Salvadora persica (Miswak): Antioxidant and Promising Antiangiogenic Insights

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Abstract

The use of plants to improve dental health and to promote oral hygiene has been practiced for centuries and persists in several communities throughout the world. *Salvadora persica* (Miswak) has a wide geographic distribution. The ancient Arabs had the habit of using it to whiten and polish the teeth. Here, we determined *in vitro* antioxidant activity, total phenols and flavonoids and evaluated antiproliferative activity of the extract of *S. persica* (Miswak). The MTT assay was used to estimate the antiproliferative activities of the extract against human hepatoma (HepG2) cancer cell line. Inhibition percentage of DPPH scavenging activity was dose-dependent and ranged from (30.7% ± 0.62) to (5.89% ± 0.98). The phenolic content was (2.7 ± 0.11) mg GAE/g while the flavonoid content was (2.70 ± 0.45) mg QE/g. Antiproliferative results of the extracts were found to be consistent with their antioxidant activity. Our extract also exhibited clear antiangiogenic activity. These findings introduce *S. persica* as the useful and novel potential anti-tumor agent for hepatocellular carcinoma (HCC) treatment.

Keywords

Salvadora persica, Antioxidants, Anticancer, Medicinal Plants, Traditional Medicine

1. Introduction

Free radicals have been found to play an important role in food rancidity, chemical materials degradation and damage of macromolecules such as lipids, nucleic acids, proteins and carbohydrates [1]. Therefore, free radicals contribute to certain human disorders such as cardiovascular diseases, diabetes, cancer and in-

flammatory diseases [2] [3]. In addition, free radicals may also cause depletion of the immune system antioxidants, a change in the gene expression and may induce the synthesis of abnormal proteins [4]. Free radicals and other reactive oxygen species (ROS) derived from normal essential metabolic processes are major contributors of oxidative damage in the human body. Human body is also significantly exposed to external sources of free radicals such as X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals [5]. ROS represents the major type of free radicals in the biological systems. They are produced through the mitochondrial electrons transport chain [3] [6]. A balance between free radicals and the antioxidative defense system is important for proper physiological functions. However, the antioxidants that naturally produced by the body might not be adequate to prevent the ROS-induced damages. Therefore, antioxidant complements are important to boost the body's own capacity to reduce or neutralize the oxidative damages [7].

Typically, an antioxidant is a synthetic or natural substance that donates an electron to a free radical and neutralizes it. Scavenging of the free radicals reduces oxidative injury and consequently protects humans against infection and degenerative diseases [8]. Synthetic phenolic antioxidants such as butylated hydroxy anisole, butylated hydroxyl toluenes, tertiary butylated hydroquinone and gallic acid esters have been used to inhibit oxidation. They serve as chelating agents that bind to metals and reduce their contribution to the oxidation process [9]. Unfortunately, these synthetic antioxidants may cause or promote negative health effects therefore, there is a strong trend to replace them with naturally occurring antioxidants to reduce the risk of many diseases that related to oxidative stress [9] [10].

Many medicinal plants used in traditional medicine are known as significant sources of natural antioxidants. These plants attract more attention for their efficiency against several diseases such as cancer, atherosclerosis, cerebral cardiovascular events, diabetes, hypertension and Alzheimer's [11]. Natural antioxidants are very efficient in blocking the process of oxidation by neutralizing free radicals and activated oxygen species. The antioxidative and pharmacological properties of medicinal plants are related to the presence of natural antioxidants such as flavonoids, tannins, coumarins, curcuminoids, xanthones, phenolics, and terpenoids. They are found in various plant products such as herbs and spices (rosemary, thyme, oregano, sage, basil, pepper, clove, cinnamon, and nutmeg) and plant extract (tea and grapeseed) [12] [13].

The use of folk medicine at the primary health care level is widespread, particularly in the Far East and GCC regions. In those areas, folk medicine is usually the first choice for most patients to treat serious diseases including cancers and various types of inflammations.

Salvadora persica (Figure 1) is the most popular plant used in the traditional medicine as a natural chewing stick and may be the first toothbrush mankind has ever known (used by Babylonians as early as 3500 BC) [14]. The use of *S. persica* is also recommended by the Islamic culture and known as Miswak or

Figure 1. (a) Miswak root; (b) Miswak roots cut into small pieces.

Sewak. It is also recommended by the World Health Organization as a chewing stick because of its efficiency for oral hygiene [15]. Most of the published studies have focused on the influence of *S. persica* on oral health revealing its mechanical properties on plaque removal [16] as well as its antibacterial [17] and antifungal effects [18].

