

Antioxidant Activity- Synergistic Effects of Thymol and Carvacrol

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Abstract: This study was conducted to evaluate the antioxidant activity-synergistic effects of Thymol and Carvacrol using Three assays DPPH, FRAP, and TEAC. In the DPPH assay Carvacrol, Thymol and Carvacrol-Thymol mixture had scavenging activity and this activity increasing by increasing concentration from 50 to 1000ppm. Carvacrol showed a strong antioxidant activity than Thymol, and no synergistic effect in their mixture at tested concentration. In FRAP assay, Carvacrol had the highest antioxidant activity as in DPPH assay result. However, the mixture of Carvacrol and Thymol showed a higher reducing power than Thymol and no synergistic effect observed. In TEAC assay, Carvacrol showed a great quenching ability of ABTS radical cation than Thymol and the mixture both. The Carvacrol content in the mixture could be responsible for this higher antioxidant activity and there was no clear synergistic effect. These findings support that essential oils always contain a mixture of different chemical compounds. In addition to the major compounds, minor compounds may make a significant contribution to the total oil antioxidant activity.

Keywords: Thymol; Carvacrol; Synergistic effect; antioxidant; Thyme essential oil.

INTRODUCTION

Essential oils are volatile, natural, complex mixtures characterized by strong odour and are formed by aromatic plants as secondary metabolites. (Ramawat and Merillon, 2013; Alvarez et al., 2019)

At present, approximately 3000 essential oils are known, 300 of which are commercially important especially for the pharmaceutical, cosmetic, perfume industries and medical properties as well as in food and beverages as flavoring agents and preservatives (Van de Braak and Leijten, 1999; Burt, 2004; Nieto et al., 2018). The latest property regard to the use of essential oils as natural antioxidants in

this context since 1938 when the earliest reports on antioxidant from spices in a patent by Maveety (Maveety, 1938).

Up to now, thousands of studies have been carried to study the antioxidant activity of several essential oils of aromatic plants. Particularly, members of *Lamiaceae* family. Such as Sage, Rosemary, Thyme, Clove, Oregano, ...etc. (Yanishlivea et al., 1999; Dang et al., 2001; Youdim et al., 2002 Wang et al., 2008 Niteo et al., 2018). Essential oils are very complex natural mixtures which contain about 20-60 components at quite different concentration. They are characterized by two or three major components at fairly high concentration (20-70%) compared to other

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components present in trace amounts. (Bakkali et al., 2008). Generally, these major components determine the biological properties such as antioxidants activities of the essential oils, and might speculate if their antioxidant effects are the result of a synergism of all molecules or reflect only the main molecules present at the highest levels (Ipeke et al., 2005; Nieto et al., 2018). Carvacrol and Thymol have been reported by many authors to have a very high antioxidant activity and they were responsible for most of the antioxidant activity of essential oils of *Lamiaceae* family members (Jukic and milos, 2005; Kulisic et al., 2005; Chizolla et al., 2008).

It is established that Thymol and Carvacrol are considered as the most common effective free radical scavengers (Jamli et al., 2012). It is thought that the hydroxyl group in Thymol and Carvacrol (Figure 1) is usually the site of hydrogen donation.

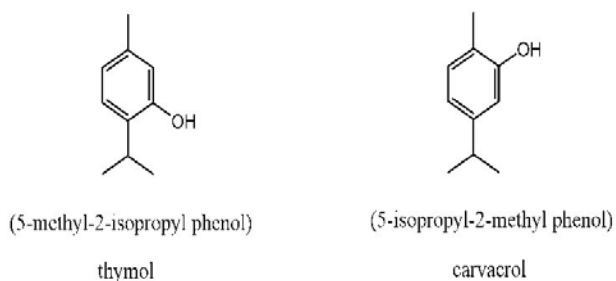


Figure: (1). Chemical structure of thymol and carvacrol

However, the literature contains conflicting observation regarding to Thymol and Carvacrol antioxidant activities. (Yanishliva et al., 1999; Youdim et al., 2002; Teixeira et al., 2013) reported that Thymol had a higher antioxidant activity than Carvacrol, whereas some other studies found that Carvacrol had a stronger antioxidant activity than obtained with Thymol (Yanishliva et al., 1999; Ruberto and Barata, 2000; Miguel et al., 2003; Jamali et al., 2012). The contradiction could be due to methodological variations in the different assays used. In addition, a synergistic

effect was observed by (Puertas-Majia et al, 2002) between Thymol and Carvacrol in mixture at ratio of 1:1 when tested in bulk oil. An increase in the antioxidant activity by 25% was noticed. On the other hand, (Miguel et al., 2003) reported that no synergistic activity was between Thymol and Carvacrol. Due to this contradiction in previous published data the objective of this study to determine the antioxidant activities of Thymol and Carvacrol and mixture (1:1 ratio) of both using DPPH, FRAP and TEAC assays.

