ESSENTIAL OIL COMPOSITION AND ANTIOXIDANT, ANTICANCER ACTIVITIES OF *ARTEMISIA SANTOLINIFOLIA* (PAMP) GROWN IN MONGOLIA

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Abstract:

Medicinal plants have always been considered a healthy source of life for all people. In Mongolia, the use of various medicinal and food plants has a long history. In recent years, interest in plant-derived food additives has grown. This study was designed to determine essential oil chemical composition and evaluate antioxidant, cytotoxic activities of aerial parts serial fractions from Artemisia santolinifolia grown in Mongolia. The chemical composition of essential oil from A. santolinifolia was determined by GC-MS analysis. Among them 49 components β-thujene (47.89%), sabinene ketone (11.78%), α-thujene (3.98%), terpinen-4-ol (2.34%) were found to be the major ones. The ethanol crude extract of A.santolinifolia was suspended in water and consequently fractionated with n-hexane, chloroform, ethyl acetate and butyl alcohol. All the fractions were examined for their antioxidant and protective effect against tert-butylhydroperoxide-induced cell death by using DPPH and MTT assays, respectively. Ethyl acetate fraction showed the highest DPPH radical scavenging activity at a concentration of 50 µg/ml by 91.67% with the IC50 value of 12.03 µg/ml. MTT results showed that ethyl acetate, butanol and water fractions demonstrated significant protective effect against the oxidative stress in HepG2 cell line. These suggest that the fractions of A. santolinifolia could hold a good potential for use in the pharmaceutical industry.

Keywords: *A.santolinifolia; essential oil composition; antioxidant and cytotoxic activities*

INTRODUCTION

Several plants were widespread for their many therapeutic and pharmaceutical virtues, especially antioxidant, anti-tumoral, and anti-infectious activities. A big part of the world's population still relies on the benefits of food for the treatment of common illnesses (Zhang, 2004).

Many natural compounds extracted from plants exhibit important biological activities (Jian-Qing Yu, 2011). A number of aromatic medicinal plants used for treating infectious diseases have been mentioned in different phytotherapy manuals due to their availability, fewer side effects, and reduced toxicity. The essential oils of these aromatic plants (Vandendool & Kratz, 1963) are responsible for their fragrance as well as biological properties (Kalemba & Kunicka, 2003). Essential oils are complex mixtures of volatile secondary metabolites that mainly consist of mono- and sesquiterpenes and are responsible for both the fragrant and biological effects of aromatic medicinal plants (Salzer, 1977; Angioni, 2003; Senatore, 2004). An important characteristic of essential oils and their constituents is their hydrophobicity, which enables them to partition in the lipids of bacterial cell membranes and mitochondria, thus disturbing the structures and rendering them more permeable (Sikkema, 1995). Essential oil plants have recently received much attention by the pharmaceutical industry due to their multiple functions, especially their antioxidant, anticancer and antimicrobial activities (Bakkali, 2008; Sylvestre, 2006).

The major constituents of many these oils are phenolic compounds (terpenoids and phenylpropanoids) (Lawrence, 2005). Nevertheless, aromatic plants producing non-phenolic essential oils are also used as spices and in folk remedies as antiseptics. (Daise Lopez-Luts, 2008). Free radicals and reaction oxygen species (ROS) are constantly produced as byproducts in the human body during cell metabolism. These harmful byproducts, if not eliminate immediately, can cause oxidative damage to functional macromolecules such as DNA, proteins, and lipids (Apel, 2004; Tang, 2004).

This increases the chance of occurrence of age-related disorders, cancer, atherosclerosis, neurodegenerative diseases, and inflammation (Tang, 2004; Cai, 2004). Consumption of antioxidants, through diet and supplements, is expected to remove ROS from the living system and provide health benefits. Several studies demonstrated that medicinal plants are a rich source of antioxidant compounds such as phenolics, flavonoids, quinones, vitamins, coumarins, and alkaloids, which can decrease the incidence of oxidative stress and associated diseases (Cai, 2004).