Chemical analysis of the roots and bark of *S. persica* (Miswak) has shown the presence of several biologically active chemical constituents such as alkaloids, chlorides, fluorides and sulfur-containing organic substances. Moderate concentrations of silica, sulfur, and vitamin C have been found with small quantities of tannins, saponins, flavonoids, and sterols [19].

Treatment of cancer using medicinal plants has played an important role in cancer therapeutics. Most clinical applications of plant secondary metabolites and their derivatives have been aimed at combating cancer [20] [21]. The discovery of novel anticancer agents from natural sources was largely based on the testing for cytotoxic activity against human cancer cell lines (*in vitro*) or using animal (*in vivo*) as model systems [22] [23]. Studies have demonstrated significant cytotoxic activity of petroleum ether extract against lung carcinoma cell line A549 and colon carcinoma cell line-HCT116. Isolated ursolicacid was more effective than oleanolic acid against HepG2, MCF7 and HCT116 but oleanolic was potent against A549 [24].

The current study investigates the total phenolic and flavonoid contents in the hydroalcoholic extract of *S. persica* roots which is one of the most traditionally plant used in the United Arab Emirates (UAE). In addition, the *in vitro* antioxidant activity of Miswak roots extract using DPPH assay will be determined. Furthermore, both *in vitro* and *ex vivo* tools are set to be used to shed more lights into the anti-cancer and antiangiogenic properties of *S. persica* root extract.

2. Material and Methods

2.1. Chemicals

All solvents were analytical grade. Ascorbic acid, ferric chloride, aluminum chloride, potassium acetate, quercetin, DPPH reagent, Folin-Ciocalteau reagent,

gallic acid, sodium carbonate, methanol, ethanol, thiazolyl blue, tetrazolium bromide and dimethyl sulfoxide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Millipore deionized water was used throughout.

2.2. Plant Samples

Protected roots of *S. persica* in vacuum plastic bags were purchased from the local market and authenticated by the United Arab Emirates University, College of Science Biology Department. A voucher specimen was deposited at the herbarium of the Biology Department. The sample was kept protected in a labeled dry plastic zipper bag.

2.3. Preparation of Plant Extract

S. persica roots were washed with tap water then cut into small pieces and crushed in a grinder. A sample of 10 g of the crushed roots was macerated in 150 ml of 70% (v/v) aqueous ethanol at 4˚C for 48 h. The resulting mixture was filtered under vacuum then concentrated under reduced pressure using a rotary evaporator at 40˚C. The extract was further dried using a TELSTAR CRYODOS freeze dryer machine until no more water can be distilled then kept at −20˚C for further analysis. A solution of 30 mg/ml of the extract was prepared in 50% ethanol for the following tests.

2.4. Determination of Total Polyphenol Content (TPC)

The total phenolic content (TPC) of *S. persica* was measured using Folin-Ciocalteau reagent according to the method described by Singleton [25]. 1 ml of the stock solution (30 mg/ml) was diluted into 10 ml using 50% ethanol. 100 μl of this solution was mixed with 200 μl of the Folin-Ciocalteau reagent and 2 ml of de-ionized water then incubated at room temperature for 3 min. 1 ml of 20% (w/w) aqueous sodium carbonate was then added to the mixture. The mixture was incubated for 1 h at room temperature. A negative control sample was also prepared using the same procedure. The absorbance of the resulting blue color was measured at 765 nm. The concentration of total phenolic compounds in the extract were expressed in mg gallic acid equivalents (GAE) per g dry weight of plant material using an equation obtained from gallic acid calibration curve (0.5 - 26 μg/ml). The samples were analyzed in triplicate.

2.5. Free-Radical Scavenging Activity (DPPH Assay)

The free radical scavenging activity of *S. persica* roots was measured using 2,2-diphenyl-1-picrylhydrazil (DPPH•) as described previously [26]. Four methanolic solutions of the *S. persica* extract (0.15, 0.30, 0.60 and 1.5 mg/ml) were prepared. A 200 μl of each sample was mixed with 3.8 ml of 60 μg/ml methanolic solution of DPPH in a test tube. The contents of the tubes were rapidly swirled then allowed to stand for 30 min at room temperature in the dark. The absorbance was measured spectrophotometrically at 517 nm. Methanol was serving as

the blank sample and a control was also assayed simultaneously. The scavenging ability of the plant extract was calculated using Equation (1).

$$\text{DPPH Scavenging activity}\left(\%\right) = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \qquad (1)$$

where Abs control is the absorbance of DPPH + methanol; Abs sample is the absorbance of DPPH radical + sample (sample or standard). The EC_{50} value (μg/ml) which is the effective concentration at which DPPH· radicals are scavenged by 50% was determined graphically. The total antioxidant activity was expressed as ascorbic acid equivalent/g dry extract using an equation obtained from ascorbic acid calibration curve (5 - 25 μg/ml). The assay was done in triplicate.

