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Evaluation of Antiproliferative and cytotoxic Activities of *Tulsi essential oils*

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Abstract: Tulsi is medicinal plant has been successfully adapted, propagated and cultivated under Egyptian conditions. The plant is promoted in relieving sore throats, toothaches, colds, coughs, laryngitits, bronchitis, nasal congestion and inflammation of the mouth and throat. They have many biological activities such as antipyretic, anti-inflammatory and antifungal activities. This study aims to evaluate cytotoxic effect of *Ocimum sanctum* (Tulsi) (Lamiacae) volatile oil on different human cell lines. volatile oils of tulsi (herb, leaves and flowers) extracted by hydro distillation were tested for their cytotoxic activities on different cancer cell lines including; HU60 (human lung adenocarcinoma), MCF7 (human breast adenocarcinoma), HEPG2 (human hepatocellular carcinoma), Hela (human cervix carcinoma) and U251(human brain tumor 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. The volatile oil of tulsi herb is the most potent evaluated volatile oil against Hela, HepG2 and U251, IC50 (0.9, 0.54 and 4.15 µg/ml, respectively . The volatile oil of tulsi organs (herb, leaves and flowers) treated human carcinoma HU60 has very little or no cytotoxicity except the volatile oil of flower and herb of tulsi it presented IC50 = (8.06 and 4.15) µg/ml, respectively against human carcinoma HU60. The findings of the present investigation demonstrated that the volatile oil of tulsi organs significantly suppresses growth and induces apoptosis in Hela, HepG-2 and HU60 cell lines.
Keywords: *Ocimum sanctum*, volatile oil, anti-cancer, cytotoxicity.

Introduction

Cancer is a dreaded disease which is best characterized by abnormal cell division and is caused by mutation of genes involved in the control of cell division. Cancer grows out of normal cells in the body. Normal cells multiply when the body needs them, and die when the body doesn't need them. Cancer appears to occur when the growth of cells in the body is out of control and cells divide too quickly. There are various factors involved in the genesis of cancer like toxic chemicals, excessive use of alcohol, exposure to environmental toxins, some poisonous plants like mushrooms and exposure to excessive sunlight, genetic problems, radiation, viruses, etc. However, the cause of many cancers remains unknown. The current standard approach of western medicine for treatment of cancer consists of an attempt to eradicate established tumor with combined treatment such as surgery, chemotherapy and radiation. However, this therapy has failed in many respects. In many cases it makes human life miserable and usually reduces the span of life. The patient remains sick due to toxic effects of radio and chemotherapies as these do not kill only cancer cells but normal cells also and produce low hematological picture and low immune syndromes making the patient prone to opportunistic infections, reduce strength and vitality. The failure of modern therapies has prompted complementary and alternative medicine scientists to investigate the plant derived safe and effective therapeutic agents. The present situation has become

too controversial that some oncologists themselves claim that cancer is not a disease, the anaerobic cell growths are meant to absorb the toxins which kill the patients. However, by surgery, chemotherapy and radiotherapy we destroy the protective mechanism and metastasis from one organ to other organ is common. The genus *Ocimum* belongs to the Lamiaceae. It is collectively called as Basil, is a diverse and rich source of aromatic essential oil. These essential oils are being used as pharmaceutical agents because of their antimicrobial, antiemetic, antidiabetic, antifertility, antiasthmatic, antistress and anticancer activity[1].The experimental studies conducted with *Ocimum sanctum* (OS) extract on fibrosarcoma cells in culture have demonstrated that *Ocimum sanctum* exhibits anticancer activity. [2]Most of the modern research on therapeutic uses of Tulsi (*Ocimum sanctum*) has confirmed that Tulsi contains hundreds of phytochemicals which possess antioxidant, adaptogenic and immune-enhancing properties. Tulsi meets the three requirements for an agent to become an adaptogen: being innocuous in nature, promotes physiological functions and induce a state of non-specific increased resistance (SNIR) in the body. As this herb has health benefitting effects by reducing stress and improving both cellular and humoral immunity, its role in prevention & treatment of cancer cases may be a new approach in therapy of cancer. Antioxidants are substances that inhibit the oxidation of our cells from toxins such as free radicals. Reactive oxygen species (ROS) including singlet oxygen ($^1O2$), superoxide ion (O2−), hydroxyl ion (OH) and hydrogen peroxide (H_2O_2) are highly reactive and toxic molecules generated in cells during normal metabolism. ROS can cause oxidative damage to proteins, lipids, enzymes, and DNA, and they have also been linked to pathogenesis of oxidative diseases. Living cells possess an excellent scavenging mechanism to avoid excess ROS induced cellular injury, however, with ageing and under influence of external stresses, these mechanisms become inefficient, and dietary supplementation of synthetic antioxidants is required[3]. In this context, aromatic plants, particularly their essential oils, are being evaluated for antioxidant activity. *Ocimum* plants contain large amounts of antioxidants other than vitamin C, vitamin E, flavonoids and carotenoids. This study aimed to evaluate the efficacy of OS essential oils against cancer cell lines and their antioxidant properties.

