The Usage of Albedo Orange Methanol and Ethanol Extraction as Anticancer Agent

Yara Moenes, Ahmed Nada\(^1\) and Aly Fahmy Mohamed\(^2\)
\(^1\)MSA University-Biotechnology department \(^1\)Faculty of Biotechnology- MSA University and Head of R&D sector VACSERA- Egypt\(^2\)

Abstract: The present study aimed to evaluate the anticancer activity of methanol/ ethanol Orange Albedo extracts where test extracts showed anti-proliferative activity. Also, the test extract viability % were concentration, IC\(_{50}\) value and cell type dependent. Also, there was a significant up / down regulation of pro/ anti apoptotic genes. Also the cell cycle profile indicated cell arrest at the G2 / M phase compared with the control accompanied with apoptotic profile significantly elevated than non treated cell control (P<0.05).

Keywords: Cancer, Albedo orange, Lung, Larynx, Pro-apoptotic, Anti-apoptotic

Introduction

1.1. Cancer is among the leading causes of death in both developed and developing countries. yet the burden of this lethal disease is more well-found in developing countries, where more than 80 percent of the world population live and the growing trend is more striking (Roshandel et al., 2014) . Cancer cells are cells that have the ability to grow uncontrollably due to some internal and external signals. Cancer results from a series of molecular events that fundamentally alter the normal properties of cells. In cancer cells the normal control systems that prevent cell overgrowth and the invasion of other tissues are disabled. These altered cells divide and grow in the presence of signals that normally inhibit cell growth; therefore, they no longer require special signals to induce cell growth and division and production of new enzymes. The irregularity in cancer cells usually result from mutations in protein-encoding genes that legalize cell division. This is often because the genes that construct the proteins that normally restore DNA damage are themselves not operation normally, because they are also altered, cancer is not a transmittable disease but hereditary. Many other causes of cancer additional than inherited alteration, which are the environmental aspect and diet like tobacco, asbestos, chemicals and others. Cancer is categorized by the type of cell that the tumor cells derived from. These types include Carcinoma , Sarcoma, Germ cell tumor, Blastoma, Lymphoma and leukemia. The indicators of cancer differ depending on the type and the site of cancer. The human body requirements intake of antioxidants, to increase the immunity, which acts as anticancer agent. Some of the fruits are considered as the major anticancer foods especially citrus fruits, because of their rich antioxidants such as phenols, vitamin C, vitamin E, beta-carotene and lipotene. These compound also known as nutraceuticals supplies health benefits due to a risk drop of chronic illness such as cancer (Zamantha Escobedo-Avellaneda., et al 2009). Citrus fruits like orange, lime fruits are composed by an outside layer (peel) formed by flavedo and albedo and an interior substance called endocarp that include vesicles with juice. Flavedo is the external colored layer of citrus fruit. Albedo is the soft, spongy white layer in any citrus fruit (Turhan et al., 2006). The traditional chemotherapy treatment induce reverse events, these side effects can vary greatly, depending on the properties of the drug. The most common side effects are nausea and vomiting, alopecia, chronic fatigue, skin and subcutaneous tissue damage, while less frequent are hepatotoxicity, neurotoxicity, cardio toxicity, nephrotoxicity and reproductive toxicity(Fernandez et al., 2012). So, the aim of the present work is to evaluate the Orange Albedo bio-derivatives extracted using different solvents as anticancer against on Laryngeal cancer (HEP-2) and lung cancer cell line (A549). And related genetic and cell cycle profile under the effect of IC50 concentration.

1.2. Methodology:

Albedo extraction

Preparation of Albedo extracts

100 gm of Orange Albedo were washed with DDW to remove unwanted materials. Orange Albedo was dried in hot oven at 40 °C for 48 hrs till dryness, followed by grinding. Powder was divided as two separate sets for differential extraction using both ethanol and methanol according to (Nair et al., 2002). The prepared powders were soaked in both methanol and ethanol for 7 days, extracts were cold centrifuged (Jouan-France) at 9000 RPM / h and...
solvents were evaporated using rotary evaporator 
(Kalantari et al., 2007 and Hasson et al., 2011). 
Extracts were sterile filtrated using 0.22 µm 
Millipore disposable sterile cup filters.

