from Readel-de Haën; diethyl ether (anhydrous) was from J.T. Baker; and Amberlite XAD-4 was from FLUKA. The following compounds were purchased: 4-(22-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES 99%) (ACROS Co.), Potassium Chloride (KCl) (S.R.L), D(+) glucose (Merck), Calcium chloride ($CaCl_2$) (S.D), Fetal Bovine Serum (FBS) (Lonza), Penicillin Streptomycin (PS) (Gibco BRL), Sodium thiopental, Silymarin (Sigma). Sodium Chloride (NaCl), Sodium Phosphate monobasic (NaH_2PO_4), Sodium Phosphate dibasic (Na_2HPO_4), Potassium Phosphate monobasic (KH_2PO_4), Ethylenebis (oxyethylenenitrilo) tetra-ethylene glycol bis (2-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA), Collagenase Type IV, Culture medium RPMI-1640, Trypan Blue, Insulin, Dexamethasone, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium-bromide (MTT) and Dimethylsulfoxide (DMSO). (Sigma Co.)

Extraction and isolation

Dried powdered leaves of *A.bunius* (500 g) were extracted by maceration using methanol (3L x 2) at room tempareture .The extract was filtered and the solvent was removed under vaccum. The crude extract (130 g) was chromatographed on a polyamide 6S column with a gradient elution of MOH: H_2O with increasing polarity. The separation was monitored by PC, and eluted fractions exhibiting similar appearances when combined, yielding 9 fractions. Repeated paper chromatography and purification over Sephadex LH-20 column lead to the isolation of six pure compounds (**1**-**6**). Fraction 2 afforded compounds **1** (25 mg) while fraction 2 afforded compound **2** (30mg), **3** (10 mg), **4** (15mg) and **5** (18 mg). Fraction **8** afforded compound **6** (7 mg).

Estimation of the Phenolic Content by the Folin–Ciocalteu Test

The total concentration of phenols in the extract was determined according to the Folin–Ciocalteu method **(Waterman and Mole, 1994*)***. The total phenol concentration was calculated from a calibration curve, using gallic acid as a standard, and the results were expressed as mg of gallic acid equivalents (mg GAE) per 100 g fresh fruit.

Biochemical Assays For total methanol extract of A. bunius leaves

Lactate dehydrogenase (LDH)

LDH leakage from cell cytosol to the culture medium has been generally used as an indicator of cell integrity. Primary culture of rat hepatocytes were incubated for 2 hours with ascending concentrations (125, 250, 500, 1000 µg) of different tested samples/mL culture medium. LDH was measured in culture medium to reflect cell viability. The LDH activity was measured by an optimized standard method according to the recommendations of the Deutsche Gesellschaft fur Klinische Chemie (Rec. GSCC(DGKC),1970).

Determination of Nitric Oxide (NO) production

Nitric oxide is very specific to liver injury and is almost always produced during liver inflammation. To test for the possible hepatoprotective effects of tested silymarin samples, different concentrations (12.5, 25, 50, 100 µg/mL) of tested samples were added to culture medium of primary hepatocyte culture that has been subjected to paracetamol injury and incubated for 2 hours. Culture medium was used for measurements of nitrite (a stable metabolite of NO) to reflect NO production by Griess method (Montgomery and Dymock, 1961). Decrease levels of NO production are indicative of hepatoprotection.

Glutathione Reductase (GR)

Reduced glutathione is the main non enzymatic antioxidant within the cell and plays an important role in the defense against oxidative stress. It is assumed that glutathione reductase depletion reflects intracellular oxidation. On the contrary, an increase in glutathione reductase concentrations could be expected to prepare the cell against a potential oxidative insult.

The content of reduced glutathione was quantified by the fluorometric assay of Goldberg and Spooner (1983). The method takes advantage of the reaction of reduced glutathione with N-(1-naphthyl) ethyenediamine. Addition to the cell culture evoke a dramatic decrease of cytosolic GR which have been pretreated for 2 hours with different concentrations (12.5, 25, 50, 100 µg/mL) of tested samples.

NB: Quercetin was used as a positive control; paracetamol (25 mM/mL) was used to induce liver injury. Each concentrations was placed in three wells so that mean of triplicates and standard deviation was used for statistical analysis.

Concentration of samples used: 100 µg sample

Statistical analysis: All data presented are based on means of triplicate absorbance determinations. Experiments were repeated twice for all samples.

