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ORIGINAL ARTICLE

# *Smirnovia Iranica* Whole Herb Extract: Antioxidant, Radical Scavenging, Anti-microbial and Anti-Cancer Effects

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**KEYWORDS**

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IC50

**ABSTRACT:** In this study, the biological activities of whole herb extract of a medicinal plant named *Smirnovia Iranica* were investigated. The extraction was performed using Supercritical $CO_2$ and phytochemical compounds, total phenolic content (TPC), total flavonoid content (TFC), antioxidant, anti-bacterial, and anti-cancer effect were determined in the extract. Based on the results, high TPC concentration (120.36 mg GAE/100 g FW) and TFC (17.41 mg quercetin/100g FW) were obtained in the extract. Besides, the extract showed significant antioxidant activity (IC50=53.97 µg $mL^{-1}$). Moreover, the extract showed a notable inhibitory effect against *Escherichia coli (*MIC=15.63 mg $mL^{-1}$) and *Salmonella enterica* (MIC=31.25 mg $mL^{-1}$). On the other hand, this extract showed the cytotoxic effect on glioblastoma cancer cell lines in the MTT assay. This is the first, yet comprehensive, scientific report about the chemical composition and pharmacological properties of the extract of *Smirnovia Iranica* whole herb. According to current results, the *Smirnovia Iranica* extract has excellent antioxidant properties for application as bioactive components for various objects such as as food supplements. The experiment confirmed the efficacy of the extracts as natural antimicrobials and suggested the possibility of employing them in drugs for the treatment of infectious diseases caused by the test organisms.

## INTRODUCTION

The species of *Smirnovia Iranica* belongs to the *Fabaceae* family that is known in Iran as Dome-Gavi. This herb has a wide range of traditional medicine use as immunomodulator, anti-bacterial, disinfection, anti-diabetic, anti-inflammatory agent. *Smirnovia* species are valuable native and compatible in central Iran regions' sand fields, which is very important in terms of forage production, soil protection, and creating a beautiful landscape and medicinal value [1]. The grown condition, such as temperature, humidity, etc., has significant impacts on the quantity of phenolic compounds in herbs [2]. In recent years, antioxidant, anti-bacterial, and anti-cancer properties of different herbs belong to *Fabacea* family, such as *Coronilla minima* [3], *Cytisus triflorus*

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[4], *Senna singueana* [5], *Caesalpinia pulcherrima (L.) Swartz* [6] and *Medicago sativa L.* [7] have been reported. Nevertheless, there is no report on the bioactive compounds, antioxidant, anti-microbial, and anti-cancer activity of *Smirnovia Iranica* species. The quantity and quality of phenolic compounds in plants used as traditional medician are useful for knowing their antioxidant potential.

Oxidation is a reaction that preserves energy for biological treatments in many organisms. Its products can play an essential role in phenomena like mutagenesis, carcinogenesis, autoimmune inflammatory diseases, neurodegenerative diseases, athero- sclerosis, etc. [8]. Compared with synthetic antioxidants, natural ones, acquired from natural sources, absorb more attention because of their higher safety with lower side effects [9, 10].

Therefore, the antioxidant activity of various natural compounds was considered, and most of them confirmed that total phenolic content (TPC) and total flavonoid content (TFC) of plant extracts had linear relation with antioxidant capacity [11]. Also, it is well demonstrated that antioxidant components, especially flavonoids, interfere with several signal transduction pathways in the cancer cells and thus inhibit proliferation, angiogenesis, metastasis, and increase apoptosis. Besides the antioxidant activity, phenolic compounds are also showed [12] anti-microbial [13] and anti-inflammatory activities [14]. Moreover, they had a significant effect in inhibiting cancer and heart diseases [15]. Based on the literature, consumption of fruits and vegetables can cause significantly reducing cardiovascular diseases and certain types of cancer risk [16].

Different techniques are used for the extraction of bio-components from various sources. The value and feature of extracted bio-components are directly affected by the extraction technique. Supercritical fluid extraction is a rapid and effective extraction method that is often used to extract non-polar bio components. This technique has been identified as a green sample preparation technique due to its high efficiency, environment-friendly, powerful, and speed [17].

There is valuable information on *Smirnovia Iranica* species that raise our knowledge to apply them in functional foods and as a mixture with medicines and different nutrients. The aim of this study was to evaluate the antioxidant activity, phytochemical composition, and the antibacterial and cytotoxic potential of the *Smirnovia Iranica* species which grown in Iran.

