Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(1): 1251-1255
Received: 21-11-2018
Accepted: 25-12-2018

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Quantitative analysis of primary and secondary metabolites of ethanol seed extract of *Origanum majorana* (Marjoram)

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Abstract
The present study was carried out to investigate the phytochemical profile of the seed extract of marjoram (*Origanum majorana*) an important ethnomedicinal plant. The seed sample of the plant was collected, washed, shade dried and powdered. The powder was successively extracted with ethanol by soxhalation method. The ethanolic seed extract was used for the qualitative and quantitative analysis to find out the phytochemical constituents in seed extract of plant. The qualitative analysis showed the presence of flavonoids, tannins, saponins, sterols, alkaloids, proteins, lipids and carbohydrates in the seed extract. The Quantitative analysis of total flavonoids (aluminium chloride colorimetric assay), total phenols (Folin ciocalteau's reagent method), lipid (rapid method) carbohydrate (anthrone method) and protein (Lowry's method) was evaluated. The results revealed the presence of total flavonoids (104.89±8.96 mg/g dry weight), total phenol (39.82±1.57 mg/g dry weight), proteins (36.83±0.01mg/g dry weight), carbohydrate (31.80±0.05 mg/g dry weight) and lipid (10.27±0.39 mg/g dry weight) respectively in seed extract. The crude seed extract from marjoram showed high concentrations of flavonoids which can be used as antibiotic for several diseases. Also, the richness in phenol in this plant can be correlated with its antioxidant property and could be a good source of natural antioxidant. The finding of this study gives the green line that traditional use of marjoram has been credited with medicinal properties.

Keywords: phytochemicals, primary metabolites, secondary metabolites, ethanol seed extract, marjoram

Introduction
The presences of phytochemical constituents in medicinal plants are useful for human health (Bargah, 2015) [1]. These Phytochemicals have medicinal activity in plants. Phytochemicals have two classes that are primary and secondary metabolites. Carbohydrates, amino acids, proteins, lipid and chlorophylls are included in primary metabolites while secondary metabolites consist of alkaloids, saponins, steroids, flavonoids, tannins and phenol (Kalimuthu and Prabakaran, 2013) [10]. Phytochemcals now have been able to isolate, identify and characterize about 70,000 chemical substances synthesized by plants (Hency, 2014) [7].
Marjoram (*Origanum majorana*) plant is an herbaceous plant and it does belong to the family *Lamiaceae*. It is also known as Sweet marjoram. It is commonly found in overall India. The genus *Origanum* has around 900 different species. It is used as a good home made medicine for chest infection, sore throat, rheumatic pain, nervous disorder, cardiovascular diseases, epilepsy, insomnia, skin care, flatulence and stomach disorder and also used as flavouring agent in food products. Recently antimicrobial, antimutagenic, antihyperglycemic, antilipidemic and antiulcer activites were identified in this plant (Tripahty *et al.*, 2016) [18]. Fungistatic and fungicidal effect of essential oil of this plant has been reported on *Verticillium dahliae* and *Penicillium aurantiogriseum*; well-known disastrous fungi (Rus *et al*, 2015) [14].

Materials and Methods
The seed of *Origanum majorana*, locally known as as marjoram was collected from Jaipur, Rajasthan during the month of May. The identification of plant was confirmed from Herbarium, Department of Botany, University of Rajasthan, Jaipur. The seeds of marjoram were washed thoroughly and shade dried for a week at room temperature. The sample was converted in powder form finely by using mortal pestle and mixer grinder and extracted with ethanol using Soxhlet method, for 12 hours at 25°C. The extract was filtered and filtrates were evaporated in a hot rotating water bath until dry and kept at - 4 °C for further use.

Qualitative phytochemical analysis of plant extract
Different qualitative chemical tests were performed for establishing profile of given extracts

for its chemical composition. The ethanolic extract was reported for the presence of various phytoconstituents such as carbohydrate, alkaloids, protein, flavonoid, glycosides, sterols, saponins, phenolic compound, tannins and flavonoids. All tests were done as per the standard procedure.

