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INVESTIGATION OF SOME ACTIVE CONSTITUENTS OF *EUPHORBIA BIVONAE* STEUD.

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Abstract:
The investigation of some active constituents of Euphorbia bivonae included terpenes, alkaloids, coumarins and anthraquinones to evaluate its economic values as medicinal plant, where sugiol and betulin compounds were isolated from the aerial parts and roots of Euphorbia bivonae, while ferruginol compound was isolated only from the roots. The total terpenes content of the aerial parts and roots of E. bivonae were 3.44 and 8.96 mg/g, respectively, which were estimated spectrophotometrically. Meanwhile, the compounds 2Piperidinone, N[4bromonbutyl] and komaroine were isolated from the aerial parts and roots of E. bivonae. While the alkaloid compounds 5,8-dihydroxy methyl-canthin-6-one and 1-methoxy-8-hydroxy-methyl-canthin-6-one were isolated from only the roots. The estimated percentages of the total alkaloids were 0.40% and 1.02% for the aerial parts and roots, respectively. The coumarin compound umbelliferone was isolated from the aerial parts and roots, and the total coumarins content of the aerial parts and roots were 0.42 and 0.49 mg/g, respectively. On other hand anthraquinone compound emodin (1, 3, 8-trihydroxy-6-methyl-9, 10 anthracenedione) was detected and isolated from the roots only, meanwhile the concentrations of total anthraquinones were 0.04 and 0.94g/100g dry material for aerial parts and roots, respectively.

Key words: *Euphorbia bivonae, terpenes, alkaloids, coumarins and anthraquinones.*

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INTRODUCTION:

Euphorbia is the largest genus in the plant family Euphorbiaceae, comprising about 2000 known species and ranging from annuals to trees. Over 33 species of this genus have been found in Egypt [1]. Somes pecies of *Euphorbia* have been used as medicinal plants for the treatment of skin diseases, gonorrhea, intestinal parasites, inflammation and wart cures [2]. Plants of this genus are known for their rich content of secondary metabolites. Indeed, numerous studies undertaken on this genus have revealed presence of triterpenes, diterpenes, steroids [2], alkaloids, flavonoids, coumarins, glycosides, gums, polysaccharides, phenols, tannins, terpenes and terpenoids [3] and macrocyclic diterpenes [4]. In addition to these substances, plants contain other chemical compounds. These can act as agents to prevent undesirable side effects of the main active substances or to assist in the assimilation of the main substances. Plants have an almost limitless ability to synthesize aromatic substances, mainly secondary metabolites of which 12,000 have been isolated, a number estimated to be less than 10% of the total [5]. These active components serve as molecules of plant defense against attack by microorganisms, insects and herbivores and at the same time also exhibit medicinal properties for treating several ailments. Scientific research has allowed us to discover a wide range of active components, of which the most important, as far as health is concerned, are essential oils, alkaloids, glycosides or heterosides, mucilage and gums and tannins [6]. From time immemorial Besides the well known skin irritant and tumor promoting tigliane, ingenane and daphnane diterpenes [7], some diterpenes of *Euphorbia* have been found to possess various activities such as cytotoxic, antitumor,antibacterial [2]. Umbelliferone and scopoletin were separated from *Euphorbia maculata* L. [8]. Biological effects of coumarins observed include antibacterial, antithrombotic and vasodilatory, antimutagenic, lipoxygenase and cycloxygenase inhibition, scavenging of reactive oxygen species, and antitumourigenic effects, including malignant melanoma.[3, 5, 6] A recent study has shown that umbelliferone inhibits the release of Cyclin D1, which is overexpressed in many types of cancer. This knowledge may lead to its use in cancer therapy [9]. Ethyl acetate extract of the aerial parts of *Euphorbia bivonae* plant have a potent cytotoxic activity in vitro against different human cell line at low concentration and showed a remarkable with dose (400 mg/kg. b. wt. orally for 10 days) against hepato and nephro toxic rats induced by over dose of paracetamol. This activity attributed with its high content of flavonoid compounds as kaempferol-3-O-α-dirhamnoside-O-β Dglucopyranoside, kaempferol-3-rutinoside, isorhamentin -3- rutinoside, kaempferol-3,7-diglucopyranoside,kaempferol-3rhamnopyranoside,kaempferol-3- glucopyranoside, kaempferol-7- rhamnopyranoside, quercetin -3-glucopyranoside, kaempferol-4`-methylether,3`, 4` dihydroxyflavone and isorhamentin) [10]. Investigation of the aerial parts and roots of the *Euphorbia bivonae* may evaluate our knowledge about the other active constituent of the plant as pharmacological bioactive agents.

