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Application of Biotechnology to Improve Biological Activity of Rosmarinus Officinalis Oil

M.A.El-Sibaie[1], M.A.Swelim[2], M.M.Amer[2] and T.M.Emam[1]
[1]Soil Microbiology Unit, Desert Research Center, Cairo, Egypt
[2]Microbiology Dept., Faculty of Science, Benha Univ., Benha, Egypt
E-Mail: tameremam7@gmail.com

Abstract

Volatile oils obtained from Rosmarinus officinalis plants treated with recommended doses of organic and mineral fertilizers (VO1); plants treated with organic, mineral and bio fertilizers (VO2); plants treated with combination of organic, mineral, bio fertilizers and Biomagic (VO3) were analyzed by Gas chromatography (GC), it was illustrated that VO3 was better than VO1 and VO2 concerning known chemical constituents. Moreover, VO1, VO2 and VO3 were screened for their antibacterial, antioxidant, antitumor and antidiabetic activities. VO3 were more effective in inhibiting the growth of tested bacteria whereas VO1 was the least inhibitive. On the other hand the antitumor results revealed that VO3 exhibited the lowest inhibitory concentration 50 (IC_{50}) against MCF-7 carcinoma cell line in comparison to the other oils. The three oils were analyzed for their antioxidant activities using 2,2-Diphenyl-1-Picrylhydrazyl (DPPH%) scavenging activity method. VO3 revealed the highest DPPH scavenging % in comparison to the other oils. The results showed that alpha glucosidase diabetes enzyme was inhibited by all the tested oils in dose dependent manner but with superiority to VO3. This study showed great prospects in effectiveness of biofertilizers in combination with Biomagic on constituents of *R.* officinalis oil and subsequently on its biological activities. Thus, this study points to investigate the combination of biofertilizers and Biomagic as a potential tool to improve biological activities of plant volatile oils.

Keywords: Rosmarinus officinalis oils, GC, Biotechnology, Antibacterial, Antitumor, Antioxidant, Antidiabetic.

1. Introduction

The growing trend of antimicrobial drug resistance in virtually all organisms responsible for hospital acquired infections has become a major public health concern worldwide, due to their rapidly evolving adaptive strategies. Microorganisms have been known to successfully flourish in hospitals and community settings [1].

Antioxidants are man-made or natural substances that may prevent or delay some types of cell damage [2]. Free radicals can cause "oxidative stress," a process that can trigger cell damage.

Oxidative stress is thought to play a role in a variety of diseases including cancer, cardiovascular diseases, diabetes, Alzheimer's disease, Parkinson's disease, and eye diseases such as cataracts and age-related macular degeneration [3].

One in four deaths in the world is due to cancer. When ranked within age groups, cancer is one of the five leading causes of death amongst both males and females and the single largest cause of death worldwide [4] **.**
By 2015 cancer morbidity may climb to around nine million world-wide. This growing trend indicates a deficiency in the present cancer therapies which include surgical operation, radiotherapy and chemotherapy.

Since the average survival rates have remained essentially unchanged despite such aggressive treatments, there is a critical need for anti-cancer agents with higher efficacy, and less side effects that can be acquired at an affordable cost [5].

Diabetes that is a most dreadful disease affecting more than 250 million people around the world have diabetes. This total is expected to rise to 380 million within 20 years. Each year a further 7 million people develop diabetes. Long term diabetes leads to other complications like retinopathy, diabetic nephropathy, diabetic neuropathy, atherosclerosis, colon cancer etc.[6]**.**

Type II diabetes is characterized by hepatic and peripheral (muscle and adipose tissue) insulin resistance. The pancreas compensates by secreting more insulin, but eventually the beta cells will fail to sustain this [7], at which stage the patient requires insulin treatment. During the stage when insulin is still produced, various other classes of drugs, in combination with lifestyle alterations, can be used to manage the disease [8]**.**

The significance of natural products in the drug discovery and development processes has been reported extensively [9].

The importance of natural products as sources of innovative therapeutic agents can be illustrated by the drugs used in the control of infectious diseases, cancer, lipid disorders, immune modulation and hypertensive diseases [10]**.**

Rosmarinus officinalis, commonly known as Rosemary, is an evergreen perennial shrub belonging to the Lamiaceae family [11]**.** Native to the Mediterranean region, Rosemary is now cultivated around the world due to its use as a natural food preservative and flavoring agent [12]**.**

Rosemary has also been used as a source of traditional medicine for centuries. Its applications have ranged from memory enhancement to the treatment of gastrointestinal diseases [13, 14].