Materials and Methods

Plant materials

Seeds of tulsi (*Ocimum sanctum* L.) were obtained from company of (J.L. Hudson seedsman P.O. Box 1058 Red Wood city California 94064 USA). The seeds of plant was cultivated in the Experimental Farm of the National Research Centre, El Nobaria, El-Bihara Governrate (150 Km Northern South of Cairo), Egypt.

Essential oil extraction

At the flowering stage of the plant freshly collected flowers, Herb and leaves of *Ocimum sanctum* were extracted by hydrodistillation for 3 h using a Clevenger-type apparatus. The extracted essential oil was dried over anhydrous sodium sulphate and the purified oil was filled in small vials, tightly sealed and stored in a refrigerator (4 °C) until further analysis.

Antioxidant properties

Chemicals;

1,1-diphenyl-2-picryl-hydrazyl (DPPH), ABTS (2,2-azino- bis(3-ethylbenzthiazolin-6-sulphonic acid) diammonium salt), peroxidase, potassium hexacyanoferrate, and trichloroacetic acid (TCA) and ferric chloride were purchased from Sigma (Sigma-Aldrich).

Free radical scavenging activity

The free radical scavenging activity of tulsi essential oils were measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH•) using the method of[4]. Briefly, 0.1mM solution of DPPH• in ethanol was prepared. Then, 1ml of this solution was added to 1ml of plant essential oils and standards solution . The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517nm in a spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The DPPH• radical concentration in the reaction medium was calculated from the following equation:

DPPH• scavenging effect (%) = $100 - [((A_0 - A_1)/A_0) \times 100]$

Where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample of essential oil[5].

Reducing power

The reducing power of essential oils was determined according to the method of[6]. Tulsi oils and standard solution in 1ml of methanol were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50 ◦C for 20 min. A portion (2.5 ml) of TCA (10%) was added to the mixture, which was then centrifuged for 10 min at 1000 × *g*. The upper layer of solution (2.5 ml) was mixed with methanol (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700nm in a spectrophotometer (Jasco V- 630 (serial No, C 317961148, Japan). Vitamin C was used as a control. Higher absorbance of the reaction mixture indicated greater reducing power.

Total antioxidant capacity

Total antioxidant activity was measured according to the method described by[7]. 0.2ml of peroxidase (4.4units/ml), 0.2ml of H_2O_2 (50µM), 0.2ml of ABTS(2,2-azino-bis(3-ethylbenz-thiazoline-6- sulfonic acid, diammonium salt, 100 µM) and 1.0ml distilled water were mixed, and kept in the dark for 1h to form a bluish-green complex. After adding 1.0ml of essential oils, the absorbance at 734nm was measured. The total antioxidant activity was calculated as follows:

Total antioxidant activity (%)= [1-(A sample/ A control)]x 100

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[8]**.** The method was carried out according to[9].