2.6. Determination of Total Flavonoids

The total flavonoids content of the *S. persica* extract was measured using the aluminum chloride colorimetric method [27]. A 500 μl of the methanolic extract solution (600 μg/ml) was mixed with 0.1 ml of 10% (w/v) aluminum chloride solution, 0.1 ml of 1 M potassium acetate solution, 1.5 ml of methanol and 2.8 ml of distilled water. The reaction mixture was thoroughly mixed and incubated at room temperature for 30 min. The absorbance was measured at 415 nm. A calibration curve of quercetin was prepared by using concentration from 1 to 25 μg/ml in methanol. The total flavonoids content was expressed as mg of quercetin equivalents (QE)/ g of the dry extract using an equation obtained from quercetin calibration curve (5 - 25 μg/ml). The assay was done in triplicate.

2.7. Cell Culture

A human HepG2 hepatocarcinoma cells were obtained from CLS Cell Lines Service (Eppelheim, Germany) was cultured in RPMI 1640 (Sigma, USA) medium containing 1% antibiotic cocktail and supplemented with 10% fetal bovine serum (FBS) f. Cells were incubated at 37˚C in 5% CO_2 humidified incubator. Cells were passaged every 2 - 3 days using 0.25% trypsin-EDTA. All the reagents were from rom GIBCO (Life Technologies, Germany).

2.8. Cytotoxicity Assay

HepG2 were seeded at a density of 5000 cells/well in a 96-well plate (Nunc), and were allowed to attach overnight. Thereafter, cells were treated with various concentrations of the plant extract for 24 hours. To assess the cytotoxic effect of the plant extract, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltratrazolium bromide (MTT) assay was carried out. Briefly, cells treated with the plant extract were exposed to tetrazolium MTT (Sigma, USA) at a concentration of 5 mg/ml. The reduced yellow MTT salt by viable active cells to insoluble purple formazan was dissolved using DMSO. The absorbance of the coloured solution was measured at a wavelength of 570 nm using Epoch microplate spectrophotometer (BioTek, USA). The obtained absorbance at 570 nm of both control and treated cells was

used to calculate percentage of cell viability. Assuming 100% viability in control cells, percentage of treated cells viability will be calculated accordingly using Equation (2).

$$\text{Percent of viable cells} = \left(\text{Abs of treated cells}\right)/\left(\text{Abs of control cells}\right) \times 100 \quad (2)$$

2.9. Assessment of Morphological Changes

HepG2 were seeded at a density of 0.25×10^6 cells/ well in a 6-well plate, and were allowed to attach overnight. After which, cells were treated without (0 μg) or with increasing concentrations of *S. persica* root extract (200, 400, 600, 800 μg) for 24 hours. The morphology of the cells was assessed using an inverted microscope.

2.10. Western Blotting

HepG2 cells were seeded at a density of 1×10^6 cells/100 mm plate and allowed to attach before being treated with *S. persica* root extract. Cells were treated with increasing concentrations of *S. persica* root extract (200, 400, 600, 800 μg) for 24 hours. Whole cell lysates were separated using 10% - 15% SDS polyacrylamide gel electrophoresis. Proteins were transferred onto PVDF membranes prior to incubation with various primary antibodies VEGF, AKT, p-AKT, (Cellsignalling technologies), p-SRC, p-P38, p-P44/42, GAPDH (Abcam) was used as loading controls. Protein bands were detected using Western Sure Chemiluminescent Substrate (LI-COR) and C-DiGit blot scanner (LI-COR).

