Annuaire de l'Université de Sofia "St. Kliment Ohridski"
Faculte de Biologie
2018, volume 103, livre 4, pp. 13-21
Youth Scientific Conference "Kliment's Days", Sofia 2017

COMPARATIVE ANALYSIS OF EFFECTS OF SESQUITERPENE LACTONES AND POLYPHENOLS FROM *INULA OCULUS-CHRISTI* L. ON NORMAL AND CANCER CELLS

R. VELEVA[1], M. BORISOVA[1], M. MILEV[1], A. KOSTADINOVA[2], B. MRAVKOV[1], J. DOUMANOV[1], V. MOSKOVA-DOUMANOVA[1], A. TRENDAFILOVA[3], M. TODOROVA[3], T. TOPOUZOVA-HRISTOVA[1]

[1] *Faculty of Biology, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria*
[2] *Institute of Biophysics and Biomedical Engineering, BAS, Sofia, Bulgaria*
[3] *Institute of Organic Chemistry with Centre of Phytochemistry, BAS, Sofia, Bulgaria*
** Corresponding author: topouzova@biofac.uni-sofia.bg*

Keywords: plant extracts, *Inula oculus-christi,* cytotoxicity, A549 cells, MDCK II cells

Abstract: Plant extracts have been widely used in traditional medicine for centuries. The experimental research on some herbs from genus Inula indicated the anti-tumor effect of certain components of plant extracts. Our previous studies have revealed stronger anti-proliferative effect of the total extracts from *Inula oculus-christi* on cancer cells compared to non-cancer cells. Our current efforts are to identify those of the extracts' components, responsible for the observed effects. The aim of this study was to establish the effects of two extracts from on cancer and non-cancer cells. One of them was enriched in sesquiterpene lactones (extract A) and second - in phenolic acids and flavonoids (polyphenols, extract B). As a model system, we used non-cancer MDCKII cell line and cancer A549 cell line. Cytotoxicity was measured spectrophotometrically by crystal violet assay and the IC50 (inhibition concentration) for both extracts on both cell lines was determined. Changes in cell morphology were observed by bright field microscopy. Determined IC50 was higher for non-cancer cells than for cancer cells. We observed milder changes in the morphology of MDCK II cells after treatment. Our results indicate stronger impacts of studied extracts on cancer cells rather than on non-cancer cell. Extracts enriched in sesquiterpene lactones showed stronger cytotoxic effect.

INTRODUCTION

The genus *Inula* (Asteraceae) comprises more than 100 species, which are widely distributed in Asia, Europe and Africa. Some species from this genus have been used as traditional herbal medicines throughout the world (M.S Ali-Shtayeh et al., 1998; Leporatti and Ivancheva, 2003; Dulger et al. 2004; Mahasneh and Talib, 2010).

Their extracts have antioxidant properties and possess the ability to protect against enzymatic and non-enzymatic lipid peroxidation in model membranes (Schinella et al., 2001). They also possess antibacterial, antifungal and anti-inflammatory activity (Ali-Shtayeh and Abu Ghdeib, 1999; Máñez et al., 1999; Lokhande et al., 2007).

Our previous studies revealed strong anti-proliferative effect of extracts from *Inula oculus-christi*, enriched in certain groups of organic compounds (flavonoid glycosides, phenolic acids, flavonoid aglycones and some combinations of them) on cancer and non-cancer cells (Veleva et al., 2016). Flavonoids are known to have anti-inflammatory, antiallergic, antibacterial, antiviral, anticancer and hepatoprotective activities (Di Carlo et al., 1999). Certain sesquiterpene lactones show anti-tumor, cytotoxic or anti-microbial effects (Rodriguez et al., 1976; Moghadam et al., 2013; G.-W. Wang et al. 2014). The aim of the current study is to establish the biological effects of *Inula oculus-christi* extract enriched in sesquiterpene lactones on normal and cancer cells and to compare its effect with that of the extract containing polyphenols (phenolic acids and flavonoids). As model systems in our study, we used non-cancer canine kidney epithelial cells (MDCKII) and human lung carcinoma cell line (A549). The cytotoxic effects of both extracts were assessed and IC50 (concentration of inhibition) was evaluated in the cases where such concentration was reached during the investigation. Morphological analysis of changes induced by the extracts was also applied and stronger cytotoxic effect of extract enriched in sesquiterpene lactones was observed.