Cancer is a general term applied to malignant diseases characterized by rapid and uncontrolled abnormal cells formation which may mass together to form a growth or proliferate throughout the body and it may progress until it causes death. Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population. Medicinal herbs have been widely used for treatment of diseases in traditional way for several generations. An interaction between traditional medicine and modern biotechnological tools is to be established towards new drug development. The interference between cell biology, in vitro assays and structural chemistry will be the best way forward to obtain valuable leads. There is considerable scientific evidence to suggest that nutritive and non nutritive plant-based dietary factors can inhibit the process of carcinogenesis effectively.

Cancer chemoprevention involves pharmacologic intervention with synthetic or naturally occurring chemicals to prevent, inhibit or reverse carcinogenesis or prevent the development of invasive cancer. Out of an estimated 250,000 higher plants, less than 1% has been screened pharmacologically (El-Shemy et al., 2007). In recent years, focus on plant research has increased all over the world. Antioxidants are a group of substances that are useful for fighting cancer and other processes that potentially lead to diseases such as atherosclerosis, Alzheimer's, Parkinson's, diabetes and heart disease (Valko et al., 2007). Unlike cytotoxic agents that damage tumor cells, antioxidants act by preventing the onset of cancer during carcinogenesis, and they are generally beneficial to cells. Oxidants such as reactive oxygen species (ROS) that include the superoxide radical, hydroxyl radical, hydroperoxyl radical and nitrogen species (RNS) such as peroxynitrite and nitric oxide damage macromolecules, including proteins, lipids, enzymes and deoxyribonucleic acid (DNA). To combat these radicals, living organisms produce enzymes (for example, catalase, superoxide dismutase and peroxidase) or rely on nonenzymatic molecules, such as glutathione, cysteine, ascorbic acid, flavonoids and vitamin K for protection (Ahmed, 2012).

Artemisia santolinifolia belongs to the genus *Artemisia* of *Asteraceae* (*Compositae*). *Artemisia* (*Wormwood*) is a large, diverse genus of plants with between 200-500 species which are mainly found in Asia, Europe and North America (Bora & Sharma, 2011). Among them 105 species grow in Mongolian Forest-Steppe and Desert-Gobi (Grubov, 1982). Many Artemisia species, which are known by such common names as mugwort, sagebrush, sagewort, and wormwood, have a vast range of biological activities, including antimalarial, cytotoxic, antifungal, antibacterial, antioxidant, and other useful effects (Bora & Sharma, 2011).

They have a characteristic scent or taste, based on monoterpenes and sesquiterpenes, which in many cases are the reason for their application in Mongolian traditional medicine (Shatar, 1998; Markova, 1985). These herbs are used worldwide in tonic, stomachic and stimulant beverages and as antiphlogistics in antiseptic oils or tinctures applied for the relief of rheumatic pains (Verezovskaya, 1991).

Mongolia is rich in essential oil medicinal plants. Mongolian traditional medicine has long history of more than 2500 years. There are about 60 clans, 200 species, 300 kinds of essential oil plants and 600 kinds of herbal plants have been registered, among of them, 150-200 kinds are commonly used (Shatar, 2000; Ligaa, 2005). Many essential oil plants have not been studied yet. It is notable to investigate their chemical compositions and biological activities by using traditional medicine (Shatar, 1989).

To our knowledge, there are no published reports on the chemical composition, biological activities of the essential oil and various fractions of *A. santolinifolia* grown in Mongolia. Therefore, the aim of this study was to determine essential oil chemical composition and describe their antioxidative and protective effect against *tert*-butylhydroperoxide-induced

cell death of various fractions from *A. santolinifolia* grown in Mongolia. Consequently, it is important to develop a better understanding of their mode of biological action for new application in human health.

2. Experimental

2.1. Chemicals

Dimethyl sulfoxide (DMSO), MTT, and DPPH were purchased from Sigma Chemical Company. Dulbecco's modified Eagle's medium (DMEM) medium, fetal bovine serum, penicillin, and streptomycin were purchased from GIBCO Co. (GIBCO BRL, Grand Island, NY, USA). The human liver cancer cell lines (HepG2) were purchased from American Type Culture Collection (ATCC, USA). All other chemicals were of analytical grade and purchased from Sigma-Aldrich (USA) and DUKSAN Co. (Korea).