2.11. Immunocytochemistry and Fluorescent Staining

HepG2 cells were seeded at a density of 3×10^4 cells/ well in an 8-chambered glass plate (Nunc™ Lab-Tek™) and allowed to attach before being treated with *S. persica* root extract. Cells were treated with two doses (600, and 800 μM) for 24 hours. After that cells were fixed with 4% paraformaldehyde followed by incubation with primary antibody for VEGFR2 (Cellsignalling technologies) and with secondary antibodies tagged with FITC (Alexa Fluor, Molecular Probes). Finally, nuclei were stained using 4, 6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml in PBS; for 25 min at room temperature). Cells were imaged using Inverted Phase Contrast Microscope model IX53 with fluorescent attachment complete with Olympus microscope high resolution digital camera model PD73.

2.12. Experimental Animals

Twelve to fourteen weeks old healthy Sprague Dawley male rats were used. To avoid physiological variations that could affect the process of angiogenesis in female rats due to estrous cycle, only male rats were used in rat aortic ring assay. The animals obtained from animal house facility of the College of the Medicine and health Sciences (UAE University). The animals were kept in well-ventilated cage with food and water provided. The animals were euthanized using CO_2 and dissected to excise thoracic aorta. All procedures were carried out according to

the guidelines of Animal Research Ethics Committee (UAEU). The present study was submitted to the institutional animal ethics committee, "Animal Research Ethics Committee" for evaluation and the present study is approved by the committee (Approval Reference # A 8-15).

2.13. Rat Aorta Ring Assay

This assay was carried out on rat aortic explants as previously described [28]. Thoracic aortas were removed from euthanized male rats, rinsed with serum free medium and cleaned from fibroadipose tissues. Totally 18 rats were used in this assay and approximately 12 to 14 rings (each ring is about 1 mm thickness) were prepared from an each aorta. The aortas were cross sectioned into small rings and seeded individually in 48-wells plate (Nunc) in 300 μL serum free M199 media (Invitrogen) containing 3 mg/ml fibrinogen and 5 mg/ml aprotinin (Sigma). Ten microliters of thrombin [50 NIH U/ml in 1% bovine serum albumin (Sigma) in 0.15 M NaCl (Sigma)] was added into each well and incubated at 37°C for 90 min to solidify. A second layer of M199 medium supplemented with 20% HIFBS (Invitrogen), 0.1% ἐ-aminocaproic acid (Sigma), 1% L-Glutamine(Sigma), 2.5 μg/ml amphotericin B, and 60 μg/ml gentamicin (Sigma) was added into each well (300 μL/well). All the extracts were added at final concentrations of 100 μg/ml. On day four, the medium was replaced with a fresh one containing the test materials. On day five, aortic rings were photographed using Olympus microscope high resolution digital camera model PD73 and subsequently the length of blood vessels outgrowth from the primary tissue explants was measured using Image J.

The inhibition of blood vessels formation was calculated using the formula:

$$\% \text{ blood vessels inhibition} = \left[1 - \left(A_0/A\right)\right] \times 100$$

where：

A_0 = distance of blood vessels growth in treated rings in μm, A = distance of blood vessels growth in the control in μm.

2.14. Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Correlation analysis of antioxidants versus the total phenolic and flavonoid contents were carried out using the regression analysis, with GraphPad Prism 6.0 and Microsoft Excel 2016. $P < 0.05$ was considered statistically significant.

3. Results and Discussion

The plant kingdom is a good source of natural antioxidants used for health promotion. The therapeutic activity of plants is mostly due to groups of secondary plant metabolites mainly antioxidant phenolics and flavonoids. These substances exhibit antioxidant capability and can be a good defense against oxidative stress from oxidizing agents and free radicals.

The goal of the current work is to show the antioxidant activity, polyphenolic and flavonoids contents of *S. persica*, a folk plant common in the UAE. The antiproliferative and antiangiogenic effects of *S. persica* extract are also studied both *in vitro* and *ex vivo*.

3.1. Plant Extraction

S. persica extract was obtained by completely evaporation of ethanol and water. The extract was dried using a rotary evaporator then a freeze dryer machine was used to remove water. The extract was obtained as an amorphous solid mixed with little oil. The yield (w/w) of the extracted Miswak roots was found to be 12.54%.

3.2. Total Polyphenol Content (TPC) of the Extract

Phenolic compounds are widely spread throughout the plants and known as aromatic secondary metabolites. They have been associated with antioxidant properties and it is likely that the antioxidant activity of the plants is due to these compounds [29]. The hydroxyl groups in phenols have shown a strong scavenging ability of free radicals. Therefore, the antioxidant activity of the plants may be attributed to the presence of total polyphenol contents [26] [30]. The phenolic compounds are commonly estimated using Folin-Ciocalteau reagent. This reagent reacts with phenolic compounds and gives a blue color complex that absorbs radiation at 765 nm and allows quantification [31].

