Callus culture, phytochemical analysis and antioxidant and cytotoxic effects of marjoram (*Origanum majorana*)

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Received: 10 March 2023
Revised: 30 March 2023
Accepted: 29 April 2023
Published: 20 November 2023

Egyptian Pharmaceutical Journal 2023, 22:545–550

Background and aim
Oxidative stress plays an essential role in the pathogenesis of several chronic diseases, including cancer, diabetes, and multiple sclerosis. Phenolic compounds are a group of plant-derived antioxidants used to treat or prevent cancer. Marjoram is an aromatic and medicinal plant found in Mediterranean countries and is used in traditional medicine. The aim of this study was to produce callus cultures, determine the in vitro antioxidant activity, total phenol and flavonoid contents, and evaluate the cytotoxic effects of marjoram extracts.

Materials and methods
Leaf, root, and stem explants were cultured in medium supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid to induce callus formation. Antioxidant activity was evaluated using a 1,1-diphenyl-2-picrylhydrazyl assay. Total flavonoid and phenolic contents were measured using colorimetric assays. An MTT assay was used to estimate the cytotoxic effects of the extracts on a hepatocellular carcinoma cell line.

Results and conclusion
Leaf explants were the most promising for callus induction. The highest frequencies of callus induction were obtained with 2,4-D at 0.25 mg/L for leaf and root explants and 0.5 mg/L for stem explants. The maximum amount of extractable phenolic and flavonoid compounds was observed in the acetone extracts. The acetone extracts had higher cytotoxic effects than the methanol extracts, but there was no significant difference in antioxidant activity. These findings support the potential of marjoram as a resource for antioxidant and anticancer agents, and provide sufficient scientific background to isolate and purify bioactive compounds for further applications.

Keywords:
anticancer, antioxidant, callus culture, marjoram, total flavonoid content, total phenolic content

Egypt Pharmaceut J 22:545–550

1687-4315

Introduction

Cancer is one of the most serious challenges in today's world. It is a major source of morbidity and mortality throughout the world. In fact, cancer is the second leading cause of death around the globe and its severity is continuing to accelerate [1]. This frightful disease is responsible for one in eight deaths worldwide – more than AIDS, tuberculosis, and malaria together [2]. Cancer is a broad term that describes a group of illnesses including abnormal cell growth with the potential to invade other tissues. Among the over 100 types of cancer affect humans, liver cancer is the sixth most commonly diagnosed cancer and the third leading cause of cancer death [3]. Despite considerable efforts in the last few decades on cancer research, treatments of cancer remain too aggressive to patient health. Today, primary treatment methods such as surgery of tumor, chemotherapy and radiotherapy often accompanied by sever side effects that significantly reduce the quality of life. Moreover, the toxicity of some treatments restricts their use and efficiency. Therefore, there is an inevitable need to discover and develop new compounds for use as anticancer agents with lower toxicity to normal cells and safer side effects.

Oxidative stress-related diseases are another major problem worldwide. It is increasingly being realized that many of today's diseases such as stroke, diabetes, arthritis, autoimmune disorders, cardiovascular diseases and neurodegenerative diseases are associated with the oxidative stress that results from an imbalance between the formation and neutralization of prooxidants [4]. These excess free radicals cause oxidative damage to various biological macromolecules such as proteins, lipids and DNA and this leads to ultimately promote cellular damage and death [5]. The toxicity of free radicals is balanced by the antioxidants

which can protect the biological systems by removing or quenching the free radicals (prooxidants).

Natural bioactive compounds, including those in marjoram, exhibit antioxidant, antimicrobial, and anticancer effects [6,7], have therapeutic potential [8,9], and are in high demand [10]. Although marjoram can be propagated using stem cuttings, poor rooting lowers its survival efficacy [11].

In vitro cell culture techniques enable medicinal plant propagation [12,13], that result in large numbers [14,15] of pathogen-free plants with desirable characteristics using transgenic technologies [16–18], and provides a platform for in vitro bioactive compound production [19]. The aim of this study was to produce callus culture, perform phytochemical analysis, and investigate the antioxidant and anticancer properties of marjoram.