2.2. Plant material

Wild plants materials used in this investigation were collected from Eastern-Mongolia, Bayanzag region of the Gobi Mountains behind the Gurvan Saikhan Mountains, in September 2011. The plants were taxonomically identified by Prof. S.Shatar from the Institute of Chemistry and Chemical Technology (MAS), Mongolia. Voucher specimen has been deposited in the herbarium section of Department of Chemistry, National University of Mongolia (NUM), Ulaanbaatar, Mongolia.

2.3. Extraction of the essential oil

The aerial parts of the freshly collected plants were finely chopped and hydro-distilled for 3 h using a Clevenger-Adams type apparatus (Adams, 1991). The yield of the essential oil produced during the steam distillation was 0.95% (v/w). The oil samples obtained were dehydrated over anhydrous sodium sulphate and stored at 4°C prior to analysis.

2.4. Extraction and fractionation of plant

Different crude extracts of wild and traditional plants as well as, fresh plants were prepared according to Ferrigni et al. (Ferrigin, 1982). The air-dried and powdered whole plant was extracted with 70% ethanol ($2L \times 3$) using sonicator under room temperature. The resultant extracts were combined and evaporated in a rotary vacuum evaporator (Buchi R-205, Switzerland) at 40°C to afford crude extracts. The ethanol crude extracts were suspended in water and then fractionated successively with n-hexane, chloroform, ethyl acetate and butyl alcohol by using the separation funnel, respectively. All the extracted materials were preserved at -20°C until analysis of biological activities.

2.5. Analysis of essential oil

A Hewlett-Packard HP 6890 gas chromatograph coupled with a DB-5 fused silica capillary column (30 m × 0.25 mm × 0.25 µm film thickness, J&W Scientific) and FID detector was used for the quantitative determination of oil components. Oven temperature was programmed as follows: 50°C for 2 min, rising to 250°C at 5°C/min. Injector temperature: 270°C. Carrier gas: He with a flow rate of 1 ml/min. Split ratio 1:10. The volume injected 1.0 µl of 1% solution (diluted in hexane). Identification of the oil components was based on their retention indices and MS, obtained from GC/MS analysis on a HP 6890/HP5973 equipped with DB-5 fused silica capillary column (30 m × 0.25 mm × 0.25 µm film thickness, J&W Scientific). The EI/MS spectra were recorded at 70eV. The mass range was 35 to 500 m/z. The essential oil components were identified on the basis of their mass data and the comparison of their relative retention index (RRI) (Vandendool & Kratz, 1963) that was obtained using various series of n-alkanes. Their EI-mass spectra were either compared with the NIST/NBS and Wiley library spectra found in the literature (Massada, 1976), or were confirmed by comparison with data published in a reference book (Adams, 2001). The percentages of the individual components were obtained from the relative GC peak areas as shown in Table 1.

2.6. Antioxidant activity measurement

2.6.1. DPPH assay

The free radical scavenging activity (antioxidant activity) has been evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, which is regarded as an easy, reliable, sensitive, and rapid method as compared with other antiradical methods (Moon & Shibamoto, 2009). A review by Moon and Shibamoto also reported that 90% of antioxidant activity measurements are conducted by this method. In vitro antioxidant activities of the plants were evaluated on the basis of their ability to inhibit H_2O_2 induced oxidative stress in yeast cells (Saccharomyces cervisiae). This method has been employed for in vivo antioxidant studies as yeast is the ideal model organism for monitoring the biological mechanism (Azevedo, 2011; Lee, 1999).

The assay was carried out according to the method of Brand-William et al. (Brand-Williams, 1995) with a little modification to investigate the free radical scavenging activity of samples. Briefly, the samples were dissolved in ethanol at the concentration of 100 mg/ml and then serially diluted by ethanol. On each well of a 96-well plate, 100 µl of samples of different concentration were mixed together with 100 µl of 60 µM DPPH prepared in ethanol. After incubation of 20-30 minutes for reaction, the absorbance of supernatants was measured at 517 nm. Ethanol was used as a negative control and α-tocopherol as a positive control.

