Bioactive Cytotoxic Agents and Chemokine Production Inhibitors in LPS-Induced Raw264.7 Macrophage Cell Line from Flowers of Crepis senecioides

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Abstract
Crepis senecioides is one of the endemic plant species found in Libya. The ethanol extract (E) of the flowers was partitioned on silica gel column with dichloromethane affording four compounds. Their structures were elucidated by the physicochemical and spectral data as germanicol acetate (1), 3',3''butyl, 8',8''carboxy) di-octyl phthalate (2), taraxasterol (3) and β-sitosterol (4). effect of the tested samples 1 and 2 on the viability of RAW macrophage 264.7 were studied. anti-cancer activity was tested on several human cell lines. Anti-inflammatory effect was also screened. Compound 1 had promising cellular cytotoxicity with IC_{50} 60.26µg/mL on HepG2. Compound 2 had a talented cellular cytotoxicity with IC_{50} 50.18 µg/mL on PC3. Both 1 and 2 exerted alike cytotoxicity with IC_{50} 78.53, 78.14 on PC3 and MCF-7 carcinoma cell lines respectively. The treatment of LPS-stimulated macrophage with 2 led to a highly significant inhibition 45.05% in the nitrite concentration in LPS-stimulated macrophage. Compound 1 had a promising cellular cytotoxicity on HepG2, while 2 exhibited an inhibition on chemokine expression in LPS-induced RAW 264.7 macrophages and had talented cellular cytotoxicity on PC3.

Keywords: Crepis senecioides; Asteraceae; Anti-inflammatory; cytotoxic effect; terpenoids; phthalate derivative.

1. Introduction
A continuous chemical and biological screening of unexplored flora is necessary for exploitation of natural local resources in national drug industry. Traditional African herbal medicine is among the most ancient natural therapies and perhaps the oldest folk medicine currently practiced (Sawandi, 2012; Brendler et al., 2010).

Plant derivatives have been used over the years to treat a wide variety of ailments, from microbial infections to various forms of neoplastic growth. The isolation and characterization of novel compounds that might serve as leads for the development of new and effective drugs from medicinal plants has now become an area of much interest worldwide (De Mesquita et al., 2011). Surely, in drug discovery or drug assessment using cell lines, researchers attempt to find compounds that lead to the triggering of apoptosis or programmed cell death in diseased cells such as cancer cells. A candidate drug is therefore introduced to the cells and its effects ascertained. The most ideal is a compound that is potent at low concentrations and discriminates between diseased and normal cells (Cochrane et al., 2008).

Cancer is a group of diseases that involve unregulated cell growth and death, tumor-promoting inflammation, induction of angiogenesis, evasion of the immune system, and activation of metastasis and invasion (Hanahan and Weinberg, 2011). Hepatocellular carcinoma and prostate cancer is a major health problem worldwide, including Egypt. In the recent past, it has become the second most prevalent cancer among men in Egypt (Soliman et al., 2010; Boyle et al., 2003; Muir et al., 1991). The pattern of cancer indicated the increased burden of liver cancer. Breast cancer occupied the second rank (Ibrahim et al., 2014).

Crepis is a genus of annual and perennial flowering plants of the family Asteraceae, encompasses around 200 species, including about 70 European representatives (Mabberley, 2008). It is distributed throughout the North hemisphere and Africa (Enke, and Gemeinholzer, 2008). The center of diversity of the genus Crepis is in the Mediterranean region. Crepis was commonly known in some parts of the world as hawks beard. The taxonomy and evolutionary relationships of Crepis were studied (Babcock, 1947; Jeffrey, 1979; Ali et al., 1967).

The genus name Crepis derives from the Greek krepis, meaning sandal, possibly in reference to the shape of the fruit (Flora of America). Al-Jabal Al-Akhder is floristically one of the richest of all the phytogeographical region of Libya. Crepis senecioides Delile one of the endemic species was found in wadi Tanezzuft (Mukassabi et al., 2012).