Cell culture
Larynx cancer (HEP-2) and lung cancer cell lines 
(A549) were obtained from R&D Sector, VACSERA- 
Egypt. The cell lines were cultured in growth medium; 
DMEM supplemented with 10% FBS and 100 
µg/mi Streptomycin and 100 IU / ml penicillin) 
and maintained according to the producer 
instructions. Grown cells were re-cultured in tissue 
culture plates (TPP-Swiss) and TC flasks according 
to the applications.

Cell viability assay
Cell viability was performed using MTT assay. As 
HEP-2 and A549 cells were pre-cultured in 96 wells 
plate as 5x10² cells/ml. The medium was changed 
frequently and the OAlb-E/M extracts were 
dispensed to the pre-cultured cells; 2 fold serially 
diluted. Plates were incubated for 24 hours (Jouan- 
France). Plates were examined under the inverted 
microscope (Hund-Germany). Dead cells were 
were washed out using Phosphate buffer saline (PBS) 
twice. Remaining viable cells were stained using 
MTT stain used as 0.5 mg/ml for 4 hrs at 37°C. 
Developed Purple colored MTT –Formazan crystals 
were dissolved in 50 µl of DMSO (ICI-UK) for 30 
minutes. Optical density represent the viability % 
were plotted against the extract concentration. IC₅₀ 
value was calculated using Master Plex - 2010 
software.

Cell viability (%) = OD of treated wells x 100 
OD of control wells

Cell Cycle Analysis
HEP-2 and A549 cells were cells pre-cultured in 25 
cm²surface area cell culture flasks and were treated 
with the IC₅₀ value of test extracts dissolved in D- 
MEM medium, for 24h. For cell cycle analyses, the 
cells were harvested and fixed gently with 70% 
ethanol in PBS, preserved at a temperature of 4°C 
overnight and then re-suspended in PBS containing 
40 µg/ml PI and 0.1 mg/ml RNase and 0.1% Triton 
X- 100 in a dark room. The cells were analyzed after 
30 min at 37°C, using a flow-cytometer (Becton- 
Dickinson, San Jose, CA, USA) set with an argon ion 
laser at a wavelength of 488 nm.

Expression of Apoptosis using Real Time PCR
Total RNA was extracted from IC₅₀ treated and 
untreated HEP-2 and A549 post 24 hrs treatment via 
RNeasy mini Kit (Qiagen - USA) according to 
manufacturer’s directions. Concentration of extracted 
RNA was estimated by a Beckman dual 
spectrophotometer (USA). The expression level of 
apoptosis associated genes; p53 (F: 5’-TCA GAT 
CCT AGC GTC GAG CCC-3’ and R: 5’-GGG TGT 
GGA ATC AAC CCA CAG-3’), Bax (F: 5’-ATG 
GAC GGG TCC GGG GAG CA-3’ and R: 5’-CCC 
AGT TGA AGT TGC CGT CA-3’) and Bcl2 (F: 
5’-GTG AAC TGG GGG AGG ATT GT-3’ and R: 
5’- 
GGA GAA ATC AAA CAG AGG CC-3’) was determined by real-time PCR. Ten ng of 
the extracted total RNA from each sample were used for 
cDNA synthesis using high capacity cDNA Reverse 
Transcriptase kit (Applied Biosystems-USA). 
The obtained cDNA was then amplified by Syber Green I 
PCR Master Kit (Fermentas- Lithuania) by Step One 
instrument (Applied Biosystems-USA), as follows;10 
min at 95°C for enzyme activation proceeded by 40 
cycles of 15 seconds at a temperature of 95°C, 20 
seconds at 55°C and 30 seconds at 72°C for the 
amplication step. Modifying in the expression of 
every target gene were stabilize relative to the mean 
critical threshold (CT) values of β-actin as 
housekeeping gene by the ΔCt technique.

