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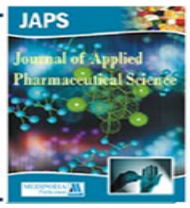

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Kalanchoe thrysiflora Harv. and *Kalanchoe marmorata* Baker; DNA Profiling, biological guided fractionation of different extracts; isolation and identification of cytotoxic compounds

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ABSTRACT

DNA profiling of two closely related ornamental plants belonging to family crassulaceae *viz. Kalanchoe thrysiflora* Harv. and *Kalanchoe marmorata* Baker were performed to establish genetic polymorphism. Biological guided fractionation of the two plant extracts to assess their cytotoxicity, had led to the isolation of one steroidal and one triterpenoidal compound from the most active dichloromethane fraction of *Kalanchoe thrysiflora*. The cytotoxicity of the isolated compounds were evaluated against normal (HFB4) and cancer (MCF7) cells. Compound 1 (3-oxo-olean-12-ene) and compound 2 (*β*-sitosterol) showed similar cytotoxic activity on MCF7 at IC_{50} 17.4 and 17.6 µg/ml respectively while on HFB4, the compounds revealed cytotoxic activity at IC_{50} 21.9 and 21.6 respectively.

Keywords: Crassulaceae, cytotoxicity, DNA profiling, *Kalanchoe thrysiflora*, *Kalanchoe marmorata.*

INTRODUCTION

Medicinal plants constitute a common alternative for cancer treatment in many countries around the world. At this time, more than 3000 plants worldwide have been reported to have anti-cancer properties. Globally, the incidence of the use of plant-derived products for cancer treatment is from 10% to 40% with this rate reaching 50% in Asiatic patients. Family crassulaceae is a large family, widely distributed in the cosmopolitan, especially southern Africa, except Australia and the West pacific. It comprises about 35 genera and 1500 species (Bailey, 1953; Bailey, 1958; Boulos 1999). *Kalanchoe*, a genus of approximately one hundred species, is native to tropical Africa but has been naturalized throughout the tropics (Chopra, Nayar & Chopra 1956; Gaind, Singla & Wallace 1981). Reviewing the available current literature, some *Kalanchoe* species were found to have important biological activities e.g. antiviral, sedative, antiulcer, immunomodulatory, antilieshmanial, anti-inflammatory, thyroid peroxidase inhibitor, cytotoxic effect, hepatoprotective, analgesic, antimicrobial, antihyperglycemic, larvicidal and insecticidal (Nguelefack *et al.*, 2006). Concerning *Kalanchoe thyrsiflora* Harv. and *Kalanchoe marmorata* Baker, no reports on biological activities could be traced in the available literature which prompted the biological investigation of their leaf extracts.

Earlier studies on different *Kalanchoe* species reported the isolation of polysaccharides, flavonoids, sterols, ascorbic acid, trace elements, organic acids, hydrocarbons, triterpenoids and bufadenolides (Liu *et al.,* 1989; Siddiqui *et al.,* 1989; Kalinowska *et al.,* 1990; Costa, Jossang & Bodo, 1996; Supratman *et al.,* 2001; Yadav & Dixit 2003; Singab *et al*., 2011).

DNA-based molecular markers are utilized in the fields of taxonomy, physiology, etc. and have been widely used for authentication of plant species of medicinal importance (Joshi *et al*., 2004). In this study, both *K. thyrsiflora* and *K. marmorata* widely used as ornamental plants are subjected to DNA profiling using RAPD (Random Amplified Polymorphic DNA) technique in an effort to compare and validate the genetic polymorphism between the plants. The initial screenings for plants used for cancer treatment are cell-based assays using established cancer cell lines, in which the toxic effects of plant extracts or isolated compounds can be measured (Alonso-Castro *et al.,* 2011). In the current study, cytotoxic screening of *K. thyrsiflora* and *K. marmorata* is followed by isolation of compounds from the most biologically active fraction.

MATERIAL AND METHODS

Plant material and extraction

Samples of *Kalanchoe thrysiflora* Harv. & *Kalanchoe marmorata* Baker were purchased from Sheben El- Kanater/ Elkalubya, Egypt, in June 2009 and the plants were kindly identified by agricultural engineer Mrs. Terease Labib, El- Orman Botanical Garden. A voucher specimen had been deposited in the herbarium, Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University.

