PHYTOCHEMICAL AND CYTOTOXICITY INVESTIGATIONS OF SALVADORA PERSICA BARK EXTRACTS

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ABSTRACT

Background/Aim: Salvadora persica is an evergreen shrub or small tree to 6-7m. Fruits have a sweet, agreeable, aromatic, slightly pungent and peppery taste. It has many biological activities such as antipyretic, anti-inflammatory and antifungal activities. This study aims to evaluate cytotoxic effect of Salvadora persica, meswak, (Salvadoraceae) extracts and isolate main compounds from the most effective extract on different human cell lines.

Materials and Methods: Extracts from meswak sticks and bark, aqueous alchoholic (crude), petroleum ether, chloroform and ethyl acetate extracts were tested for their cytotoxic activities on human hepatocellular carcinoma, breast, lung and colon carcinoma cell lines. Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan. In addition, two triterpenes were isolated from the petroleum ether extract, most active extract, partitioned from aqueous alchoholic crude extract using thin layer and column chromatographic technique. Elucidation of the chemical structure of two triterpenes was established based on their spectroscopic data (MS, 1HNMR, 13CNMR& IR).

Results: The petroleum ether extract is the most potent evaluated extract. It presented IC 50=43.6 µg/ml against human hepatocellular carcinoma cell line-HepG2, IC50= 44.3 µg/ml against human breast carcinoma cell line-MCF7, 19.87 µg/ml against lung carcinoma cell line-A549 and 10.2 µg/ml against colon carcinoma cell line-HCT116, however the other extracts showed weak activities. Ursolic was more effective than oleanolic acid against HepG2, MCF7 and HCT116 (IC 50= 26.32, 18.73 & 20.4 µg/mL, respectively) while oleanolic was potent against A549 (IC50= 19.5 µg/mL).

Conclusion: petroleum ether extract and ursolic acid showed cytotoxic activity against all tested human cell lines. Petroleum ether extract was superior against HCT116 and A549 while ursolic acid was efficient against HepG2 and MCF7.

Keywords: Salvadora, triterpenes, anti-cancer, cytotoxicity.

INTRODUCTION

It is generally accepted that a diet of large amount of vegetables, fruits, and other plant products lowers cancer incident, there is still a need to identify the most effective constituents of the diet, as well as to elucidate their mechanisms of action (1). Approximately one-third of the women with breast cancer developed metastasis and ultimately died of the disease. MCF-7 cell has become a prominent model system for the study of breast cancer as it relates to the susceptibility of the cells to apoptosis. Further, it has become increasingly important in the prevention or treatment of a number of major solid tumors, particularly metastatic and drug-resistant breast cancer (2).

Many oleanane and ursane triterpenoids are reported to have interesting biological, pharmacological, or medicinal activities similar to those of retinoids and steroids, such as anti-inflammatory activity, suppression of tumor promotion, suppression of immunoglobulin synthesis, protection of the liver against toxic injury, induction of collagen synthesis, and induction of differentiation in leukemia or teratocarcinoma cells (3, 4). Cancer is a general term applied to a series of malignant diseases which may affect many different parts of the body. If the process is not controlled, it may progress until it causes the death of the organism (5). In addition, cancer is one of the major causes of death in developed countries, together with cardiac and cerebrovascular diseases (6). Currently, much commonly used anti-cancer therapeutics represents broadly cytotoxic agents. These agents have been frequently discovered using cell-based cytotoxicity assays.

Aqueous extracts of miswak enhances the growth of fibroblasts and inhibit the growth of carcinogenic bacteria (7), use of persica mouthwash resulted in improved gingival health and lower carriage rate of cariogenic bacteria when compared with the pre-treatment values. It also has many biological activities, such as antipyretic action (8) while methanol extract exhibited antifungal activity against Candida albicans and Saccharomyces cerevisiae (9) as well as anti-ulcer activity (10, 11), in addition, root’s extract has hypocholesterolemic activity (12). Benzylamides isolated from Salvadora persica have significant inhibitory effect on human collagen-induced platelet aggregation, and a
moderate antibacterial activity against Escherichia coli (13). The present study was designed to investigate the inhibitory effect of different Salvodora extracts on different human cancer cell lines and to isolate main compounds of efficent extract.

**MATERIALS AND METHODS**

All solvents were purchased from Merck, Germany. NMR spectra were recorded on 1H-NMR (Joel ECA, 500MHz), 13C-NMR (Joel ECA, 125MHz) and mass-spectroscopy (Finningen, model 3200 at 70eV).

**Plant and extraction**

*Salvadora persica* L. (Salvadoraceae) was collected in April 2009 from Aswan botanical garden, Egypt. It was identified and authenticated by Dr. Mohamed Nabil el-Hadidi, Professor of Plant Taxonomy, Cairo University.