Statistical Analysis
All experiments were passed out three independent 
results. Were statistically examined by one 
way investigation of variance (ANOVA) and were 
presented as mean ± SD. The variation as considered 
statistically significant at p<0.05.

RESULTS
The IC₅₀ of test extracts was calculated and it was 
clear that it was cell type dependent as HEP-2 cells 
were significantly affected than A549 (P<0.05) 
and there was a no significant difference regarding the 
solvents (P>0.05), [Fig. 1]. Viability % of treated 
A549 and HEP-2 cells monitored using MTT assay. 
Data recorded revealed that viability was 
conversation dependent but not extract type 
dependent (P>0.05), [Fig. 2]. Regarding the 
anticancer potentials of test extracts, data recorded 
revealed that the effect was cell type and extract 
dependent as pro-apoptotic gene; Bax was 
significantly elevated (P<0.05) post treatment of A549 
cell line with OAlb-E than OAlb-M while the HEP2 
cells were affected almost equally as gene fold 
increase was insignificantly changed in OAlb-E than 
OAlb-M extract HEP2 cell line treatment (P>0.05). 
In the same time apoptosis inducing gene was 
significantly affect post treatment of A549 cell 
in the treatment with OAlb-M than OAlb-E (P<0.05) 
and not insignificantly affected (P>0.05) in case of HEP- 
2 cell line [Fig. 3]. Also, Anti-apoptotic gene BCI-2 
was significantly down regulated compared with the 
cell control. Also, the related cell cycle arrest profile, 
data recorded revealed that the cell cycle profile was
cell type and extract type dependent as Alb-M was significantly effective on the cell arrest G2-M phase than OAlb-E in A549 cell line treated also a apoptotic cells were detected in a significant way compared with the cell control and the OAlb-M induced a significantly apoptotic pattern than OAlb-E extracts in case of A549 cell line. The same pattern was noticed in case of HEP-2 cell line. Also, apoptotic cell % in HEP 2 cell line was significantly elevated than in A549 (P<0.05) [Fig.4]

Discussion
The present work aimed to investigate the anticancer potentials of orange Albedo extract both methanolic and ethanolic extract aiming to manage fruit waste control and make pharmaceutical application of fruit wastes, there is a very little data about in vivo anticancer activity of plant origin extracts, while in vitro was recorded. So, it was important in the present work to refer to the in vitro anticancer potentials where the cytotoxic effect of test extracted materials was monitored to determine the IC50 against lung (A549) and larynx (HEP-2) cancer cell lines. Citrus fruits are the main source of important phytochemical nutrients and for long have been valued for their wholesome nutritious and antioxidant properties. It is scientifically proven that oranges being rich in vitamins and minerals polyphenols and flavonoids those have many health benefits. Moreover, it is now appreciated that other biologically active, non-nutrient compounds found in citrus fruits such as phytochemical antioxidants, soluble and insoluble dietary fibers are known to be helpful in reducing the risk for cancers, many chronic diseases like arthritis, obesity and coronary heart diseases (Crowell, 1999). The report of Crowell is agree with our reported data that Albedo extract showed a clear anticancer potentials as there was a significant up regulation and down regulation of both pro-apoptotic and anti-apoptotic genes namely P53, Bax and BCL-2 respectively in both cancer cell lines used. Also, the anticancer potentials may be attributed contents of Albedo extract as it contains vitamins, especially vitamin C, phytochemical compounds like liminoids, synephrine, hesperidin flavonoid, polyphenols, pectin etc. It is clear that antioxidant content of which the flavonoids those are excellent radical-scavengers of the hydroxyl radical as reported by Cillard and Cillard (1986); Da Silva et al. (1991); Macheix et al., (1990); Bombardelli and Morazzoni (1993); Di Majo et al.(2005), and Tripoli et al. (2007). Also, the anti-Cancer potentials of Citrus flavonoids can prevent cancer through selective cytotoxicity, antiproliferative actions and apoptosis (Elangovan et al., 1994 and Hirano et al., 1994). Flavonoids are antimutagenic, thus protects the DNA from damage by their ability to absorb ultraviolet light (Stapleton and Walbot, 1994). They neutralize free radicals that promote mutations when they are generated near DNA. This has been shown in mice body irradiated with X-ray (Shimoi et al., 1994). Flavonoids can also protect the DNA by interacting directly with the tumoral agents, as in the induced chromosomal aberrations by bleomycin (Heo et al., 1994). The inhibitory effect of citrus flavonoids on tumoral development and cell proliferation by rat malignant cells, in cardiac and hepatic tissue of syngenetic rats has been reported (Bracke et al., 1989). The ability to function as such by citrus flavonoids are based on cell mobility inhibition (Bracke et al., 1989 and 1991). Oranges are also rich in iron, chlorine, manganese, zinc, sodium, phosphorous, iodine, calcium, folic acid, potassium, pectin, beta-carotene and amino acids and fiber. Flavonoids are another phytochemicals found in citrus fruits.