MATERIALS AND METHODS:

General experimental procedures

The NMR measurements were carried out on A JEOL Ex-270 NMR spectrometer apparatus (270 MHZ for ^{1}H NMR). EI-Mass measurements were carried out on finningan SSQ 7000. Ultraviolet spectrophotometric analysis (UV) using Schimadzu UV 240 spectrophotometer. The IR spectra were recorded on a Bruker Vector 22 FTIR. GC-MS analysis was carried out on a Hewlett-Packard 6890 gas chromatograph fitted with a fused silica HP-5MS capillary column (30 m × 0.25 mm; film thickness 0.25 µm). The oven temperature was programmed from 60ºC at 3ºC/min. Helium was used as carrier gas at a flow rate of 2 ml/min. The gas chromatograph was coupled to a Hewlett-Packard 6890 mass selective detector. The MS operating parameters were: ionization voltage, 70 eV; ion source temperature 200ºC.

Plant material

Euphorbia bivonae Steud. was collected at full flowering stage from Ageba area, Mersa-Mattruh during April 2011. The aerial parts and roots of the plants were separately, air dried, ground to fine powder and kept to be used for different analysis.

Extraction of *Euphorbia bivonae* aerial parts and roots

Each of the aerial parts and roots powder (2 Kg for each part separately) of *Euphorbia bivonae* was extracted several times with 95% ethanol at room temperature. The solvent was evaporated under vacuum to give 172.35 g and 203.23 g of aerial part and roots extract, respectively. The obtained dry extracts were suspended in water followed by extraction with petroleum ether (60-80 ºC), chloroform and n-butanol for four times consecutively.

Investigation of terpenes

Separation and identification of terpenes

The petroleum ether fraction for each of the aerial parts and roots was evaporated under reduced pressure (50ºC) to yield 39.14 g and 50.08g of sticky residue for each of the aerial parts and roots,

respectively. Each residue was applied separately on the top of a silica gel column (60 - 120 mesh). Gradient elution was first carried out using petroleum ether, followed by adding ethyl acetate gradually to increase polarity. Each fraction of was collected and monitored by TLC using pre-coated silica gel 60F 254, (E-Merck) plates and petroleum ether: chloroform (70:30 v:v) as solvent system and p-anisaldehyde as spray reagent. Similar fractions were pooled together, each of the collected fractions of the aerial parts and roots was applied on preparative TLC using the solvent system petroleum ether: chloroform (70:30 v:v), where the investigation of comparative TLC revealed the presence of two isolated compounds (A_1 and A_2) in the petroleum ether residue of the aerial parts and three isolated compounds (B_1, B_2 and B_3) in the petroleum ether residue of roots of *Euphorbia bivonae* [11,12].

The separated spots were detected by p-anisaldehyde spray reagent, where they gave pale violet with R_f = 0.16 (A_1) and pale brown with R_f = 0.10 (A_2), respectively, for the two compounds (A_1 and A_2) of the aerial parts. While the separated spots of the roots gave reddish violet with R_f = 0.51(B_1), pale violet with R_f = 0.16 (B_2) and pale brown with R_f = 0.10 (B_3), respectively, for three compounds (B_1, B_2 and B_3), which indicated that the compounds (A_1 and A_2) of the aerial parts were similar to the compounds (B_2 and B_3), which were separated from the roots. The pure compounds were identified by Mass spectrometric analysis (MS) and ^{1}H-NMR.

Investigation of alkaloids

Separation and identification of alkaloids

The chloroform fraction for each of the aerial parts and roots was evaporated under reduced pressure (50ºC) to yield 30.05 g and 43.11g of sticky residue for each of the aerial parts and roots, respectively. Each residue was applied separately on the top of a silica gel column (60 - 120 mesh). Gradient elution was first carried out using the pure chloroform, followed by adding ethanol gradually to increase polarity. Each fraction of was collected and monitored by TLC using precoated silica gel 60F 254, (E-Merck) plates using chloroform: methanol (90:10 v:v) and Chloroform : methanol (70:30 v:v) as solvent systems and then the developed chromatograms of air-dried and sprayed with Dragondorff 's reagent. Similar fractions were pooled together, each of the collected fractions of the aerial parts and roots was applied on preparative TLC using the solvent system chloroform: methanol (90:10 v:v) [13]. The investigation of comparative TLC revealed the presence of two isolated compounds (C_1 and C_2) in the chloroform residue of the aerial parts and three isolated compounds (D_1, D_2 and D_3) in the chloroform residue of the roots.