Test for alkaloids
10 mg extract of seed was taken and few drops of Wagner's reagent were added and the formation of a reddish brown colour indicates the presence of alkaloids (Geetha and Geetha, 2014) [4].

Test for protein
Seed Extract was treated with few drops of concentrated HNO_3, formation of yellow indicates the presence of proteins (Godghate *et al*., 2012) [5].

Test for reducing sugar
1 ml of Fehling's solution was added to 0.5 mg of extract and boiled in a water bath. The formation of brick red precipitate indicates the presence of reducing sugars (Geetha and Geetha, 2014) [4].

Test for flavonoid
10 mg of extract was taken and few drops of 10% lead acetate solution were added. Appearance of yellow precipitate indicates the presence of flavonoids (Geetha and Geetha, 2014) [4].

Test for Saponin
5 ml of extract was diluted with 20 ml distilled water and shaken well in a test tube for 15 min. The formation of foam to a length of 1cm indicated the presence of saponins and steroids (Geetha and Geetha, 2014) [4].

Test for glycoside
0.5 mg of extract was dissolved in 1 ml of water and then aqueous NaOH solution was added. Formation of yellow color indicates the presence of glycosides (Geetha and Geetha, 2014) [4].

Test for steroids and sterols
5 mg of extract was dissolved in 2 ml of chloroform and equal volume of concentrated sulphuric acid was added along the sides of the test tube. The upper layer turns red and lower layer turns yellow with green fluorescence, indicating the presence of the steroids and sterols compound, in the extract (Geetha and Geetha, 2014) [4].

Test for Tannins
5 mg of extract was taken and 0.5 ml of 5% ferric chloride was added. The development of dark bluish black color indicates the presence of tannins (Geetha and Geetha, 2014) [4].

Test for phenol
The extract is treated with 5ml of water and 5% aqueous ferric chloride solution. The Blue or green coloration is observed (Kokate, 1994) [11].

Quantitative analysis of phytochemical constituents
Determination of total phenolic compounds (McDonald *et al*., 2001) [13].
Total Phenolic Content was estimated using Folin-Ciocalteu reagent method with modifications. 1 ml of plant extract was mixed with 5ml Folin-Ciocalteu reagent and 7% Sodium carbonate solution in Distilled Water. The mixtures were allowed to stand for 30 min. at room temperature. The absorbance was measured at 760 nm using UV-visible spectrophotometer against a blank which was composed of the same reagents except test extract. The standard calibration curve was prepared using 1.0, 1.5, 3.0, 6.0, 12, 24 µg/ml solutions of Gallic Acid in methanol (R^2 = 0.998). Total Phenolic content in the seed extract was expressed as Gallic Acid Equivalents (mg of GAE/g dry weight of extract) which is a common reference compound.

Detemnination of flavonoid content (Woisky and Salatino, 1998) [20].
The aluminium chloride colorimetric method was modified from the procedure reported by Woisky and Salatino. Quercetin was used to make the calibration curve. 10 mg of quercetin was dissolved in 80% ethanol. The standard solution and sample (0.5ml) were separately mixed with 1.5 ml of 95% ethanol. 0.1 ml of 10% aluminium chloride was added to all tubes. Blank was prepared by using distilled water instead of sample. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm.

Determination of lipid (Jayaraman, 1980) [9].
The test samples were dried, powdered and 100mg was macerated with 10 mL distilled water, transferred to a conical flask containing 30 mL of chloroform and methanol (2/1:v/v). This mixture was evenly mixed and left for overnight incubation at RT in dark for over all extraction. Further 20 mL of chloroform (2 mL of water added) was mixed with it and centrifuged, now the 2 layers were separated. The lower layer contained all the lipids (in chloroform), was collected in the pre-weighed glass vials. The colored layer separated in methanol contained all the water soluble materials. The thick medium layer was discarded in each test sample. The chloroform layers dried in vacuo and weighed. Each of this treatment was repeated thrice and the mean values were calculated.