The flavonoids have strong inherent ability to modify the body’s reaction to allergens, viruses and carcinogens. They show anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activity. Quercetin, myricitin, rutin, tangeritin, naringin and hesperidin are found amongst the common flavonoids in citrus fruits. Quercetin is a flavonoid and more specifically a flavonol that constitutes the aglycone of the glycoside rutin. Quercetin is found to be the most active due of the flavonoids and many medicinal plants owe much of their activity due to their high Quercetin content. It has been suggested that flavonoids-rich diet has an adverse effect on the proliferation of cancer cells. Various mechanisms have been proposed to explain the anti-proliferative activity of flavonoids, for example, it has been proposed that the anti-proliferative effects of flavonoids are mainly mediated by the inhibition on several kinases and kinase inhibitors involved in cell-cycle arrest and apoptosis.

Cyclin-dependent kinases (CDKs) have been recognized as key regulators of cell cycle progression. Alteration and deregulation of CDK activity are pathogenic hallmarks of neoplasia [86]. Besides, the dysregulation of the checkpoints at both G1/S and G2/M of the cell cycle also play an important role in the development of malignant neoplasm. Citrus flavonoids including apigenin, diosmin, rutin, tangeretin have been demonstrated to inhibit proliferation of different kinds of cultured human cancer cell lines including human squamous cell, carcinoma cell line (HTB-43), MDA-MB-435 ER- human breast cancer cells, MCF-7 ER+ human breast cancer cells, DU-145 androgen receptor-negative human prostate cancer cells, HT-29 human colon cancer cells, DMS-114 human lung cancer cells, and SK-MEL5 human melanoma cells. In the study of Kawaii et al, twenty-seven Citrus flavonoids were examined for their anti-proliferative activities.
against tumor including lung carcinoma A549 and gastric TGBC11TKB cancer cells and normal human cell lines. The result showed that luteolin, natsudaidain, quercetin, tangeretin, eriodictyol, nobletin, and 3, 3', 4', 5, 6, 7, 8-heptamethoxyflavone were suggested to be potential anti-cancer agents.

In addition, Angst et al. reported that quercetin caused significant apoptosis and reduced tumor cell proliferation in a nude mouse model. It was reported that the antiproliferative characteristics of hesperedin was inducing the expression and transcriptional activity of PPARγ and promoted p53 accumulation and down regulated constitutive NF-κB activity in a PPARγ-dependent and PPARγ-independent manner in NALM-6 cells (Aranganathan et al.).