The fresh succulent green leaves of *K. thyrsiflora* and *K. marmorata* (2.5 Kg each) were cut into smaller pieces and homogenized in a mechanical blender with (20% W/V) distilled H_2O. Half Kg of *K. thyrsiflora and K. marmorata* Baker were cut also into smaller pieces and homogenized in a mechanical blender with ethanol. The homogenized aqueous and ethanolic extracts were then filtered through muslin and lyophilized to yield dark brown residue of the aqueous extract**s** of *K. thyrsiflora* (53.7 g**)** and *K. marmorata* **(**93.8 g) and ethanol extracts (15 g, 26.4 g) respectively. The lyophilized extracts of *K. thyrsiflora and K. marmorata* were dissolved in least amount of H_2O then partitioned using dichloromethane (2 L), ethyl acetate (2 L) and *n*-butanol (700 ml) then each fraction is concentrated under reduced pressure to afford (5.3 g, 0.7 g) of dichloromethane fraction, (1.7 g, 2.1 g) of ethyl acetate fraction and (1.1 g, 1.9 g) of *n*-butanol fraction respectively.

Random Amplified Polymorphic-DNA-PCR

Samples of fresh leaves of both *Kalanchoe* species was stored at -70^{0}C, freeze dried and ground under liquid nitrogen to fine powder (50 mg each) prior to DNA isolation. DNA was extracted using the CTAB (1% (w/v) N-cetyl-N, N, N-trimethylammonium Bromide) method (Doyle, Doyle 1987).

Ten oligonucleotide primers of arbitrary sequences were used in this study with the following sequence:**OPB-12** (5'-CCTTGACGCA-3'); **OPB-18** (5'-CCACAGCAGT-3'); **OPB-09** (5'-TGGGGGACTC- 3'); **OPB-15** (5'-GGAGGGTGTT-3'); **OP0-09** (5'-TCCCACGCAA-3'); **OPB-01** (5'-GTTTCGCTCC- 3'); **OPO-02** (5'-ACGTAGCGTC- 3'); **OPA-02** (5'-TGCCGAGCTG-3'); **OPA-06** (5'-GGTCCCTGAC-3') and **OPA-11** (5'-CAATCGCCGT-3').

RAPD bands were treated as presence or absence, without considering their percentage. For estimating genetic distance among the tested samples; each of the DNA bands was treated as a unit character. Genetic similarity (GS) was analyzed according to the equation of Jaccard (Jaccard 1908**)**: $GS=2\ N_{ab}/\ (N_a+N_b)$; where, N_{ab} is the number of shared fragments between plants a and b; N_a is the number of scored fragments of plant a and N_b is the number of scored fragments of plant b.

Cytotoxic screening of different plant extracts

One mg of each of the aqueous extract, alcohol extract, dichloromethane fraction, ethyl acetate fraction and *n*-butanol fraction of both *Kalanchoe* species were subjected to Sulphorhodamine-B (SRB) assay of cytotoxic activity at concentration of 0-50 µg/ml were tested using tumor cell line MCF7 (Breast carcinoma cell line). The two isolated pure compounds (**1** and **2**) from the dichloromethane fraction of *K. thyrsiflora* were tested for their cytotoxic activity using both Breast carcinoma cell line (MCF7) and normal melanocyte cell line (HFB4). Both cell lines were obtained from the American Type Culture Collection at National Cancer Institute, Cairo, Egypt. Cells were used when 90 % confluence was reached in T25 flasks. Adherent cell lines were harvested with 0.025 % trypsin. Viability was determined by trypan blue exclusion using the inverted microscope. Cells were seeded in 96-well microtiter plates at a concentration of $5x10^4$ to 10^5 cell/well in a fresh medium and left to attach to the plates for 24 hrs. After 24 hrs, cells were incubated with the appropriate concentration ranges of drugs, completed to total of 200 µl volume/well using fresh medium and incubation was continued for 24 and 48 hrs. Control cells were treated with vehicle alone. For each drug concentration, 4 wells were used. Following 24 and 48 hrs treatment, the cells were fixed with 50 µl cold 50% trichloroacetic acid for 1 hr at 4 ^{0}C. Wells were washed 5 times with distilled water and stained for 30 min at room temperature with 50 µl 0.4 % SRB dissolved in 1 % acetic acid. The wells were then washed 4 times with 1 % acetic acid. The plates were air-dried and the dye was solubilized with 100 µl/well of 10 mM tris base (pH 10.5) for 5 min on a shaker (Orbital shaker OS 20, Boeco, Germany) at 1600rpm.The optical density (O.D.) of each well was measured spectrophotometrically at 564nm with an ELISA microplate reader (Meter tech. Σ 960, U.S.A.). The mean background absorbances was automatically subtracted and mean values of each drug concentration was calculated. The percentage of cell survival was calculated as follows:

Survival fraction = O.D. (treated cells)/ O.D. (control cells).