The total phenolic content for the ethanolic extract of *S. persica* roots was determined using gallic acid as a standard and results were expressed as gallic acid equivalents (mg/g). The calibration curve showed linearity for gallic acid in the range of 0.5 - 26 μg/ml, with a correlation coefficient (R^2) of 0.989. *S. persica* extract showed low to moderate total polyphenols (2.7 ± 0.11 mg GAE/g). Total polyphenols contents of *S. persica* collected from southern and middle regions in Saudi Arabia were 7.9 ± 0.07 and 5.7 ± 0.04 mg/g respectively [1].

3.3. The DPPH Radical Scavenging Activity

The antioxidant activity of the *S. persica* extract was determined according to the method reported by Lim [26]. In this method, the antioxidant activity of the extract is measured based on its ability to reduce the stable DPPH radical. DPPH radical (DPPH•) is a stable radical and can gain an electron or hydrogen radical and form a stable diamagnetic molecule producing a color change from blue to yellow [32]. DPPH color change from blue to yellow has been widely used to measure the radical scavenging activity because of its stability, simplicity, and reproducibility [33]. The measured remaining amount (%) of DPPH• after 30 min indicates the free radical scavenging capacity of the ethanolic extract of *S. persica* roots. The total antioxidant activity was expressed as ascorbic acid equivalent/g dry extract.

The calibration curve of ascorbic acid showed linearity in the range of 5 - 20
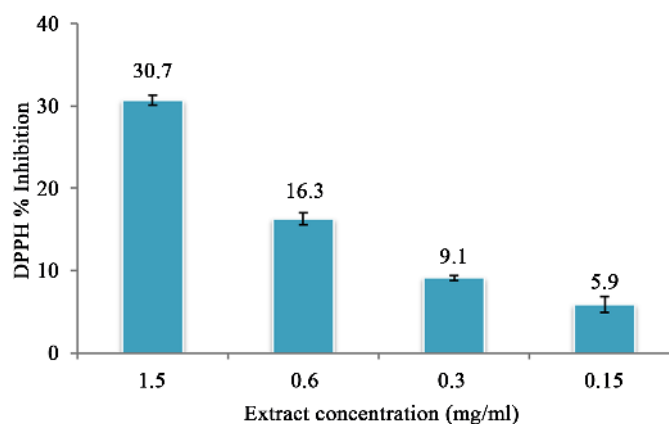
Figure 2. DPPH radical scavenging activities of *S. persica* extract.

μg/ml, with a correlation coefficient (R^2) of 0.989. The ethanolic extract of *S. persica* exhibited a moderate DPPH scavenging activity at the measured concentrations and ranged from (30.7% ± 0.62%) at 1.50 mg/ml to (5.89% ± 0.98%) at 0.15 mg/ml as shown in **Figure 2**. The percentage inhibitions of DPPH scavenging activity in S*. persica* extract were dose-dependent. It has been reported that there is a positive correlation between DPPH scavenging activity and total polyphenol contents. Therefore, the obtained percentage inhibitions of DPPH scavenging activity of *S. persica* extract are consistent with its total polyphenols content indicates the antioxidant activity of *S. persica* plant might be related to its content of phenolic compounds.

Moreover, DPPH radical scavenging ability of *S. persica* extract was evaluated as half maximal effective concentration EC_{50} (μg/ml) value. Low EC_{50} values indicate high potency of the plant and high free radical scavenging activity. The scavenging activity exerted by *S. persica* is (EC_{50} = 3.24 mg/ml) suggesting that the presence of the phenolic compounds contributed significantly to the antioxidant activity of the tested plants. Therefore, the antioxidant activity of *S. persica* may be related to the presence of the phenolic compounds. These findings are consistent with reported works that showed a positive correlation between the total phenolic content and the antioxidant activity of some plant extract [26] [30] [34].

3.4. Total Flavonoids Content

Flavonoids and their derivatives exhibit different biological activities including anticancer activity. Flavonoids are the most common and widely distributed group of phenolic compounds found in the plant significantly contributes to their antioxidant properties [34]. Flavonoids' anticancer activity is attributed to their potent antioxidant effects including metal chelation and free-radical scavenging activities [35]. Aluminum chloride assay was used to determine the flavonoids content in which the aluminum ion, Al (III), forms a complex with the carbonyl and hydroxyl groups of flavones and flavonols producing a yellow color measured spectrophotometrically at 415 nm [36]. Flavonoid content was calculated

from the regression equation of quercetin calibration curve and was expressed as quercetin equivalents. The basic structure of flavonoids is made of two benzene rings (A and B) which are linked to a pyrone ring (C). Flavonoids are divided into different classes based on their molecular substitutes [37].