Crepis species are used as food plants by the larvae of some Lepidoptera species including the broad-barred white moth and the fly Tephritis Formosa (White, 1984). Seeds of Crepis species are an important food source for some bird species (Buckingham and Peach 2005). The leaves and tender stem of C. commutate and C. vesicaria are eaten raw, boiled, steamed or browned in salads. The leaves of C. rueppelli Sch. Bip was used for diarrhea (Wabe et al., 2011).

The genus Crepis is a wealthy source of sesquiterpenes lactone-type guaianolides (Pant and Rastogi, 1979; Zidorn, 2008; Ghosh et al., 1985). Phenolics reported from the genus Crepis encompass isoluteolin
identified in the leaves of *C. senecioides* Delile and *C. tectorum* L. (Harborne, 1978).

Moreover, luteolin and caffeoyl quinic acid and caffeoyl tartaric acid derivatives were reported (Zidorn et al., 2008).

Reviewing the current literature nothing could be traced on either the chemical profile or the biological potentialities of *C. senecioides* grown in Libya. In our ongoing research for endemic plant in Libya, we investigated the chemical profile of the dichloromethane soluble fraction of the ethanolic extract of flowers of *C. senecioides* as well as evaluate the effect of its extract (E) and the main isolated phytochemicals, compounds 1 and 2 on chemokine expression in LPS-induced RAW 264.7 macrophages. Furthermore, provide insights into their possible cytotoxic activities.

2. Materials and methods

2.1 Plant Material

The flowers of *Crepis senecioides* Delile subsp. *senecioides* (Asteraceae) were obtained from the shrubs growing in Benghazi-Libya. Plant collection was carried out from April to May 2013. The identity of the plant material was kindly verified by Dr. Mohamed Al-Gebali, botanist specialist. Voucher specimens (Number 1-12-2014-1) are kept at the herbarium of the Department of Pharmacognosy, College of Pharmacy, Cairo University.

2.2 Material for phytochemical study

Material, solvent systems and spray reagents for chromatographic studies included Silica gel H (Merck, Darmstadt, Germany) for vacuum liquid chromatography (VLC), silica gel 60 (70-230 mesh ASTM; Fluka, Steinheim, Germany), Thin-layer chromatography (TLC) was performed on silica gel GF254 precoated plates (Fluka) using the following solvent systems: S<sub>1</sub>, n-hexane-ethyl acetate (90:10 v/v); S<sub>2</sub>, n-hexane-ethyl acetate (80:20 v/v) and S<sub>3</sub>, dichloromethane-methanol (95:5 v/v); S<sub>4</sub>. The chromatograms were visualized under UV light using Ultraviolet lamp (λ max =254 and 330 nm, Shimadzu), a product of Hanovia lamps for localization of spots on chromatograms before and after spraying with p-anisaldehyde/sulfuric acid spray reagent. Optical rotation values were measured on an ATAGO POLAX-D, No. 936216 (AEAGO Co., LTD., Japan) polarimeter with a 1dm cell (ATAGO901048). HR-ESIMS was recorded with the JEOL JMX-AX 505, HAD mass spectrophotometer at an ionization voltage of 70 eV. IR spectra were observed as KBr discs using Jasco FT/IR-460 plus, Japan Infrared Spectrophotometer. ¹H NMR and ¹³C NMR spectra were recorded on a Bruker high performance digital FT-NMR spectrophotometer operating at 400 MHz (¹H) and 100 MHz (¹³C) and chemical shifts are given in δ (ppm) relative in CDCl₃ (δ 7.26 and 77.24 ppm for ¹H and ¹³C NMR respectively).

2.3 Extraction and fractionation

The air-dried powdered flowers of *C. senecioides* (500g) were extracted by percolation using cold method in room temperature with ethanol 95% (4 x 500mL) till exhaustion (for one week). The ethanol extract was evaporated under reduced pressure to yield (49g) dried extract. The dried residue was suspended in water (150 mL) and partitioned successively between dichloromethane (6 x 500 mL). The dichloromethane fraction was evaporated to yield (6g).