Carbohydrate estimation (Hedge and Hofreiter, 1962) [8].
Carbohydrate content was estimated by the anthrone method. Taken 1ml of sample was mixed with 4ml of anthrone reagent. It was then incubated in boiling water bath for 8 minutes and the absorbance was read at 630 nm against a reagent blank. The estimation was done in triplicates and the results were expressed as mg/gdw sample.

Estimation of protein (Lowry *et al*., 1951) [12].
Protein content was estimated by the Lowry method. 2 ml of the sample was taken and added 5ml of freshly prepared alkaline copper reagent was added and after 10 minute incubation on room temparture, Then add, 0.5ml of Folin – Ciocalteau reagent. Mixed well and incubated at room temperature for 30 minute in dark. Reagent blank was also prepared. The blue colour developed was read at 660 nm.

Results
In the present study, qualitative analysis of ethanolic seed extract of Marjoram revealed the presence of phytochemical constituents such as alkaloids, phytosterols, glycosides, tannins and phenolic compounds, flavonoids, saponins, carbohydrates and Protein were present in ethanolic seed extract of Marjoram (Table 1).

Table-2 and Figure- 3 showing the quantitative study of primary metabolites reveals various chemical constituents and table-3 and fig.- 6 also showing the quantitative study of secondary metabolites reveals various chemical constituents present in the plant.

The amount of protein was determined with Lowry method using BSA as a standard compound and the protein was expressed as mg/ml BSA equivalent using the standard curve equation: y = 0.007x - 0.007 (R^2 = 0.996) (Figure 1). where y is absorbance at 660 nm and x is protein amount in the seed extracts of Marjoram was 36.83 mg/gdw (Fig. 3).

The amount of carbohydrate was determined with anthrone method. Glucose was used as a standard compound and the carbohydrate was expressed as mg/ml glucose equivalent using the standard curve equation: y = 0.003x - 0.108 R^2 = 0.989 (Fig. 2); where y is absorbance at 630 nm and x is carbohydrate amount in the seed extract of Marjoram was 31.80 mg/gdw (Fig. 3).

The lipid content was determined with chloroform and methanol. The lipid was found 10.27 mg/gdw in ethenolic seed extract of marjoram (Fig. 3).

Table 1: Preliminary phytochemical screening of Marjoram seed extract

S. No.	Phytochemical Components	Results
1	Alkaloid	++
2	Protein	+++
3	Carbohydrate	+++
4	Flavonoid	+
5	Saponin	+
6	Glycoside	+
7	Steroids and Sterol	+++
8	Tannin	+++
9	Phenol	+++

(+ ++) Heavy Present, (++) Medium Present, (+) Slight Present, (-) Absent.

Table 2: Determination of Primary metabolites

S. No	Primary metabolites	Quantity (mg/g)
1	Protein	36.83 ± 0.01
2	Carbohydrate	31.80 ± 0.05
3	Lipid	10.27 ± 0.39

Values are expressed by mean ± SD (n=3)