MATERIALS AND METHODS

Plant extracts

Wild growing *I. oculus-christi* was collected in full flowering stage in July 2016 from western Rhodope Mountains in Bulgaria. The plant was identified by Dr. Ina Aneva (Institute of Biodiversity and Ecosystem Research, BAS, Sofia). A voucher specimen (SOM 1360) has been deposited in the Herbarium of the Institute of Biodiversity and Ecosystem Research, BAS, Sofia, Bulgaria.

Air-dried flowers of *I. oculus-christi* were subsequently extracted with chloroform and methanol at room temperature (twice each). The crude extracts were obtained after filtration and evaporation of the solvents under vacuum. The chloroform extract was further subjected to column chromatography on Silica gel using $CHCl_3$/Acetone mixtures as eluent to give the fraction enriched in sesquiterpene lactones (extract A). The methanol extract was subjected to column chromatography on Sephadex LH 20 using methanol as eluent to afford fraction containing phenolic acids and flavonoids (Extract B). The final concentration of extracts in medium was presented in μg/ml.

Cell cultures

Human lung carcinoma cell line (A549) and non-cancer kidney epithelial cells (MDCKII) were used in this research. Cells were growth at standard conditions (humidified atmosphere with 5% CO_2, at 37°C) in Dulbecco's modified eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% (v/v)

antibiotic–antimycotic solution (streptomycin 100 μg/ml, penicillin 100 U/ml and amphotericin B 0.25 μg/ml).

25 cm^2 'CELLSTAR®' flasks were used to maintain the cells and Nunk™ 96-well plates were used to plate the cells for the experiments,

For experiments cells were sub-cultivated with initial concentration of $1x10^5$ cells per ml and 24 hours later they were treated with both extracts for either 24 or 48 hours.

Crystal violet assay

Cells were treated for 24 and 48 hours with both extracts to determine their cytotoxicity. For the Extract A (enriched in sesquiterpene lactones) ten different concentrations were tested (10 μg/ml; 20 μg/ml; 30 μg/ml; 40 μg/ml; 50 μg/ml, 60 μg/ml, 70 μg/ml, 80 μg/ml, 90 μg/ml, 100 μg/ml) and for Extract B (with phenolic acids and flavonoids) six different concentrations (10 μg/ml; 20 μg/ml; 30 μg/ml; 40 μg/ml; 50 μg/ml, 60 μg/ml) were used.

Cell morphology after treatment was observed under inverted microscope (XDS-2A, China) after crystal violet staining of the cells. On the 24th and on the 48th hour of treatment, the cells were washed with PBS and fixed with 4% formaldehyde for 20 min. Plates were washed with distilled water and 1% crystal violet solution was added to every well for 20 min at room temperature. All pictures were taken by a digital camera (DV-130, China).

Crystal violet assay was used to track also the cytotoxicity. After photo documentation of the samples, they were washed three times and air dried. Protein-bound dye was solubilized with 10 % acetic acid. Optical density, corresponding to the cell count in every well was measured by Epoch Microplate Spectrophotometer with the Gen5 Data Analysis software at wavelength of 570 nm. As a control were used cells grown at the same conditions but without the extracts in the culture medium and one empty well was left for calibration of the spectrophotometer. The values were calculated as percent from the control (untreated cells) and after graphical representation of data Microsoft Excel was used to determinate the IC_{50}.

RESULTS AND DISCUSSION

Cytotoxicity of the studied plant extracts was evaluated in concentration range 10-100 μg/ml for sesquiterpene lactones and 10-60 μg/ml for polyphenols and for 24 – 48 h of treatment. Cancer A549 cells and non-cancer MDCK II cells demonstrated different sensitivity toward extract A (enriched in sesquiterpene lactones) in contrast to their susceptibility toward extract B (enriched in phenolic acids and flavonoids).

Extract A showed strong effects on both cell lines in concentrations greater than 50 μg/ml. It was observed a reduction of cell viability after 24h of treatment and this effect was much stronger for cancer cells (A549). The amount of the survived normal MDCKII cells was triple than A549 cells (fig 1A) at the highest used concentration. Similar tendency was found after 48h of treatment (fig. 1B). Interestingly, more prominent differences in the sensitivity of the two model cell types used (e.g. at 20 μg/ml extract concentration quantity of cancer cells was 55% of the control, while those for normal MDCKII cells was 90%) were detected at concentrations up to 60 μg/ml.