The fresh miswak bark and sticks were cut into small pieces and allowed to dry at room temperature for ten days. Then it was ground to powder in a ball mill. The powder (500g) was exhaustively extracted with alcohol (ethanol, 70%), and then concentrated under reduced pressure using rotary evaporator to a small volume (100ml). The crude aqueous ethanolic extract was fractionated using gradient solvents, petroleum ether, chloroform, ethyl acetate and crude alcoholic extracts.

**Cytotoxic assay**

Cell viability test was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) to purple formazan (14).

**Cell lines**

Different cancer cell lines including; HCT116 (human colon adenocarcinoma), MCF7 (human breast adenocarcinoma), HEPG2 (human hepatocellular carcinoma) and A549( lung carcinoma) were obtained from Cancer Center, Karolinska Institute, Stockholm, Sweden. VERO (green African monkey kidney) cells were obtained from Vac Sera Center, Egypt.

**MTT assay**

The following procedure was done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). The method was carried out according to Thabrew et al. (1997) (15). Plant extracts were prepared in 0.4% DMSO as a solubilizer.

Cells were batch cultured for 10 days, then seeded at concentration of 10x10^3 cells/well for all cell line and 6x10^3 for VERO in fresh complete growth medium in 96-well microtiter plastic plates at 37 °C for 24 h under 5% CO2 using a water jacketed Carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of extracts to give a final concentration of (100-50-25-12.5-6.25-3.125-0.78 and 1.56 µg/ml). Cells were suspended in RPMI 1640 medium (for HepG2) containing 1% antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000µg/ml Streptomycin Sulfate and 25µg/ml Amphotericin B) and 1% L-glutamine in 96-well flat bottom microplate at 37 °C under 5% CO2. After 48 h of incubation, medium was aspirated, 40µl MTT salt (2.5µg/ml) were added to each well and incubated for further four hours at 37°C under 5% CO2. To stop the reaction and dissolving the formed crystals, 200µL of 10% Sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37°C. A positive control which composed of 100µg/ml of Annona cherimolia extract was used as a known cytotoxic natural agent that gives 100% lethality under the same conditions.

The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595nm and a reference wave length of 620nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. DMSO is the vehicle used for dissolution of plant extracts and its final concentration on the cells was less than 0.2%. The IC50 was then defined as the concentration of the extract that produced a 50% decrease in cell viability relative to the control which was wells exposed to the solvent without any extract.

The percentage of change in viability was calculated according to the formula:
\[
\text{Percentage of Viability} = \left(\frac{\text{Reading of extract}}{\text{Reading of negative control}} - 1\right) \times 100
\]

A probit analysis was carried for IC50 determination using SPSS 11 program and the data are expressed as mean ± SEM. A probability value of P<0.05 was considered to be statistically significant.

Selectivity index(SI) of each extract with different cell lines was calculated as follow,
\[
\text{SI}= \frac{\text{IC}_{50} \text{ of VERO cell}}{\text{IC}_{50} \text{ of extract on different tested cell line}}
\]

**Isolation of triterpenes**

The potentiality of ursolic acid in therapeutics has drawn the scientific attention during last decade (16). This fact is evidenced by the exponentially increasing...
pharmacological research on the anti-inflammatory (17), anti-cancer (18), and other activities of ursolic acid (19). Ursolic acid itself is shown to have antioxidant properties (20, 21).

The aqueous alcoholic extract of S. persica was successively extracted with petroleum ether, chloroform and ethyl acetate. The petroleum ether extract was used for triterpene isolation by silica gel column chromatography. A part of petroleum ether extract (5g) was eluted by gradient petroleum ether: ether, ten fractions were collected. Fraction three was further purified by thin layer chromatographic F254 using n-hexane: ethyl acetate (5:1) as solvent system, and then tested for purity with different solvent systems, n-hexane: ethyl acetate: MeOH: water (10: 5: 2.5: 1), CHCl3: MeOH: Et2O (90: 5: 5). Rf's values were 0.4 and 0.49 for oleanolic and ursolic acids, respectively in CHCl3: MeOH: Et2O (90: 5: 5). The spots were revealed with iodine or p-anisaldehyde or vanillin in H2SO4. The isolated triterpenes were characterized by spectrometric methods; 1H-NMR, 13C-NMR and Mass spectrometry.

RESULTS

Different meswak extracts; crude extract, petroleum ether, chloroform and ethyl acetate, were screened for their cytotoxic activity against different cell lines, HePG2, MCF7, HCT116 and A549 as well as against VERO cell. The isolated main components, ursolic acid and oleanolic acid, of the most effective extract (petroleum ether) were screened with the same cell lines. Incubation of cell line with DMSO, negative control, didn't show any mortality through the inocubation period.