Also reported the antiproliferative effects of hesperetin. They found that supplementation with hesperetin (20mg/kg body weight) lowered the proliferating cell nuclear antigen (PCNA) labeling index and suppressed the formation of aberrant crypt foci (ACF) in the rats with colon cancer. Nobletin, tangeretin and sinensetin are the main PMFs found in Citrus. Lee et al. revealed that PMF-mediated induction of GADD45α partially underlies the anti-proliferative effect of PMF on colorectal cancer cells. Nobletin was found to act as an anti-carcinogenic compound through anti-proliferative activity, induction of apoptosis and cell cycle deregulation. It was reported that tangeretin suppressed breast cancer proliferation by up-regulating p53/p21 proteins and inducing G1/S phase cell cycle arrest. Glycogen synthase kinase-3β (GSK-3β) is phosphorylated by Akt, and GSK-3 itself is involved in the regulation of cell proliferation, anti-apoptotic pathways, and cell cycle progression. Nuclear factor kappa B (NF-κB) transcription factors regulate several important physiologic processes of cell, e.g., cell growth, and apoptosis.

Thus, inhibition of NF-κB activation offers a potential strategy for treatment of different malignancies. Apigenin was found to induce pancreatic cell death through inhibition of GSK-3β/ NF-κB signaling pathway. COX, an inflammatory enzyme induced by cytokines, catalyzes the conversion of AAD to PGEs. COX-2 expression is associated with tumor growth. Murakami et al. reported that nobletin showed the greatest anti-proliferative activity by suppressing the expression of COX-2 in vitro, and inhibiting dimethylbenz anthracene (0.19 mmol)/TPA (1.6 mmol)-induced skin tumor formation. Ras, a small G-protein, physiologically directs cell proliferation and cell cycle via regulation of mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling cascade. Dysregulation of Ras/MEK/ERK signaling has been reported to cause tumorigenesis and gliomas. Aoki et al. found that nobletin suppresses the cell proliferation by inhibiting Ras activity and MEK/ERK signaling cascade probably via a Ca2+-sensitive PKC-dependent mechanism. Hsiao et al. also reported that nobletin suppressed cell proliferation in HL-60 AML cells via inducing cell-cycle arrest at the G0/G1 phase by suppressing extracellular signal-regulated kinase (ERK) activity. In another study, the nobletin was shown to inhibit the proliferation of human pancreatic cancer cells (PANC-1) by inducing apoptosis via up-regulation of the pro-apoptotic protein bax and down-regulation of the anti-apoptotic proteins bcl-2 and p53. 17β-estradiol (E2) is involved in the pathogenesis of several types of cancers. Bulzomi et al. reported that naringenin can bind to ERα (estrogen receptor α) as an antagonist, thereby limiting the effect of E2 in promoting cellular proliferation. Regarding the effect of citrus extract suppression on the tumor metastasis Citrus flavonoids that have been reported to have anti-metastasis effects on tumors mainly through MMPs include the total flavonoids, naringin, hesperidin, kaempferol, tangeretin, naringin, naringenin, and nobletin. Iishiwa et al. reported that the flavonoids isolated from Citrus depressa Hayata including tangeretin, 6-demethoxytangeretin, nobletin, 5-demethylnobletin, 6-demethoxynobletin, and sinensetin suppressed the interleukin 1 (IL-1) induced production of pro MMP-9/progelatinase B in rabbit synovial cells in a dose dependent manner, among these flavonoids, nobletin is most effectively in suppressing proMMP-9 production along with the decrease in its mRNA. Arivazhagan L et al. reported that tangeretin treatment significantly suppressed matrix metalloproteinase (MMP)-2, MMP-9 in DMBA-induced animal models. Lee et al. f. Naringenin as flavonoids was evaluated for its anti-metastasis effects by Lentini et al. They found that oral administration of naringenin to C57BL6/N mice inoculated with B16-F10 cells reduces the number of lung metastases. Park et al. reported that Citrus flavonoids isolated from Korean Citrus aurantium L have anti-invasive effect through the inhibition of MMP-2 expression in A549 cells in a dose-dependent manner. Chen et al investigated the anti-metastatic activity of kaempferol and its