Particularly, these two compounds have been found in the Libyan Thyme (*Thymus Capitatus*). Growing wide in Al-jabal Al-Akhdar region.

MATERIALS AND METHODS

Chemical and reagents: Folin-Ciocalteu reagent, anhydrous sodium carbonate, butylated hydroxyanisole (BHA), sodium acetate trihydrate, 2,4,6-Tripyridyl-5-Triazin (TPTZ), gallic acid, Potassium Persulphate, Disodium Hydrogen Phosphate. Ferric Chloride Hexahydrate, Thymol, Carvacrol were purchased from Sigma-Aldrich (Poole, Dorset, UK). 2,2-Diphenyl-1-Picrylhydrazyl free radical (DPPH[•]), 2,2-Azino-Bis (3-Ethylbenzothiazoline-6-Sulfonic acid) Diammonium salt (ABTS) and (±)-6-Hydroxy-2,5,7,8-Tetramethylchromane-2-Carboxylic acid (Trolox) were obtained from Merck chemicals Ltd. (Darmstadt, Germany).

DPPH radical scavenging activity assay: The radical scavenging activity of Thymol and Carvacrol was measured using the stable radical 2,2-Diphenyl-1-Picrylhydrazyl as described by (Chizzola et al., 2008). Each sample (4 ml) dissolved in ethanol at different concentrations (50, 250, 500, 1000 ppm) was mixed with DPPH[•] (1 ml, 0.1 mM). BHA was used as a standard at the same concentration at the samples. The reduction of the DPPH[•] free radical measured after incubation at room temperature for 30 mins. the absorbance was read at a

wavelength of 517 nm against a blank (4 ml ethanol mixed with 1 ml of (0.1mM) DPPH[•] solution).

DPPH[•], a purple coloured, stable free radical is reduced to yellow coloured diphenylpicrylhydrazine when antioxidants are added. The inhibition ratio (%) was calculated from the following equation.

$$\% \text{inhibition} = \frac{[\text{absorbance of control} - \text{absorbance of tested samples}]}{\text{absorbance of control}} \times 100$$

The assay was conducted in triplicate, and results were calculated as mean \pm standard deviation (SD).

Ferric Reducing Antioxidant Power (FRAP) assay:

The total antioxidant potential of Thymol and Carvacrol was determined by means of the ferric reducing antioxidant power assay using the Benzi and Strain (1996) method. For the assay, serial dilutions of Thymol and Carvacrol in Ethanol (100ml) were prepared at (50,100,250,500,1000 ppm), and mixture of both compounds at the same concentration as each one individually was made and tested for synergism effect. A working reagent was prepared by mixing acetate buffer (25 ml, PH 3.6) with TPTZ (2.5 ml of 10 mM) in HCL (40 mM) and freshly prepared solution of 20 mM FeCl₃.6H₂O (2.5 ml). the diluted sample (50 μ l) was mixed with working reagent (3 ml). After 8 min of incubation at 37c° the absorbance was measured at wavelength of 593 nm.

A standard curve (Figure 2) was prepared using different concentrations of Trolox in ethanol (50-1000 mg/ml).

The results were corrected for dilution and expressed as mM of Trolox equivalents/l of samples.

The experiment was performed in three replications and results were expressed as mean \pm standard deviation (SD).