Dichloromethane fraction (6g) was chromatographed on 60 g silica gel in a vacuum liquid chromatography column (VLC) (15x5 cm). Gradient elution was performed using n-hexane, n-hexane/chloroform/ether mixtures, chloroform, chloroform/ethyl acetate mixtures, ethyl acetate, ethyl acetate/methanol mixture and methanol. The polarity was increased by 5 % every 200 ml till 100% methanol. Fractions of 200 ml each were collected and monitored by TLC to yield four main fractions (A–D). Fraction A (45-50% chloroform/n-hexane) was re-chromatographed over a silica gel 60 column, using n-hexane/ethanol acetate (9:9: 0.1 v/v) as eluent, to give compound 1 (34 mg, white needle crystals, R<sub>t</sub> = 0.89 in S<sub>3</sub>). Fraction B (60-70 % chloroform/n-hexane) was re-chromatographed over a silica gel 60 column, using n-hexane/ethanol acetate (9:9: 0.1 v/v) as eluent, to give compound 2 (23 mg, oily, R<sub>t</sub> = 0.63 in S<sub>3</sub>). Fraction C (85-90 % chloroform/n-hexane) was purified in the same way using n-hexane/ethanol acetate (9:8:0.2 v/v) as an eluent to give compound 3 (90 mg, White amorphous, R<sub>t</sub> = 0.61 in S<sub>3</sub>). Fraction D (20% ethyl acetate/chloroform) was similarly purified using n-hexane/ethyl acetate (9:8:0.2 v/v) as eluent to give compound 4 (63 mg, white needle-shaped crystals, R<sub>t</sub> = 0.57 in S<sub>3</sub>).  

**Compound 1:** Was isolated as white microcrystalline powder (60 mg), m.p. 278-281°C; [α]²⁵D =+5.8 (2c,CHCl₃ ) . Negative HR-EIMS showed a molecular ion peak at m/z; 467.75408 [M-1]. ¹H NMR (400 MHz, CDCl₃) δ : 4.88 (1H, s, H-19), 4.48 (1H, m, H-3α), 2.06 (3H, s, CH₃CO), 1.10 (3H, s, CH₃-27), 1.04 (3H, s, CH₃-26), 0.97 (3H, s, CH₃-25), 0.96 (3H, s, CH₃-23), 0.93 (3H, s, CH₃-29), 0.89 (3H, s, CH₃-30), 0.87 (3H, s, CH₃-24), 0.76 (3H, s, CH₃-28). ¹³C NMR (100 MHz, CDCl₃) δ : 38.6 (C-1), 24.1 (C-2), 81.0 (C-3), 38.4 (C-4), 55.6 (C-5), 18.2 (C-6), 34.5 (C-7), 40.8 (C-8), 51.1 (C-9), 37.5 (C-10), 21.1 (C-11), 26.5 (C-12), 38.3 (C-13), 43.3 (C-14), 27.9 (C-15), 37.8 (C-16), 34.3 (C-17), 142.7 (C-18), 129.8 (C-19), 32.7 (C-20), 33.7 (C-21), 37.7 (C-22), 28.3 (C-23), 16.8 (C-24), 16.4 (C-25), 17.1 (C-26), 14.9 (C-27), 25.6 (C-28), 31.6 (C-29), 29.5 (C-30), 171.0
Breast adenocarcinoma (MCF-7), prostate carcinoma (PC3), and murine macrophage (RAW 264.7) (ATCC, USA) were used in the cytotoxicity assays for endotoxin using Pyrogent® Ultra gel clot assay, and they were found endotoxin free. All experiments were performed at least in triplicate.

### 2.4 Material for biological screening

#### 2.4.1 Cell culture

Several human cell lines were used in testing anti-cancer activity including: hepatocellular carcinoma (Hep-G2), breast adenocarcinoma (MCF-7), prostate carcinoma (PC3), and RAW murine macrophage (RAW 264.7) (ATCC, VA, USA). Cells were routinely cultured in DMEM (Dulbeco's Modified Eagle's Medium), except RAW 264.7 cells, which were grown in RPMI-1640 at 37 ºC in a humified air containing 5% CO₂. Media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 U/mL penicillin G sodium, 100 U/mL streptomycin sulphate, and 250 ng/mL amphotericin B. Monolayer cells were harvested by trypsin/EDTA treatment, except for RAW 264.7 cells, which were collected by gentle scraping. The tested samples were dissolved in dimethyl sulphoxide (DMSO, 99.9%, HPLC grade) and diluted 1000-fold in the assays. In all the cellular experiments, results were compared with DMSo-treated cells. Sample dilutions were tested before and after the MTT assay. Fifty µl of cell culture material was obtained from Lonza (USA), and all chemicals were from Sigma (USA).