With increasing of the extract concentration differences subsided.

Our results are in agreement with those obtained from other authors (Moghadam et al., 2013; G.-W. Wang et al. 2014). It is known that lactones produced and synthesized from different sources have anti-tumor effects. For example, N-(3-oxododecanoyl)-homoserine lactone (C12) produced from *Pseudomonas aeruginosa*, has shown additional function to inhibit tumor growth independent of both pro- and anti-apoptotic Bcl-2 proteins (Zhao et al. 2016). It has been shown that sesquiterpene lactones obtained from *Inula sp.* possess strong cytotoxic effect against several cancerous cell lines (G.-W. Wang et al. 2014).

Extract B (enriched in phenolic acids and flavonoids) demonstrated milder cytotoxic effect, compared to extract A. Significant differences in the survival of both cell types after 24 or 48 hours of treatment were not detected. More than 50 % of cells survived short-term treatment without significant morphological changes even at highest used concentration of 60 μg/ml (Fig. 1C). In comparison with extract A, the cell survival for the cancerous A549 cells was similar to non-cancer MDCKII cells in this range of concentrations (see Fig. 1 A and C). Similar effect was observed on the 48th hour of treatment with the extract B at concentration up to 60 μg/ml. The cytotoxic effect was stronger as compared to 24 h, but the sensitivity of cancer and non-cancer cells was analogous (Fig.1C and D).

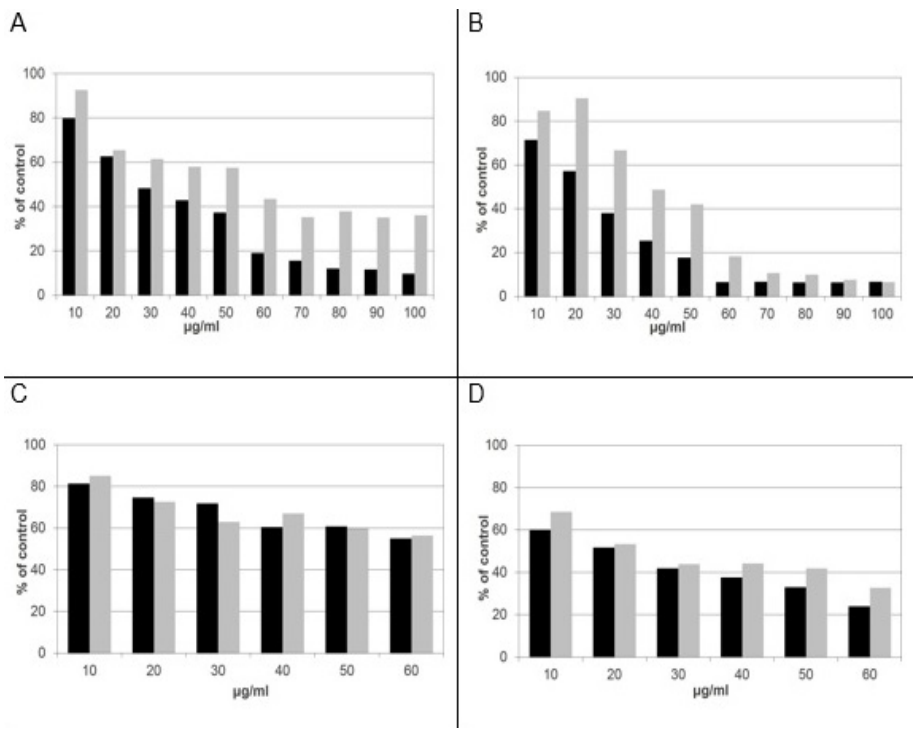

Fig. 1 Cytotoxic effects of extracts on cancer and non-cancer cells after 24 hours (1A and 1C) and 48 hours (1B and 1D) of treatment. Effects of lactones are represented on 1A and 1B, those of phenolic acids and flavonoids – on 1C and1D. *Results for A549 cells are depicted with black bars, for MDCKII cells – with gray.*

It was previously established that polyphenolic acids have oncoprotective properties. They are a heterogeneous class of substances, antioxidants and they are proven to possess anti-proliferative action (Kampa et al. 2003). Both used cell lines are permanent, with high rate of proliferation and this could explain the absence of clear time-dependent effect during 48 hours of treatment with extract B and similar sensitivity of both cell types.