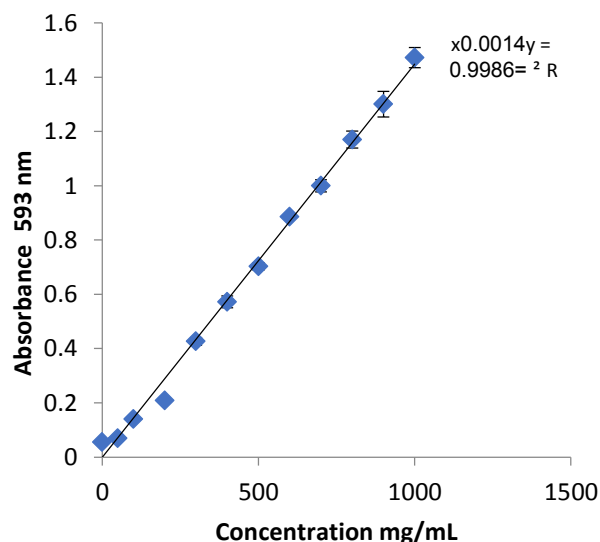


Figure: (2). (FRAP) Standard curve for trolox at range of concentrations (50-1000 mg/mL) measured against absorbance at 593nm. Results are triplicates (\pm SD).

Trolox equivalent antioxidant capacity (TEAC) assay:

Preparation of Phosphate buffer Saline solution (PBS) PH 7.4: PBA was Prepared by mixing Disodium Hydrogen Phosphate NaH₂PO₄ (81 ml of 5 mM) with sodium dihydrogen phosphate NaH₂PO₄ (19 ml of 5mM), and Sodium Chloride (0.9 g) was added to the mixture.

The TEAC assay modified by Re et al., (1999) was used to assess the amount of free radicals that can be scavenged by the antioxidants (Carvacrol and Thymol). Tested samples were diluted in 100 ml ethanol and various concentration were made (20, 30, 40, 50, 70 ppm) for Carvacrol, Thymol, and mixtures of both compounds for the synergism effect. The ABTS radical cation was formed by reacting ABTS (7 mM) and Potassium Persulfate (2.45 mM) after incubation at room temperature, in the dark, for 12-16 hours the stock solution was diluted twenty times with Phosphate buffer Saline solution (PBS) until and absorbance of (0.7 \pm 0.02) at 734 nm was reached. ABTS solution (2 ml) was added to each of tested samples (40 μ l) and mixed thoroughly by Vortex. The reactive

mixture was allowed to stand at room temperature for 1 min, and the absorbance was immediately recorded at 734nm. Only one reagent blank was made, ABTS (2 ml) mixed with PBS (40 µl), and used for all measurements when taking readings for either samples or standards. Trolox standard solution of varying concentrations (0-100 µl/ml). In absolute Ethanol was prepared and assayed in the same conditions. A standard curve was plotted and used to calculate the results (Figure 3). Results were expressed in terms of µg Trolox equivalent /l.

The assay was carried out in Triplicate, and results were calculated as mean ± standard deviation (SD).

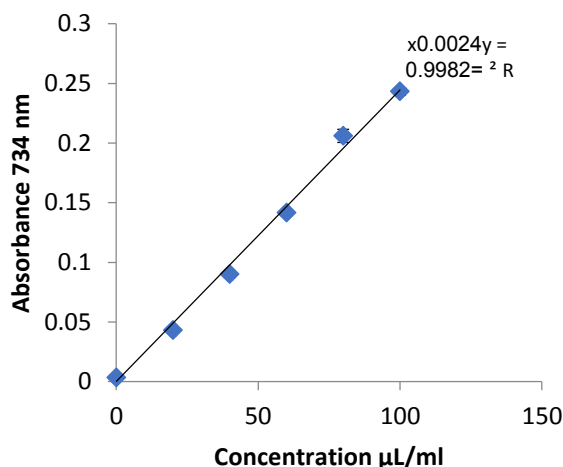


Figure: (3). (TEAC) Trolox standard curve for varying concentrations (0-100 µL/ml) measured against absorbance at 734 nm. Results are triplicates (± SD).

Statistical Analysis:

The results were analyzed by Two-Ways ANOVA using F-test (Montagomry,2000).

RESULTS AND DISCUSSION

The antioxidant activities of Thymol, Carvacrol and the mixture of both were investigated using DPPH , FRAP and TEAC assays.

In the DPPH assay (Table 1) and (Figure 4)

show that Carvacrol, Thymol and Carvacrol:Thymol mixture had scavenging activity, and this activity was highly significant (p<0.001) by increasing the concentration from 50 to 1000 ppm.