#### 2.4.2 Viability of Immune Cells

The effect of the tested samples on the growth of Raw macrophage 264.7 was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Hansen et al., 1989). The yellow tetrazolium formazan crystals, which are solubilized by the addition of a detergent. Cells (5x10^3) were incubated with various concentrations of the tested samples at 37 ºC in a FBS-free medium, before submitted to MTT assay. The absorbance was measured with an ELISA reader (BioRad, München, Germany) at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data are expressed as the mean percent of viable cells as compared to DMSO-treated cells. Treatment of macrophage with 1000 µM recombinant macrophage colony-stimulating factor (M-CSF, Pierce, USA) was used as positive control.

#### 2.4.3 Nitrite assay

The accumulation of nitrite, an indicator of NO synthesis, was measured in the culture medium by the Griess reaction (Gerhauser et al., 2002). RAW 264.7 were grown in phenol red-free RPMI-1640 containing 10% FBS. Cells were incubated for 24 h with bacterial lipopolysaccharide (LPS, 1µg/mL) in presence or absence of different tested samples extract (25µg/mL), compounds (12.5 µg/mL), or DMSO. Fifty µl of cell culture supernatant were mixed with 50 µl of Griess reagent and incubated for 10 min. The absorbance was measured spectrophotometrically at 550 nm. A standard curve was plotted using serial concentrations of sodium nitrite.

#### 2.4.4 Cytotoxicity assay

Antiproliferative activity against various tumor cell lines, hepatocellular carcinoma (Hep-G2), breast adenocarcinoma (MCF-7) and prostate carcinoma (PC3) was estimated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells (Hansen et al., 1989). The relative cell viability was expressed as the mean
percentage of viable cells relative to the respective DMSO-treated cells (control). The half maximal growth inhibitory concentration IC_{50} value was calculated from the line equation of the dose–dependent curve of each sample.

2.4.3 Statistical analysis
MTT assay data were analyzed by using two-factorial analysis of variance (ANOVA), including first-order interactions (two-way ANOVA), followed by the Turkey’s post hoc test for multiple comparisons. P < 0.05 indicated statistical significance.

3. Results
3.1 Identification of the isolated compounds
Four compounds were isolated from the dichloromethane soluble fraction of the ethanolic extract of the flowers of C. senecioides through consecutive column chromatographic separations. On the basis of physiochemical and chemical analyses as well as comparison with published data (Goad and Akihisa 1997; Kushiro et al., 2000; Nguyen et al., 2002; Sultan et al., 2010), their structures were identified as two triterpene, germanicol acetate 1, traxasterol 3, phthalate derivative, [1(3-butyl-8-carboxy) octyl, 8(3-butyl-8-carboxy) octyl] phthalate 2 and β-sitosterol 4. (Fig. 1)

Germainicol acetate (5α-Olean-18-en-3β-ol acetate) (1): was isolated as white microcrystalline powder (34 mg), m.p. 278-281°C. Negative HR-EI/MS showed a molecular ion peak at m/z: 467.75408 [M-H]. H-NMR spectrum of 1 showed 9 methyl singlet signals in them one was at δ 2.06 (3H, s) due to an acetate methyl signal. The singlet peak at δ 4.88 (1H, s) was identified as H-19 proton signal attached on an olefinic double bond (Table 1). The methyl group in acetate residue was appeared at δ 21.6 and the chemical shift of C-3 carbon was appeared to downfield at δ 81.3 ppm in the 13CNMR spectrum (Table 1). As a result, compound 1 was identified as 3β-acetoxy-olean-18-ene (Nguyen et al., 2002). Inspection of Table 1 shows that for germanicol acetate (1) the tentative values of C-1 to C-14 agree reasonably well with those deliberate by Beierbeck and Saunders (1975) and also with those obtained for lupeol (Wenkert et al., 1978). Compound 1 previously reported in Euphorbia species (Biesboer et al., 1982; Hemmers et al., 1988). Germanicol acetate previously demonstrated some slight cytotoxic activity against Jurkat cells after 72 h; however the activity was minimal compared to the standard cytotoxic compound vincristine (Luz et al., 2016)