The IC_{50} values (the concentrations of drug required to produce 50% inhibition of cell growth). The experiment was repeated 3 times for cell line used then repeated after one week (Skehan *et al.*, 1990). For more convenient selection of the least toxic compounds among the most active, a special parameter-SX (selectivity Index) has been introduced. It is a selectivity indicator of tested substances towards tumor cells, SX = [IC_{50} (HFB4)/IC_{50} (MCF7)] × 100. The SX represents the ratio of IC_{50} obtained in toxicity testing using normal melanocytes cell line (HFB4) to IC_{50} of same compound in cytotoxicity test using breast cancer cell line (MCF7), multiplied by 100. The SX value above 100 indicates that the cytotoxic effect of tested substance is greater towards cancer cells while SX value 100 or below would suggest that concentration of compound for achieving therapeutic effect is similar or lower than the concentration causing toxic effects. Obviously, the most desirable substances would have SX values distinctly higher than 100 (Lima *et al.*, 2005).

Isolation of dichloromethane fraction of *Kalanchoe thyrsiflora* Harv.

The dichloromethane fraction of *Kalanchoe thyrsiflora* Harv.(5.27g) was solubilized in *n*-Hexane and fractionated over a glass column (55cm L x 5cm D) which was packed with silica gel 60-120 (100g) for column adopting the wet method. Elution was done using *n*- Hexane with increasing polarity by adding acetone. Finally the column was washed with acetone (100%) and methanol to ensure perfect elution process. Similar fractions (50 ml) were pooled together after screening by TLC (silica gel 60-120) plates using *n*-Hexane: ethyl acetate (different ratios) guided by UV and vanillin-H_2SO_4 spraying reagent to yield 7 subfractions.

Fractions IV (2.16 g) and V (1.06 g) showed major spots on TLC after spraying with vanillin- H_2SO_4 using *n*-Hexane: ethyl acetate (96:4) solvent system. Further purification of these fractions was done by applying them on a column (35 cm L x 2 cm D) packed with silica gel 60-120 (40g) as adsorbent and *n*-Hexane with an increasing ratio of dichloromethane as an eluent. Two major compounds 1 (fraction IV) and 2 (fraction V) were isolated and tested for their cytotoxic activity. Identification of these compounds was performed using physicochemical as well as spectral properties, IR, ^1H and ^{13}C NMR analysis.

^1H spectra run at 300 MHz and ^{13}C spectra were run at 75.46 MHz in deutrated dimethylsulphoxide (DMSO-d_6) and $CDCl_3$. tetramethylsilane (TMS) as internal standard. Chemical shifts are quoted in δ and were related to that of the solvents.

Compound 1:3-oxo-olean-12-ene

IR (cm^{-1}): 2944 (aliphatic C-H), 1706 (carbonyl group), 1610 (olefinic carbon at [C-12]), 1380 (geminal dimethyl group). ^1H-NMR ($CDCl_3$) δ (ppm): 2.3 (2H, *m*, H-1), 2.5 (2H, *m*, H-2), 5.1(1H, *t*, H-12), 0.8 (3H, *s*, Me-23), 0.83 (3H, *s*, Me-24), 0.86 (3H, *s*, Me-25), 1.01(3H, *s*, Me-26), 1.04(3H, *s*, Me-27), 1.08 (3H, *s*, Me-28), 1.06 (3H, *s*, Me-29), 1.13 (3H, *s*, Me-30). ^{13}C-NMR ($CDCl_3$) δ (ppm): 39.8 (C-1), 34.8 (C-2), 217.8 (C-3), 47.5 (C-4), 59.2 (C-5), 19.7 (C-6), 32.6 (C-7), 46.88 (C-8), 55.3 (C-9), 39.3 (C-10), 23.7 (C-11), 121.5 (C-12), 145.3 (C-13), 41.9 (C-14), 28.5 (C-15), 26.6 (C-16), 33.4 (C-17), 47.4 (C-18), 46.9 (C-19), 32.2 (C-20), 36.7 (C-21), 37.19 (C-22), 27.02 (C-23), 21.5 (C-24), 16.8 (C-25), 15.3 (C-26), 26.2 (C-27), 29.7 (C-28), 34.2 (C-29), 25.9 (C-30).