The secret behind the preservative and therapeutic abilities of rosemary lies in its essential oil and extract. While the chemical composition of these secondary metabolites varies considerably depending on ecological conditions, all rosemary essential oils and extracts contain biologically active compounds that make them unique [15].

R. officinalis have strong antioxidant compounds in its essential oil and extract that is making *R. officinalis* a plant of great interest in today's food and medical industries [16].

Many studies have reported on the anticancer mechanisms of Rosmarinus officinalis, Rosemary has displayed significant anti proliferative activities against several human cancer cell lines [17]**.**

Antidiabetic activities of *R.* officinalis oil inhibited lipid peroxidation and activated antioxidant enzymes, the oil also promoted insulin secretion [18].

The present work aimed to investigate the effect of using a combination of bacterial and fungal biofertilizers, namely; Azotobacter chroococcum and Vesicular Arbuscular Mycorrhiza (VAM) as well as addition of non-hormonal growth promoter (Biomagic) on Rosmarinus officinalis oil components and subsequently on its biological activities.

2. Materials and methods

2.1 Extraction of essential oils from different treated-*R. officinalis* plants

The plant material of *Rosmarinus officinalis* treated with recommended doses of mineral + organic fertilizers (group 1); plants treated with mineral + organic + bio fertilizers, (namely; *Azotobacter chroococcum* and VAM) (group 2); plants treated with mineral + organic + bio fertilizers and the Biomagic (microbial non-hormonal foliar spray) (group 3) were cut into pieces (100 g) and put separately in flasks containing double distilled water.

A continuous steam distillation extraction head was attached to the flask, after steam distillation, the oils were collected and dried over anhydrous sodium sulfate [19].

2.2 Analysis of essential oils

The analysis of the essential oils was performed using GC–MS (type Hewlett Packard 5890 USA). The components were identified based on the comparison of their relative retention time and mass spectra with those of standards, NBS75K library data of the GC–MS system and literature data.

The results were also confirmed by the comparison of the compounds elution order with their relative retention indices on non-polar phases reported in the literature [20].

2.3Antimicrobial activity of different *R.officinalis oils*

2.3.1 Bacterial species

A total of eight bacterial type strains; namely Staphylococcus aureus (ATCC 6538), Staphylococcus epidermidis (ATCC 12228), Enterococcus faecalis (ATCC 29212), Proteus mirabilis (ATCC 12453), Proteus vulgaris (ATCC 8427), Klebsiella pneumoniae (ATCC 13883), Pseudomonas aeruginosa (ATCC 27853) and Salmonella typhimurium (ATCC 14028) obtained from American Type Culture Collection (ATCC) were used in the present study.

Each strain was immediately cultured on Mueller-Hinton agar (Bio-rad, France) for 24 h at 37°C.

2.3.2 Disc-diffusion assay

Antibacterial tests were carried out by disc diffusion method as demonstrated by [21] using 100 µl of suspension containing 10^6 cfu/ml of tested bacteria spread on Mueller- Hinton agar medium (Bio-rad, France).

The discs (6 mm in diameter) were impregnated with 20µl of oil/disc) placed on the inoculated agar. Ciprofloxacin (20 µg/disc was used as a positive reference standard). The inoculated plates were incubated at 37°C.

Antibacterial activity was evaluated by measuring the zone of inhibition against tested bacteria after 24 hours of incubation. Each assay in this experiment was carried out in triplicate.

2.3.3 Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration values of tested essential oils were determined using two-fold serial dilutions of essential oils by the agar dilution method as described previously by [22].

MIC values were determined as the lowest concentration of tested oils where absence of growth was recorded. Each test was repeated at least twice.

2.4 Antioxidant Assay

The antioxidant activity of oils was determined by the DPPH free radical scavenging assay in triplicate and average values were considered.

2.4.1 DPPH Radical Scavenging Activity

Freshly prepared (0.004%w/v) methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was prepared and stored at 10°C in the dark.

A methanol solution of the test compound was prepared. A 40 µL aliquot of the methanol solution was added to 3ml of DPPH solution.

Absorbance measurements were recorded immediately with a UV-visible spectrophotometer (Milton Roy, Spectronic 1201).