The obtained results demonstrated that extract A (enriched in sesquiterpene lactones) has significantly stronger effect on both cell lines compared to the extract B (containing phenolic acids and flavonoids). Etract A in concentration range 10-60 µg/ml had more specific cytotoxic effect on the cancerous cell line rather than the non-cancerous. These results support many other studies on lactones from different sources showing anti-cancer effects (Martins, et al. 2015).

The IC_{50} for both extracts for 24h and 48h of treatment (See Table 1) were also determined based on the results in Fig. 1.

It was observed that the extract A had equal IC50 for A549 cells independently of the treatment time. For MDCKII, IC_{50} on 24th hour was considerably higher compared to 48th hour of treatment, suggesting sensibilisation of these cells with time.

Extract B (enriched in phenolic acids and flavonoids), on the other hand, did not demonstrate such strong dose-dependent activity and did not reach IC_{50} during the first 24 hours of treatment for both cell lines. On the 48th hour the cell viability was lower and IC_{50} of both cell lines were almost the same (Table 1).

Table 1. IC_{50} of both extracts for cell lines A549 and MDCKII.

IC_{50}	Sesquiterpene lactones		Flavonoids and phenolic acids	
	24h	48h	24h	48h
A549	25µg/ml	25µg/ml	-	22µg/ml
MDCK II	57µg/ml	43µg/ml	-	23µg/ml

The changes in the cell morphology could be indicative for the cell stress levels or injuries occurred. This was the reason to evaluate the morphological changes at each of the concentrations used for both extracts. The cells used in the current research demonstrated typical epithelial-like morphology, when they were grown at normal conditions. A549 cells were with cobblestone morphology, while MDCK II cells form dense monolayer with many adherent and tight junctions between cells (Fig. 2 Controls).

The observed reduction in cell number after application of extract A was confirmed by direct observation of cell monolayers. A549 cells demonstrate heavily altered morphology – they are shrunken with pale cytoplasm and strongly dyed nuclei, also a lot of debris are visible (Fig. 2). On the other hand,

the monolayer of MDCKII cell line is slightly compromised, cell contacts are preserved but the nuclei seem to be normal.

The morphological changes in A549 on the 48th hour of treatment are similar to those, observed on 24th hour. MDCKII also exhibit alterations caused by the extract A. Some vacuoles around the nuclei and near the cell periphery are detected as well as changes in the cell shape, indicating amendment in cytoskeleton. Further investigations could reveal the molecular mechanisms of these alterations and whether non-cancer MDCKII would survive the treatment with the sesquiterpene lactone enriched extracts.

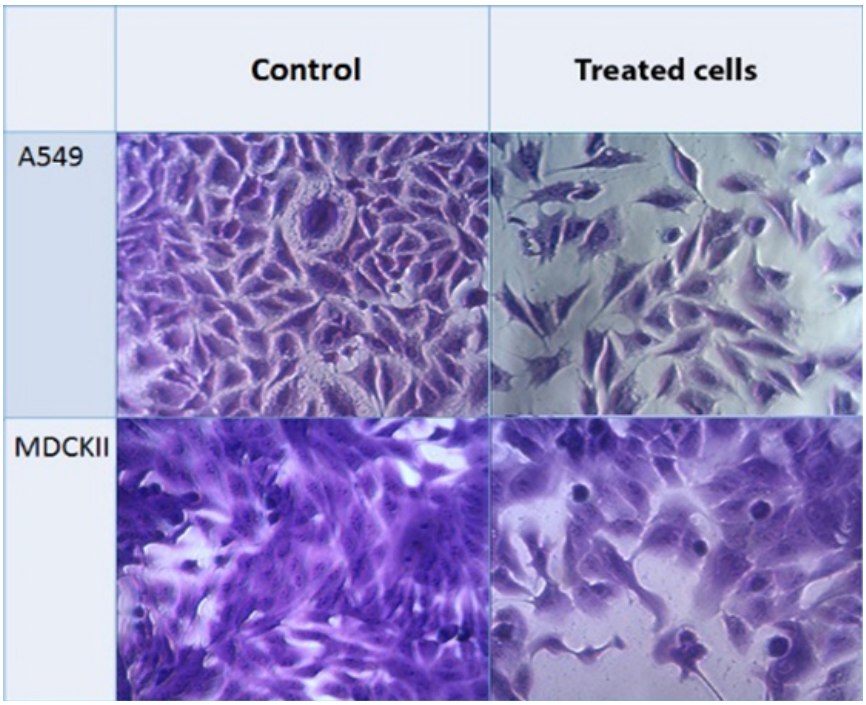

Fig. 2 Typical morphological changes of cancer (A549) and non-cancer cells (MDCK II) after treatment with extract A (enriched in sesquiterpene lactones)

