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A comparison of some phenolic compounds and essential oil contents in bacterial-gall contaminated and non-contaminated Rosemary (*Rosmarinus officinalis*) plants

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Abstract

Rosmarinus officinalis is one of the most important medicinal and aromatic plants around the world. Essential oil as a weapon against biotic and abiotic stresses plays an important role in essential oil bearing plants. In this study the essential oil content and constituent of plants containing bacterium-gall on the shoots and crown was compared with non-contaminated plants. Essential oil percentage was extracted by hydro-distillation using Clevenger trap. Identification of the essential oil compounds was done by GC/MS. Results in essential oil percentage showed that, contaminated plants were relatively lower than that of non-contaminated plants per 100 grams of dry leaves. GC/MS also, verified that α- pinene and Camphor, respectively were 1.43% and 7.02%, more in contaminated plant than non-contaminated plant. In addition, Berbenone (18%), was the most common constituent which had been observed only in contaminated rosemaries. The analysis of leaf extract using HPLC, showed that the content of rosmarinic acid of healthy plants was significantly lower than that of contaminated plants. In addition, contaminated plants had total phenol and flavonoid compounds as well as antioxidant activity in the highest amount. Based on the obtained results, it can be concluded that, gall bacteria had influence on the phenolic components which are known as secondary metabolites in this medicinal plant and phenolic derivatives may be modified in quantity and quality by the affection of this biotic stressor, this could be considered as an application for further usage in metabolic culture.

Introduction

The function of plants secondary metabolites can be classified as mediators in the interaction of the plant with its environment, such as plant-insect, plant-microorganism and plant-plant interactions (Harborne & Williams., 2001). It has been proven for years that *Agrobacterium tumefaciens* is a gall producer and is considered an invader for most of the cases. It attacks the DNA of the alive cells, and promotes growth of the cells by nonstop cell division mechanism to induce a cancer-like type in plants (Gelvin., 1990), but the plant defense system against bacterium gall has not studied much well. It is clear that the defense responses of host plants arose by mediating altered defense gene expression. The active resistance of plants to this enormous cell division is often expressed by the Hypersensitive Reaction (HR) of challenged plant cells. An author believes that this sensitivity is characterized by Reactive Oxygen Species known as ROS (Ingram., 1987). ROS have two different roles; first, they may exacerbate the harmful oxidative effect of infection or second, participate in the defense response by being toxic to the invading pathogen. The first happens mostly when the pathogen effect is serious and the resistance mechanisms against the pathogen are not abundant, thus the plant leads to death (Kumar et al., 2009). Monoterepens comprise the major components of the essential oil of the mint family (Lamiaceae) including Rosemary, mint, peppermint and etc. Monoterepen biosynthesis in Rosemary is specifically localized in the glander trichomes (McCaskill et al., 1992) and originates in the leucoplasts of the secretory trichome cells, where essence accumulates in its maximum amount (Turner et al., 1999). Isoprenoids are a large group of