Compound 2: β-sitosterol

IR (cm^{-1}): 3377.8 (OH group), 2921.4 (aliphatic C-H), 1645.7 (olefinic carbon at [C-5]), 1382.1 (geminal dimethyl group). ^1H-NMR ($CDCl_3$) δ (ppm) 3.2 (1H, *m*, H-3), 5.1(1H, *t*, H-6), 0.8 (3H, *s*, Me-18), 1.6 (3H, *s*, Me-19), 1.2 (3H, *d*, *J* =6.5 Hz, Me-21), 1.06 (3H, *d*, *J* =6.7 Hz, Me-26), 0.9 (3H, *d*, *J* =6.7 Hz, Me-27), 1.1 (3H, *t*, *J* =7.4 Hz, Me-29).^{13}C-NMR ($CDCl_3$) δ (ppm) 38.85 (C-1), 31.19 (C-2), 79.1 (C-3), 41.7 (C-4), 124.5 (C-5), 121.8 (C-6), 32.7 (C-7), 33.4 (C-8), 47.7 (C-9), 37.2 (C-10), 23.78 (C-11), 39.7 (C-12), 46.9 (C-13), 59.1 (C-14), 26.6 (C-15), 28.1 (C-16), 55.2 (C-17), 15.7 (C-18), 17.9 (C-19), 38.6 (C-20), 17.4 (C-21), 34.8 (C-22), 27.34 (C-23), 47.3 (C-24), 29.7 (C-25), 18.4 (C-26), 21.5 (C-27), 26.2 (C-28), 16.8 (C-29).

RESULTS AND DISCUSSION

DNA profiling and genetic polymorphism

Genetic diversity as revealed by RAPD using ten decamer primers were used to detect the genetic variability. Each of the ten primers had successfully directed the amplification of a genome-specific fingerprint of DNA fragments. The ten primers (*OPB-12*, *OPB-18*, *OPB-09*, *OPB-15*, *OPO-09*, *OPB-01*, *OPO-02*, *OPA-02*, *OPA-06* and *OPA-11*) of arbitrary sequences generated 92 fragments in *K. thyrsiflora*, while 75 fragments were generated in *K. marmorata* with a total of 167 fragments as shown in Table (1).

The obtained RAPD-PCR products for the two *Kalanchoe* species using ten decamer primers are represented in Fig. (1). The ten primers had produced multiple band profiles with a number of amplified DNA fragments ranging from 17, when OPA-06 was used with *K. marmorata* and 13 fragments when OPA-02 was used with *K. thyrsiflora*. Whereas, the least number of fragments was 2 being produced by OPB-15 in *K. marmorata* and 6 being produced by OPB-09 in *K. thyrsiflora*. From total fragments of 167 bands, 111 were polymorphic representing a level of polymorphism of 67.66 %. With the highest degree of similarity (60.86%) recorded using primer OPA-02 and the lowest degree of similarity (0%) recorded using primer OPB-09 as shown in Table (1). It can be concluded that the most relevant fragment resulting from the successful combination of template and primer was that produced by OPB-09 and OPA-11 RAPD primers. Such primers could be used to discriminate between the two *Kalanchoe* species depending on their low values of similarity coefficients and high level of polymorphism. However, the other estimated RAPD primers, which produce high values of similarity coefficient and low levels of polymorphism, could be used in the identification of different *Kalanchoe* species.

Table. 1: Total numbers of RAPD-PCR fragments, distribution of monomorphic and polymorphic bands and similarity coefficients generated by ten decamer arbitrary primers in *K. thyrsiflora* Harv. (*K.T.*) and *K. marmorata* Baker (*K.M.*).