Table 3: Determination of Secondary metabolites

S. No	Secondary metabolites	Quantity (mg/g)
1	Flavonoid	39.82 ± 1.57
2	Phenols	104.89 ± 8.96

Values are expressed by mean ± SD

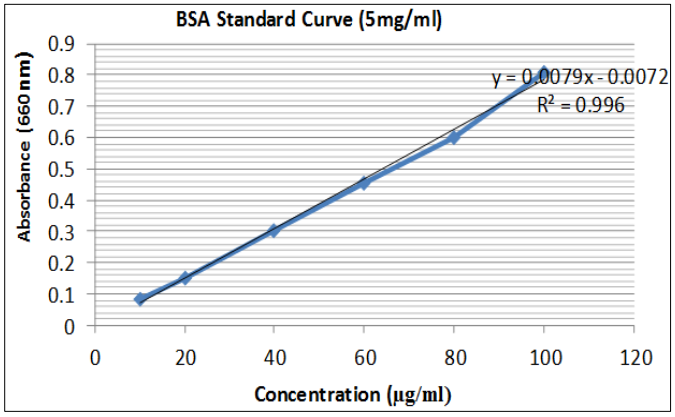

Fig 1: BSA standard curve for total protein estimation

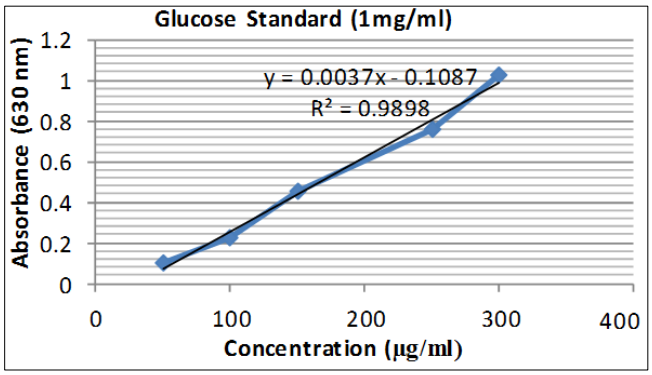

Fig 2: Glucose Standard Curve

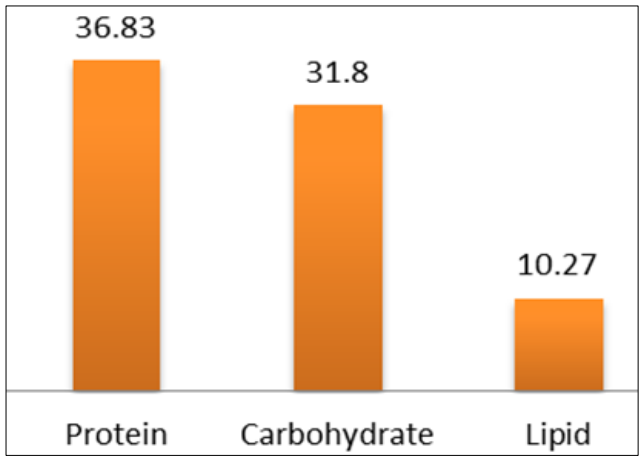

Fig 3: Primary metabolites in seed of marjoram

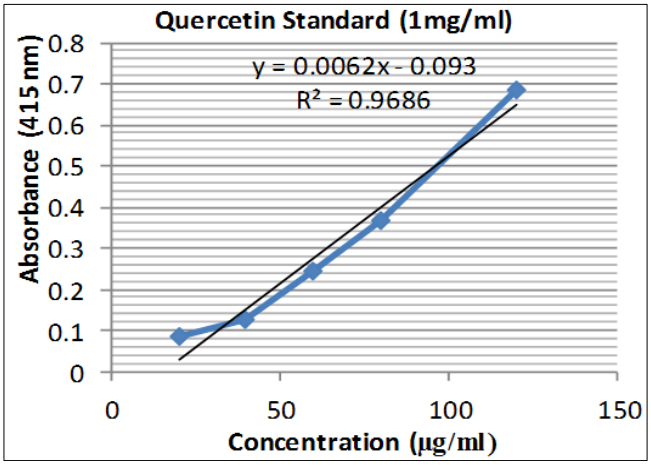

Fig 4: Quercetin Standard Curve

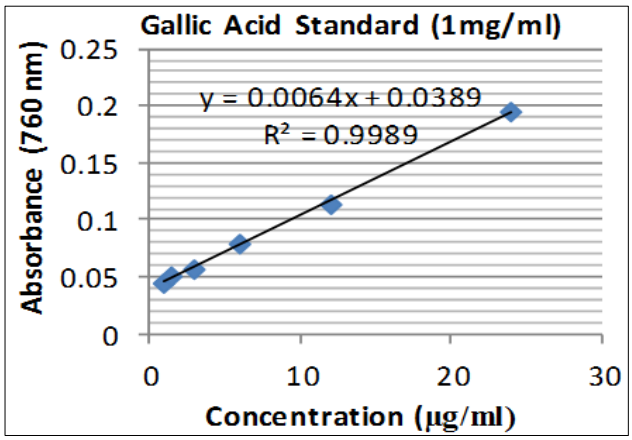

Fig 5: Gallic Acid Standard Curve

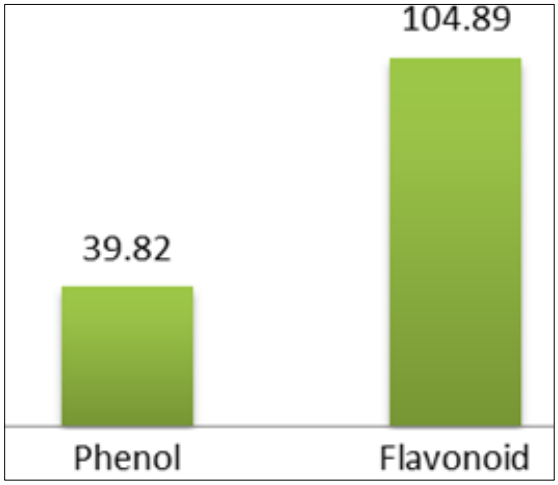

Fig 6: Secondary metabolites in seed of marjoram

The amount of total flavonoid was determined with aluminum chloride assay. Quercetin was used as a standard compound and the total flavonoid was expressed as mg/ml quercetin equivalent using the standard curve equation: y = 0.006x - 0.093 (R^2= 0.968) (Figure 4); where y is absorbance at 415 nm and x is total flavonoid content in the extracts of Marjoram expressed in mg/gdw. The total flavonoid was 104.89 mg/gdw in the ethanolic seed extract.

The total phenol content was determined with Folin-Ciocalteu reagent. Gallic acid was used as a standard compound and the total phenols were expressed as mg/ml gallic acid equivalent using the standard curve equation: y = 0.006x + 0.038, (R^2= 0.998) (Figure 5); where y is absorbance at 760 nm and x is total phenolic content in the extract of Marjoram expressed in mg/gdw. The total phenolic content was found in the ethanolic seed extract of Marjoram (39.82 mg/gdw).

Discussion

The results obtained from the present study showed that the extract of Marjoram contain amounts of phenolic and flavonoid compounds. The Flavonoids and phenols are major compounds that act as antioxidants or free radical scavengers (Bhandary *et al.