The separated spots were detected by using Dragendorff's spray reagent, where they spots gave orange color after sprayed with R_f = 0.62 (C_1) and with R_f = 0.51 (C_2), respectively, for the two compounds (C_1 and C_2) of the aerial parts. While the separated compounds of the roots gave at R_f = 0.62 (D_1), at R_f = 0.51 (D_2), at R_f = 0.40 (D_3) and R_f = 0.27 (D_4), respectively. The pure compounds were identified by GC-MS, ^{1}H-NMR, and Mass spectrometric analysis (MS).

Reagent for alkaloids [14]

Dragendorff's reagent, Mayer's reagent, Wagner's reagent

Investigation of coumarins and anthraquinones

Separation and identification of coumarins and anthraquinones

N-butanol fraction of the aerial parts and roots were preparative separately on the paper chromatography [3MM, Butanol: Acetic acid: Water (BAW) 4:1:5]. The bands were cut and eluted separately by 70% methanol, while the impure bands for each the aerial parts and roots were further purified using preparative Thin Layer Chromatography (TLC) with chloroform: methanol/ (8.5:2.5), then the separated band was cuts and eluted by 70% methanol. The investigation of comparative TLC revealed the presence of two purified spots different in nature. one spot is blue in color in 365 daylight and under UV light and turned into fluorescence blue upon exposure to ammonia vapor appears in each of the aerial parts and roots with the same R_f 0.89 in each part. This color spot demonstrated that the compound nature is coumarin. On the other hand the other spot was orange in color in 365 daylight and under UV light and turned into red upon exposure to ammonia vapor appears at roots only with R_f 0.92. This color spot demonstrated that the compound nature is anthraquinone. The compounds was identified with Mass spectrometric analysis(MS) and ^{1}H-NMR and IR.

Estimation of the total terpenes of *Euphorbia bivonae* parts: according to Indumathi et al.[15]

Estimation of the total alkaloids of *Euphorbia bivonae* parts: According to British, Pharmacopoeia [16].

Estimation of the total coumarins of *Euphorbia bivonae* parts: According to Osório et al [17].

Estimation of the total anthraquinones of *Euphorbia bivonae* parts: According to Koshioka and Takino [18].

RESULTS AND DISCUSSIONS:

Investigation of terpenes at *Euphorbia bivonae* parts

The R_f values and the obtained colors of the isolated terpene compounds of the aerial parts and roots of *E. bivonae* were confirmed that the separated two compounds (A_1 and A_2) of the aerial parts were similar to that compounds (B_2 and B_3), which were separated from the roots. So it could be concluded that the compounds (A_1& B_2) are the same compound, and (A_2 & B_3) are the same compound.

Compound (A_1)

Compound (A_1) (28 mg) was soluble in chloroform and methanol. It exhibited the following spectral data:

MS (70 e.v., El-MS) m/z: 300 (M^+,100%), 285.4 (90%), 257 [M-isopropyl]+ (10%), 244.2 (15%), 201 (36%), 187.3 (20%) and 66.2 (16%).

^{1}H-NMR ($CDCl_3$, 300 MHz) showed signals for an isopropyl group presented by a pair of non equivalent secondary methyl groups at δ 1.16 ppm and 1.23 ppm (3H,d,J=7.1 Hz) corresponding to 3H16 and 3H17, respectively, and one proton septet at δ =3.42 ppm (1H15, septet). Two aromatic protons at δ 7.07 ppm (1H11,s) and 7.75 ppm (1H14,s). Three quaternary methyl groups (3H,s) at δ 0.95 ppm, 1.10 ppm and 1.31 ppm corresponding to 3H18, 3H19 and 3H20 respectively. The proton at H5 is coupled with the 2 protons of H6 and appeared at δ 1.34 ppm (1H,dd) while the protons of H6 showed signals at δ 2.54 ppm (2H,dd). Three methylene groups, the protons of which showed signals between δ 1.54 ppm corresponding to 2H1, 2H2 and 2H3. The above spectral data, of compound (A_1) showed that it could be identified as the diterpenoid sugiol ($C_{20}H_{28}O_2$), where these results were coincide with that reported by Abou Hussein [19] for sugiol (Table 1). Sugiol is a bioactive against inflammation [20].