Primer Code	RAPD Fragments			Monomorphic Fragments	Polymorphic Fragments	Similarity Coefficients
	K.T.	K.M.	Total			
OPB-12	8	3	11	1	9	18.18181818
OPB-18	8	7	15	4	7	53.33333333
OPB-09	6	6	12	0	12	0
OPB-15	10	2	12	2	8	33.33333333
OPO-09	11	5	16	3	10	37.5
OPB-01	7	5	12	2	6	33.33333333
OPO-02	9	5	14	2	10	28.57142857
OPA-02	13	10	23	7	9	60.86956522
OPA-06	10	17	27	4	19	29.62962963
OPA-11	10	15	25	2	21	16
Total	92	75	167	27	111	32.33532934

Table. 2: IC_{50} of different extracts, fractions & isolated compounds from *K. thyrsiflora* Harv. and *K. marmorata* Baker on breast carcinoma cell line (MCF7)

	K. thyrsiflora Harv. IC_{50} (ug/ml)	*K. marmorata* Baker IC_{50} (ug/ml)
Alc. Ext.	18.1	18
Aq. Ext.	22.5	17.6
CH_2cl_2 fraction	4	7.4
Ethyl acetate fraction	16.7	17.8
n-butanol fraction	19.2	22.2
Compound 1	-	17.4
Compound 2	-	17.6

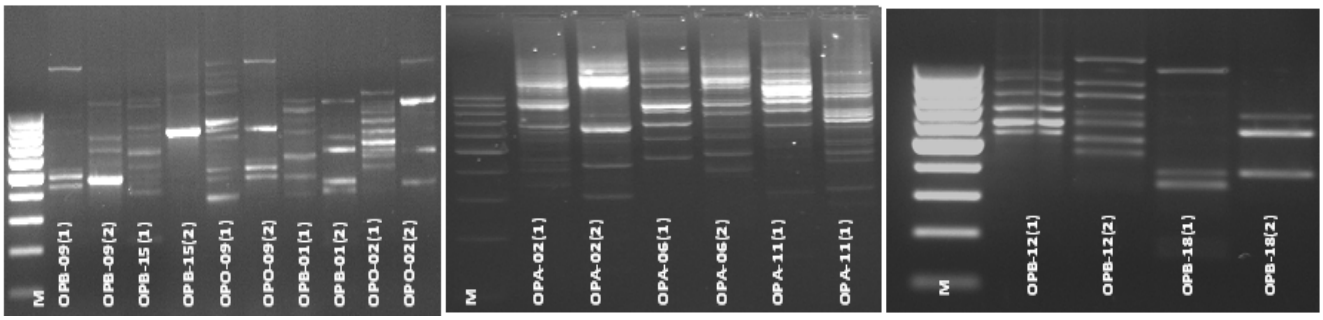


Fig. 1: The obtained RAPD-PCR products for *Kalanchoe thyrsiflora* Harv. (1) & *Kalanchoe marmorata* Baker (2) using ten decamer primers.

Cytotoxic activity

By comparing the cytotoxic activity of different extracts and fractions of both plants *K. thyrsiflora* and *K. marmorata* (Table 2 & Figure 3) on Breast carcinoma cell line (MCF7), it was observed that the aqueous extracts of both plants exhibited moderate cytotoxic activity at IC_{50} 22.5 µg/ml and 17.6 µg/ml respectively while that of the alcohol extracts of both plants showed the same cytotoxic activity at near IC_{50} of 18.1 µg/ml and 18 µg/ml respectively. The *n*-butanol fractions of *K. thyrsiflora* and *K. marmorata* exhibited moderate cytotoxic activity at IC_{50} 19.2 µg/ml and 22.2 µg/ml respectively almost similar to the aqueous and alcoholic extracts while the ethyl acetate fractions showed higher cytotoxic activity at IC_{50} 16.7µg/ml and 17.8µg/ml respectively than the aforementioned extracts. Concerning the dichloromethane fraction, *K. thyrsiflora* exhibited the highest cytotoxic activity at IC_{50} 4µg/ml in comparison with *K. marmorata* at 7.4 µg/ml as compared to standard doxorubicin which showed cytotoxic activity on MCF7 at IC_{50} 2.97 µg/ml (Figure 2). From the previous data, it was obvious that the most potent cytotoxic fraction was dichloromethane fraction of *Kalanchoe thyrsiflora* Harv. which was subjected to further isolation of its components. This active fraction led to the isolation of two pure isolated compounds which were further tested for their cytotoxic activity on both breast carcinoma cell line (MCF7) and normal melanocyte cell line (HFB4). Compound 1 (3-oxo-olean-12-ene) and compound 2 (*β*-sitosterol) showed similar moderate cytotoxic activity on MCF7 at IC_{50} 17.4 and 17.6 µg/ml respectively. While on HFB4, showed cytotoxic activity at IC_{50} 21.9 and 21.6 respectively (Table 2 & Figure 3).