Materials and methods

Surface sterilization of seeds and explant preparation

Marjoram was washed with running water, surface-disinfected with 70% ethanol for 1 min, and soaked in 10% NaOCl for 10 min The seeds were then washed four times with sterile distilled water and allowed to dry on sterile filter paper. Aseptic seeds were cultured on Murashige and Skoog (MS) medium [20] without phytohormones and allowed to germinate. The medium was fortified with 3% sucrose and solidified on 0.7% agar. The pH of the culture medium was adjusted to 5.8 before adding the gelling agent, followed by autoclave sterilization at 121°C for 25 min The cultures were incubated at 25±2°C with a light/dark cycle of 16 h/8 h under white fluorescent light. Leaves, stems, and roots (0.5–1 cm in length) from 2–3-week-old seedlings were used as explants.

Callus induction

All three explant types were cultured separately on MS medium fortified with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.25, 0.5, 0.75 and 1.0 mg/L). The medium composition and culture conditions were the same as those described above. After four weeks, explants with successfully induced calli were scored.

Preparation of extracts

Fresh callus originated from either leaves, stems or roots were dried in oven at 40°C, for 1 d. The three dried sample types were ground into fine powders. Methanol and acetone were used as the solvents to prepare the extracts. Five grams of each of the three samples was drenched separately in 50 ml of each solvent and maintained in a shaker for 2 d. The obtained mixtures were filtered through Whatman filter paper No. 1. The filtrates were evaporated to near dryness and the outcome viscous powders were resolved in the same solvent. The obtained extracts were stored in the dark at 4°C.

Total phenolic content

The total phenolic content of the extracts was measured using the Folin–Ciocalteu method, as described previously [21]. The absorbance was read at 765 nm. The quantity of total phenolic compounds was determined from a gallic acid standard curve and expressed as mg gallic acid equivalents/100 g fresh weight (mg GAE/100 g fresh weight).

Total flavonoid content

Total flavonoid content was determined using the aluminum chloride colorimetric method as previously described [22]. Absorbance was read at 420 nm and the flavonoid content was determined from a quercetin standard curve and expressed as mg quercetin equivalents/100 g fresh weight basis (mg QE/100 g fresh weight).

Cell lines and culture

The hepatocellular carcinoma cell line Huh7 was gifted from C.M. Rice, Rockefeller University, NY, USA and kept in a humidified cell incubator containing 5% CO_2 and 95% air at 37°C with Dulbecco's Modified Eagle's Medium (DMEM) fortified with 1% penicillin-streptomycin, 1% non-essential amino acids, and 10% fetal bovine serum (FBS).

MTT assay

Viability and proliferation were evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-+0 diphenyltetrazolium bromide] test [23]. Huh7 cells were seeded into 96-well plates at a density of 2×10^4 cells/well. Cells were incubated in a humidified cell incubator containing 5% CO_2 and 95% air at 37°C with DMEM medium supplemented with 1% penicillin-streptomycin, 1% non-essential amino acids, and 10% FBS for 24 h. After incubation various concentrations of extracts (100, 50, 25, and 12.5 μg/ml) were added as treatment in addition to cisplatin as a positive control. The plates were further incubated for 48 h, the media were renewed, and the cells were incubated with 30 μl of MTT solution at 5 mg/ml for 4 h at 37°C in a humidified CO_2 incubator. Addition of 100 μl dimethyl sulfoxide (DMSO) was added to each well by pipetting for color development, and the plates were incubated for

Table 1 Effect of different concentrations of 2,4-D on the frequency of callus induction from various explants of marjoram

	Callus induction frequency (%)		
Concentration of 2,4-D (mg/L)	Leaf	Root	Stem
0.00	0.00	0.00	0.00
0.25	96.67±3.33 A-a	83.33±6.67 A-b	60.00±10.00 B-c
0.50	83.33±3.33 B-a	63.33±3.33 B-b	83.33±6.67 A-a
0.75	80.00±5.77 B-a	60.00±5.77 B-b	66.67±8.82 B-b
1	66.67±6.67 C-a	46.67±3.33 C-b	63.33±8.82 B-a

Values were represented as mean ± standard error of three independent experiments. Values accompanied with the same capital letters within a column or the same lowercase letters within a row are significantly different ($P<0.05$). 2,4-D: 2,4-dichlorophenoxyacetic acid.

10 min Finally, the absorbance was read at 490 nm using a microplate reader.

Statistical analysis

All experiments were conducted in triplicate, and the data are presented as the mean values ± standard errors of triplicate measurements. Statistical analysis of the data was performed by Analysis of Variance (ANOVA) using IBM SPSS statistics subscription software, and a probability value of $P<0.05$ was considered to represent a statistically significant difference among mean values.