Compound (A_2)

Compound (A_2) (19mg) was soluble in chloroform and methanol. It exhibited the following spectral data:

MS (70 e.v., El-MS) m/z: 442 (M^+, 12%), 410.02 (46%), 411 (100%), 401.1 (34%), 382.2 (33%), 230 (96%), 208 (78%), 190.1 (91%), 172.3 (31%), 135.5 (27%), 118.2 (14%), 105 (15%), 92 (22%).

1H-NMR ($CDCl_3$, 300 MHz) showed six singlet methyl groups at δ 0.71, 0.84, 0.96, 0.97, 1 , 1.62 (6H,s) and one isopropenyl moiety at δ 1.77(3H,d,J=5.3 Hz), two diastereotopic protons for a methylene group (attached to hydroxyl) at δ 3.31 and 3.78 ppm (H28 and H28', dd, J =10.7,4.3) and two exocyclic methylene protons at δ 4.66, 4.56 (H29 and 29', s). Five proton-signals at δ 4.57 (s, H29), 4.48 (s, H29'), 3.80 (dd, J = 10.7, 4.3, H28), 3.31 (dd, J=10.7, 3.7, H28') and 3.18 (dd, J = 12.2, 6.87, H3). The above spectral data, of compound (A_2) showed that it could be identified as the triterpenoid Betulin (C30H50O2) (Table 1), where these results were coincide with that reported by Ayatollahi et al.[21]. Betulin has recently been reported as a cytotoxic agent for several tumor cell lines [22].

Compound (B1)

Compound (B_1) (23mg) was soluble in chloroform and exhibited the following spectral data:

MS (70 e.v., El-MS) m/z: 286 [M]+ (100%), 270.6 (60%), 227.1 [M-isopropyl]$^+$ (15%), 201.1 (22%), 189.2 (42%), 175.1 (40%), 66.1 (24%).

^{1}H-NMR ($CDCL_3$, 300 MHz) showed signals for an isopropyl group at δ 1.33 ppm (3H16, d, J=6.9Hz), 1.20 (3H17, d, J=6.9Hz) and 3.14 ppm (1H15 septet). Two aromatic protons at δ 6.91 ppm (1H,s) and δ 6.69 ppm (1H,s) respectively. Three quaternary methyl groups at δ 0.95 ppm (3-H18,s), 0.96 ppm (3H19,s) and δ 1.14ppm (3H20,s). H5 proton showed a signal at δ 1.40 ppm (1H,dd). 2H6 protons at ä 1.75 ppm (2H,m) and 2H7 protons at δ 2.86 ppm (2H,m). The protons of three methylene groups corresponding to 2H1, 2H2 and 2H3 at δ 1.42 to 2.17 ppm. Hence compound (B_1) could be identified as the diterpenoid ferruginol ($C_{20}H_{30}O$) (Table 1), where these results were coincide with that detected by Abou Hussein[19] for ferruginol. Son et al. [23] reported that the ferruginol is strongly inhibited colon, lung, and breast human tumors and oncogene transformed cells with GI50 2–5 µg/mL.

The obtained results indicated that the isolated compounds sugiol and Betulin were present in both the aerial parts and roots, while ferruginol compound was isolated only from the roots.

Investigation of alkaloids

The obtained results indicated that the detected compounds (C_1 and C_2) in the aerial parts were the same compounds of D_1 and D_2, which were detected in the roots. While the alkaloid compounds D_3 and D_4 were detected only in roots.

Compound (C_1)

Compound (C_1) (9mg) was soluble in chloroform, ether, ethanol with few drops of chloroform. It was identified by using GC-MS with Willey libraries.

The alkaloid compound (C_1) was identified as 2Piperidinone, N[4bromonbutyl] ($C_9H_{16}BrNO$) (Table 1) with retention time (min.): 13.70 and molecular weight, 233. The mass spectrum of the compound revealed that, M/Z (relative Abundance %); 233 (M^+, 4%), 150.2 (10%), 135.1 (34%), 111.2 (16%), 85.1 (31%), 71.1 (44%) and 57.1 (100%). where these results were coincide with that reported by Ilani et al. [24].