In Vitro Bioassay on Primary Culture of Rat Hepatocytes Monolayer:

Animals: Wistar male rats (250-300 g), obtained from the animal house of the NRC (National Research Center, Cairo).

Isolation and Preparation of Rat Hepatocytes Monolayer Culture:

A primary culture of rat hepatocytes was prepared according to (Seglen, 1976) method, which was modified by (Kiso *et al*., 1983) using a waster male rat (250-300 gm) The rat was obtained from the animal house of the NRC (National Research Center, Cairo). Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Guide, 1985).

IC_{50} Determination on Rat Hepatocytes Monolayer Culture:

After 22-24 hours, the rat hepatocyte monolayer was washed twice with Phosphate Buffer Saline (PBS). In order to determine IC_{50}, different concentrations were prepared for each sample (100 – 1000 µg /mL). After two hours of cells incubation with the extract, cell viability was determined using the MTT assay. The assay was performed according to the method of (Mosmann, 1983) modified by Carmichael *et al*., (1987). Absorbance of formasan crystals produced by viable cells was read at 540 and 630 nm dual wave-length using the Automatic Kinetic Microplate Reader (Labsystems Multiskan RC reader). Each experiment was repeated three times and the mean absorption of each concentration was calculated. A graph plotted with X-axis showing the different concentrations of the extract used and the y-axis showing the absorbance percentage of viable cells. The IC_{50} was graphically determined from the concentration that yielded an absorption coinciding with the 50% of cells that received no extract.

Evaluation of Hepatoprotective Activity:

The primary rat hepatocyte monolayer was prepared as above. Different concentrations were prepared from phospholipids fraction (12.5-100 µg /mL) using serial dilutions technique by dissolving in DMSO (1% maximum concentration). For each concentration, three replicates were carried out; in addition to positive control that was 50 µg/ mL Silymarin. The plate was incubated for 2 hrs at 37 ºC and 5% CO_2, then washed twice with PBS. A 200 µL of 25 mM paracetamol was added to each well. After one hour of cells incubation with the paracetamol, cell viability was determined using the MTT assay. The concentration of the extract that was able to

protect the cells from the hepatotoxic effect of paracetamol by hundred percent was considered hepatoprotective.

Results and discussion

Column fractionation of the methanol extract from the leaves of *A. bunius* on a polyamide 6S using a water- methanol step gradient elution, repeated paper chromatography and purification on Sephadex LH 20 afforded six compounds (**1**-**6**) .Compound **1** gave chromatographic, UV, hydrolytic data, ^{1}H and ^{13}C NMR spectral data closely similar to those reported to coraligin (Nawwar *et al.* 1994). While compounds **2**, **3**, **4** and **5** gave chromatographic, UV, and ^{1}H NMR data typical gallic acid, ferulic acid, ellagic acid and Vicinin II respectively (Chanwitheesuk *et al.* 2007; Rajput A.P. and Patel M. K., 2001 ; Osman *et al* 2009; Nawwar *et al* 1994 ; Ye *et al* 2007; Xie *et al* 2003).

Compound **6** was obtained as yellow amorphous powder. It showed chromatographic properties (dark purple on PC under UV light, turning yellow when fumed with ammonia vapor, high R_f values in organic solvents, and no development in aqueous solvents) , hydrolytic experiment (2N HCl, 1hr, 100 ˚C) and UV spectral analysis (Experimental) characteristic for a flavone aglycone. In the negative ion HRESI-MS, a peak corresponding to $[M-H]^-$ was observed at *m/z* 537.077521, showing the molecular formula of **6** to be $C_{30}H_{18}O_{10}$. ^{1}H NMR spectrum (CD_3OD) showed two signals at δ_H 13.09 and 12.96 due to chelated phenolic hydroxyls and two doublets at δ H 7.52 (*d*, 2H, *J* = 8.7 Hz, H-2'/H-5'); 6.78 (*d*, 2H, *J* = 8.7 Hz, H-3'/H-5') assigned to an aromatic system. Signals of a trisubstituted aromatic ring containing one oxygenated carbon were observed at δ H 7.95 (*d*, 1H, *J* = 2.3 Hz, H-2'), 7.12 (*d*, 1H, *J* = 8.7 Hz, H-5') and 7. 90 (*dd*, 1H, *J* = 8.7 and 2.3 Hz, H-6'), along with doublets of a tetrasubstituted aromatic ring containing three oxygenated carbons at δH 6.2(*d*, 1H, *J* = 2.0 Hz, H-6) and 6.40 (*d*, 1H, *J* = 2.0 Hz, H-8).The signals at δC 102.7(C-3), 101.5. (C-3"), 97.8 (C-8) and 119.0 (C-3'") in the ^{13}C NMR spectra, were compatible with the structure of two flavone moieties with the link C-8→C-3'" (Markhan *et al.*, 1987). These data and the additional signals of quaternary carbons detected in the ^{13}C NMR spectra were compared with previous report of the biflavone amentoflavone (Yue and Kang, 2011). Analysis of the HSQC spectra of **6** permitted to verify correlations which were important to distinguish the carbons and the respective hydrogens. So, this spectra was important in the identification of the aromatic carbons of 1,4-disubstitued system of B ring of Unity I. In the same way it contributed to recognize the resonances of aromatic carbons of B ring of Unity II. Analysis of the HMBC correlation spectra are in agreement to those of amentoflavone (Figure 1) (Markham et al., 1987). Here we report on the first isolation of the biflavonoid