Cell lines

Different cancer cell lines including; HU60 (human lung adenocarcinoma), MCF7 (human breast adenocarcinoma), HEPG2 (human hepatocellular carcinoma), Hela (human cervix carcinoma) and U251(human brain tumor cell line) were obtained from Cancer Center, Karolinska Institute, Stokholm, 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). Cells were batch cultured for 10 days, then seeded at concentration of $10x10^3$ cells/well for all cell line and 6x103 for VERO in fresh complete growth medium in 96-well microtiter plastic plates at 37 ºC for 24 h under 5% CO_2 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 essential oils 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), 1% antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000µg/ml Streptomycin Sulfate and 25µg/ml Amphotericin B) and 1% Lglutamine in 96-well flat bottom microplate at 37 ºC 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 37ºC 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 37ºC. 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 ess. oils and its final concentration on the cells was less than 0.2%. The IC_{50} was then defined as the concentration of the essential oil that produced a 50% decrease in cell viability relative to the control which was wells exposed to the solvent without any essential oils. The percentage of change in viability was calculated according to the formula: (Reading of essential oil / Reading of negative control) -1) x 100.

A probit analysis was carried for IC_{50} 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 oil with different cell lines was calculated as follow, SI= IC_{50} of VERO cell/ IC_{50} of oil on different tested cell line

Results

The genus *Ocimum* belongs to the Lamiaceae family. *Ocimum* shows a pungent, aromatic flavor and is commonly cultivated for culinary purposes for treating various types of diseases like lowering blood glucose, treats cold, fever, parasitic infestations on the body, inflammation of joints and headaches[10].The three tested essential oils extracted from Tulsi parts significantly scavenged the DPPH radicals at all tested concentrations. Both herb and leaves essential oils scavenged radicals more flowers (IC_{50}=18.21 and 10.51 µg/ ml for herb and leaves oils, respectively) while flower essential oil produced the lowest radical scavenging against DPPH radicals, Table (1).

Table (1): antioxidant properties of Tulsi essential oils

Test / Essential oil	Essential oils IC_{50} of different antioxidant tests (µg/ ml)		
	Radical scavenging	**Reducing power**	**Total antioxidant capacity**
Herb essential oil	18.21±1.03 ^{a}	25.11±2.54	15.40±1.05 ^{c}
Flowers essential oil	23.15±1.42	30.62±1.00 ^{b}	17.46±0.98 ^{c}
Leaves essential oil	10.51±2.01	14.31±0.96	11.70±2.11
Vitamin C	16.50±1.17 ^{a}	29.00±2.05 ^{b}	77.48 ±1.06

Data are presented as mean of triplicates ± standard deviation. Data were analyzed by ANOVA one way followed with Post Hoc for multiple comparisons. Groups have the same letter have no significant difference between them. IC_{50} is a concentration that reproduces 50% inhibition.

On the other hand, Tulsi essential oils showed reduction capability effect in potassium ferricyanide-ferric chloride system. Tulsi leaves oil was the powerful reduction agent (IC_{50}=14.31 µg/ ml) followed with herb oil (IC_{50}=25.11 µg/ ml) whereas flowers oil and vitamin C produced the same effects (IC_{50}= 30.62 and 29 µg/ ml, respectively).

Total antioxidant capacity of three essential oils was determined using ABTS-peroxidase assay. The oils exhibited total antioxidant capacity more than vitamin by many times as mentioned in Table (1). Leaves oil had the highest capacity as antioxidant agent (IC_{50}= 11.70 µg/ ml) while the medium level was recorded for herb essential oil (IC_{50}= 15.04 µg/ ml) as well as the lowest capacity was the flower oil IC_{50}= 17.46 µg/ ml) whereas vitamin C showed IC_{50}= 77.48 µg/ ml, Table (1).

Tulsi essential oils were tested for their effect on cell viability on Ehrlich cells.