Compound (C_2)

Compound (C_2) (15mg) was soluble in chloroform, ether, ethanol with few drops of chloroform. This compound was identified by using GC-MS with Willey libraries.

The alkaloid compound (C2) was identified as komaroine ($C_{20}H_{18}N_2$) (Table 1) with retention time (min.): 45.56 and molecular weight, 286. The mass spectrum of the compound revealed that, M/Z (relative Abundance %); 286 (M^+, 70%), 257.2 (36%), 201.2 (44%), 105.1 (100%) and 55.0 (62%).

Compound (D_3)

Compound (D_3) (20mg), was soluble in chloroform, and ethanol with few drops of chloroform. Compound (D_3) was identified using ^{1}H-NMR and EI-Mass spectrum.

MS (70ev, EI-MS) showed an ion peak at m/z 280 calculated for $C_{16}H_{12}N_2O_3$. Fragment ions m/z 280 (8%), 278.2 (30%), 219.2 (6%), 195.1 (3%), 166.2 (12%), 150 (18%), 147 (100%), 112.1 (16%), 80.2 (10%), 72.2 (22%), 57 (28%).

^{1}H-NMR spectrum of compound (D_3) showed signals at δ ppm 4.2 (2H, d, J =5Hz, CH_2OH), 4.5 (1H, bs, OH), 5.5 (1H, bs, OH), 6.2 (2H, d, J= 5.1 Hz, CH_2OH), 7.8 (1H, t, J=7.6-4.8 Hz, H10), 7.6 (1H, d, J= 4.2 Hz, H11), 7.9 (1H, d, J=7.8 Hz, H9), 8.0 (1H, S, H4), 8.1 (1H, d, J=4.9 Hz, H1), 8.5 (1H, d, J=4.8 Hz, H2).

The obtained results of the compound (D_3) concluded that it could be 5,8-dihydroxy methyl-canthin-6-one alkaloid (Table 1).

Compound (D_4)

Compound (D_4) (19mg) was soluble in chloroform, and ethanol with few drops of chloroform. . Compound (D_4) was identified using ^{1}H-NMR and EI-Mass spectrum.

MS (70ev, EI-MS) showed a molecular ion peak (M^+) at m/z 280 calculated for $C_{16}H_{12}N_2O_3$. Fragment ions m/z 280 (14%), 277.1 (29%), 245.2 (6%), 219.1 (8%), 187.1 (17%), 165.2 (48%), 148 (100%), 113.1 (8%), 83.2 (16%), 70.1 (22%), 56.2 (40%).

^{1}H-NMR spectrum of compound (D_4) showed signals at δ ppm 3.8 (2H, d, J =5.2 Hz, CH2), 4.2 (3H, S, OCH_3), 4.9 (1H, bs, OH), 6.8 (1H, d, J= 9.8 Hz, H5), 7.0 (1H, d, J=7.5 Hz, H9), 7.4 (1H, t, J= 7.5,5.1 Hz, H10), 7.6 (1H, d, J=10.7 Hz, H4), 7.8 (1H, d, J=7.7 Hz, H11), 7.9 (1H, S, H2). These results indecated that the compound (D_4) was identified as 1-methoxy-8-hydroxy-methyl-canthin-6-one alkaloid (Table 1).

The obtained results concluded that the compounds 2Piperidinone, N[4bromonbutyl] and komaroine were detected in the aerial parts and roots of *E. bivonae* but the 5,8-dihydroxy methyl-canthin-6-one and 1-methoxy-8-hydroxy-methyl-canthin-6-one alkaloid compounds were detected only in the roots.