## MATERIALS AND METHODS

### *Materials*

All chemicals used were of analytical grade or higher. Gallic acid, DPPH, Vitamin C, BHT, aluminum chloride and Sodium carbonate were purchased from Sigma–Aldrich (Gillingham, UK). Whatman® cellulose chromatography papers 1 Chr sheets, (20 × 20) cm (GE Healthcare Life Sciences, UK) were used for paper chromatography.

### *Plant material and chemicals*

First Dome-Gavi whole herb (*Smirnovia Iranica*) (DGWH) picked by hand from Zanjan Province (36.6452°N 48.4599°E) in May 2020, quickly moved to a laboratory, and stored at 4 °C. After cleaning, the leaves were dried in the shade. Then a laboratory mill was used to powder the dried leaves into fine particles. Subsequently, the powder was passed through the sieve with 50 mesh, and the samples were stored at -18°C.

### *Supercritical fluid extraction*

The DGWH powder was mixed with 80% ethanol at a ratio of 1:35, w $v^{-1}$ for 24 h in a shaker at room temperature. The supercritical $CO_2$ was used to extract (system Suprex MPS / 225 Multipurpose) at 35°C and pressure of 100 bar. After 30 minutes, the obtained solution was centrifuged (10 minutes, 3500 G), and the supernatant was collected and passed through the Whatman NO. 1. Lastly, the solvent was eliminated using a vacuum evaporator. The obtained extract was freeze-dried and stored (4°C) until the next analysis.

### *Phytochemical composition analysis*

The extract's phytochemical compounds were determined using high-performance liquid chromatography apparatus (waters 2695, USA) equipped with a PDA detector (waters 996, USA). Millennium32 software was used for data acquisition and integration. The used column was a

C18-Waters (15 cm×4.6 mm). Solvent A was methanol, and solvent B was distilled water in a gradient manner when the flow rate was 1Ml $min^{-1}$. The temperature was adjusted at 25°C and the wavelength at 195-400 nm. The quantification was performed using the linear calibration curves of standard compounds [18].

### *Determination of TPC*

Total phenolic contents of the extract were determined using the Folin–Ciocalteu's reagent (FCR) method reported by Folin–Ciocalteau with some modification [19]. Briefly, 100 µL of the powdered extract was poured into a tube; later, distilled water was added until the volume reached 0.5 mL, 0.25 mL of FCR, and 1.25 mL, 20% w $v^{-1}$ of sodium carbonate was then combined with the sample. Next, it was held for 40 min at 25ºC, and subsequently, the UV–vis spectrophotometer (Shimadzu UV-VIS 1601, Japan) was used to read the absorbance of the sample 725 nm. Distilled water was used as blank. The TFC of the extract was reported as gallic acid equivalent (GAE).100$g^{-1}$ fresh weight of the herb (FW).

### *Determination of TFC*

Based on the procedure described by Wijekoon et al., (2011), the colorimetric method was employed to discover the amount of flavonoid components [20]. The reaction mixture consisted of 1 mL extract sample and 1 mL (1 mg) in 1 mL aluminum chloride. The prepared solution was mixed on a magnetic stirrer for 15 min. Subsequently, the absorbance was determined at 430 nm by spectrophotometer (Shimadzu UV-160A).

### *DPPH assay*

The DPPH assay was used to determine the antiradical activity of the DGWH extract [21]. Samples were prepared by suspending DPPH solution (1000 µL, 0.012g 100 $mL^{-1}$) at various concentrations. After incubation in the dark for 2hr, the samples' absorbance was recorded at 517 nm. The blank sample was pure methanol, and the positive control was BHT and vitamin C. The following equation was employed to calculate radical scavenging activity.

$$\%DPPH = (Ab\text{-}As/\ Ab) \times 100 \quad (1)$$

Where Ab and As are the absorbance of the control and sample, respectively.

IC50 was also calculated (extract concentration with 50% DPPH inhibition) was calculated by plotting the inhibition (%) against the extract concentration [24].

### *Anti-microbial activity*

The sensitivity of different pathogenic microorganisms into the extract was evaluated according to the Agar dilution method. The bacteria species were including both Gram-positive (*Staphylococcus aureus*, ATCC 29737) and Gram-negative (*Escherichia coli,* ATCC 25922) and *Salmonella typhi,* ATCC 13076), which purchased from the Iranian Research Organization for Science and Technology (IROST). To determine anti-microbial activity, $10^6$ CFU $mL^{-1}$ suspensions were prepared from each microorganism's new culture. Mueller-Hinton broth (MHB) as media was mixed with determined concentrations of the extract. 180µL of prepared media and 20µL of bacterial suspension were transferred to a 96-well cell culture plate. MHB alone was used as a negative control sample that confirms the sterility of media. After incubation at 37°C for 18h, the changing color of each well was determined after the addition of 20µL of trininazolium chloride solution (5 mg $mL^{-1}$) that determine the growth of microorganisms. The minimum inhibitory concentration (MIC) was recognized based on the first well without changing color. The concentration of extract in this well was considered as MIC. The wells without red color were recultured on Mueller-Hinton agar for 24h at 37ºC. The minimum concentration of the well without bacterial growth was considered as MBC [10].