The scavenging capacity (SC) of the sample was calculated using the following formula:
SC (%) = $[1\text{-}A_S/A_C]$ 100

Where, A_S = is the net absorbance of the sample, A_C = is the net absorbance of negative control. The IC_{50} value of a sample is the concentration of sample at which 50% activity of DPPH (absorbance) is inhibited. It was calculated by linear regression.

2.7. Cell lines and culture

Human hepatoma HepG2 cells were obtained from the ATCC. HepG2 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. The cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

2.8. MTT assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) colorimetric method was used for determining cytotoxic activity of samples as describing by Mosman (Mosman, 1983). The MTT colorimetric assay is an established method of determining viable cell number in proliferation and cytotoxicity studies. This assay is based on the cleavage of the yellow tetrazolium salt, MTT, to form a soluble blue formazan product by mitochondrial enzymes, and the amount of formazan produced is directly proportional to the number of living, not dead cells, present during MTT exposure. Since the MTT assay is rapid, convenient, and economical, it has become a very popular technique for quantification of viable cells in culture (Sylvester, 2011).

HepG2 cells (1×10^4 cells/well) were plated in a 96-well plate for 24 hour and then treated with 20 µg/mL and 40 µg/mL concentrations of the extracts dissolved in DMSO. DMSO concentration (0.2%) in DMEM was equal to all groups including the control group. For cellular treatments, HepG2 cells were cultured in DMEM without fetal bovine serum to reduce direct interaction between the phytochemicals and fetal bovine serum. After 24 hour incubation, the cells were washed by Dulbecco's phosphate buffered saline two times and treated with 250 µM *t*-BHP for 3 hour. The cell viability was evaluated by using the EZ-Cytox cell viability assay kit (Daeil Lab Service). Epigallocatechin-3-gallate (EGCG, Sigma Aldrich) at 10 µM dose was used as comparative control.

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was carried out by Student's *t*-test. $P<0.01$ was considered statistically significant.

3. Results and discussion

3.1. Chemical composition of essential oil

The essential oil composition was determined by GC-MS analysis. A total of 49 components were identified, representing 95% of the total oil composition. The monoterpenoides made up the largest component of the oil and had many representative volatiles. The oxygenated monoterpenes (74.46%), monoterpene hydrocarbons (2.63%), sesquiterpenes (2.51%), and whereas sesquiterpenes were weakly sesquiterpenoides (0.85%). The main constituents were found to be β-thujene (47.89%), sabinene ketone (11.78%), α-thujene (3.98%), terpinen-4-ol (2.34%).

These differences might have been derived from local, climatic and seasonal factors. The percentage content of the individual components, the retention times and percent yields are summarized in Table 1.

Table 1: Chemical components of essential oil monoterpenes from *A. santolinifolia* grown in Mongolia

№	**Monoterpenes**	Percentage, %	№	**Monoterpenes**	Percentage, %
1	α-Thujene	0.11	9	α-Terpinene	0.22
2	Benzaldehyde	0.10	10	*p*-Cymene	0.93
3	α-Pinene	0.10	11	Limonene	0.10
4	Camphene	0.10	12	*cis*-Ocymene	0.18
5	Sabinene	0.18	13	γ-Terpinene	0.55
6	β-Pinene	0.10	14	Terpinolene	0.11
7	Myrcene	0.11		**Total monoterpenes (%)**	**2.63**
8	α-Phellandrene	0.14			

Table 2: Chemical components of essential oil monoterpenoides from *A. santolinifolia* grown in Mongolia