amentoflavone and the flavone viccinin from *A.bunius* species (Tchinda *et* al., 2006).

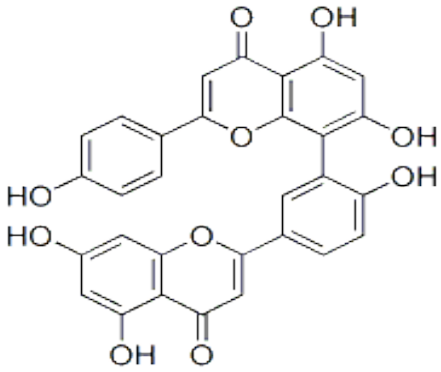


Figure (1): Compound **6** (Amentoflavone).

Spectral data of compounds (1-6) isolated from *Antidesma bunius* leaves

Corilagin (1-*O*-galloyl 3,6-*O*-hexahydroxydiphenoyl-*β*-glucopyranoside) (1)

1, was obtained as white amorphous powder of R_f values (x 100): 55 (H_2O), 59 (AcOH-6), 32 (BAW). It showed a M*r* of 633 in negative ESI-MS, corresponding to a molecular ion $[M-H]^-$ at *m/z* =633 . UV Spectral Data λ_{max} (MeOH): 218 nm ^{1}H- NMR spectral data (DMSO-d_6) δ (ppm), β glucose moiety: 6.17 (d, *J*=7.3 Hz, H-1), 3.85 (dd, *J*=7.3, 2.3 Hz, H-2), 4.56 (s, H-3), 4.19 ((s, H-4), 4.21 (t, *J*=7.3 Hz, H-5), 4.32 (dd, *J*=11.2; 6.2 Hz, H-6), 3.94 (dd, *J*=11.2; 6.2 Hz, H-6´´), 6.98 (s of galloyl protons), 6.46, 6.53 (s of hexahydroxydiphenoyl protons).^{13}C-NMR spectral data (DMSO-d_6) δ (ppm) β-$B_{1,4}$ glucose moiety: 118.7 (C-1),71.7 (C-2), 77.7 (C-3), 64.0(C-4), 76.4 (C-5), 62.2 (C-6),galloyl : 94.42 (C-1), 109.0 (C-2,6), 145.6 (C-3,5), 139.0 (C-4), 164.9 (C=O) hexahydroxydiphenoyl carbons: 123.0, 123.9(C-1,1´), 106.1, 106.9 (C-2, 2´), 143,9, 144.2, 144.8, 144.9 (C-3,3´, 5,5´) 135.4, 135.6 (C-4, 4´), 115.6, 115.8 (C-6,6´), 166,8, 167.3(C=O)).

Gallic acid (3, 4, 5-trihydroxybenzoic) (2)

2, was isolated as needle crystals of R_f values (x 100): 0 (H_2O), 9(AcOH-6), 48 (BAW). UV Spectral Data λ_{max} (MeOH): 273. ^{1}H- NMR (DMSO-d_6) δ (ppm): 6.87(s, H-2 and H-6).