The calibration curve of quercetin showed linearity in the range of 1 - 25 µg/ml, with a correlation coefficient (R^2) of 0.999. The total flavonoid contents of the ethanolic Miswak extract in terms of quercetin equivalent was 2.70 ± 0.45 mg QE/g extract.

It has been reported that there is a positive correlation between EC_{50} (DPPH· scavenging) and total flavonoids content. As the obtained free radical scavenging activity and total flavonoids content are low this may indicate that flavonoids may participate in the free radical scavenging activity of this plant extract. The presence of kaempferol, quercetin, quercetinrutin, and a quercetinglucoside in Miswak extracts has been reported [19].

3.5. *S. persica* Affected Cell Morphology and Viability

After pre-culturing with *S. persica* extract (200, 400, 600, 800 µg) for 24 h, HepG2 cells were observed by an inverted microscope to study the morphological changes. As shown in **Figure 3(b)**, cells shrunk, became more slender in shape and lost their ability to attach, showing signs of cell death starting at the lower
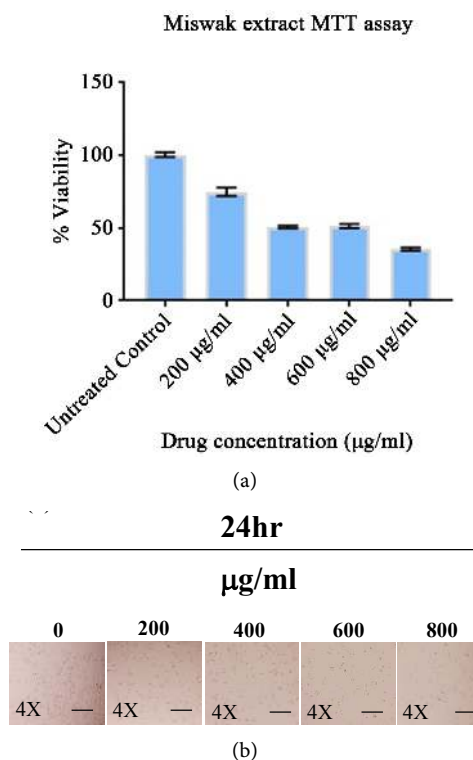

Figure 3. Antiproliferative effect of *S. persica* root extract: (a) MTT analysis of HepG2 cells post treatment with increasing doses of *S. persica* root extract for 24 h; (b) Morphological analysis of HepG2 cells post treatment with increasing doses of *S. persica* root extract for 24 h.

dose of 200 μg/ml. We also found that the cell metabolism was affected in a dose-dependent manner as the HepG2 cells reached the viability of 70%, 50% and 40% as depicted using the MTT assay in Figure 3(a). Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm. When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. The cellular mechanism of MTT reduction into formazan involves reaction with NADH or similar reducing molecules that transfer electrons to MTT [38] [39] [40]. Hence, this result reflects the ability of *S. persica* extract to strongly inhibit the viability of HepG2 cells.