compounds that play essential role in pigments, antioxidants, membrane component, communications and defense in plants (Harborne et al., 1991). By the combination of a pyruvate molecular and a D-Glyceraldehyde- 3- phosphate, the biosynthesis of isoprene (C5), monoterepens (C10), diterpens (C20) and tetraterepens (C40), complete up via a mevalonate independent pathway (Lichtenthaler., 1999 ; Eisenriech et al., 1997). Rosmarinic acid is the dominant hydroxy cinnamic acid ester accumulated in Boraginaceae and Lamiaceae plants, as well as medical plants like thyme, oregano, savory, peppermint (Lu & LY., 1999). RA (Rosmarinic acid) exhibited various pharmacological activities including prevention of oxidation of low density lipoprotein, inhibition of murine cell proliferation activity and of cyclooxigenase (Matsuno et al., 2002). The biological activity of RA is described as antibacterial, anti-viral, antioxidative, and anti-inflammation and even more recently RA or its salts were reported to have anti HIV activities (Chen et al., 1999). Galls, which are produced by *Agrobacterium tumefaciens,* tumorized the plant cells by T-DNA in the plasmid of the bacteria that could be a biotic stress and produce signaling factors as an invader. Indeed, one of the earliest stages in the interaction between *Agrobacterium sp*. and a plant is the attachement of the bacterium to the surface of the plant cell. A plant cell becomes susceptible to *Agrobacterium* when it is wounded. The wounded cells release phenolic compounds that activate the virolence- region of the bacterial plasmid (Binns., 1988) and thus polyphenolic compounds, like flavonoids (Rosmarinic acid), phenolic or non-phenolic essential oils and antioxidant enzymes secretion. In this study, rosemary shrubs on campus in Gorgan University of Agricultural Sciences and Natural Resources; (E: 54º 23' 42.71" N: 36.5º 33.23", Height of sea level (m): 6, average of 30 recent years rainfall (mm): 601mm, mean of 30 recent years temperature: (Max. 22.8°C, Min.12.7°C), which were attacked by gall-bacteria (unpublished data from plant pathology faculty in this university), were examined for phenolic and essential oil compound compared to uncontaminated plants.

Materials and Methods

- **Extraction, Isolation procedure and Essential oil percentage**

Fresh leaves of contaminated and non-contaminated rosemaries (*Rosmarinus officinalis* L.) were collected and then were shade dried at room temperature for 7 days (Khorshidi et al., 2009). The dried samples were powdered and used for essential oil extraction. A 500 ml bubble was filled with 40 g of powdered leaves and 250 ml of tap water. The essential oil of samples was extracted for about 3 hours using Clevenger via distilled water method and extracted oil was stored in refrigerator until analysis. To evaluate the antioxidant producing agents and activity, methanolic extract was obtained from each contaminated and non-contaminated plant samples. Thus, One gram of dried leaf was powdered and then added to 10 cc 80% methanol (HPLC grade methanol) and shook for 24h. After filtration of plant material, methanolic extract was obtained.

- **Total phenol**

Total phenolic compounds were determined by the Folin-ciocalteau method with some modifications (Raggazi et al., 1973). 20µl of methanolic extract of samples was mixed with 1.16 ml of distilled water and 100µl of Folin-ciocalteau, the mixture was kept in dark for 1-8 minutes. Then, 300 µl of sodium carbonate was added. After 2 h of incubation at room conditions, the absorbance of reaction was measured at 760 nm. The standard curve was prepared using 50 to 250 mg/ml solutions of Gallic acid in methanol: water (1:1 v/v). The value of total phenol was expressed in terms of gallic acid equivalent (mg/g).

- **Total flavonoid**

Flavonoid content of the extraction was determined by 0.5 ml of plant extraction solution (10% w/v) was separately mixed with 1.5 ml of methanol, 0.1ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate, and 2.8 ml of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm using (model: UNICO 2800) Spectrophotometer; the calibration curve was prepared by quercetin solutions at concentrations of 12.5 to 100 µg/ml (Ebrahimzadeh et al., 2008).

- **DPPH radical-scavenging activity**

The stable 1, 1-diphenyl-2-picryl hydroxyl radical (DPPH) was used to determine free radical scavenging activity of the extract (Brand-Williams et al., 1995). One milliliter of plant methanolic extraction was added to an equal volume (1:1 v/v) to methanol solution of 100 µM DPPH (for 100µM DPPH: 4 mg of DPPH purple powder saturated in 100 ml of methanol). After 15 min at dark room temperature the absorbance was recorded at 517 nm. Control sample also, contains 1 ml of methanol and 1 ml of DPPH solution. The percentage of DPPH radical-scavenging activity was calculated according to the formula bellow:

DPPH radical scavenging activity(%) $= \frac{A_c - A_s}{A_c} \times 100$

A_c= control sample, A_s=extract sample

- **Rosmarinic acid**

According to (Angelov et al., 2007) with a modification, about 0.5 g of fresh Rosemary leaves were crushed in a mortar mixing (Made in Belgium) with (1:10) HPLC grade methanol. The obtained solution was sonicated for 10 min at room temperature. Then the mixture shook for 12 h. The mixture was centrifuged for 10 min in 3500 rpm. Finally the supernatant was filtered through 0.2µm syringe filter and stored at -20 °C until further analysis. The analyses of samples were repeated three times using HPLC, Merck-Hitachi-L7100, consisting of an UV detector Hitachi system and column oven L-2300 Hitachi. The instrument was equipped with a Reverse Phase column (RP) C-18: (250×4.6mm, 5 µm particle size) using 80:20 HPLC methanol and deionized distilled as a mobile phase. The flow rate was set at 0.4 ml/min throughout the isocratic phase. The system was set on 280 nm. The column temperature was maintained at 25°C and the injection volume was 10µl. Quantification was performed by calibration curve, using the available standards. Particularly, rosmarinic acid (3, 4-dihydroxyphenyllactic acid) standard curve was considered by 25, 50,100,200 mg/ml concentrations of rosmarinic acid (Sigma-aldrich HPLC grade).

- **Analysis of the essential oil contents**

Essential oil in contaminated and non-contaminated plants were analyzed by 5975C, MODE EI, mass selective detector, connected with a 7890N, AGILENT gas chromatograph. Data processing system was conducted with NIST 14 mass spectral library (National Institute of Standards and Technology), which is an evaluated collection of Electron Ionization (EI) and mass spectral. The system provided the ability to detect the breakdown of the essential oil in contaminated and non-contaminated rosemaries. Separation was achieved by a fused- silica capillary, HP 5MS, column (30m×0.25mm) and Film thickness was 0.25µm. Helium was used as a carrier gas at a flow rate of 1ml/min. The injection volume was 1µl and the injection temperature was 260°C and split (50.1) technique was used during injection. The MS used a mass quadrupole detector temperature at (270°C) and had an ionization voltage of 70 ev and temperature program was 60 °C for 4 min, then 3 °C/min to 100 °C, then 4 °C/min to 225 °C.

- **Data analysis**

Data analysis was done using SAS software (version 9) in complete randomized design and the mean value was compared via LSD test at 5% probability.

Results

- **Essential oil percentage**

It has been observed that the essential oil percentage (each 100g) of NCR (non-contaminated rosemary, NCR= 1.62±0.07) was 2.5 fold more than CR (contaminated Rosemary, CR= 0.63±0.05) (Table 1).
This result may reveal that contaminated plants were more probable of being under risks of tissue damages caused by bacterial invasion. Some authors found that total essential oil of pepper mints subjected to severe abiotic stress was significantly reduced as stress increased (Charles et al., 1990). Moreover, total oil yield was strongly correlated with shoot biomass and leaf area, he concluded that under severe condition smaller leaves and/or fewer leaves produced by plants. So that, total oil production consequently reduced. While on the other hand, stressors may cause a higher oil gland density as a result of stress- induced reductions in leaf area and that could provide the reason for the observed higher oil content per unit leaf dry weight. Some scientists examined rosemary's morphological and phytochemical changes under abiotic stress (Delfine et al., 2005). They found that stem biomass, whole plant weight and leaf dry mass reduced by the sever water deficient consequently total yield of monoterpenes reduced as the water stress increased. Unlike the percentage of the essence in each 100 gram, the mean of three replications of each two groups of plant show a significant difference in antioxidant activity (CR=70.27±0.24 and NCR= 40.81±0.12) (Table 1). Interestingly there`s 1.7 folds increase in antioxidant amount in Contaminated plants rather than NCR. Flavonoid contents were also 1.6 folds more in bacterial contaminated plants than healthy ones (Table 1). This amount is significantly ($P< 0.05$) stronger in bacterial plants. Bacterial invasion had a significant influence on increasing antioxidant, total phenol and flavonoids contents because of the induced biotic stress that caused ROS involvement (Decker., 1995). In this study, HPLC analysis showed that the rosmarinic acid content of contaminated plants was significantly higher than that of non-contaminated plants (CR= 40.15 rather than NCR=34.42) (Table 1). As Charles et al. (1990) mentioned, stressors may cause changes in gland frequency then it could provide the reason for the examined higher polyphenolics compounds per gram of leaf fresh weight.