Ferrulic acid (3)

3, isolated as colourless cubic crystals of R_f values (x 100): (H_2O), (AcOH-6), (BAW). UV Spectral Data λ_{max} (MeOH): 320.0, a shoulder at 293.1 and 232.6nm). The ^{1}H-NMR spectral data of **(3)** (Acetone-d_6, room temp.) δ (ppm): 7.5 (d, *J* = 16 Hz, H-α), 7.15 (br s, $\Delta\nu_{1/2}$ = 4 Hz, H-2), 7.05

(dd, *J* = 8 Hz and *J* = 2 Hz, H-6), 6.94 (d, *J* = 8 Hz, H-5), 6.36 (d, H-β) and 3.84 (s, OMe)].

Ellagic acid (4)

4, was isolated as amorphous powder of R_f values (x 100): 44 (H_2O), 55(AcOH-6), 72 (BAW). UV Spectral Data λ_{max} (MeOH): 255, 362. ^{1}H- NMR (DMSO-d_6) δ (ppm): 7.48 (S, H- 5 and H-5').

Vicenin II (Apigenin-6, 8-di-*C*-*β*-D-glucopyranoside) (5)

5, was obtained as yellowish white amorphous powder of R_f values (x 100): 34 (H_2O), 48 (15% AcOH), 13 (BAW). It showed a M*r* of in negative ESI-MS, corresponding to a molecular ion $[M-H]^-$ at *m/z* = . UV Spectral Data λ_{max} (MeOH): 270 nm, 351 nm, + NaOMe: 274 nm, 404 nm, + NaOAC: 274 nm, 342 nm, 381 nm, + NaOAC + H_3BO_3: 272 nm, 349 nm, 351 nm, +Al Cl_3: 273 nm, 349 nm, 445 nm, +Al Cl_3 + HCl: 273 nm, 349 nm, 403 nm. ^{1}H- NMR Spectral Data (DMSO-d_6) δ (ppm), apigenin moiety: 6.79 (s, H-3), 7.98 (d, *J*= 8.5 Hz,H2',6'), 6.85 (d, *J*=8.5 Hz, H-3',5'), glucose moieties: 4.73 (d, *J* = 8.69 Hz, H-1"), 4.66 (d, *J* = 8.0 Hz, H-1'"), 3-4 multiplet. ^{13}C-NMR Spectral Data (DMSO-d_6) δ (ppm) apigenin moiety: 164.0 (C-2), 102.5 (C-3), 180.2 (C-4), 158.0 (C-5), 108.2 (C-6), 161.1 (C-7), 105.1 (C-8), 155.0 (C-9), 103.7 (C-10), 121.5 (C-1'), 129.0 (C-2'), 115.8 (C-3'), 160.8 (C-4'), 115.8 (C-5'), 129.0 (C- 6'), glucose moieties: 73.6 (C-1"), 73.2 (C-2"), 79.1 (C-3"), 70.6 (C-4"), 81.8 (C-5"), 61.2 (C-6"), 74.5 (C-1'"), 69.7 (C-2'"), 78.8 (C-3'"), 70.8 (C-4'"), 79.1 (C-5'"), 60.8 (C-6'").

Amentoflavone (3′, 8′′- Biapigenin) (6)

6, was isolated as amorphous yellow powder. Negative ESI MS (M-H) *m/z*= 537.077521 corresponding to the chemical formula: C_{30} H_{18} O_{10}. UV MeOH : 269, 291 (s) ,332; MeOH + NaOMe 275, 295 (s), 379; MeOH + NaOAc : 274 , 380; NaOAc+ H3BO3:270 , 339 ; MeOH + AlCl3 ; 279,303 345,; MeOH + $AlCl_3$ + HCl : 279, 303 , 345, 384.^{1}H NMR (400 MHz in MeOD): Unity I: δ 6.46 (*s*, 1H, H-3); 6.41 (*s*, 1H, H-6); 7.52 (*d*, 2H, *J* = 8.7 Hz, H-2'/H-5'); 6.78 (*d*, 2H, *J* = 8.7 Hz, H-3'/H-5'); Unity II: δ 6.49 (*s*, 1H, H-3); 6.2(*d*, 1H, *J* = 2.0 Hz, H-6); 6.40 (*d*, 1H, *J* = 2.0 Hz, H-8);7.95 (*d*, 1H, *J* = 2.3 Hz, H-2'); 7.12 (*d*, 1H, *J* = 8.7 Hz, H-5'); 7. 90 (*dd*, 1H, *J* = 8.7 and 2.3 Hz, H-6').^{13}C NMR (400MHz in $CDCL_3$): Unity I: δ 163.4 (C-2); 100.8 (C-3); 181.5 (C-4); 160.8 (C-5); 97.3(C-6); 163.4 (C-7); 97.8(C-8); 156.9 (C-9); 102.8 (C-10); 120.7 (C-1'); 126.7 (C-2'/C-6'); 114.3 (C-3'/C-5'); 160.0 (C-4'); Unity II: 163.7 (C-2); 101.5 (C-3); 181.8 (C-4); 160.0 (C-5); 97.6(C-6); 163.6 (C-7); 92.5 (C-8); 156.9 (C-9); 102.81 (C-10); 120.8(C-1'); 126.4 (C-2'); 119.0 (C-3'); 158.4 (C-4'); 114.8 (C-5'); 130.2 (C-6').