The decrease in absorbance at 515 nm was determined continuously, with data being recorded at 1 min intervals until the absorbance stabilized (16 min). The absorbance of the DPPH radical without antioxidant (control) and the reference compound ascorbic acid were also measured.

All the determinations were performed in three replicates and averaged. The percentage inhibition (PI) of the DPPH radical was calculated according to the formula: PI = ({(*A*C- *A*T)/ *A*C} x 100] (1); Where *A*C = Absorbance of the control at t = 0 min and *A*T = absorbance of the sample+DPPH at t = 16 min [23].

2.5 Evaluation of cytotoxic effects of different R. officinalis oils

2.5.1 Mammalian cell lines MCF-7 cells

Human breast cancer cell line (MCF-7) was obtained from VACSERA Tissue Culture Unit.

2.5.2 Chemicals used

Dimethyl sulfoxide (DMSO), crystal violet and trypan blue dye were purchased from Sigma (St. Louis, Mo., USA). Fetal Bovine serum, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin and 0.25% Trypsin-EDTA were purchased from Lonza.

2.5.3 Crystal violet stain (1%)

It composed of 0.5% (w/v) crystal violet and 50% methanol then made up to volume with ddH_2O and filtered through a Whatmann No.1 filter paper.

2.5.4 Cell line Propagation

The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50µg/ml gentamycin.

All cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and were subcultured two times a week.

2.5.5 Cytotoxicity evaluation using viability assay

For cytotoxicity assay as demonstrated by [24, 25], the cells were seeded in 96-well plate at a cell concentration of 1×10^4 cells per well in 100µl of growth medium.

Fresh medium containing different concentrations of the test sample was added after 24 h of seeding.

Serial two-fold dilutions of the tested chemical compound were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette.

The microtiter plates were incubated at 37ºC in a humidified incubator with 5% CO_2 for a period of 48 h. Three wells were used for each concentration of the test sample.

Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. After incubation of the cells for at 37°C, various concentrations of oils were added, and the incubation was continued for 24 h and viable cells yield was determined by a colorimetric method.

In brief, after the end of the incubation period, media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 minutes.

The stain was removed and the plates were rinsed using tap water until all excess stain is removed. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates were measured after gently shaken on Microplate reader (TECAN, Inc.), using a test wavelength of 490 nm.

All results were corrected for background absorbance detected in wells without added stain.

Treated samples were compared with the cell control in the absence of the tested compounds.

All experiments were carried out in triplicate. The cell cytotoxic effect of each tested compound was calculated**.**

The optical density was measured with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [1-ODt/ODc)]x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells.

The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified compound.

The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA).

2.6 In-vitro antidiabetic Assay

α-glucosidase *(*Saccharomyces cerevisiae*)* and 3, 5, di-nitro salicylic acid (DNS) were purchased from Sigma-Aldrich, Bangalore. P-nitro-phenyl-α-D-glucopyranoside (p-NPG), sodium carbonate (Na_2CO_3), sodium dihydrogen phosphate, di-sodium hydrogen phosphate were purchased from Hi-Media, Mumbai.

α-glucosidase inhibitory activity of different oils was carried out according to the standard method with minor modification [26].

In a 96-well plate, reaction mixture containing 50 µl phosphate buffer (100 mM, pH = 6. 8), 10 µl alpha-glucosidase (1 U/ml), and 20 µl of varying concentrations of extracts and fractions (1000 to 7.81 µl/mL) was pre incubated at 37°C for 15 min.

Then, 20 µl P-NPG (5 mM) was added as a substrate and incubated further at 37°C for 20 min. The reaction was stopped by adding 50 µl Na_2CO_3 (0.1 M).

The absorbance of the released p-nitrophenol was measured at 405 nm using Multiplate Reader. Acarbose at various concentrations 1000 to 7.81 μg/ml) was included as a standard.

Without test substance was set up in parallel as a control and each experiment was performed in triplicates.

The results were expressed as percentage inhibition, which was calculated using the formula, Inhibitory activity (%) = $(1 - As/Ac) \times 100$, where, As is the absorbance in the presence of test substance and Ac is the absorbance of control.

3. Results

3.1 Oil constituents of different rosemary oils

The results of the chemical analyses of the different rosemary essential oils under study are presented in Table **(**1).

There were variations in the percentage of constituents of the three oils. Rosmarinic acid was the most abundant component in the three oil (31.06, 36.55 and 38.67 %) for VO1, VO2 and VO3 respectively.