Table 1. Essential oil, antioxidant activity and rosmarinic acid of Contaminated and Non Contaminated Rosemary (CR and NCR)

	Essential oil (per 100 g of Dry leaves)	DPPH activity	Total phenolic compounds	Total flavonoid	Rosmarinic acid
	Percentage (%)	Percentage (%)	mg/g GAE	mg/g Que	mg/g Fw
CR	0.63±0.05	70.27±0.24	28.7±0.012	24.0±1.4	40.15±0.0003
NCR	1.62±0.07	40.81±0.12	20.2±0. 11	16.4±0. 6	34.42±0
$LSD_{5\%}$	0.26	0.74	0. 32	4. 4	0.0009

- **Essential oil Constituents**

GC-MS conclusion identified 37 compounds of essential oil in non-contaminated and 30 for contaminated plants. Some of these compounds were found in both groups but some of essential oil constituents in contaminated and non-contaminated plants were quite different (Table2, 3 and 4).

Table 2. Area percentage in Non-contaminated and Contaminated Rosemary (CR, NCR)

Number of constituents	Compound	CR	NCR
		Area%	Area%
1	Tricyclene	0.11	0.14
2	alpha.-Pinene	13.13	11.7
3	Camphene	2.66	2.80
4	verbenene	0.17	0.39
5	beta.-Myrcene	1.2	1.47
6	alpha.-Terpinene	0.46	0.85
7	1,8-Cineole	17.5	18.81
8	gamma.-Terpinene	0.88	1.59
9	alpha .-terpinolene	0.97	1.7
10	Linalool	3.34	3.69
11	chrysanthenone	0.67	1.35
12	Camphor	10.91	3.89
13	Pinocarvone	0.4	0.63
14	4-Terpineol	1.41	1.66
15	Camphene	1.29	0.95
16	geraniol	1.31	6.89
17	Borneol, acetate	6.22	6.08
18	trans-Caryophyllene	2.31	1.53
19	alpha.-Humulene	0.48	0.32
20	Caryophyllene oxide	0.65	0.71

Table3. Exclusive constituents of essential oil in Non-contaminated Rosemary (NCR)

Number of constituents	Compund	NCR
		Area%
1	alpha-Thujene	0.19
2	2-.beta.-pinene	1.73
3	alpha.-Phellandrene	0.31
4	Terpineol, Z-.beta.-	0.57
5	(S)-cis-Verbenol	0.08
6	Verbenone	23
7	beta.-Citronellol	0.35
8	Z-Citral	0.46
9	Myrtenyl acetate	0.18
10	Pulespenone	0.12
11	trans-Carane	0.08
12	p-Mentha-1(7),8-diene	0.21
13	Geranyl acetate	0.91
14	Methyleugenol	0.58
15	caryophylla-3,8(13)-dien-5.beta	0.08
16	Ethyl amyl ketone	0.49
17	Alpha-terpineol	3.28

Table 4. Exclusive constituents of essential oil in Contaminated Rosemary (CR)

Number of constituents	Compound	CR
		Area%
1	beta.-Pinene	1.14
2	3-Octanone	1.01
3	l-Phellandrene	0.2
4	Borneol	7.71
5	Pinocamphone	1.14
6	p-Menth-1-en-8-ol	2.31
7	Myrtenol	0.4
8	2-Pinen-4-one (Berbenone)	18.2
9	2,6-Octadien-1-ol, 3,7-dimethyl	0.13
10	delta-Cadinene	0.13