The total phenolic content of *A.biunius* leaves was estimated at 90 mg/ml of gallic acid equivalent (GAE) per 100 g plant extract using the Folin-Ciocalteu method.

Antioxidant Biochemical Assays for Total Methanol Extract of *A. biunius* Leaves:

***Hepatoprotective effect against Paracetamol*:** The sample showed the highest therapeutic index of 80 similar to that reference control of quercetin (Table1.). .

***Concentration of Glutathione Reductase Activity*:** The sample induced significant endogenous intracellular production of GR (67.88/10^6 cells) (1.00µg/mL) into culture medium which reflects antioxidant capacity that differs with the reference control quercetin (Table1.).

***Effect of the sample on the inhibition of Nitric Oxide*:** Results revealed dose dependant decrease in the levels of NO in culture medium of hepatocytes exposed to injury. The maximal effect was achieved at 100µg/mL, the maximum tested concentration (Table1.).

Table1. Antioxidant Biochemical Assays for Total Methanol Extract of *A. biunius* Leaves.

Sample	LDHL	HP	T I %	GR	NO
MEABL	>1000	<12.5	>80	67.88	7.95±2.2
Quercetin	1000	12.5	80	80.51	11.11±1.7
Silymarin	500	50	10	62.52	10.97±1.9

LDH : Lactate dehydrogenase leakage after 2h (µg/mL).
HP : Hepatoprotection ((µg/mL).
TI % : Therapeutic index %.
GR : Glutathione reductase /10^6 cells (1.00µg/mL)
NO : Nitric Oxide (µmol nitrite/10^6cells) (100µg/mL).

Results obtained in the hepatocyte culture model presented in this study revealed:

1) A comparative analysis of the potential hepatoprotective effect assumed.
2) An exploration of the preventive effect against oxidative stress by GR assay as an intracellular screening system for testing antioxidant properties.
3) The role of Ant. Sample **MEABL** in protecting hepatocytes from nitric oxide, a specific factor of liver injury, by inhibiting the production of nitrite, a stable metabolite of NO.\

Hepatotoxicity

IC_{50} of **MEABL**, **1** and **2** were higher than 1000 µg/mL .This result revealed that the samples exhibited high margin of safety after 2hrs treatment of the monolayer of rat hepatocites. Sample **5** exerted hepatotoxic effects on the hepatocytes revealed by an IC_{50} of 125 µg/mL as shown in figure (2).