Both compounds 3-oxo-olean-12-ene and *β*-sitosterol showed selectivity index (SX) at 125.86 and 122.72 respectively indicating that the cytotoxic effect of both isolated compounds is mostly selective towards cancer cells than normal cell (Popiołkiewicz *et al.*, 2005). It was observed that the dichloromethane fraction exhibited higher cytotoxic activity than the two isolated compound which may be due to the synergistic

activity of other components present in the dichloromethane fraction that may affect its cytotoxic activity.

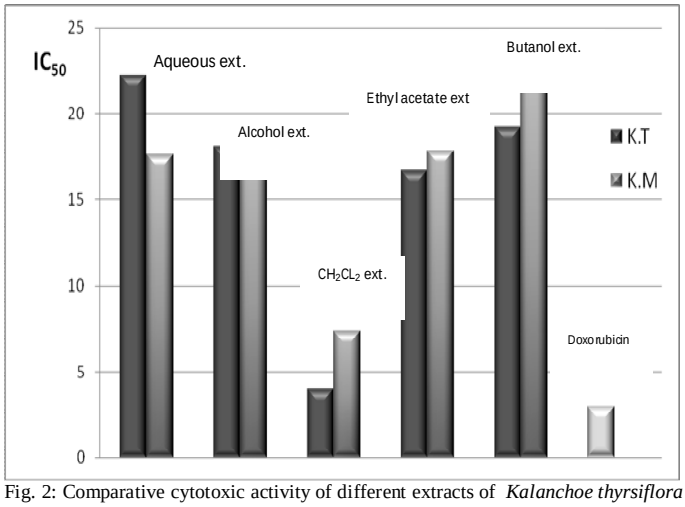


Fig. 2: Comparative cytotoxic activity of different extracts of *Kalanchoe thyrsiflora* Harv. (*K.T.*) and *Kalanchoe marmorata* Baker (*K.M.*) on MCF7.

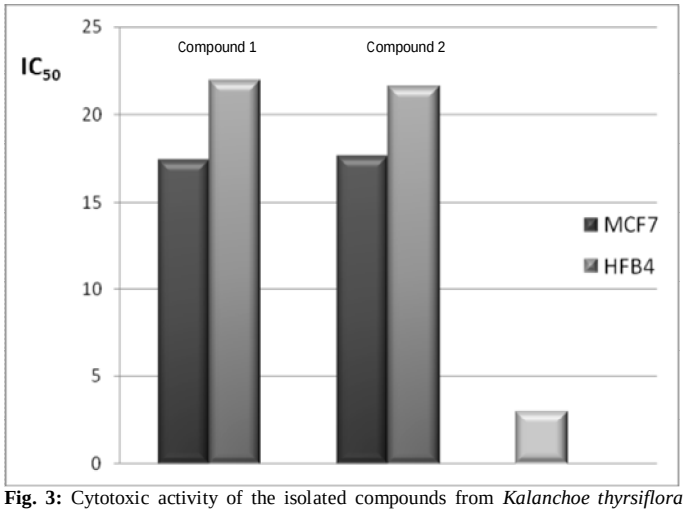
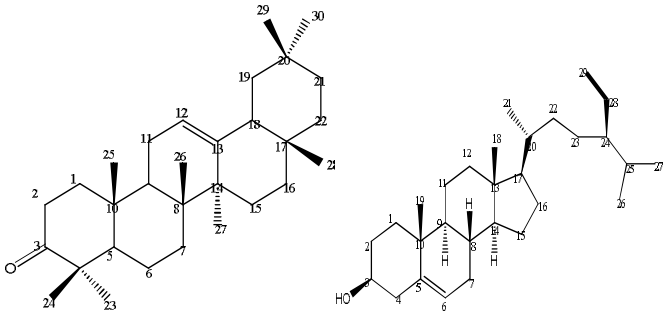


Fig. 3: Cytotoxic activity of the isolated compounds from *Kalanchoe thyrsiflora* Harv. on MCF7 & HFB4.