The lower cytotoxicity of the extract B was confirmed by analysis of the morphology after treatment. The non-cancer MDCKII cells did not show significant morphological changes after 24h application of the whole range of concentrations of extract B (enriched in phenolic acids and flavonoids) (Fig. 3). The cancerous A549 cells also did not show significant changes in the morphology of the survived cells, although reduced cell number was clearly observed.

On the 48th hour of treatment with sub-toxic concentration of 10µg/ml of extract B, A549 cells completely recover their morphology and even some mitotic figures can be seen demonstrating that this concentration is not harmful

to the cells and they continue undergoing cell division. On the other hand, the concentrations over IC_{50} led to visible changes in cell shape, and density of the cytoplasmic and nuclear staining was different (not shown). Many cells were shrunk and cell debris were observed between them, suggesting an intensive cell death. The MDCKII cells sustained their morphological alterations after 48 hours treatment within the whole concentration range of extract B. After the higher concentration applied, the cells started to express some more changes: the cytoplasm was pale, the nuclei were pyknotic and some vacuoles into the cytoplasm were visible. These alterations are typical for cellular stress response and might be reversible if stress factors are eliminated.

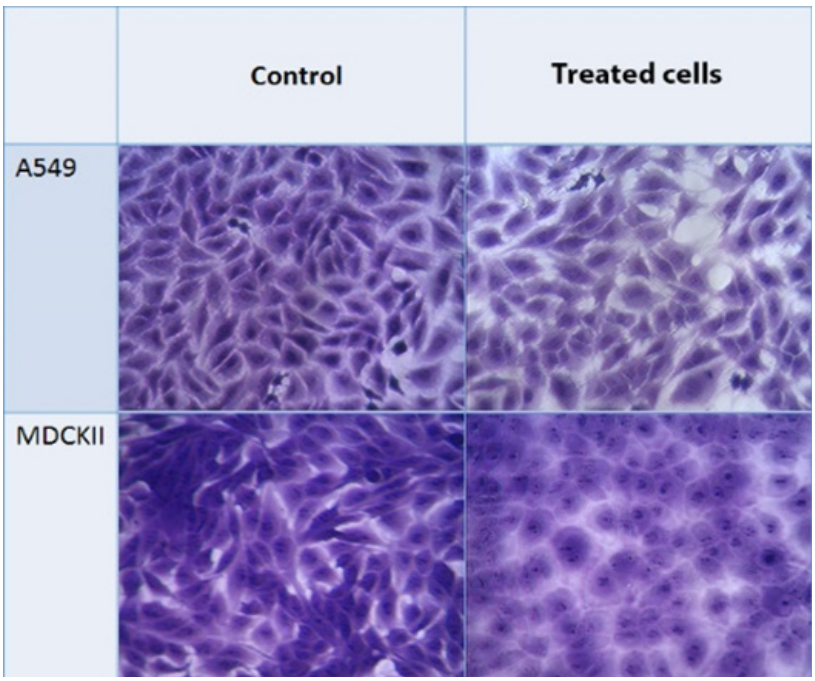

Fig. 3 Typical morphological changes of cancer (A549) and non-cancer MDCK II cells after treatment with extract B (enriched in phenolic acids and flavonoids)

CONCLUSIONS

Our studies supported the findings that sesquiterpene lactones possess anti-cancer activity. The extract of *Inula oculus-christi* enriched in lactones was cytotoxic to the cancerous A549 cell line. The extract, enriched in phenolic acids and flavonoids was cytotoxic only at higher concentrations and longer treatment without significant differences between cancerous and non-cancerous cells. The selection of a particular sesquiterpene lactone or a combination of lactone and polyphenol could be a promising basis for future investigations of the mechanisms of the anti-tumour action of plant extracts.

Acknowledgments: This work was supported by Sofia University Science fund by grant N80-10-2/12.04.2017 and National Science fund, grant N DN09/11/16.12.2016.