Table:(1). DPPH activity (% bleaching of DPPH solution) of thymol and carvacrol, and a (1:1) mixture of both

Groups	Concentration ppm			
	50 (A)	250 (B)	500 (C)	1000 (D)
thymol (a)	31.80±0.6	43.36±0.5	60.24±1.7	82.72±1.5
	AB***	AB***	AC***	AD***
	AC***	BC***	BC***	BD***
	AD***	BD***	CD***	CD***
	ab***	ab***	ac***	ab***
	ad***	ac***	ad***	ac***
	ad***	ad***	ad***	ad***
carvacrol (b)	37.43±0.9	49.04±1.6	62.07±1.2	93.19±0.3
	AB***	AB***	AC***	AD***
	AC***	BC***	BC***	BD***
	AD***	BD***	CD***	CD***
	ba***	ba***	bc***	CD***
	bc***	bc***	bd***	ba***
	bd***	bd***	bd***	bc***
Thymol+carvacrol (c)	30.44±0.6	39.96±1.1	55.21±1.3	76.94±0.3
	AB***	AB***	AC***	AD***
	AC***	BC***	BC***	BD***
	AD***	BD***	CD***	CD***
	cb***	ca***	ca***	ca***
	cd***	cb***	cb***	cb***
	cd***	cd***	cd***	cd***
BHA (d)	92.61±0.4	93.65±0.5	94.16±0.4	94.42±0.5
	AC*	0.5		
	AD**		da***	da** dc**
	da***	da***	db***	
	db***	db***	dc***	
	dc***	dc***		
	dc***	dc***		

A,B,C and D indicate concentrations per group, a, b, c and d indicate groups per concentration. * means significant at 0.05, ** means significant at 0.01 and *** means significant at 0.001. Results are the means of triplicates ±SD.

However, this activity was lower than that obtained from BHA positive control at the same concentration tested. Carvacrol showed a stronger antioxidant activity (p<0.001) than Thymol at four concentrations used. The results are in good agreement with those obtained by (Yanishlivea et al., 1999; Ruberto

and Barata 2000; Miquel et al., 2003 jamali et al., 2012). Regarding to synergistic effect, the result of this study revealed no synergistic effect in the mixture of Carvacrol:Thymol at the tested concentrations, compared with Carvacrol and Thymol individually. These results are in good agreement with the obtained by (Miguel et al., 2003).

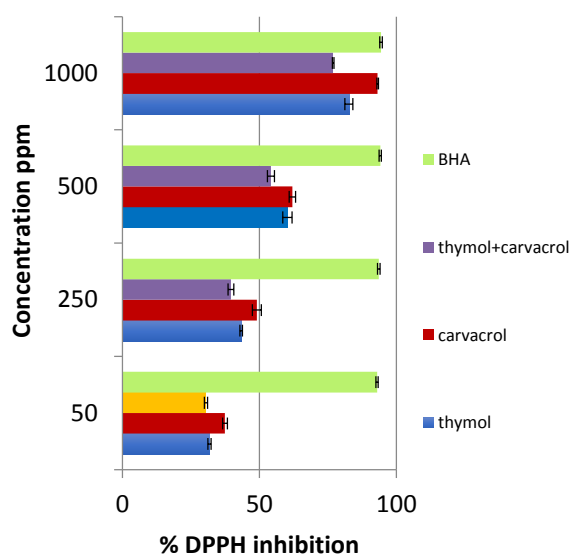


Figure: (4). Free radical scavenging activity of thymol and carvacrol, and a (1:1) mixture of both at different concentrations in the DPPH assay. Data expressed as percentage of bleaching of DPPH solution. Results are the means of triplicates \pm SD. BHA= butylated hydroxy-anisole.

Table 2 and Figure 5 summarize the reducing power of Thymol and Carvacrol and a 1:1 mixture of both as observed in the FRAP assay.

The results showed the same pattern as the DPPH assay. Results with Carvacrol, which had the highest antioxidant activity ($p < 0.001$) compared with Thymol and the mixture of both. However, the mixture of Carvacrol and Thymol in this assay showed a higher reducing power than Thymol at each concentration used. There was no synergistic effect observed between Carvacrol and Thymol.