Data presented in Table 1 showed the weak activity of meswak crude extract against different cell lines while fractionation of this extract to three fractions according to solvent polarity showed favorable effect. The three fractions showed cytotoxic effect greater than the crude extract. Petroleum ether extract was more potent than the other two extracts against all tested cell lines. It showed potent effect in inhibiting human colon carcinoma cell lines- HCT116, IC50=10.2µg/mL, also it was similar to oleanolic acid on human lung carcinoma cell lines- A549, IC50=19.87 and 19.5µg/mL for petroleum ether and oleanolic, respectively. Petroleum ether extract is potent extract against HCT116 as compared to all tested meswak extracts and isolated compound.

Ursolic acid was the best inhibitor for HePG2 and MCF7 cell lines comparable to crude extract, fractions and oleanolic acid (IC50= 26.32 and 18.73 µg/mL for HePG2 and MCF7, respectively). The weakest tested one against all tested human cellular carcinoma cell lines was crude extract then chloroform extract followed by ethyl acetate extract. Chloroform extract and ethyl acetate extract were alike in their effect against MCF7 (49.3 and 48.53 µg/mL, respectively). Data presented in Table 1 showed the effect of different tested extracts on VERO cell.

Interpretation of triterpenes

The isolated triterpenes were well characterized by spectrometric analyses. Spectral analyses of the two isolated triterpenes showed that the two isolated compounds have nearly the same signals in 1HNMR and IR but they are different in 13CNMR in the characteristic signal C-19 which carries the methyl group in compound 1 while the same methyl group presented in C-20 in compound 2.

1H-NMR analysis revealed that 5.3 (s,1H, H-12), 4.82 (1H, t, OH), 4.41 (t, 1H, H-3), , 3.64 (m, 2H, CH2OH), , 1.07 (s, 3H, CH3), 0.95 (s, 3H, CH3), 0.91 (d, 3H, CH3), 0.94 (s, 3H, CH3), 0.63 (s, 3H, CH3); 13C NMR (CDCl3, 125 MHz): δ C (for C-3, 12,13,18,23,24,25,26,27,28 and C-30): 89.8, 120, 141.5, 43.4, 24.8, 16.4, 15.4, 17.3, 23.9, 33.1 and 24.8. These results are coincided with those of Bhawana,2011(24) and Sun Liu et al., 2010(25).
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Table 1: Cytotoxic activity of *S. persica* extracts against different human cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Crude extract</th>
<th>Petroleum ether fraction</th>
<th>Chloroform fraction</th>
<th>Ethyl acetate fraction</th>
<th>Ursolic acid</th>
<th>Oleanolic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µg/mL)</td>
<td>MCF7</td>
<td>HCT116</td>
<td>A549</td>
<td>VERO</td>
<td>MCF7</td>
<td>HCT116</td>
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<tr>
<td>HePG2</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
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<tr>
<td>(Selectivity index, SI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Crude extract</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Petroleum ether fraction</td>
<td>43.6±1.56</td>
<td>44.25±1.66</td>
<td>10.2±1.41</td>
<td>19.87±1.20</td>
<td>379±36.35</td>
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</tr>
<tr>
<td>(8.67)</td>
<td>(8.56)</td>
<td>(37.16)</td>
<td>(19.87)</td>
<td></td>
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<tr>
<td>Chloroform fraction</td>
<td>81±1.38</td>
<td>49.3±1.32</td>
<td>86.9±0.88</td>
<td>58.8±1.65</td>
<td>859±100.24</td>
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<tr>
<td>(10.6)</td>
<td>(17.43)</td>
<td>(9.88)</td>
<td>(14.60)</td>
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<tr>
<td>Ethyl acetate fraction</td>
<td>55.67±1.12</td>
<td>48.53±95</td>
<td>30.92±1.75</td>
<td>26.48±1.41</td>
<td>366±9.15</td>
<td></td>
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<tr>
<td>(6.57)</td>
<td>(7.54)</td>
<td>(11.84)</td>
<td>(13.82)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ursolic acid</td>
<td>26.32±1.32</td>
<td>18.73±0.94</td>
<td>20.4±1.65</td>
<td>22.43±1.51</td>
<td>57±10.11</td>
<td></td>
</tr>
<tr>
<td>(2.17)</td>
<td>(3.04)</td>
<td>(2.79)</td>
<td>(2.54)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Oleanolic acid</td>
<td>36.87±1.14</td>
<td>26.19±1.45</td>
<td>24.2±1.36</td>
<td>19.50±1.72</td>
<td>103.5±22</td>
<td></td>
</tr>
<tr>
<td>(2.80)</td>
<td>(3.95)</td>
<td>(4.28)</td>
<td>(5.31)</td>
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All values are expressed as IC<sub>50</sub> (inhibitory concentration 50%) ± SEM, n=3, SPSS 11, followed by T-test, SI= Selectivity index

DISCUSSION

Drug discovery from medicinal plants has played an important role in the treatment of cancer and, indeed, most new clinical applications of plant secondary metabolites and their derivatives over the second half of the present century have been applied toward combating cancer (26, 27).