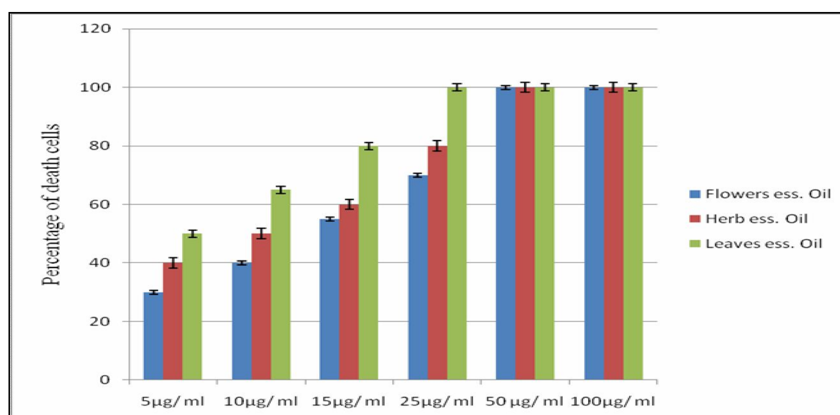

Fig. (1) Inhibition percentage of cell viability of Tulsi essential oils on Ehrlich cells

Tulsi essential oils were screened for their cytotoxic activity against different cell lines, HePG2, HU60, MCF7, Hela, U 251 as well as against VERO cell. Incubation of cell line with DMSO, negative control, didn't show any mortality through the incubation period. Flower essential oil showed potent effect against HePG2 (IC_{50}= 0.47 µg/ ml) followed with Hela cell line (IC_{50}= 1.83 µg/ ml), HU60 (IC_{50}= 8.06 µg/ ml) and then U251 (IC_{50}= 12.15 µg/ ml) while it showed weak effect against MCF7 (IC_{50}= 300 µg/ ml), Table (2). It exhibited high selectivity to HePG2, liver carcinoma cells (SI= 1634.04) while the weak selectivity was observed with Hela cell line, cervix carcinoma.

Table (2): Cytotoxic effect of Tulsi essential oils against different cell lines

Cell line / Essential oil	IC_{50} (µg/ ml) followed with selective index					
	HePG2	HU60	MCF7	Hela	U251	Vero
Flower essential oil	0.47±0.01[a] (1634.04)	8.06±0.11 (95.29)	300±1.06 (2.56)	1.83±0.97 (419.67)	12.15±1.14 (63.21)	768±3.42
Herb essential oil	0.54±0.00 [a] (1620)	4.15±0.05 (210.84)	500±2.45 (1.75)	0.90±0.05 (9722)	1.00±0.06 (875)	875±4.13
Leaves essential oil	0.91±0.03 (1098)	0.00±0.00	450±0.98 (2.22)	5.62±1.01 (177.94)	10.00±0.03 (100)	>1000±5.32

All values are expressed as IC_{50} (Inhibitory concentration of 50 of tested cells)± SD followed by T-test, SI= Selectivity index. Groups have the same letter have no significant difference between them. IC_{50} is a concentration that reproduces 50% inhibition.

On the other hand, herb essential oil showed high cytotoxic effect on HePG2 (IC_{50}= 0.54 µg/ ml) and it produced nearly the same effect on cervix carcinoma, Hela, and brain tumor cell line, U251, (IC_{50}= 0.9 and 1 µg/ ml, respectively) whereas the weak inhibitor effect was recorded against breast cancer cell line, MCF7 (IC_{50}= 500 µg/ ml). It produced high selective index with cervix carcinoma, Hela, (SI= 9722).

As mentioned in Table (2), leaves essential oil exhibited preferable effect on vero cell (IC_{50}>1000 µg/ ml) which means that leaves oil has less toxic effect on healthy cells. Also it significantly inhibited liver carcinoma cells (IC_{50}= 0.9 µg/ ml) but it inhibited cervix carcinoma and brain tumor cell line in a lower effect (IC_{50}= 5.62 and 10 µg/ ml, respectively) as compared to HePG2. Generally, it is evidence from the mentioned data that all oils highly inhibited HePG2 and the most potent one is flowers essential oil while the effective one on lung carcinoma cell line, HU60, is herb oil also the effective oil on cervix carcinoma is herb essential oil, the same trend was observed with brain tumor cell line, U251.