### *Anti-cancer activity*

### *Cell Culture*

The rat C6 and U87 human glioblastoma cell lines were provided by the Pasteur Institute of Iran (National Cell Bank of Iran, Tehran, Iran). Cells were cultured in DMEM-F12 culture media in a monolayer manner with the addition of 10% heat-inactivated fetal bovine serum, 100 µg $mL^{-1}$ penicillin, and 100 µg $mL^{-1}$ streptomycin (Gibco, Grand Island, USA). Then, they were placed in

an incubator with a humidified atmosphere of 95% air and 5% $CO_2$ for 24 h at 37°C.

### *MTT assay*

The anti-cancer activity of different doses of DGWH extracts on glioblastoma cell lines C6 and U87 determined by the MTT assay kit (Sigma-Aldrich). Briefly, the glioblastoma cell lines were seeded at $10^4$ cells/well and cultured (24h) in 96-well plates. The cells were exposed to prepared mediums containing different concentrations of extract and then incubated for 72h. In the next step, the MTT solution was added to each well. After four h of incubation (37°C), the formazan crystals formed were dissolved in 100μL acid/alcohol (0.04NHCl in isopropanol) by mixing. A microplate reader was used to determine the optical density of samples (570nm). The evaluation of cell viability percentage was performed in comparison to the non-treated cell. Cytotoxic compounds can damage and destroy cells, thus decreasing the reduction of MTT to formazan.

### *Statistical analysis*

Results were expressed as the mean ± standard error of mean (SEM). The data generated from quantitative assays for phytochemicals were subjected to ANOVA using SAS software (the SAS system for windows 9.0, English). Comparison among mean values was made by Least Significant Difference (LSD) to test significant differences at $P < 0.05$. Linear regression analysis was used to calculate $IC_{50}$ values. Linear correlations were analyzed by using regression in R software.

## RESULTS AND DISCUSSION

### *TPC and TFC*

Phenolic compounds have an essential role in the color, flavor, and taste of the food product. Moreover, they show good anti-microbial and antioxidant properties and can denature enzymes [22]. Another functional property of the Phenolic compounds is binding to the substrate, such as minerals, vitamins, and carbohydrates, and preserves them from microorganism's activities [23].
Chemical composition analysis of the DGWH extract by HPLC is presented in Table 1.

**Table 1.** Major phenolic and flavonoid compounds of DGWH extract analyzed by HPLC.

| Compound | Concentration (mg/100 g DE*) |
|---|---|
| Phenolic compounds | |
| **2,5-Dihydroxybenzoic acid** | 24.71 |
| **chicoric acid** | 81.42 |
| **Rosmarinic Acid** | 14.23 |
| Flavonoid compounds | |
| **Rutin** | 12.29 |
| **Apigenin** | 5.12 |

These result revealed that the phenolic and flavonoid compounds in the concentration of mg/100 g FW and matched with the standard as analyzed by HPLC were Rutin (12.29), Apigenin (5.12), 2,5-Dihydroxybenzoic acid (24.71), chicoric acid (81.42), Rosmarinic Acid [14.23]. The estimated amount of TPC in 100 g of DGWH extract was about 120.36 mg.
The TPC of DGWH extract were close to whole-plant extracts of *Torilis leptophylla L.* (121.9 mg GAE/100 g FW) [24], higher than *Dysphania ambrosioides* (L.) (87.96 mg GAE/100 g FW) [25], pomegranate (11.62-21.03 mg GAE/100 g FW) [26], Bouhattam (52.69 mg GAE/100 g FW) [27], raw apple honey (9.9 mg GAE/100 g FW) [28] and Basil (60 mg GAE/100 g FW) [29]. It also showed a lower TPC value comparable to that of red onions (310 mg GAE/100 g FW) [30], *A. sessilis* (292 mg GAE/100 g FW) [31], and *Phoenix dactylifera L.* (178 mg GAE/100 g FW) [32]. Results suggest that this extract can act as a natural source of antioxidants for preventing oxidation Due to the considerable quantities of phenolic compounds. These findings may give valuable insights for the producers who provide foodstuff which exposed to oxidation.
Flavonoids can act as excellent free radical scavengers in oxidation conditions, so they attract attention [33]. Furthermore, flavonoids can act in various areas, such as