A number of naturally derived agents have been entered into clinical trials after various preclinical trials. The discovery of novel antitumour agents from natural sources was largely based on the testing for cytotoxic activity against cancer cell lines grown either in vitro or using in vivo models (28, 29).

*Salvadora persica* bark and sticks were extracted with aqueous alcohol then the crude extract was fractionated with petroleum ether, chloroform and ethyl acetate and screened for their cytotoxic activity against different human cellular carcinoma, HePG2, MCF7, HCT116 and A549 cell lines. NO mortality was observed with negative control containing cell lines with DMSO, solubelizer, that refers to viability of cell lines through the incubation period which meaning that experimental condition without FCS for 48h didn't synchronize or hamper the cell cycle. Crude extract was the weakest extract against all tested cell lines while the most effective extract was petroleum extract followed by ethyl acetate fraction and finally chloroform fraction. The activity of petroleum ether extract may be due to their triterpenoid compounds. According to the obvious mentioned results main compounds of petroleum ether, superior extract, were isolated and elucidated as triterpenes. The isolated ursolic acid and oleanolic acid also were screened for their cytotoxic activity against the same cell lines. Ursolic acid showed potent activity than oleanolic acid against HePG2, MCF7 and HCT116 while oleanolic acid was best inhibitor for A549 than ursolic acid.

UA and OA both belong to pentacyclic triterpenoid acids. They have a similar molecular structure, but have different sites of the methyl group on the E ring: if the methyl group at C<sub>19</sub> of UA is moved to C<sub>20</sub>, it becomes OA, they are distributed widely in plants. The toxicity of UA and OA is low and their distribution in plants is extensive. Besides their anti-tumor activity, they also possess immuno-regulatory and liver-protective effects. Therefore, they have a bright future in clinical application (30).
Ursolic acid (UA, 3β-hydroxy-urs-12-en-28-oic acid), a pentacyclic triterpenic acid, exists abundantly in plant kingdom as a constituent of medicinal herbs. Ursolic has been reported to display a remarkable spectrum of biochemical activities to influence processes that are dysregulated during cancer development. These include inhibition of tumorigenesis, tumor promotion, invasion, metastasis, angiogenesis and induction of tumor cell differentiation (31). In addition, Hsu et al., 2004 (32) have reported that UA inhibits the cell proliferation of human lung cancer cell line A549 and provided a molecular understanding for this effect. Mode of action studies revealed that UA blocked the cell cycle progression in the G1 phase.

Oleanolic acid is one of the most important triterpenes, which is found widely distributed in Nature, along with its glycosides (33). Possessing multiple biological effects, such as inhibiting the proliferation of human leukemia-HL60 cells in culture. OA can induce apoptosis of HL60 cells by the death-receptor pathway (34).

Oleanolic acid has hepatoprotective, anti-stomach ulcer, hypoglycemic, anti-hyperlipidemic, anti-hypertensive, cardiotoxic, anti-dysrhythmic, anti-aggregation of blood platelet, anti-cancer, protection of renal toxicity, anti-inflammatory, anti-microbial and anti-fertility activities with low toxicity (35). OA had been shown to act at various stages of tumor development, including inhibition of tumorigenesis, inhibition of tumor promotion, and induction of tumor cell differentiation. It effectively inhibited angiogenesis, invasion of tumor cells and metastasis (36).

OA has inhibition effect on human colon carcinoma cell line HCT15 due to changes in cell morphology, cytotoxicity and cell cycle in-vitro (37). It also decreased the proliferative ability of high potentially metastatic lung cancer cell line PGCL3 in-vitro also OA increases the percentage of apoptosis of lung cells carcinoma. The mechanism of inhibition of OA on human lung cancer cells may be due to inhibition of the adhesion, migration and the cathepsin B secretion of the cells (38). Anti-tumor effect of OA may be due to stimulating secretion of NO and TNF-α release and was able to upregulate iNOS and TNF-α expression (by reverse transcription-polymerase chain reaction) through NF-κB transactivation which may be the mechanism for its anti-tumor effect (39). The presence of ursolic acid and oleanolic acid in petroleum ether extract may be responsible for its cytotoxic effect.

In conclusion, present study demonstrate a significant cytotoxic activity of petroleum ether extract against lung carcinoma cell line-A549 and colon carcinoma cell line-HCT116. Isolated ursolic acid was more effective than oleanolic acid against HepG2, MCF7 and HCT116 but oleanolic was potent against A549 (IC50= 19.5µg/mL).

REFERENCES


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أbeer Y Ibrahim

يرجى أعد صيغة النص بشكل صحيح وفعال لأتمكن من قراءته والرد عليه بشكل صحيح.