№	**Monoterpenoides**	Percentage, %	№	**Monoterpenoides**	Percentage, %
1	Linalool	0.97	19	Cuminaldehyde	0.10
2	α-Thujene	3.98	20	Neral	0.10
3	β-Thujene	47.89	21	Carbotane acetone	0.16
4	Sabinene ketone	11.78	22	*cis*-Sabinene hydrate acetone	0.31
5	*cis*-Pinene hydrate	0.32	23	*trans*-Myrtanol	0.70
6	Myroxyde	0.35	24	Carvone	+
7	i-3-Thujanol	0.44	25	Estragol	+
8	*trans*-p-Ment-2-ene-1-ol	0.73	26	Geraniol	+
9	Camphor	0.30	27	Bornyl acetate	0.1
10	Isopulegol	0.25	28	Thymol	0.01
11	Pinocarvone	0.11	29	Mentyl acetate	0.1
12	Neomenthol	0.25	30	Carvacrol	0.21
13	Terpinen-4-ol	2.34	31	Verbenyl acetate	0.15
14	*p*-Cymene-8-ol	0.11	32	α-Terpenil acetate	0.13
15	α-Terpineol	0.21	33	Eugenol	0.10
16	Myrtenol	0.43		**Total monoterpenoides (%)**	76.46
17	Nerol	0.10			

Table 3: Chemical components of essential oil sesquiterpenes from *A.santolinifolia* grown in Mongolia

№	**Sesquiterpenes**	Percentage, %	№	**Sesquiterpenes**	Percentage, %
1	α-Copaene	0.10	8	Germacrene-D	0.25
2	β-Longipinene	0.12	9	Ar-Curcumene	0.10
3	cis-α-Bergamotene	0.14	10	β-Selinene	0.20
4	β-Caryophyllene	0.20	11	Bicyclo bergamotene	1.10
5	Humulene	0.10	12	δ-Cadinene	0.1
6	β-Farnesene	0.10			
Total sesquiterpenes (%)					**2.51**

Table 4: Chemical components of essential oil sesquiterpenoides from *A.santolinifolia* grown in Mongolia

№	**Sesquiterpenoides**	Percentage, %			
1	*E*-Nerolidol	0.05		**Total sesquiterpenoides**	**0.85%**
2	Spathulenol	0.35			
3	Caryophyllene oxide	0.10			
4	Oplopanone	0.35			
Total determined comounds					**76.45%**

3.2. Antioxidant activity

DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron-donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (Brand-Williams, 1995).

Antioxidant activities of the different fractions from *A. santolinifolia* were tested by the DPPH radical scavenging assay. The effect of antioxidant on DPPH radical scavenging was thought to be due to their hydrogen donating ability or radical scavenging activity. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form DPPH (non radical) with the loss of this violet color (Molyneux, 2004).

Among all fractions the ethyl acetate fraction showed strong free radical scavenging activity in DPPH assay system with IC_{50} value of 12.03±2.5%. The scavenging activity of the various fractions in decreasing order was: Ethyl acetate (EtOAc)> n-hexane (Hex) > n-buthanol (BuOH) > ethanol (EtOH) > water (H_2O) > chloroform ($CHCl_3$) (Fig 1).

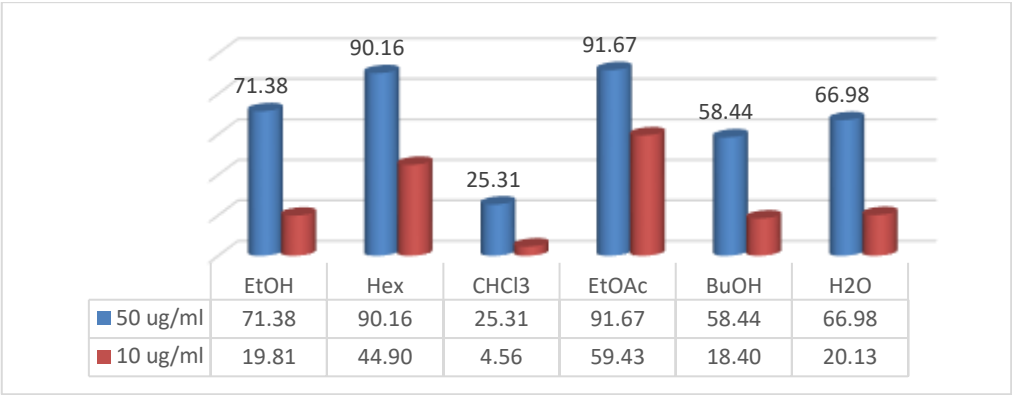

Fig 1: Free radical scavenging activity (%) by the DPPH assay of serial fractions of *A. santolinifolia* at a concentration of 50 and 10 µg/ml

3.3. Protective effect against *t*-BHP induced cell death

To investigate the protective effect against *t*-BHP induced cell death of different fractions from *A.santolinifolia*, we evaluated its effect on a selection of HepG2 (human liver cancer cell line) cell lines by MTT assay.