1,8-(3′,3′′-butyl-8′,8′′-carboxy)di-octylphthalate (2): was obtained as colorless oil with specific rotation of -1.49 (c 0.28, MeOH). The molecular formula was determined to be C34H32O6 [M-H]; 591.4537 by HR-ESIMS. H-NMR spectra showed phthalate moiety at δ 7.73 (2H, dd, J = 8.8, 2.0 Hz) and δ C 128.8; 7.55(2H, dd, J = 8.8, 2.4 Hz) and δ C 130.9 and the quartenary carbon at δ C 132.5. The hydroxylated methine were evidenced at δ 4.25 ppm. From interpretation of HSQC and HMBC spectral data, the structures was connected to provide one alkylated nonanoate moiety which was ester-linked with the phthalate moiety as evidenced from the correlations between the carbonyl groups at δ 167.8 (2C) and the hydroxylated methine protons at δ 4.25. The numbers of severely overlapped methylene and methine groups were counted from the molecular formula deduced from the MS data. From the interpretations of H and 13CNMR data (Table 2) compound 2 could be tentatively identified as 1,8-(3′,3′′-butyl-8′,8′′-carboxy)di-octylphthalate (Sultan et al., 2010). Compound 2 could be considering as petrochemicals, have been thought to be environmental pollutants or component of the soils and sediments. Phthalate derivatives were previously isolated from the heartwood of Petrocarpus angolensis (Bezuidenhoudt et al., 1980), Aloe vera (Lee et al., 2000), Hypericum hyssopifolium (Cakir et al., 2003) and Strculia guttata seeds (Katade et al., 2006).

Taraxasterol (3): The analysis of the 1H-NMR spectrum of 3 (Table 1) confirmed the presence of seven methyl groups, of which six were singlets δ (0.76, 0.86 (2 CH3), 0.92, 0.99 and δ 1.01 and one was displayed as doublet at δ 1.00 (J = 7.0 Hz). Moreover, the presence of an axial hydroxymethine (δ 3.20, dd, J = 11.2, 4.8 Hz) and two exomethylene protons (δ 4.65, br s) could be observed. The 13CNMR spectrum (Table 1) showed the presence of thirty signals, one olefinic at δ c 107.1, and seven methyl groups. The referred data indicated the molecular formula C30H50O (six degrees of unsaturation), pointing to a pentacyclic triterpene alcohol with a terminal double bond. The structure was elucidated on the basis of its physical and spectroscopic features and by comparing it with the previously reported data as taraxasterol (Mahato and Kundu,1994; Kushiro et al., 2000).