Results

Callus induction

The percentages of cultures of each explant source showing calli in MS medium fortified with and without 2,4-D are summarized in Table 1. All explants cultured on MS medium without 2,4-D served as controls and did not induce callus formation. 2,4-D is indispensable for callus induction. Callus induction frequency ranged from 46.67±3.33 to 96.67±3.33. Among the different concentration of 2,4-D used, the best callus induction was seen at the lowest 2,4-D concentration (0.25 mg/L) used for leaf and root explants. The percentage of cultures showing callus induction decreased with an increase in 2,4-D to 1.00 mg/L. Stem explants showed the maximum frequency of callus induction in MS medium fortified with 0.50 mg/L of 2,4-D. After four weeks, callus formation was observed in all the 2,4-D treatment groups and explant types, although the percentage of callus formation varied significantly. ANOVA showed a significant effect of explant type on callus induction. Leaf explants showed a higher ability to induce callus formation than that of root and stem explants.

Total phenolic and flavonoid contents

The total phenolic and flavonoid content of the extracts were detected spectrophotometrically and presented as gallic acid (mg GAE/100 g fresh weight) and quercetin (mg QE/100 g fresh weight) equivalents, respectively (Tables 2 and 3). The total phenolic content of the extracts ranged between 110.00±5.77 to 216.67 ±6.67 mg of GAE/100 g of extract (Table 2). The maximum amount of extractable phenolic compounds was observed in the acetone extracts compared with that of the methanol extracts. Using the same extraction solvent, there were no significant differences between callus extracts originating from different explants.

Table 2 Effect of different solvent extracts on total phenolic content of marjoram

	Total phenolic content (mg GAE/100 g fresh weight)	
Samples	Methanol extract	Acetone extract
Leaf derived callus	133.33±8.82 A-a	213.33±6.67 A-b
Root derived callus	110.00±5.77 A-a	216.67±6.67 A-b
Stem derived callus	116.67±8.82 A-a	210.00±5.77 A-b

Values were presented as mean ± standard error of three independent experiments. Values accompanied with the same capital letters within a column or the same lowercase letters within a row are significantly different ($P<0.05$). GAE: gallic acid equivalents.

Table 3 Effect of different solvent extracts on total flavonoid content of marjoram

	Total flavonoid content (mg QE/100 g fresh weight)	
Samples	Methanol extract	Acetone extract
Leaf derived callus	30.00±5.77 A-a	106.67±3.33 A-b
Root derived callus	33.33±6.67 A-a	103.33±3.33 A-b
Stem derived callus	26.67±3.33 A-a	96.67±8.82 A-b

Values were represented as mean ± standard error of three independent experiments. Values accompanied with the same capital letters within a column or the same lowercase letters within a row are significantly different ($P<0.05$). QE, quercetin equivalents.

The concentrations of flavonoids varied from 26.67 ±3.33 to 106.67±3.33 mg of QE/100 g of extract (Table 3). Similar to phenolic content, the acetone extracts contained higher levels of flavonoids than that of the methanol extracts. In addition, within the same extraction solvent, there was no significant difference in the total flavonoid content of callus extracts

Table 4 Antioxidant activity of different extracts of marjoram

	1,1-Diphenyl-2-picrylhydrazyl inhibition (%)	
Samples	Methanol extract	Acetone extract
Leaf derived callus	86.33±2.73 A-a	87.00±2.08 A-a
Root derived callus	85.00±2.31 A-a	84.67±3.38 A-a
Stem derived callus	84.67±2.60 A-a	83.33±2.96 A-a
Ascorbic acid	91.67±1.67	

Values were represented as mean ± standard error of three independent experiments. Values accompanied with the same capital letters within a column or the same lowercase letters within a row not significantly different ($P<0.05$).

Table 5 Cytotoxic effect of different extracts of marjoram on the human hepatoma-derived HuH7 cell line

	Cytotoxicity assay (IC50, μg/mL)	
Samples	Methanol extract	Acetone extract
Leaf derived callus	320.00±11.55 A-a	183.33±14.53 A-b
Root derived callus	353.33±18.56 A-a	210.00±15.28 A-b
Stem derived callus	333.33±12.02 A-a	216.67±17.64 A-b
cisplatin	56.67±6.67	

Values were represented as mean ± standard error of three independent experiments. Values accompanied with the same capital letters within a column or the same lowercase letters within a row are significantly different ($P<0.05$).

originating from the three types of explants (leaf, root, and stem).