These cell lines were submitted to growing concentrations of *A.santolinifolia* fractions for 24 hour following by the treatment of t-BHP for 3 hour. As shown in Fig 2, the ethyl acetate, butanol and water fractions of plant showed significant protective effect against t-BHP induced cell death in HepG2 cells. The protective effect of the different fractions in decreasing order was: butanol > water > ethyl acetate > ethanol extract > hexane > chloroform (Fig 2).

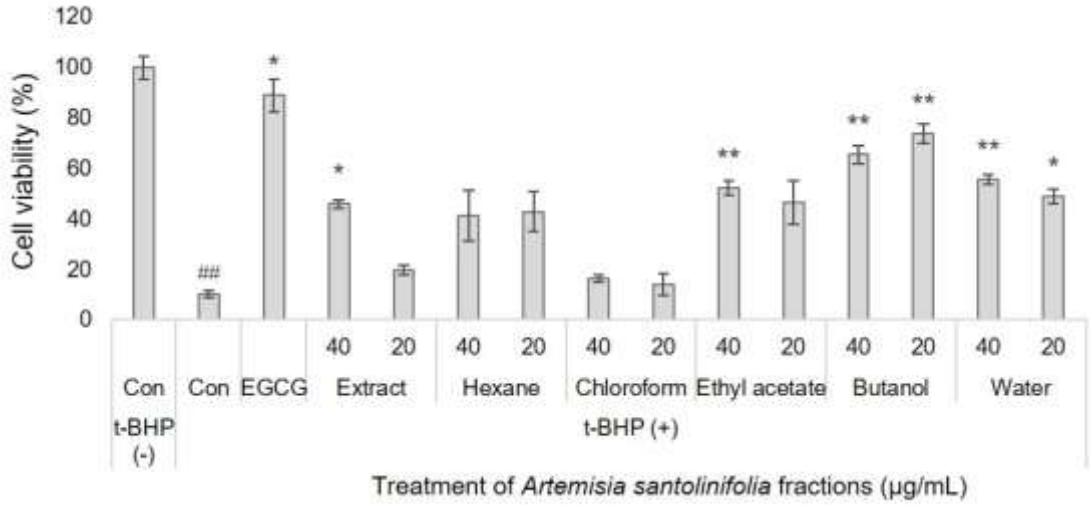

Fig 2: Cell viability (%) of fractions from *A. santolinifolia* protecting t-BHP induced cell death in HepG2 cell line.

Cells were treated with the fractions for 24 hours at a concentration of 40 µg/mL and 20 µg/mL following by the treatment of 250 µM *t*-BHP for 3 hours. *P < 0.01,
**p < 0.001, significant with respect to *t*-BHP alone treated group; ## p < 0.001,
significant with respect to vehicle-treated control

4. Conclusions

In recent years, interest in plant-derived food additives has grown. Plant extracts might substitute synthetic food antioxidants, which may influence human health when consumed chronically (Martinez-Tome, 2001). The results of our study confirmed the importance of the investigated plants in our nutrition.

This study on essential oil chemical composition and biological activities of *A. santolinifolia* grown in Mongolia were not well performed before. Essential oils hydrodistilled from *A. santolinifolia* were found to be rich in β-thujene (47.89%), sabinene ketone (11.78%), α-thujene (3.98%) and terpinen-4-ol (2.34%).

The antioxidant activity of the ethyl acetate and hexane fractions were moderate than other fractions. The results clearly showed that the n-butanol, water and ethyl acetate fractions presented significant protective effect against t-BHP induced cell death in HepG2 human cancer cell lines tested. The results of this work also demonstrate the potential of *A. santolinifolia* various fractions as a new antioxidant and protective agents for human health.

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