Isolation and identification of compounds *1 and 2*

Identification of compound 1: 3-oxo-olean-12-ene

Compound 1 (0.8g) was obtained as white crystals (*n*-hexane). It gave a dark purple spot on TLC when sprayed with vanillin-H_2SO_4 using *n*-hexane: ethyl acetate (90:10) solvent system with R_f 0.54. Analysis of the IR spectrum revealed presence of carbonyl group at 1706 cm^{-1}, olefinic carbon at 1610 cm^{-1} (C-12) and geminal dimethyl group at 1380 cm^{-1}. The ^{1}H-NMR spectrum displayed 8 singlet signal at δ (0.8-1.13) indicating the presence of eight methyl groups. An intense peak at δ 5.1 (1H, *t*) indicating the olefinic proton at H-12. The nature of the ketonic functional group was confirmed in the ^{13}C-NMR by a signal at δ 217.8 (C-3) also presence of 121.5 & 145.3 ppm indicating olefinic carbons at C12 & C13 respectively, this data is in close agreement with the data reported in the literature to be 3-oxo-olean-12-ene (Figure 4) (Lima *et al.*, 2005; Caceres- Castillo *et al.*, 2008).



3-oxo-olean-12-ene (Compound 1) *β*-sitosterol (Compound 2)

Fig. 4: Chemical structure of compound **1** and **2.**

Identification of compound 2: β-sitosterol

Compound 2 (0.1 g) was isolated as white powder (*n*-Hexane: CH_2CL_2 70:30) with R_f 0.2. It gave buff color under UV that turned dark purple spot on TLC after spraying with vanillin-H_2SO_4 using *n*-hexane: ethyl acetate (90:10) solvent system. IR spectrum of 2 exhibited an intense absorption band at 3377.8 cm^{-1} indicating presence of hydroxyl group and olefinic carbon at 1645.7 cm^{-1}(C-5). The ^{1}H-NMR spectrum showed the presence of C-24 ethyl sterol nucleus by the existence of six methyl groups at δ (0.8-1.6). Moreover, a signal at δ 5.1 (1H, *t*) corresponding to an olefinic proton at H-6, an oxygen bearing methine proton at δ 3.2 (1H, *m*). The ^{13}C-NMR spectrum displayed signals for 29 carbons that were assigned for the six methyl groups [δ 15.7 (C-18), 17.2 (C-19), 17.4 (C-21), 18.4 (C-26), 21.5 (C-27), 16.8 (C-29),], one oxygenated at δ 79.1 (C-3), vinylic carbon at δ [124.5(C-5), 121.8 (C-6)]. This physical and spectral data of compound 2 were identical with those published for *β*-sitosterol (Figure 4) (Jayaprakasha *et al.*, 2007; Moghaddam *et al.*, 2007).

CONCLUSION

A comparative DNA profiling of the leaves of both *K. thyrsiflora* and *K. marmorata* was performed in order to contribute to the present pharmacognostical knowledge on these species. A 67.66% polymorphism was attained using ten different primers with the most relevant primer used for discrimination being OPB-09 and OPA-11 RAPD primers.

The biological study of different leaf extracts of both *Kalanchoe thyrsiflora* Harv. and *Kalanchoe marmorata* Baker. revealed cytotoxic activity of different extracts, and also the cytotoxic activity of pure isolated compounds according to biological guided fractionation. The phytochemical investigation of the most potent cytotoxic fraction; dichloromethane fraction of *Kalanchoe thyrsiflora* Harv., had led to isolation of two pure compounds 3-oxo-olean-12-ene and *β*-sitosterol. Although the isolation and purification of compounds with promising cytotoxic activities from active plant extracts has been extensively researched, testing on *in-vivo* systems remains a significant step. Continuing research should include studies on the mechanisms of cytotoxic action of each fraction as well as the determination of optimal doses.

REFERENCES

Alonso-Castro, A.J., Villareal, M.L., Salazar-Olivo, L.A., Sanchez, M.G., Dominguez, F. & Garcia-carranca, A. Mexican medicinal plants used for cancer treatment: Pharmacological, phytochemical and ethnobotanical studies. Journal of Ethnopharmacology 2011; 133: 945-972.

Bailey, L.H. The standard Cyclopedia of Horticulture1. The Macmillan Company, New York, (1953) 578-581.

Bailey, L.H. The standard Cyclopedia of Horticulture2. The Macmillan Company, New York (1958) 1731-1732.

Benson, L. Plant Classification.Heath and company, Boston (1957) 241-244.

Boulos, L. Flora of Egypt.Al Hadara Publishing, Cairo, Egypt (1999) 238.