3.2 In vitro antibacterial activity of different rosemary oils

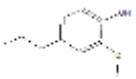
The results of antibacterial activity of tested essential oils are presented in Table (2).

The three tested oils revealed antibacterial activity in concentration of 20 µl/ml. The essential oil VO1 exhibited the lowest inhibition zone with range (12.2-17.6 mm); while inhibition zones obtained for VO2 ranged from (13.3-21.9 mm). VO3 showed the highest inhibition zone range (15.4-22.3 mm).

Interestingly, VO3 possessed a higher antibacterial effect than Ciprofloxacin against Klebsiella pneumoniae.

VO1, VO2 and VO3 revealed MIC ranges (15.63-125), (1.95-62.5) and (0.98-31.25) µl /ml respectively Table (3).

Table (1) Percentages of volatile oil components of different rosemary oils.

Oil constituents	Structure	*RT	*VO1	*VO2	*VO3
α-Pinene		6.32	2.14	2.31	3.17
Camphor		10.92	5.90	7.47	8.56
β-Pinene		11.34	1.38	2.18	2.15
1,8 Cineole		14.77	12.61	14.83	15.90
Linalool		15.32	11.41	12.77	16.11
Limonene		16.34	3.67	4.48	6.08
Terpineol		17.32	3.18	2.22	3.13
Borneol		17.96	5.91	4.23	3.18
Bornyl acetate		18.32	8.86	1.60	2.25
Y- Terpinene		18.99	6.90	5.17	0.47
Eugenol		19.1	3.98	2.35	0.31
Rosmarinic acid		19.8	31.06	36.55	38.67
Total Components			97.00	96.16	99.97
Unknown			3.00	3.84	0.03

*RT: Retention Time.

Table (2) In vitro antimicrobial activities of rosemary oil tested at 20µl/ml and expressed as inhibition zone diameter (mm) in the form of mean ± SD.

Extract code / Tested micro.	VO1	VO2	VO3	Ciprofloxacin
Gram Positive Bacteria	**Mean of inhibition zone (mm) ± SD**			
Staphylococcus aureus(ATCC 6538)**	13.6± 0.58	13.9± 0.58	17.3± 0.58	21.45± 1.2
Staphylococcus epidermidis (ATCC 12228)	15.4± 0.63	16.1± 0.63	19.32± 0.63	22.8± 0.72
Enterococcus faecalis (ATCC 29212)	12.2± 1.2	13.3± 1.2	15.4± 1.2	19.3± 1.5
Gram negative Bacteria				
Proteus mirabilis (ATCC 12453)	13.5± 1.5	15.2± 1.5	18.3± 1.5	21.2± 1.2
Proteus vulgaris (ATCC 8427)	*NIZ	NIZ	10.3± 1.3	20.4± 0.63
Klebsiella pneumoniae (ATCC 13883)	16.2± 0.63	21.9± 0.63	23.1± 0.63	21.35± 1.5
Pseudomonas aeruginosa (ATCC 27853)	17.6± 0.58	19.8± 0.58	22.3± 0.58	19.8± 2.1
Salmonella typhimurium (ATCC 14028)	15.6± 1.2	17.2± 1.2	19.2± 1.2	24.8± 1.5

*NIZ: No Inhibition Zone

**ATCC : American Type Culture Collection

Table (3) In vitro antibacterial activities as MICS (µg/ml) of different rosemary oils against tested microorganisms.

Extract code / Tested micro.	VO1	VO2	VO3	Ciprofloxacin
	Minimum inhibitory concentration (µl/ ml)			
Gram Positive Bacteria				
Staphylococcus aureus (ATCC 6538)**	125	62.5	15.63	1.95
Staphylococcus epidermidis (ATCC 12228)	62.5	31.25	3.9	0.98
Enterococcus faecalis (ATCC 29212)	125	31.25	31.25	3.9
Gram negative Bacteria				
Proteus mirabilis (ATCC 12453)	125	31.25	7.81	0.98
Proteus vulgaris (ATCC 8427)	*ND	ND	125	1.95
Klebsiella pneumoniae (ATCC 13883)	31.25	1.95	0.98	1.95
Pseudomonas aeruginosa (ATCC 27853)	15.63	1.95	0.98	1.95
Salmonella typhimurium (ATCC 14028)	15.63	15.63	3.9	0.49

*ND: Not Detected

**ATCC : American Type Culture Collection

3.3 In vitro antioxidant activity of different rosemary oils

In this study, the level of antioxidants activity of volatile oils was evaluated using percentage of DPPH scavenging. Results in Fig (1a) revealed that the percentage of DPPH radical scavenging followed a concentration dependent manner.