β-sitosterol (4): Compound 4 was obtained as white crystals recognized as β-sitosterol on the basis of its physical and spectroscopic data (Table1). The molecular formula (C29H48O) was deduced from the NMR spectra and confirmed by the negative HR-ESIMS which showed [M-H] at m/z 413. Furthermore, the 1H-NMR spectrum revealed the presence of multiplets at δ 3.51 and 5.34 that are characteristic signals for H-3α and for the olefinic proton H-6 of 3 β-hydroxy sterols. Two methyl singlets at δ 0.68 and 1.01 ppm were also observed and assigned to CH3-18 and CH3-19. The 13CNMR showed twenty nine carbon signal including six methyls, eleven methylenes, ten methane and three quaternary carbons. Comparison of all these physical and spectroscopic data with those described in the literature for β-sitosterol, confirmed the identity of compound 4 (Rajput and Rajpu
promising multi-targeting agents in the treatment of certain cancers and inflammatory diseases. Due to their potential and can inhibit cancer cell proliferation senecioides Nitrous oxide (NO) generation is a known inflammatory index. The accumulation of nitrite, an indicator of NO exerted as double strength as Treatment of different human cancer cell lines with (ME) led to a non-cytotoxic effect with IC3.4 Antiproliferative activity for use in both chemoprevention and chemotherapy of cancer (Yadav et al., 2010). Triterpenoids in their free and ability to act at various stages in the process of carcinogenesis, namely to block NF-αB activation, induce apoptosis and inhibit proliferation, invasion, metastasis and angiogenesis, these compounds may be considered to toxicitiy (Bishayee et al., 2011; Thoppil and Bishayee, 2011). The multifunction of triterpenoids makes them 3.2 Viability of Immune Cells Using MTT assay, the effect of the tested samples on the viability of RAW macrophage 264.7 was studied after 48 h of incubation. As shown in Fig.2, the treatment of macrophage with the extract led to a non-significant induction in the macrophage growth, while sample 1 and 2 had neither cytotoxic nor proliferative effect in macrophage compared to untreated macrophage.

3.3 Nitrite assay Nitrous oxide (NO) generation is a known inflammatory index. The accumulation of nitrite, an indicator of NO synthesis, was measured by Griess assay. The cell were treated with bacterial lipopolysaccharide of E.coli for overnight to induce inflammation cascade in the macrophages, before submitted to 24 h- treatment of a safe dose of the tested samples, E (25 µg/mL) and isolates 1, 2 (12.5 µg/mL). The LPS-stimulated macrophage generated a high significant amount of NO (P<0.001) as predicted by nitrite concentration in the macrophage culture media. The treatment of LPS-stimulated macrophage with extract (E) and sample 1 resulted in insignificant inhibition of nitrites (P>0.05), while sample 2 led to a highly significant inhibition (45.05%) in the nitrite concentration in LPS-stimulated macrophage (P<0.001), as shown in Fig. 3.

3.4 Antiproliferative activity Treatment of different human cancer cell lines with (ME) led to a non-cytotoxic effect with IC50 237.48, 116.118 and 207.28 µg/mL on the cell growth of Hep-G2, PC3 and MCF-7 respectively (Table 2). Compound 1 depressed remarkably the cell growth of Hep-G2 cells which, exhibited a promising cellular cytotoxicity and exerted as double strength as 2 with IC50 60.26 µg/mL. Moreover, it exhibited a significant cytotoxic effect against human prostate cancer PC3 cells with IC50 50.18 µg/mL. Both compounds 1 and 2 exerted alike cytotoxicity with IC50 78.53 µg/mL, 78.14 µg/mL on PC3 and MCF-7 carcinoma cell lines respectively (Fig. 4 - 5).

4. Discussion Biomes are generally created by the interplay of temperature and precipitation in a given region on the Earth. Several studies have demonstrated that extracts from some herbal medicines or their mixtures have anticancer potential and can inhibit cancer cell proliferation in vitro and/or in vivo (Bonham et al., 2002). Crepis senecioides, one of the endemic species was found in the wadi Tanezzuft (Mukassabi et al., 2012). Phytochemical investigation of the dichloromethane soluble fraction of the ethanol extract of flowers of C. senecioides afforded four compounds two triterpenoids of oleanane and ursane nucleus, one sterol in addition to one phethalate ester derivative.

Triterpenoid alcohols is usually restricted to the compounds with a 6-membered E-ring (Oleanane or ursane skeletons), but also 5-membered ring such as hopanoids. Triterpenoids have proven to be particularly useful to assign sources of organic sediments and petroleum. The most common triterpenoid class in sediments is the hopanoids. Indeed they may even be the most abundant natural product on earth (Oyo-Ita et al., 2010). Oleananes, ursanes, frenanes, lupanes, and their derivatives, widely distributed mainly as the oxygenated forms in many varieties of higher plant species (Jacob et al., 2005; Pant and Rastogi, 1979; Ghosh, et al., 1985; Mahato and Sen, 1997; Jacob et al., 2004; Jacob et al., 2007; Simoneit, 2008). Their characterization in chemotaxonomic studies can provide key information of flora changes (Killops et al., 1995).