anti-cancer, anti-inflammatory, anti-allergic anti-oxidative agents [34]. The total extracted phenolic and flavonoid content of plants is generally affected by factors such as genotype, agronomic practices, harvesting time, postharvest conditions, and arid climatic conditions, and the solvent's polarity [2, 35]. The Total flavonoid content for DGWH (17.41 mg quercetin/100 g FW) was higher than thyme (4.10 mg quercetin/100 g FW) [36] and Parsley (14.1 mg quercetin/100 g FW) [29] while lower than *Cinnamon zeylanicum* (62.1 mg quercetin/100 g FW) [37] and mulberry (250.1 mg quercetin/100 g FW) [30].

***Antioxidant activity***

The antioxidant activity of different components is commonly determined by The DPPH assay [38]. The results showed a clear trend between the concentration and antioxidant activity of DGWH extract (Table 2). IC50 is a suitable indicator for determining the antioxidant activity of different extracts. It must be mentioned that IC50 is related to antioxidant activity reversely so, high IC50 leads to low antioxidant activity. The DGWH extract had higher IC50 compared to BHT and vitamin C. It may be due to the lower concentration of components with antioxidant activity in DGWH extract. Similar to the results previously reported by Noreen et al. (2017) [39].

**Table 2.** The obtained $IC_{50}$ (μg $mL^{-1}$) of DGWH extract.

| Sample | $IC_{50}$ (μg $mL^{-1}$) |
|---|---|
| **DGWH extract** | 23.02 |
| **Vit. C** | 9.04 |
| **BHT** | 12.98 |

Taherkhani (2017) also studied the antioxidant activity of Artemisia Aucheri and reported that the IC50 values of this herb growing wild in Iran were 15.7 mg $mL^{-1}$, which is much higher than DGWH extract [40].

***Anti-microbial activity***

The MIC and MBC assessment results of the DGWH extract are presented in Table 3. As a negative control, Tween 80 did not show any bactericidal and inhibitory effects on both spoilage and pathogen bacteria. *Esherchia coli* ATCC 25922 were the most sensitive species against DGWH extract at concentrations lower than the synthetic antibiotic. Compared to the extract, both Gentamicin and Rifampin are used as the reference drug showed a more effective bactericidal effect. DGWH extract in a concentration of 250 mg $mL^{-1}$ could prevent the growth of *Staphylococcus aureus* ATCC 29737. In contrast, for inhibition of *Staphylococcus aureus* ATCC 29737 higher amount of the extract (1000 mg $mL^{-1}$) was needed. The obtained MBC for *Salmonella enterica subsp. Enterica* ATCC 13076 was 31.25 mg $mL^{-1}$.

**Table 3.** Anti-bacterial activity of DGWH extract

| Microorganisms | MIC (mg $mL^{-1}$) | | | MBC (mg $mL^{-1}$) | | |
|---|---|---|---|---|---|---|
| | DGWH extract | Antibiotic control | | DGWH extract | Antibiotic control | |
| | | Gentamicin | Rifampin | | Gentamicin | Rifampin |
| ***Esherchia coli* ATCC 25922** | <15.63 | 62.5 | 31.25 | <15.63 | 62.5 | 31.25 |
| ***Staphylococcus aureus* ATCC 29737** | 250 | 3.9 | 62.5 | 1000 | 54.3 | 71.5 |
| ***Salmonella enterica subsp. Enterica* ATCC 13076** | 31.25 | 62.5 | 62.5 | 62.50 | 145 | 62.5 |

The anti-microbial mechanism of extracts is mostly related to their ionic strength. Gonelimali, Lin [41] stated that the extracts' presence could start a decrease in cytoplasmic pH. These phenomena destroy the structure and integrity of the bacterial cell membrane and finally dead the cell. Sun, Hao [42] inscribed that the anti-bacterial activity of the blueberry extract is associated with cells' death because of the role of extract in inhibited gene transcription to disrupt cell membrane structure and energy transport.

Many studies confirmed the anti-microbial activity of phenolic compounds, like Pandey's work on *Thalictrum foliolosum*. Aunjum, Biswas [43], Oscar, Antonio [44], and Hadadi, Nematzadeh [45] also observed similar experimental behavior for three herbs belonging to *Zingiber genus*, *Hyptis suaveolens (L.)* and six medicinal plants collected from Iran extracts.