3.6. *S. persica* Modulates the Essential Angiogenic Markers

Back in 1970s, Dr. Judah Folkman has elegantly proposed the notion of anti-angiogenic therapy via cutting the blood supply off cancer cells and consequently depriving them of nutrients and eventually leading to their death [41]. Angiogenesis, the formation of new blood vessels, is known to play a central role in the progression of many solid tumors, like HCC [42] [43]. Data presented here, propose that *S. persica* root extract may be a potential inhibitor of angiogenesis. Herein, key markers involved in the tumor angiogenic process have been scrutinized. Vascular endothelial growth factor (VEGF) is known as one of the most important regulators of angiogenesis [44]. The functioning of VEGF is mediated by three structurally-related receptor tyrosine kinases; VEGFR1, VEGFR2 and VEGFR3. Among those, VEGFR2 has emerged as the primary receptor of VEGF and the major mediator of VEGF-induced pro-angiogenesis signaling in endothelial cells [45] [46]. The dynamic interaction between VEGF/VEGFR2 activates several intracellular key signaling molecules, including Extracellular signal-regulated kinase (ERK) 1/2, P38 mitogen-activated protein kinase (MAPK), Src family kinase, and Protein kinase B (PKB), also known as Akt, which are responsible for angiogenesis, vascular permeability, invasion, finally leading to tumor metastasis [47] [48] [49]. In this study, we demonstrated that an *S. persica* root extract inhibited the protein expression of VEGF in a dose-dependent manner (Figure 4(a)). Immunofluorescence data presented here revealed that, *S. persica* root extract significantly modulates the expression of the active VEGF receptor, VEGFR2 (Figure 4(b)). We next performed the protein expression analysis using western blot to determine whether *S. persica* root extract regulated the activity of MAPK/AKT cascades including AKT, ERK1/2 MAPK (p-P44/42) and p38 MAPK. As shown in Figure 4(a); the extract markedly decreased the phosphorylation of AKT, p38 MAPK and ERK1/2 MAPK. This emphasis on the inhibitory effect of *S. persica* root extract on the activation of these prime pathways. The levels of total AKT was also down regulated at higher doses of *S. persica* root extract. Riesterer *et al*. [50] reported that VEGF controls the protein stability of the serine-threonine kinase PKB/Akt. Similarly, in the present study, the inhibition of VEGF by *S. persica* root extract resulted in a specific decrease of Akt protein level. Src kinase, a non-receptor tyrosine kinase, is
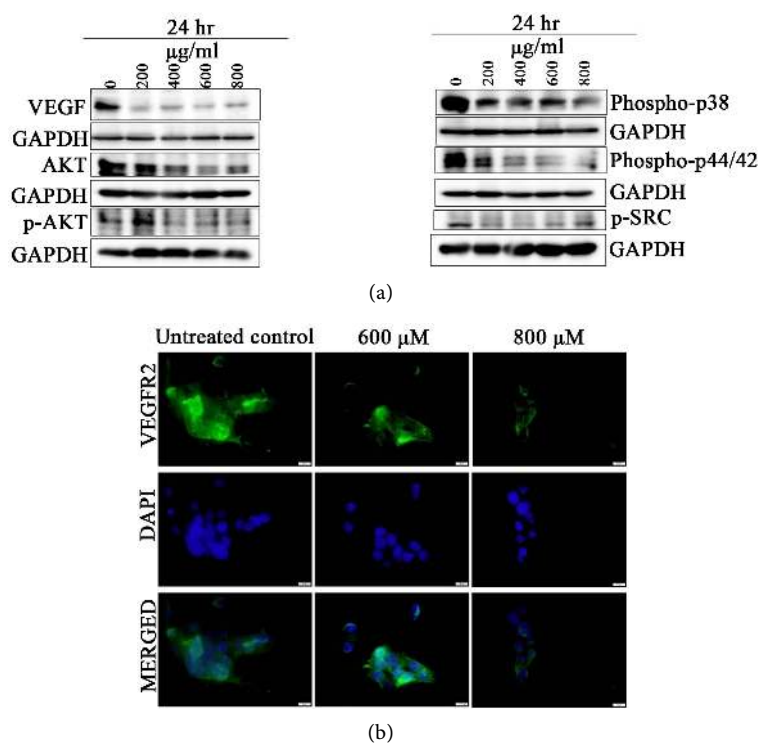
Figure 4. Inhibitory effect of *S. persica* root extract on angiogenesis related markers: (a) Western blot analysis of key essential markers in angiogenesis in HepG2 cells post treatment with increasing doses of *S. persica* root extract for 24 h; (b) Representative images of immunofluorescence assay of pre-treated HepG2 cells in two doses *S. persica* root extract of (600 μM and 800 μM) groups. Cells were immunostained with antibody against VEGFR2. VEGFR2-positive cells were stained green (Alexa Fluor® 488) and the nucleus stained blue (DAPI). Scale bar, 20 μm.

highly activated by binding of the SH2 domain to a tyrosine autophosphorylation site on VEGFR in endothelial cell [51] [52]. I the present study we found that even at a low dose of *S. persica* root extract, the activation of Src kinase is significantly inhibited. Thus, the ability of *S. persica* root extract in anti-angiogenesis of hepatocellular carcinoma was briefly demonstrated by studying the expression of the mentioned key angiogenic markers in HepG2 cells followed by exposure of the extract at different doses.