Triterpenol esters, may be useful for assessing early diagenesis of global higher plant terrestrial during river transport. There are minor compounds occurring with the dominant triterpenoids such as taraxerol. Pentacyclic triterpenes are biomarkers of specific higher plant subspecies, while the triterpenol esters are indicators of early diagenetic alteration of higher plant detritus (Oyo-Ita et al., 2010). Biogenetically oleananes with 18 double bond (olean-18-enes) are believed to be consequent from squalene. While it has been well recognized that squalene is the biological precursor of all triterpenes, olean-18-enes may be considered as the immediate precursors to olean-12-enes and olean-13-enes (Gonzalez et al., 1981).

Triterpenoids have been shown to possess numerous biological activities and display various pharmacological effects such as anti-inflammatory and anticancer properties, combined with relatively low toxicity (Bishayee et al., 2011; Throppil and Bishayee, 2011). The multifunction of triterpenoids makes them promising multi-targeting agents in the treatment of certain cancers and inflammatory diseases. Due to their ability to act at various stages in the process of carcinogenesis, namely to block NF-κB activation, induce apoptosis and inhibit proliferation, invasion, metastasis and angiogenesis, these compounds may be considered for use in both chemoprevention and chemotherapy of cancer (Yadav et al., 2010). Triterpenoids in their free and esterified forms are compounds with low polarity, and are therefore found in abundance in such plant parts as
surface cuticle waxes (Jäger et al., 2009).

Phthalates are used in a large variety of products, from enteric coatings of pharmaceutical pills and nutritional supplements to thickness control agents, gelling agents, film formers, stabilizers, dispersants, lubricants, binders, emulsifying agents, and suspending agents. Phthalates and phthalic acid derivatives such as Di-n-octyl phthalate are important chemicals, used as plasticizers in polymers, which are toxic to humans (Chen and Sung, 2005) as well as plants (Saama et al., 2003). These chemicals have been thought to be environmental pollutants and detected in soils, sediments, terrestrial and marine waters (Namikoshi et al., 2006) but that their levels are low because they are subjected to relatively rapid photochemical and biological degradation (Hurford et al., 1989).

The occurrence of phthalates has been reported from Plantago major (Romeh, 2013) Limonium bicolor (Wei and Wang, 2006), Dracaena cochiniensis (Wei et al., 1998), and Caesalpinia sappan (Sarumathy et al., 2011). Dioctylphthalate isolated from the dried seeds of Nigella glandulifera (Nguyen et al., 2007) also reported in Chinese tomato fruit and cabbage. Dioctylphthalate is reported to have antimicrobial activity (Shafaghat et al., 2012) and identified as inhibiting melanogenesis (Nguyen et al., 2007). However, there are reports that phthalate esters are naturally produced extracellularly by microorganisms such as bacteria, fungi and yeasts (Heudorf et al 2007; Roy et al., 2006; Ljungvall et al., 2008).

Production of excessive NO has been associated with a range of inflammatory diseases including arteriosclerosis, ischemic reperfusion, hypertension and septic shock (Pacher et al., 2007). The immune system plays an important role in the development and progression of cancer. Macrophages can be pro-inflammatory or anti-inflammatory. Infiltration of macrophages can account for >50% of the tumor mass in some cancers, aid in metastasis by inducing angiogenesis, and signify a poor prognosis.

Inflammation is considered to be closely associated with a number of diseases, such as infection, and environmental, autoimmune and chronic diseases. Following injury, inflammation is one of the main mechanisms involved in the repair of tissue and consists of a cascade of cellular and microvascular reactions that help remove damaged tissue and generate new tissue. The cascade includes elevated permeability in microvessels, the attachment of circulating cells to the vessels in the environs of the injury site, the migration of several cell types, macrophage/monocyte chemotaxis, the release of inflammatory mediators, cell apoptosis, as well as the growth of new tissue and blood vessels (Ware and Matthay, 2000; Lee and Downey, 2010).