Discussion

The essential oil of Lamiaceae species such as *Ballota nigra, Lamium maculatum, Marrubium vulgare and Galeopsis tetrahit* , *Ocimum sanctum* and *O. gratissimum* proved significant antioxidant properties[11,12]. The three tested essential oils extracted from Tulsi parts significantly scavenged the DPPH radicals at all tested concentrations. Both herb and leaves essential oils were potent radical scavenger than flowers oil also Tulsi essential oils showed reduction capability effect, leaves oil was the powerful reducing agent followed with herb oil and then flowers oil. The oils exhibited total antioxidant capacity more than vitamin C by many times. Leaves oil showed the highest antioxidant capacity followed with herb essential oil whereas vitamin C produced the lowest capacity. The antioxidant activity of the Ocimum essential oil might be due to the presence of mono and sesquiterpenes[13]. Our results are in accordance with those of [14] who studied the antioxidant activities of *Ocimum sanctum* in order to preserve the packed food from rancidity (decomposition of fats, oils and other lipids by hydrolysis and/ or oxidation). By taking battery of tests to assess the state of rancidity, it was found that *Tulsi* extract can be used as a preservative. Aqueous extract of *Tulsi* was added to improve shelf life of a soybean product called 'Tofu'. The shelf life of 'Tofu' increased from normal 3-4 days to 7-8 days[15].

Tulsi essential oils were screened for their cytotoxic activity against different cell lines, HePG2, HU60, MCF7, Hela, U 251 as well as VERO cell. It showed to be safe to negative control. Flower essential oil showed potent effect against HePG2, Hela cell line, HU60 and then U251 whereas the weak effect was against MCF7

with high selectivity to HePG2, liver carcinoma cells and low selectivity with Hela cell line, cervix carcinoma. The herb essential oil showed high cytotoxic effect on HePG2, cervix carcinoma, Hela, and brain tumor cell line with high selective index with cervix carcinoma while leaves essential oil significantly inhibited liver carcinoma cells. All oils highly inhibited HePG2 and the most potent one is flowers essential oil while herb oil was effective on lung carcinoma cell line and brain tumor cell line.

The observed cytotoxicity might be due to the presence of various chemical components in the oil including monoterpenes such as camphor. Studies have shown that monoterpenes exert antitumor activities and suggest that these components are a good source of cancer chemo preventive agents[16]. Our results were agree with[17] on *Ocimum sanctum* who tested it for cytotoxicity by measuring splenic leukocyte natural killer (NK) cells activity against K-562 cell line, showed that pretreatment with Imu-21, for seven days, can increase NK cell activity in mice. The possible mechanism is probably due to activation of mature NK cells or precursor cells which were previously not active.

Herbal anti-cancer compounds are unique in their feature of having anti-oxidant and immunostimulant activity preventing cancer growth indirectly along with a direct cytotoxic effect towards malignant and/or other apoptotic cells. Human fibrosarcoma cells in culture treated with an ethanolic extract of *O. sanctum* induced cytotoxicity at 50 µg/ml and above. Morphologically the cells showed shrunken cytoplasm and condensed nuclei[18]. The cells also showed depleted intracellular glutathione and increased levels of lipid peroxidation products. Administration of aqueous and ethanolic extracts of *O. sanctum* to mice bearing Sarcoma-180 solid tumors mediated a significant reduction in tumor volume and an increase in lifespan. These observations clearly indicate *O. sanctum* extracts possess anticancer activity[19].

Conclusion

Ocimum sanctum (Tulsi) essential oils may have an alternative or complementary natural medicine devoid of alarming deleterious side-effects caused to the cancer and could have beneficial effects in many diseases as their antioxidant properties.

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