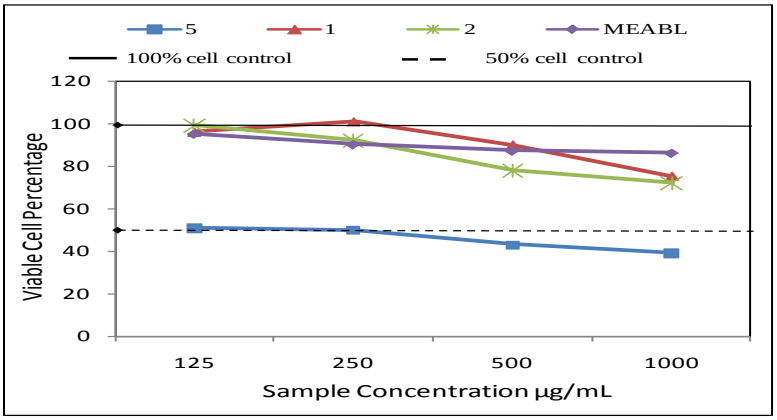
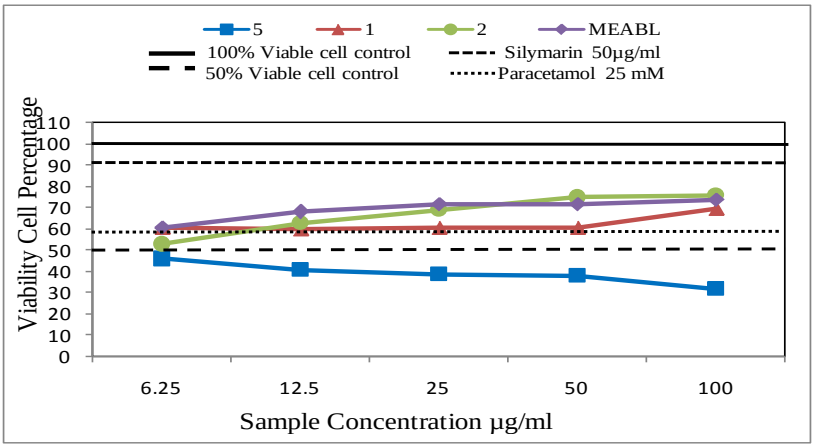


Figure (2): Viability of Monolayer of Rat Hepatocytes after 2 hrs Treatment with Different Concentrations of **MEABL** and Compounds **1**, **2** and **5** using MTT Colorimetric Assay. Each Point Represents the Mean (n=3).

Hepatoprotective Activity

Sample **5** did not show any hepatoprotective activity on the hepatocyte cells against the toxic effect induced by the paracetamol. For samples **1**, **2** and **MEABL** their hepatoprotection were shown at 6.5, 12.5 and <12.5 µg/mL respectively, as shown in figure 3.



Figures (3): Viability of Monolayer of Rat Hepatocyte after 2 hrs Treatment with Different Concentrations of **MEABL** and Compounds **1**,**2** and **5** Followed by Treatment with 25 mM Paracetamol for 2hr. in Comparison with 50 µg/ml Silymarin as Control Using MTT Colourimetric Assay. Each Point Represents the Mean (n=3).

Conclusion

In this study, we report the isolation and identification of six phenolic compounds. Among them, the biflavone amentoflavone and the C glycoside viccinin II are isolated for the first time from *A. bunius species. In vitro* antioxidant biochemical assays of the leaves extract revealed a relatively high antioxidant potency expressed by increased LDH leakage from cell cytosol and GR and high depression of NO level in comparison with qurecetin. The study showed that *A. bunius* leaves in addition to compound 1

and 2 have no toxicity on the hepatocytes and possess high hepatoprotective activity in the *in vitro* assay. *A. bunius* bioactivity could be attributed to its estimated high phenolic content (90 mg/ml of gallic acid equivalent (GAE) per 100 g plant extract. .

References:

Bringmann G., Schlauer J.and Risher H. (2000). " Revised Structure of Antidesmone, an Unusual Alkaloid form Tropical *Antidesma* Plants (Euphorbiaceae)". Tetrahedron; **56**: 3691-5.

Butkhup L.and Samappito S. (2008)**.** "An Analysis on Flavonoids Contents in Mao Luang Fruits of Fifteen Cultivars (*Antidesma bunius*), Grown in Northest Thailand". Pak. J. Biol.l Sci., **11** (7): 996-1002.

Carmichael J., DeGraff W., Gazdar A., Minna J. and Mitchell J. (1987). " Evaluation of Tetrazolium-based Semi Automated Colorimetric Assay: Assessment of Chemosensetivity Testing. Cancer Res., **47:** 936-942.

Chanwitheesuk A., Teerawutgulrag A., Kilburn J.D. and Rakariyatham N. (2007). " Antimicrobial Gallic Acid from *Caesalpinia mimosoides* Lamk ". Food Chem., **100** (3): 1044.

Gertsner J. (1938). " A Preliminary Checklist of Zulu Names of Plants with Short Notes". Bantu Stud*;* **12**: 215-36.

Goldberg D.M. and Spooner R.J. (1983). " Glutathione Reductase . In: Bergmeyer HU, Bergmeyer J GraBl M. (ed). Methods of Enzymatic Analysis", 3rd ed., . Verlag Chemie, Weinheim, **111**, 258-265.