The anti-microbial mechanism of phenolic compounds is not elucidated, while their activity depends on concentration [46].

***Anti-cancer activity***

Herbal plants are potentially useful candidates for treating cancer; they can synthesize a wide diversity of chemical compounds that may influence biological functions. Furthermore, the historically asserted chemo-preventive properties of aromatic plants are partially attributed to their volatile extracts. These extract contain a wide variety of active phytochemicals, such as flavonoids, monoterpenes, and polyphenols, among many others [21]. In this study the effect of extract of *Smirnovia Iranica* was investigated with a cancer cell line. Following cell culture with the extract, the cells were examined by MTT assay and results checked for consistence with a microscopic study.

A quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells is MTT Assay. Based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-y1)-,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product insoluble in water.

In this study, DGWH extract's effect on cell viability in glioblastoma cancer C6 and U87 cells were evaluated using the MTT assay. The result showed treatment with DGWH extract at the dose of 31.25 to 1000 µg significantly inhibited both C6 and U87 cells (Figure 1).

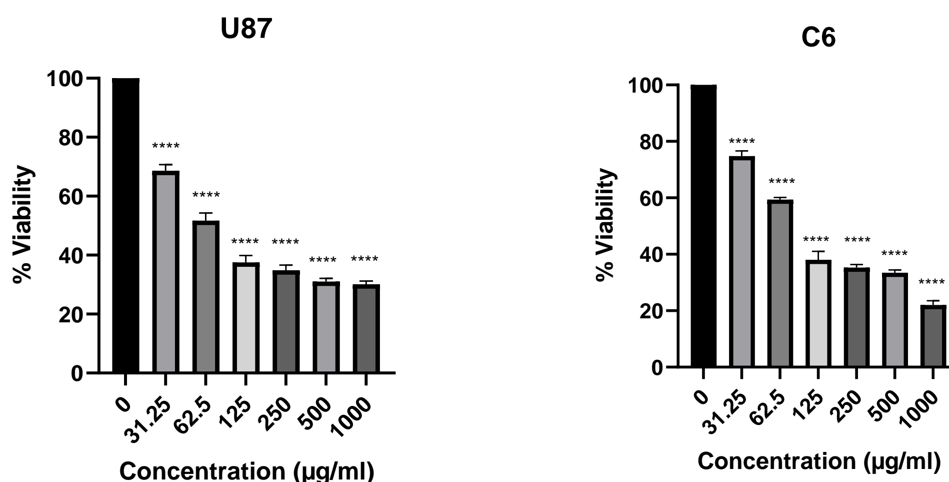


**Figure 1.** Treatment with DGWH extract at the dose of 31.25 to 1000 µg, C6 and U87 cells.

Glioblastoma is the most malignant brain tumor for which effective therapies are not available because of tumor heterogeneity, drug resistance, and blood-brain barrier complications [47]. The application of natural compounds and food supplements which have potential anti-cancer properties attract more research interest in recent years [48]. Our study revealed that DGWH extract contains antioxidant components such as Flavonoid and phenolic compounds. Previous studies have determined that these compounds significant anti-cancer activities due to the cytoprotective role against oxidative stress [49]. Oxidative stress is a physiological situation

wherein high levels of reactive oxygen species (ROS) and free radicals are produced. ROS can trigger several signaling pathways associated with the carcinogenesis process and damage DNA [50].

## CONCLUSIONS

As expected, our experiments demonstrate that DGWH extract shows a sufficient value of phenolic and Flavonoid compounds. The extracts also showed adequate antioxidant and anti-microbial activity. There is a satisfactory agreement between the extract's phenolic and flavonoid content and anti-microbial and antioxidant activity. Also, it was proposed that DGWH extract protects cells from free radical damage through antioxidant effect and inhibits tumorigenesis. Overall, these results would seem to suggest that DGWH extract is a new source of phenolic and flavonoid compounds that had great potential to use in different pharmaceuticals approaches.

### *Study limitations*

The present study limitations included small sample size, short term period for pick up the *Smirnovia Iranica* herb, the high distance between lablatory and location of sampleing, lack of control and consideration of other factors that can be effective in quality and quantity of phenolic componds in the herbs.

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## ETHICAL CANSIDERATION

The study project had approved by the research ethics committee of Semnan University of Medical Sciences (approval ID: IR.SEMUMS.REC.1397.292).

### *Conflict of interest*

The authors declared no potential conflicts of interest concerning the research, authorship, and publication of this article.

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