Investigation of coumarins

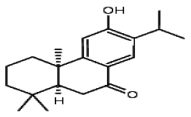
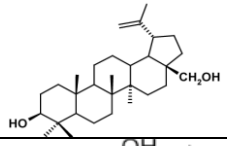
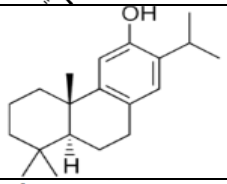
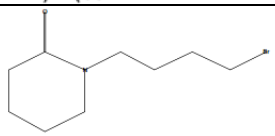
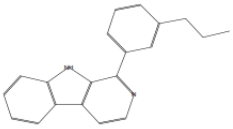
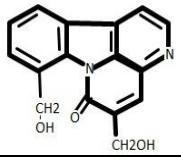
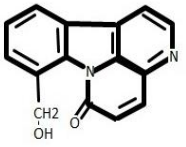
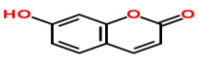
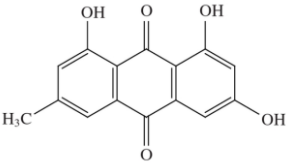
The coumarin compound (E) (21mg) was identified by using, EI-MS and ^{1}H-NMR spectra. EI-MS m / z: 162 (100%), 84.1 (18%) 65.2 (70%) and 45.2 (52%). ^{1}H NMR (400 MHz, DMSO-d6) δ ppm 7.86 (1H, d, J= 4.54 Hz, H4), 6.25 (1H, d, J 4.50 Hz, H3),7.54(1H,d,J=3.8 Hz, H5), 6.78 (1H, dd,J=3.24 Hz, H6), 6.81(1H,d,J= 1.54 Hz,H8),10.56(1H, s, OH7). Comparing the obtained data with the spectral data reviewed in literature [9] the component could be identified as umbelliferone (Table 1).

Investigation of anthraquinones

The anthraquinones compound (F) (24mg) was identified by using, IR and ^{1}H-NMR spectra. Infrared of the compound showed band at 3383.41 cm-1 that assigned to á-hydroxyl groups. Characteristics bands for anthraquinone compound 1665.87 and 1622.21 cm-1 were assigned to the free carbonyl group stretch and the conjugated carbonyl group, respectively. Band at 1476.45 cm-1 was probably assigned to a skeletal ring stretches. ^{1}H-NMR spectrum illustrated characteristic features for an aromatic pure compound that support IR analysis and get close to the structure of emodin. The first signal was at δ 2.5 ppm that assigned to the three proton of methyl group, followed by signals for by signals for aromaticity that are detailed as follows:

Singlet signal at δ 6.5 ppm that assigned to the á-proton to hydroxyl group at position 7(7-H). Doublet signal at δ 6.8 ppm that assigned to the á-proton to hydroxyl group at position (2-H).The protons para to hydroxyl group were more deshielded 4H is the most down field exhibited at δ 7.55 ppm, leaving 5H as singlet signal at 7.22ppm. The three phenolic protons appears at 13, 12.52 and 11.65 ppm, respectively. From the obtained data the compound could be identified as emodin (1, 3, 8-trihydroxy-6-methyl-9, 10 anthracenedione) (Table 1).Yi reported [25] that emodin displayed the capability of reactive oxygen species (ROS) and facilitating arsenic cytotoxicity in both leukemia and solid tumor cell lines.

Table 1: The Chemical structures of the isolated active compounds

Compound No.	Compound Name	Chemical Structures
(A_1)	Sugiol	
(A_1)	Betulin	
(B_1)	Ferruginol	
(C_1)	2Piperidinone, N[4bromonbutyl]	
(C_2)	Komaroine	
(D_3)	5,8-dihydroxy methyl-canthin-6-one	
(D_4)	1-methoxy-8-hydroxy-methyl-canthin-6-one	
(E)	Umbelliferone	
(F)	Emodin	

The total terpenes content

The total terpenes content of the aerial parts and roots were 3.44 and 8.96 mg/ gm, respectively, which were estimated spectrophotometrically.

The total alkaloids content

The percentage of the total alkaloids was 0.40% and 1.02% for the aerial parts and roots, respectively.

The total coumarins:

The total coumarins content of the aerial parts and roots were 0.42 and 0.49 mg/ gm, respectively, which were estimated spectrophotometrically.

The total anthraquinones:

The concentrations of total anthraquinones in 70% acetone extract of the aerial parts and roots were 0.04 and 0.94g/100g dry plant, respectively, which was estimated using chrysophanol as standard.

CONCLUSION:

The obtained results concluded that *Euphorbia bivonae* contained sugiol and betulin compounds in the aerial parts and roots, beside ferruginol compound only in the roots. It also contained 2Piperidinone, N[4bromonbutyl] and komaroine in

the aerial parts and roots, beside the alkaloid compounds 5,8-dihydroxy methyl-canthin-6-one and 1-methoxy-8-hydroxy-methyl-canthin-6-one in the roots. It contained also umbelliferone as coumarin compound in two parts and anthraquinone compound emodin in roots only. The total terpenes, alkaloids, coumarins and anthraquinones were estimated. So there are worthy motives to consider that there are necessity to further studies on the biological activity to benefit of this plant in the medical field.

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