Antioxidant potential

The DPPH free radical-scavenging activities of various marjoram extracts are shown in Table 4. Each extract showed a distinct antioxidant activity compared to the other extracts. The percentages of radical-scavenging activities of the extracts ranged between 87.00±2.08 to 83.33±2.96. All extracts exhibited antioxidant properties and the ability to scavenge free radicals. Statistical analysis showed no significant differences between the methanol and acetone extracts, indicating similar antioxidant activities. This activity was slightly less than that of the reference antioxidant ascorbic acid (91.67±1.67).

Cytotoxic effects

The cytotoxic activity of the six marjoram extracts against the human cancer cell line Huh7 was investigated using the MTT assay. The 50% inhibitory concentration (IC50) of each extract was determined and presented in Table 5. The results showed that all extracts reduced cell viability in a dose-dependent manner after 48 h of incubation. The cytotoxicity of acetone extracts against Huh7 cell line in terms of IC50 values ranged from 183.33 ±14.53 to 216.67±17.64, and were notably stronger than those of methanol extracts. Cisplatin was used as a positive control, and its IC50 value was 56.67±6.67. Analysis of variance ($P<0.05$) showed no significant difference between callus origins in both the methanol and acetone extracts.

Discussion

In the present study, we observed that all three types of explants promoted callus formation to varying degrees (Table 1), implying that different types of explants are suitable for callus induction in marjoram. It was also noted that leaf explants showed the highest frequency of callus induction compared with both root and stem explants. These varied effects could be attributed to differences in endogenous hormone levels. Different parts contain different growth regulators at different levels, which may have varying effects on calli [12]. Callogenesis is affected by multiple factors and is mainly controlled by the balance between endogenous and exogenous growth regulators [24]. This might also be a possible explanation of another observation that there were differences in the optimum concentration of 2,4-D required for maximum callus induction for the explants used. While 0.25 mg/L 2,4-D was optimum for the callusing of leaf and root explants, maximum callus induction was obtained for stem explants when 0.5 mg/L 2,4-D was used. Consistent with our results, numerous studies have demonstrated the superiority of 2,4-D over other auxins in callus induction in several plant species [25,26]. However, depending on the species or explant, the concentration is a critical factor controlling the maximum frequency of callus induction.

The total phenolic and flavonoid contents of the samples were quantified spectrophotometrically (Tables 2 and 3). Our results were quantitatively different from those of previous studies [27,28]. Phenolic content may differ, as various plant parts possess different amounts of bioactive compounds. In addition, the geographical origin of the species, drying time, and extraction method may also have direct effects on their contents. Sometimes, agroclimatic conditions are solely responsible for the existence of different amounts of bioactive compounds in natural resources [29].

Solvent type may also influence the phenolic content of the extracts. Solvents differ in their extraction abilities relying on their polarity and chemical structure. Different solvent extracts have different amounts of soluble phytoconstituents; hence, they have varying phenolic contents [5–8]. In this study, both types of

solvent extracts contained considerable amounts of phenolic compounds, but the acetone extracts contained much higher amounts than the methanol extracts. This suggests that most phenols and flavonoids in marjoram are soluble in acetone. The varying solubilities of phenolic compounds in different solvents can be explained by solvent polarity [28]. Moreover, phenolics may be associated with other plant metabolites such as carbohydrates and proteins.

The antioxidant activity of the marjoram extracts was evaluated based on the scavenging activity of DPPH radicals (Table 4). Our results showed that all marjoram extracts had the same ability to scavenge free radicals, and consequently, the same antioxidant power. Interestingly, these results were unexpected, as there is sufficient proof to support that the antioxidant property of plant materials is well correlated with the content of phenolic compounds [30–32], and our results indicate a statistically significant higher content of total phenolics and flavonoids in acetone extracts compared to that in methanol extracts. This suggests that other phytoconstituent(s) are extractable in methanol and are responsible for its antioxidant properties. Although DPPH is one of the most commonly used methods to detect the presence of antioxidant potential in extracts, a single assay is not sufficient to quantify antioxidant activity owing to the complex nature of bioactive compounds in natural extracts.

The MTT assay is a sensitive, simple, and reliable tool for investigating the cytotoxicity of plant-based products [33]. The cytotoxic effects of marjoram extract on the human cancer cell line HuH7 are presented in Table 5. The results revealed prominent cytotoxicity against the cancer cell line, especially with the acetone extracts. Our results agree with those of previously reported investigations that highlighted the cytotoxic effect of marjoram against various types of cancer cells, such as fibrosarcoma, leukemia, and lung cancer cells [26,34–36].

Acknowledgements

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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