Hui W.H. and Sung M.L. (1968). "An examination of the Euphorbiaceae of Hong Kong. II. The Occurrence of Epitaraxerol and other Triterpenoids". Austr. J. Chem., **21**(8) 2137 - 2140.

Kiso Y., Tohkin M.and Hikino H. (1983). " Assay Method for Antihepatotoxic Activity using Galactosamine-Induced Cytotoxicity in Primary-Cultured Hepatocytes". J. Nat. Prod., **46(**6): 841-847.

Magsino M. (2003). " Don't Laugh, Bignay is Hard Wood Philippine Daily Inquirer" . pp: A1 –A4.

Markham K. R., Sheppard C. and Geiger H. (1987). " ^{13}C NMR Studies of some Naturally Occurring Amentoflavone and Hinokiflavone Biflavonoids ". Phytochem. **26**: 3335-3337.

Micor J.R.L., Deocaris C.C. and Mojica E.E.(2005)**.** " Biological Activity of Bignay [*Antidesma bunius* (L.) Spreng] Crude Extract in *Artemia salina*". J. Med..Sci., **5**(3): 195-198.

Montgomery H.A.C. and Dymock F.G. (1961). " The Determination of Nitrite in Water". Anal., **86**: 414–416.

Mosmann T. (1983). "Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxic Assay". J. Imm. Meth, **65**: 263-271.

Nawwar M.A.M.and Hussein S.A.M., Merfort I. (1994). "NMR Spectral Analysis of Polyphenolics from *Punica granatum* ". Phytochemistry, **36**: 793-798.

NIH Guide for the Care and Use of Laboratory Animals. NIH *Publication* Number. 85-23, Revised 1985, U.S. Department of Health, Education and Welfare. Research Triangle Park, North Carolina.

Osman H., Abd. Hamid S., Al–Douh M. H. and Fang-Lim A. (2009). Indirect benzylation of Ferulic Acid catalysed by TBAI. j. Nat. Appl. Sci. **13** ` (2): 259- 264.

Palgrave K.C. (2002). " Trees of Southern Africa ". In: Moll EJ, Ed. Cape Town: Struik Publishers.

Rajput A.P. and. Patel M.K. "Chemical Investigation and Biological Activity of Phytoconstituents from Methanol Extract of *Abutilon indicum* Leaves ". J. Chem. Pharm. Res., 2012, **4**(8):3959-3965.

Rec.GSCC (DGKC) (1970). J. Clin. Chem. Clin. Biochem, **8**, 658.

Roger B. (2004). History of Fruits in the East Asia /Pacific region. 17th Congress of the Indo-Pacific Prehistory Association, Cambridge.

Seglen P.O. (1976). " Preparation of Isolated Rat Liver Cells ". Methods Cell Biol., **13**, 29-83.

Steenkamp V., Mokoele T.L. and Van Rensburg C.E.J. (2009). "Toxicicity Testing of two Medicinal Plants, *Bridelia micrantha* and *Antidesma venosum* ". The Open Toxicol. J., **3**, 35-38.

Tchinda A.T., Teshome A., Dagne E., Arnold N. and Wessjohann L.A. (2006). "Squaline and Amentoflavone from *Antidesma lacinatum* ". Bull. Chem. Soc. Ethiop. **20** (2), 325-328.

Xie C., Veitch N. C., Houghton P. J.and Simmonds M. S. J. (2003) . " Flavone C-Glycosides from *Viola yedoensis* Makino ". Chem Pharm Bull, **51** (10): 1204-1207.

Ye G., Peng H., Fan M.and Huang C.G. (2007). " Ellagic Acid Derivatives from the Stem Bark of *Dipentodon sinicus* " . Chem. Nat. Compd. **43** (2), 125-127.

Yue, S.-M.and Kang W.-Y. (2011). " "Lowering Blood Lipid and Hepatoprotective Activity of Amentoflavone from *Selaginella tamariscina In vivo* ". J. Med. Pl. Res. **5**(14), 3007-3014.

Waterman P.G. and Mole S. (1994). " Analysis of Phenolic Plant Metabolites ". Blackwell Scientific Publications, Oxford.

Watt J.M. and Breyer-Brandwijk M. G. (1962**)**. "The Medicinal and Poisonous Plants of Southern and Eastern Africa ", 2nd ed. London; Livingstone.