In tables 3 and 4 some contents of these two groups are separately shown. Some authors (Choi et al., 2000) showed that α- terpinen derivatives like γ- terpinen or terpineol have high antioxidant capacities. But according to their studies they contain no hydroxyl groups then the antioxidant capacity must be explained by their structural factor (except, terpinene-4-ol). In considering the necessity role of terpinen deriveties, in this study, the area percentage of α-terpinen is 0.85 in non Contaminated plants while in Contaminated plants is 0.46 and γ-terpinen also in Non-contaminatedplants are more than contaminated plants (Table 2). As it is examined, some essential oil contents likeBerbenone (18%), Bornoel (7.71%), α-pinene (13.13%), Camphore (10.91%), Camphene (1.29%), Bornoel acetate (6.22%), trans Caryophellene (2.31%) and α-humulen (0.48%) could be increased with phenolic compounds in contaminated plants(Table 2,4). Sesquiterpens are also the most diverse group of isopernoids.. Sesquiterpenes are found in both CR and NCR, which they are: α-caryophyllene (humulene), ß-caryophyllene and α- humulene. Interestingly, trans-caryophyllene (2.31%) and α-humulen (0.48%) are more in CR to NCR respectively, (1.53% and 0.32%). As an overall conclusion we assess that sesquiterpenes seem to be produced more in CR while monoterpenes like, α-terpinen (0.85% in NCR , 0.46% in CR), γ-terpinen (0.88% in CR and 1.59 in NCR), α-terpinolene (0.97% in CR and 1.7% in NCR) and Verbenone (23%) in NCR could be found (Table 2,3). These differences may refer to the action of defense mechanism in plants which interact with this pathogen invasion, but a noticeable increase in camphor or camphene as bicyclic monoterpenoids might be according to more stability in peroxidation chain requirements (Grabman., 2005). Besides chemical composition of an essential oil, extraction methods and the system used to determine antioxidant activity influence the results (Tsao & McCallum., 2010). Some investigations confirmed good antioxidant activity for rosemary (Schwarz et al., 1996). Although, it is not clear up to now whether terpenoids take an antioxidative tasks in plants (Baratta et al., 1998), but it is probable particularly taking into consideration.

Discussion

Antioxidant components played an important role in plant resistance and defense against microbial infections which are intimately connected with ROS (Rakotoarison et al., 1997). Generally, plants have evolved different phytochemicals and enzymes as antioxidants, for example: hydrophilic compounds (ascorbate, glutathione, phenolic compounds, and flavonoid like rosmarinic acid in this study) and also hydrophobic (alpha-tocopherols) metabolites known as antioxidative compounds (Roberts & Gordon., 2003). Although, antioxidants mostly manifest synergistic plant responses to unfavorable factors but some of the main flavonoid like rosmarinic acid may be even higher and more effective than that of ascorbate or alpha-tocopherols (Rice-Evans et al., 1997; Ryabushkina., 2005). In this study the production of polyphenolics (total phenol, flavonoid, rosmarinic acid) in contaminated rosemary plants were exacerbated by the gall producer bacteria. The main essential oil terpenoid constituents were α- pinene, Camphor, Camphene, Borneol acetate which respectively were 1.43%, 7.02%, 0.34%, and 0.14% more in CR than NCR. In addition, some contents like Berbenone (18%), as the most common constituent, was found in CR that was not identified in NCR by GC/MS analysis. This is while, the most detected content in NCR was Verbenone in 23 percent. We found that the effect of biotic stress on terpenoids showed variable area percentage. The yield of isopernoids may be one of the commercial interests that can be usefully manipulated through the bacterial contamination and can be important for future metabolic extractions. In this study, we reveal that essential oil contents in contaminated plants are different from non-contaminated plants, but the future question is that how these differentiations can be used in metabolic culture.

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