molecular mechanism of action in human U-2 osteosarcoma (U-2 OS) cells. They found that kaempferol influenced the expression and enzymatic activities of MMP-2, MMP-9 and urokinase plasminogen activator (uPA) through attenuating the MAPK signaling pathways including ERK, JNK and p38 by decreasing DNA binding ability of AP-1. Among the PMFs, tangeretin has been reported as a potential anti-metastatic agent. In addition, Lai et al. that oral administration of Gold Lotion (GL); an extract of multiple varieties of Citrus peels containing abundant flavonoids, including a large percentage of PMFs, effectively suppressed the prostate cancer of human by mechanistic down-regulation of the
protein levels of MMP-2 and MMP-9. AKT, a serine/threonine kinase protein, is a downstream target of PI3K, and it plays a pivotal role in cell migration, growth, and anti-apoptotic events in various types of cells [113]. It was reported that the FAK/PI3K/Akt is involved in the regulation of MMP-2 and MMP-9 activities on different cell types. In addition, NF-xB has been known to translocation to the nucleus and regulates the expressions of multiple genes involved in MMP-2/MMP-9 secretions. In AGS cells, nobiletin showed the inhibitory effect on the invasion and migration. The FAK/Ras/PI3K/AKT signaling pathway is a possible mechanism of the inhibitory effects of nobiletin on AGS cells, including the increased protein level of cytoplasmic IkB which exerts inhibitory effects on the transcription factor NF-xB, subsequently decreasing MMP-2 and MMP-9 activities. Hepatocyte growth factor (HGF), and its receptor, c-Met activation has recently been shown to play important roles in cancer invasion and metastasis in a wide variety of tumor cells. Besides, the activation of mitogen-activated protein/extracellular signal-regulated kinase (MEK/ERK) is well known to be associated with tumor invasion and metastasis. Shi et al. reported that nobiletin attenuates HGF-induced HepG2 cells metastasis involving both ERK and PI3K/Akt pathways and are potentially useful as anti-metastatic agents for the treatment of hepatoma. In HT-1080 cells, nobiletin was also evidenced directly inhibited MEK activity and decreases the sequential phosphorylation of ERK, exhibiting the antitumor metastatic activity by suppressing MMP expression. In respect to bone cancers, Tan et al. reported naringin inhibits migration and invasion of human chondrosarcoma via down-regulation of vascular cell adhesion molecule-1 (VCAM-1) by increasing miR-126. Finally, it can be concluded that Citrus extracts could be used as a source of pharmaceutical importance and can be used as anticancer derivative its mode of action based on the suppression of cell proliferation and cell arrest at different growth phases under the up regulation and down regulation of pro and anti-apoptotic genes. Also, it is recommended to arrange for a wide range of evaluation of anticancer potentials either In vivo or In vitro, characterization of different Albedo extract containments and their mode of application against different cancer cell lines evaluation. Also, evaluation the biochemical pattern post administration of Albedo extracts or their fraction.

Acknowledgements

I would like to express my special thanks of appreciation to everyone helped me a lot through the whole journey as well as Dr. Aly Fahmy who gave me the opportunity to start my project in his lab and learned me different technologies. I am really thankful to him and his team. Also, I would like to greatly thank to prof. Dr Ahmed Nada for his support and encouragement. Finally I would also like to thank my parents and friends and appreciate their continuous support.

### 2. References


10. Arivazhagan L, Pillai SS. Tangeretin, a citrus pentamethoxyflavone, exerts cytostatic effect via p53/p21 up-regulation and suppresses metastasis in 7, 12-dimethylbenz (α).


58. *Journal of Food and Nutrition Research* 350


86. Miyake Y, Yamamoto K, Morimitsu Y, Osawa T. Isolation of C-glucosyllflavone from lemon peel and antioxidative activity of flavonoid


110. Schulte-Hermann R, Timmermann-Trosiener I, Barthel G, Bursch W. DNA synthesis, apoptosis, and phenotypic expression as determinants of growth of altered foci in rat liver during...