3.7. *S. persica* Inhibits Microvessel Growth *in Vitro*

The rat aortic ring model, first described in the early 1980s [53], has proven to be a practical and cost effective assay of angiogenesis. The aortic ring model is an *ex vivo* assay of angiogenesis that combines advantages of both *in vivo* and *in vitro* models [54]. The vessels that grow out from aortic rings convert smooth muscle cells and pericytes to associate with the endothelial cell tube, implicating that they are anatomically analogous to neovessels *in vivo* [55]. The antagonistic effect of *S. persica* root extract in the process of angiogenesis was measured in the presence of VEGF in a dose dependent manner (300 μg/ml and 400 μg/ml). As shown in (Figure 5), *S. persica* root extract inhibits VEGF-induced rat aortic

(a)
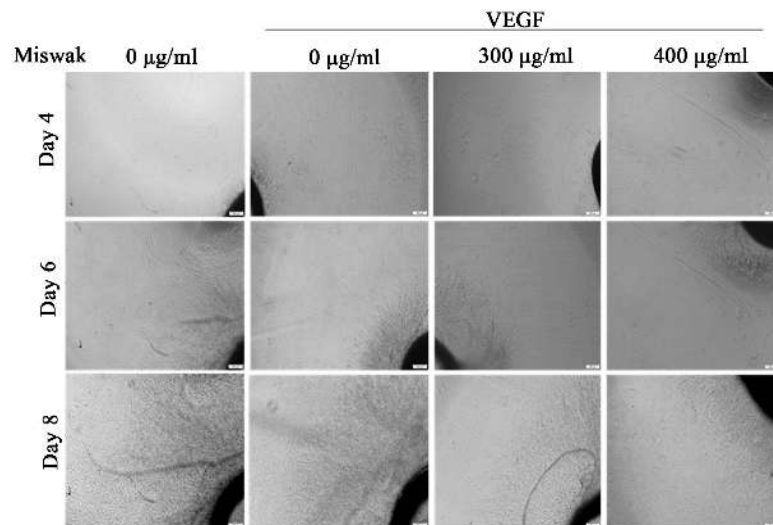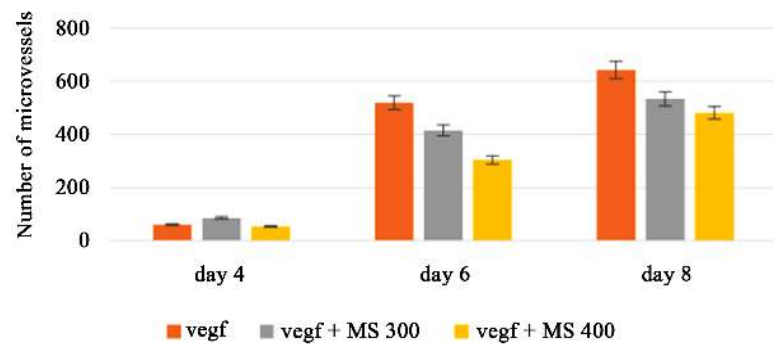
(b)

Figure 5. *S. persica* root extract inhibits microvessels sprouting in aortic ring assay in a dose-dependent manner. (a) Representative micrographs of sprouting microvessels from aortic ring grown in the absence or presence of *S. persica* root extract with or without VEGF treatment; (b) Quantification of the number of the sprouting microvessels from aortic rings grown in the presence or absence of *S. persica* root extract L. with or without VEGF treatment.

ring microvessel sprouting showing a profound effect on day 6. Compared to the control, which was induced by VEGF, the VEGF-induced drug treated aortic rings showed a commendable inhibition in neovascularization in this assay. Since the cell of the aortic outgrowth is not undergoing any modifications or multiple passages in culture, microvessels developed in this assay are essentially indistinguishable from microvessels formed during angiogenesis *in vivo*. Further in-depth investigations are underway to unravel the molecular mechanism underlying its anti-angiogenic activities.

4. Conclusion

In summary, natural antioxidants have many important applications in health promotion, food preservation, food flavoring and cosmetics. They are preferred

over synthetic antioxidants because they are safer for consumption and more environmentally friendly. The present study, investigated the antioxidant activity, polyphenolic content and anti-cancer activity of *S. persica*. We reported here that *S. persica* extract is rich in antioxidants and in polyphenols, which merited further investigations. Herein, *S. persica* was shown to exhibit antiangiogenic and antiproliferative activities, a discovery that makes this species a promising source of anticancer agent development especially for solid tumors such as liver cancer, and hence worthy of further investigation. Isolation of active compounds and exploring their mode of action against tumors by using *in vivo* experimental models would make an important future study.

Acknowledgements

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