Table:(2). FRAP analysis of thymol and carvacrol, and a (1:1) mixture of both at different concentrations. Results expressed in terms of mM trolox equivalent

Groups	Concentration ppm				
	50 (A)	250 (B)	500 (C)	1000 (D)	
thymol (a)	284.6 \pm 1.	306.23 \pm 1	556.9 \pm 0.	906.4 \pm 3.	
	9	.0	6	8	
	AB***	AB***	AC***	AD***	
	AC***	BC***	BC***	BD***	
	AD***	BD***	CD***	CD***	
	AE***	BE***	BE***	CE***	
	ab***	ab***	ab***	ab***	
	ac***	ac***	ac***	ac***	
	852.3 \pm 1.	950.81 \pm 1	1278.1 \pm 1	1478.1 \pm 1	
	2	.2	.1	.0	
carvacrol (b)	AB***	AB***	AB***	AB***	
	AC***	AC***	AC***	AC***	
	AD***	AD***	AD***	AD***	
	AE***	AE***	AE***	AE***	
	ba***	ba***	ba***	ba***	
	bc***	bc***	bc***	bc***	
	583.95 \pm 2	622.4 \pm 1.	754.47 \pm 0	1169.7 \pm 0	
	.3	1	.6	.6	
	Thymol+ carvacrol (c)	AB***	AB***	AB***	AB***
		AC***	AC***	AC***	AC***
AD***		AD***	AD***	AD***	
AE***		AE***	AE***	AE***	
ca***		ca***	ca***	ca***	
cb***		cb***	cb***	cb***	

A,B,C, D and E indicate concentrations per group, a, b, and c indicate groups per concentration. * means significant at 0.05, ** means significant at 0.01 and *** means significant at 0.001. Results are the means of triplicates \pm SD.

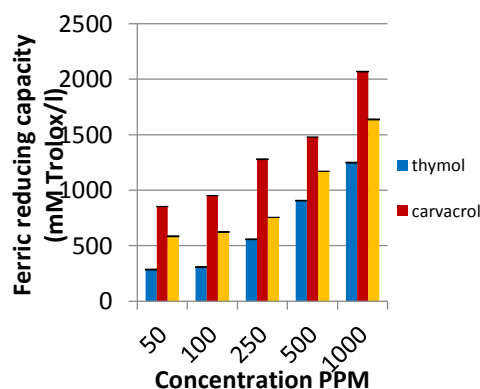


Figure: (5). FRAP analysis of thymol and carvacrol, and a (1:1) mixture of both at different concentrations. Data expressed in terms of mM trolox equivalent. Results are the means of triplicates (\pm SD)

The TEAC Assay had also the same pattern behavior of Carvacrol, Thymol and the mixture of both (in ratio of 1:1). Results are presented in (Figure 6) and (Table 3). Carvacrol showed a great quenching ability of ABTS radical cation ($p < 0.001$) than Thymol and the mixture of both at all concentrations tested. The mixture showed higher antioxidant activity than Thymol ($p < 0.001$). The Carvacrol content in the mixture could be responsible for this higher antioxidant activity and there was no clear synergistic effect.

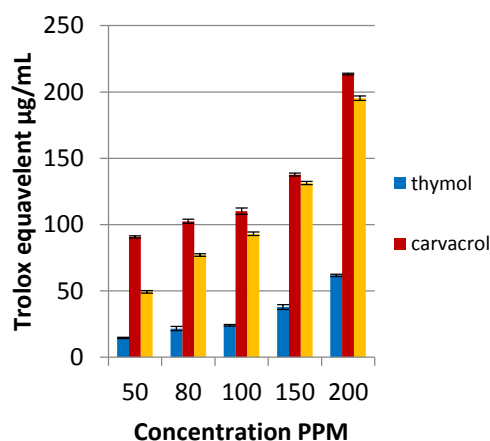


Figure: (6). TEAC values of thymol and carvacrol, and a mixture (1:1) of both at different concentrations. Results are expressed as µg/mL trolox equivalent and they are the means of triplicates ±SD

The results obtained from the three assays (DPPH[•], FRAP and TEAC) were in accordance with earlier published data on the strong antioxidant activity of Carvacrol as compared to Thymol (Ruberto and Barat 2000; Bozin et al., 2006). Furthermore, the results of current study correspond with results obtained by Undger et al., (2009) who found that there was no synergistic effect between Carvacrol and Thymol. On the other hand, the results of the present study disagree with those reported by Puertas-Mejia et al., (2003) who reported a synergistic effect between Thymol and Carvacrol. For these reasons, it is very difficult to attribute the antioxidant effect of a total essential oil to one or several active principles, be-

cause on essential oil always contains a mixture of different chemical compounds. In addition to the major compounds, minor compounds may make a significant contribution to the total oil antioxidant activity.