*, 2012) [2]. The higher level of lipids in seed extract indicates their probable drought tolerance capacity and physiological characteristics under necessarily needed in the ecological adaptation to the semi-arid environment. The presence of higher protein level in the plant points towards their possible increased food value or that a protein based bioactive compound could also be isolated in future (Talreja, 2011) [19]. In phytochemical screening of occimum sanctum, menthe arvensis and Leonotis nepotifolia presence of alkaloids, carbohydrates, flavonoids, phytosterols, proteins, steroids, terpenoids, phenols, saponins, quinones, coumarins and glycosides was observed (Salve and Kakade, 2018; Sangole and Sangole, 2017) [15, 16]. Similarly in oregano plant (*Origanum vulgare* L.) and lavender (L. *angustifolia*) ferulic, rosmarinic, p-coumaric and caffeic acid were found as main component (Spiridon *et al.*, 2011) [17]. More than 10 compounds were identified in extract of Thymus algeriensis, which mainly comprise of phenolic compounds and five flavonoids. (Boutaoui *et al*, 2018) [3]. In a diferent study, six species of Leucas were analysed for presence of various phytochemical compounds in their extracts. The screening of extracts revealed presence of phenols, tannins, flavonoids, carbohydrates and glycosides (Geethika and Kumar, 2017) [6]. These researches show that plants of lamiliacea family comprise of medicinally important primary and secondary compounds as observed for origanum majorana in this study.

Conclusion

In the present study, it was found that Marjoram seed extract is source of primary and secondary metabolites, which have many medicinal properties such as antidiuretic, anti-inflammatory, antianalgesic, anticancer, anti-viral, anti-malarial, anti-bacterial and anti-fungal activities that may be useful for pharmaceutical and food industry.

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