The results indicated that the VO3 gave the highest percentage range of DPPH radical scavenging (2.10 - 89.68 %). The ICs_{50} of oil antioxidant activity were 65.45, 55.81, 33.82 and 29.22 µl/ ml for VO1, VO2, VO3 and ascorbic acid respectively Fig (1b).

3.4 In vitro antitumor activity of different rosemary oils

The cytotoxic activity of VO1, VO2 and VO3 were examined against MCF-7 at different concentrations. The results of the three oils showed various inhibitory effects in a dose dependent manner against breast carcinoma cell Fig (2a). The highest inhibitory effect was detected for VO3 against MCF-7 cell line with cancer inhibitory range of 8.42-92.45%, followed by VO2, while VO1 exhibited the lowest cancer inhibitory range. The lowest IC_{50} value was detected for VO3 (54.24 µl /ml against MCF-7 Fig (2b).

3.5 In vitro antidiabetic activity of different rosemary oils

The maximum inhibitory percentage against α-glucosidase was detected for VO1, VO2 and VO3 as 54.87, 62.14 and 86.32 at concentration 1 mg/ ml Fig (3a).

While the descending trend of IC_{50} antidiabetic activity was VO1 > VO2 > VO3, the ICs_{50} values were detected as 385.1, 115.34, 51.92 and 30.59 µl/ml for VO1, VO2, VO3 and acarbose, respectively as illustrated in Fig (3b)