Caceres-Castillo, D., Mena-Rejon, G.J., Cedillo-Rivera, R. & Quijano, L. 21*β*-Hydroxy-oleanane-type triterpenes from *Hippocratea excels*. Phytochemistry 2008; 69: 1057-1064.

Chopra, R.N., Nayar, S.L.& Chopra, I.C. Glossary of Indian medicinal products. The Council for Scientific and Industrial Research, New Delhi (1956) 147.

Costa, S.S., Jossang, A. & Bodo, B. "4'"- Acetylsagittatin A, a kaempferol triglycoside from *Kalanchoe streptantha.* Journal of Natural Product 1996; 59: 327-329.

Doyle, J. & Doyle, J.L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletin 1987; 19: 11-15.

Gaind, K.N., Singla, A.K. & Wallace, J.W. Flavonoid glycoside of *Kalanchoe spathulata*. Phytochemistry 1981; 20: 530-531.

Jaccard, P. Nouvelles recherché sur la distribution florale.*Bulletin de la Societe Vaudoise des Sciences Naturelles,* 1908; 44: 223-70.

Jayaprakasha, G.K., Mandadi, K.K., Poulose, S.M., Jadegoud, Y., NaganaGowda, G.A. & Patil, B.S. Inhibition of colon cancer cell growth and antioxidant activity of bioactive compounds from *Poncirus trifoliate* (L.) Raf. Bioorganic & Medicinal Chemistry 2007; 15: 4923-4932.

Joshi, K, Chavan, P., Warude, D.& Patwardhan, B. Molecular markers in herbal drug technology. Current Science 2004; 87: (2) 159.

Kalinowska, M., Nes, W.R., Crumley, F.G. & Nes, W.D. Stereochemical differences in the anatomical distribution of C-24-alkylated sterols in *Kalanchoe daigremontiana*. Phytochemistry 1990; 29: 3427–3434.

Lima, F.V, Malheiros, A., Otuki, M.F., Calixto, J.B., Yunes, R.A., Filho, V.C. & Monache, F.D. Three new triterpenes from the resinous bark of *Protium kleinii* and their antinociceptive activity. Journal of Brazilian Chemical Society 2005; 16 (3B): 578–582.

Liu, K.C.S, Yang, S.L., Roberts, M.F.& Phillipson, J.D. Eupafolinrhamnosides from *Kalanchoe gracilis*. Journal of Natural Products 1989; 52: 970-974.

Moghaddam, F.M., Farimani, M.M., Salahvarzi, S. & Amin, G. Chemical constituents of dichloromethane extract of cultivated *Satureja khuzistanica*. Evidence-based Complementary and Alternative Medicine 2007; 4(1): 95-98.

Nguelefack, T.B., Nana, P., Atsamo, A.D., Dimo, T., Watcho, P., Dongmo, A.B., Tapondjou, L.A., Njamen, D., Wansi, S.L. & Kamanyi A. Analgesic and anticonvulsant effects of extracts from the leaves of *Kalanchoe crenata* Andrews Haworth (Crassulaceae). Journal of Ethnopharmacology 2006; 106: 70–75.

Popiołkiewicz, J., Polkowski, K., Skierski, J.S. & Mazurek, A.P. *In vitro* toxicity evaluation in the development of new anticancer drugs genistein glycosides. Cancer Letters 2005; 229: 67-75.

Siddiqui, S., Faizi, S., Siddiqui, B.S. & Sultana, N. Triterpenoids and phenanthrenes from leaves of *Bryophyllum Pinnatum*. Phytochemistry 1989; 28: 2433-2438.

Singab, A.B., El-Ahmady, S.H., Labib, R.M. & Fekry, S.S. Phenolics from *Kalanchoe marmorata* Baker, Family Crassulaceae. Bulletin of Faculty of Pharmacy, Cairo University 2011; 49 (1): 1-5.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S. & Boyd, M.R. New colorimetric cytotoxicity assay for anticancer-drug screening. Journal of the National Cancer Institute 1990; 82(13): 1107-1112.

Supratman, U., Fujita, T., Akiyama, K. & Hayashi, H. Insecticidal compounds from *Kalanchoe daigremontiana* × *tubiflora*. Phytochemistry 2001; 58: 311–314.

Yadav, N.P. & Dixit, V.K. Hepatoprotective activity of leaves of *Kalanchoe pinnata*. Journal of Ethnopharmacology 2003; 86: 197–202.