In summary, the flowers of C. senecioides possess an ability to accumulate terpenoid ester and phthalates derivative as a cleanup technology from sediment and environmental pollution. Triterpenol esters are indicators of early diagenetic alteration of higher plant detritus. Moreover, triterpenoid ester display a significant antiproliferative effect especially on hepatocellular carcinoma we could demonstrate that di-n-octylphthalate ester in pharmacologically pertinent doses reduces the expression of crucial inflammatory mediators, NO in RAW 264.7 macrophages.

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Table 1: NMR data of compounds 1, 3, 4 (CDCl3, 1H 400MHz, 13C 100MHz; δ in ppm, J in Hz)

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<td>0.93(3H,d,J=6.7Hz)</td>
<td>19.7</td>
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<td>-</td>
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<td>1.00(2H,d, J=7.0)</td>
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<tr>
<td>C=O</td>
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<td>171.0</td>
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<tr>
<td>H2C=O</td>
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Table 3. Results of cytotoxic activity of the ethanol extracts of *C. senecioides*.

<table>
<thead>
<tr>
<th>Tested Human cell line</th>
<th>Paclitaxel</th>
<th>Extract 1</th>
<th>Extract 2</th>
<th>Extract 3</th>
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<tr>
<td>Liver carcinoma</td>
<td>0.59</td>
<td>237.48</td>
<td>60.26</td>
<td>122.14</td>
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<tr>
<td>Prostate carcinoma PC3</td>
<td>0.63</td>
<td>116.18</td>
<td>50.18</td>
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<tr>
<td>Breast carcinoma</td>
<td>0.38</td>
<td>207.28</td>
<td>78.14</td>
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</table>

Table 4: Cytotoxicity of the ethanolic extract and the isolated compounds 1, 2 of *C. senecioides* on hepatocellular (HEPG2), prostate (PC3) and breast carcinoma cell line (MCF-7).

<table>
<thead>
<tr>
<th>Conc µg/mL</th>
<th>HEPG2</th>
<th>PC3</th>
<th>MCF-7</th>
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<tbody>
<tr>
<td></td>
<td>Extract 1</td>
<td>1</td>
<td>2</td>
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<tr>
<td>50</td>
<td>74.47±5.42</td>
<td>69.87±2.44</td>
<td>81.15±5.89</td>
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<td>25</td>
<td>89.12±6.29</td>
<td>79.83±2.30</td>
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<td>6.25</td>
<td>92.69±6.62</td>
<td>98.81±4.14</td>
<td>101.48±6.58</td>
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</tbody>
</table>

Conc = concentration
Fig 1: The isolated compounds from the ethanolic extract of flowers of *C. senecioides*

Fig 2: Effect of the tested samples, compounds 1, 2 and ethanolic extract of flowers of *C. senecioides* on the viability of the Macrophage RAW264.7 growth, as assayed by MTT viability test. The viability was expressed as % of control.
Fig. 3: Effect of the treatment with *C. senecioides* flower ethanol extract (50 µg/mL), isolated compounds 1, 2 (10 µM) and DMSO on nitrite accumulation as an index for NO generation from LPS-stimulated and untreated macrophage RAW264.7 investigated by the Griess assay in comparison with the nitrite level of control cells (untreated with LPS) and positive control (DMSO+LPS-treated cells) Nitrites were expressed as (µmole/mg protein, Mean±SE).

Fig. 4A: Effect of the tested samples, compounds 1, 2 and methanolic extract of flowers of *C. senecioides* on the viability of human Hep-G2 carcinoma cells. Fig 4B: Effect of the tested samples, on the viability of human PC3 carcinoma cells. Fig 4 C: Effect of the tested samples, on the viability of human MCF-7 carcinoma cells, as assayed by MTT viability test. The viability was expressed as % of control.
Fig. 5: IC₅₀ (µg/ml) of the tested samples compounds1, 2 and the methanolic extract of flowers of *C. senecioïdes* on various human cancer cells as assayed by MTT viability test.