REFERENCES

1. Ali-Shtayeh M. S, Abu Ghdeib S. I. 1999. Antifungal activity of plant extracts against dermatophytes. *Mycoses,* 42 (11-12): 665–672.
2. Ali-Shtayeh M.S., Yaghmour R.M., Faidi Y.R., Salem K., Al-Nuri M.A. 1998. Antimicrobial activity of 20 plants used in folkloric medicine in the Palestinian area. *Journal of Ethnopharmacology*, 60(3):265-271.
3. Di Carlo G., Mascolo N., Izzo A.A., Capasso F. 1999. Flavonoids: old and new aspects of a class of natural therapeutic drugs. *Life Sci,* 65: 337–353.
4. Dulger, B., Gonuz, A. 2004. Antimicrobial activity of certain plants used in Turkish traditional medicine. *Asian Journal of Plant Sciences,* 3 (1):104-107.
5. Eloy Rodriguez G.H.N., Towers J.C. Mitchell. 1976. Biological activities of sesquiterpene lactones. *Phytochemistry*, 15 (11):1573-1580.
6. Kampa M., Alexaki V.I., Notas G., Nifli A.P., Nistikaki A., Hatzoglou A., Bakogeorgou E., Kouimtzoglou E., Blekas G., Boskou D., Gravanis A., Castanas E. 2004. Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells: potential mechanisms of action. *Breast Cancer Research,* 6(2):R63-74.
7. Leporatti M.L., Ivancheva S. 2003. Preliminary comparative analysis of medicinal plants used in the traditional medicine of Bulgaria and Italy. *Journal of Ethnopharmacology,* 87(2–3):123-142.
8. Lokhande P.D., Gawai K.R., Kodam K.M., Kuchekar B.S., Chabukswar A.R., Jagdale S.C. 2007. Antibacterial activity of isolated constituents and extract of roots of Inula racemose. *Research Journal of Medicinal Plant,* 1 (1): 7-12.
9. Máñez S., Recio M.C., Gil I., Gómez C., Giner R.M., Waterman P.G., Ríos JL. 1999. A Glycosyl Analogue of Diacylglycerol and Other Antiinflammatory Constituents from Inula viscosa. *J. Nat. Prod.,* 62 (4):601–604.
10. Martins G.G., Lívero F.A., Stolf A.M., Kopruszinski C.M., Cardoso C.C., Beltrame O.C., Queiroz-Telles J.E., Strapasson R.L., Stefanello M.É., Oude-Elferink R., Acco A. 2015. Sesquiterpene lactones of Moquiniastrum polymorphum subsp. floccosum have antineoplastic effects in Walker-256 tumor-bearing rats. *Chemico-Biological Interactions,* 228:46-56.
11. Moghadam M.H., Naghibi F., Atoofi A., Rezaie M.A., Irani M., Mosaddegh M. 2013. Cytotoxic Activity and Apoptosis Induction by Gaillardin. Z. *Naturforsch.,* 68c:108–112
12. Schinella G.R., Tournier H.A., Prieto J.M., Mordujovich de Buschiazzo P., Ríos J.L. 2001. Antioxidant activity of anti-inflammatory plant extracts. *Life Sciences,* 70: 1023–1033.
13. Talib W.H., Mahasneh A.M. 2010. Antiproliferative activity of plant extracts used against cancer in traditional medicine. *Sci. Pharm*, 78(1): 33-45.

14. Veleva R., Moskova-Doumanova V., Todorova M., Trendafilova A., Doumanov J., Topouzova-Hristova T. 2016. Cytotoxicity of flavonoid glycosides, flavonoid aglycones and phenolic acids from Inula oculus-christi L. on mammalian cell lines. *J. BioSci. Biotechnol.,* 5(3): 219-224.
15. Wang G.-W., Qin J-J., Cheng X.-R., Shen Y.-H., Shan L., Jin H.-Z., Zhang W.-D. 2014. Inula sesquiterpenoids: structural diversity, cytotoxicity and anti-tumor activity. *Expert Opin. Investig. Drugs* 23(3):317-345
16. Zhao G., Neely A.M., Schwarzer C., Lu H., Whitt A.G., Stivers N.S., Burlison J.A., White C., Machen T.E., Li C. 2016. N-(3-oxo-acyl) homoserine lactone inhibits tumor growth independent of Bcl-2 proteins. *Oncotarget*, 7(5): 5924–5942.