Table:(3). TEAC values for thymol and carvacrol, and a (1:1) mixture of both at different concentrations. Results are expressed as µg/mL trolox

Groups	Concentration ppm			
	50 (A)	250 (B)	500 (C)	1000 (D)
thymol (a)	14.5±0.4	21.67±1.	24±0.6	
	AB***	6	AC***	37.86±1.9
	AC***	AB***	BC***	AD*** BD***
	AD***	BC***	CD***	CD***
	AE***	BD***	BE***	CE***
	ab***	BE***	ab***	ab*** ac***
	ac***	ab***	ac***	
		ac***	ac***	
carvacrol (b)	90.72±0.	102.5±1.	110.11±2.	
	9	6	5	137.75±1.2
	AB***	AB***	AB***	AB*** AC***
	AC***	AC***	AC***	AD***
	AD***	AD***	AD***	AE***
	AE***	AE***	AE***	ba*** bc***
	ba***	ba***	ba***	
	bc***	bc***	bc***	
Thymol+ carvacrol (c)	49.28±0.	77.03±0.	93.06±1.3	131.36±1.
	9	9	AB***	2
	AB***	AB***	AC***	AB*** AC***
	AC***	AC***	AD***	AD***
	AD***	AD***	AE***	AE***
	AE***	AE***	ca***	ca*** cb***
	ca***	ca***	cb***	
	cb***	cb***	cb***	

A,B,C, D and E indicate concentrations per group, a,b, and c indicate groups per concentration. * means significant at 0.05, ** means significant at 0.01 and *** means significant at 0.001. Results are the means of triplicates ±SD.

CONCLUSION

Carvacrol and Thymol were studied to see if there were any synergistic effects. Carvacrol and Thymol gave strong antioxidant activity. However, this effect was great with Carvacrol. There were no synergistic effects between Carvacrol and Thymol.

ETHICS

The authors declare no conflict of interest.

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التأثير المضاد للأكسدة - المدعم لمركبي الثايمول والكارفاكروول

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المستخلص: أجريت هذه الدراسة لتقييم التأثير المضاد للأكسدة-المدعم لمركب الثايمول، والكارفاكروول، وذلك باستخدام ثلاثة اختبارات هي DPPH و FRAP و TEAC. أعطى اختبار DPPH، والكارفاكروول، والثايمول، ومخلوطهما نشاطاً كاسحاً وهذا النشاط يزداد بزيادة التركيز من 50 إلى 1000 جزء في المليون (ppm). الكارفاكروول أعطى تأثيراً أقوى كمضاد للأكسدة من الثايمول، ولم يكن هناك تأثير مدعم لمخلوطهما عند التركيزات المختبرة، أما في اختبار FRAP كان أعلى تأثير مضاد للأكسدة لمركب الكارفاكروول كما في حالة اختبار DPPH. ولكن مخلوط الكارفاكروول، والثايمول أظهر قوة اختزالية أقوى من الثايمول، أيضاً لا يوجد تأثير مدعم. أما في اختبار TEAC، الكارفاكروول أظهر قدرة كبيرة على كسح كاتيونات الشقوق الحرة لمركب ATBS أعلى من الثايمول وكذلك مخلوطهما. وقد يرجع هذا التأثير إلى مركب الكارفاكروول في المخلوط. كما بينت النتائج عدم وجود تأثير مدعم لكل من مركبي الكارفاكروول، والثايمول. هذه النتائج تعزز أن الزيوت الطيارة دائماً هي خليط من مركبات كيميائية عديدة، وأنه بالإضافة إلى المركبات الرئيسية فإن المركبات التي توجد بنسب ضئيلة يمكن أن تساهم بدرجة كبيرة في التأثير على النشاط المضاد للأكسدة لهذه الزيوت.

الكلمات المفتاحية: كارفاكروول، ثايمول، مضادات الأكسدة، الزيوت الطيارة، الزعتر.