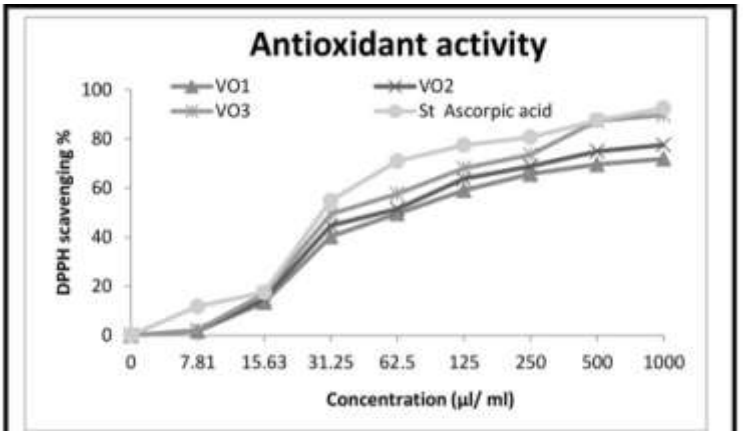


Fig (1a) The in vitro antioxidant activity of different rosemary oil

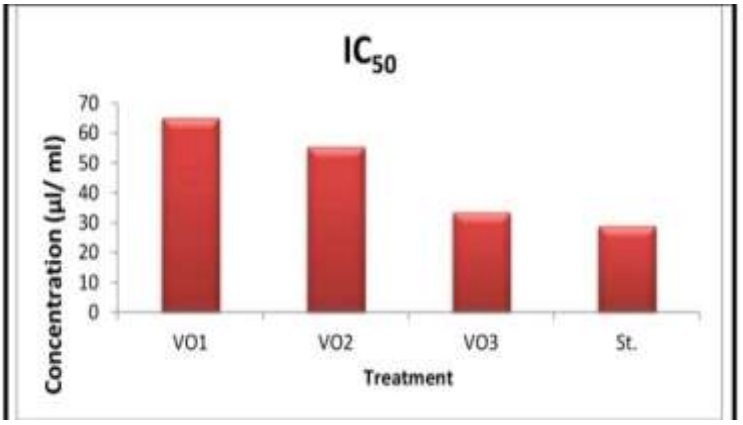


Fig (1b) The IC_{50} values of different rosemary oil

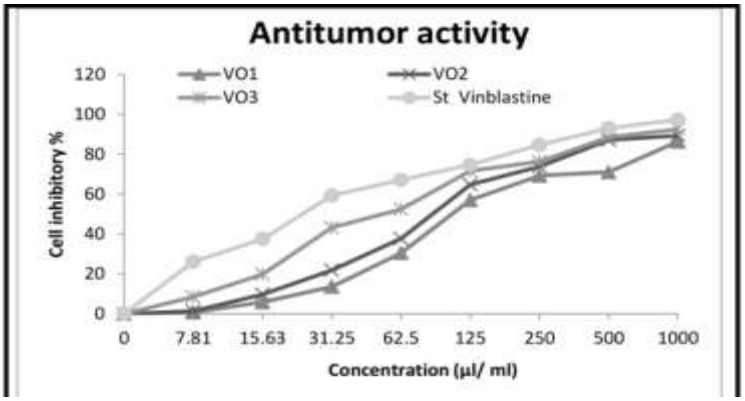


Fig (2a) The in vitro antitumor activity of different rosemary oil

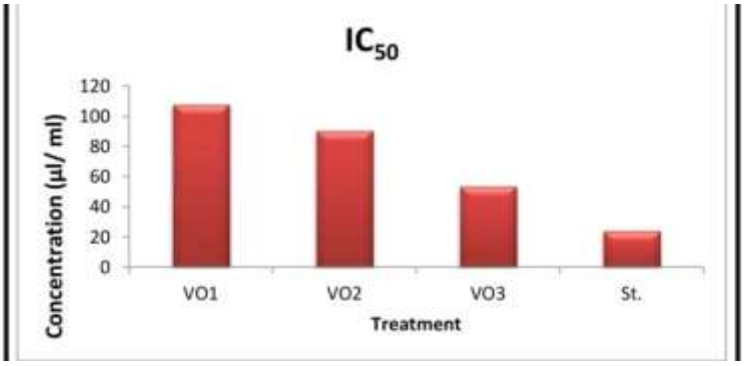


Fig (2b) The IC_{50} values of different rosemary oil

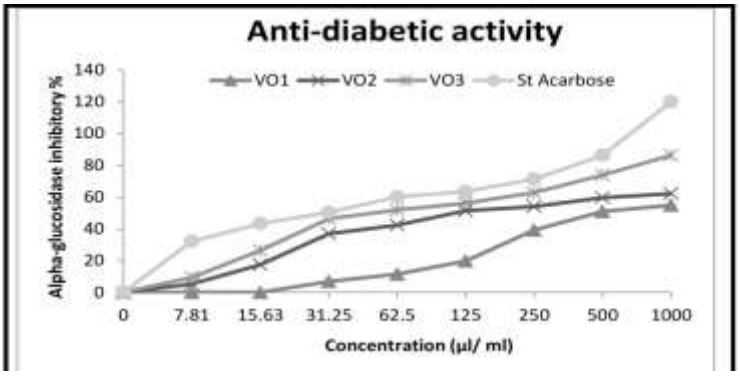


Fig (3a) The in vitro anti diabetic activity of different rosemary oil

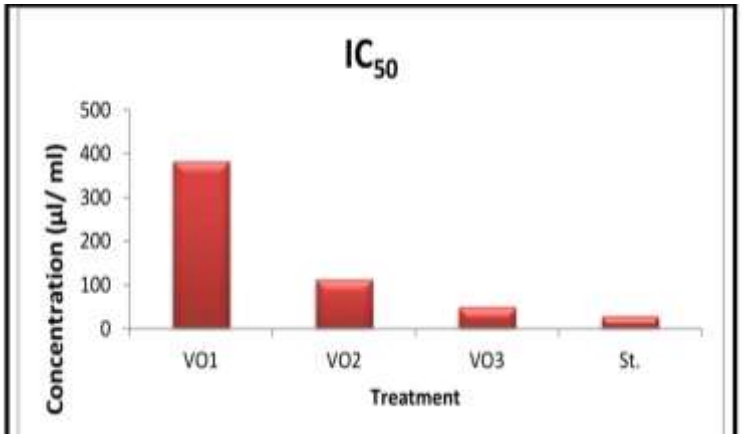


Fig (3b) The Ic50 values of different rosemary oil

4. Discussion

Remarkably, *Rosmarinus officinalis* has displayed hepatoprotective, antispasmodic, anticarcinogenic, antitumorigenic, antimicrobial, anti-inflammatory, and antioxidant properties [27]. It has also shown antidiabetic and neuroprotective activities, among others [28]. These biological properties have made rosemary a potential new therapeutic agent in the treatment of many diseases.

In the current study GC analysis of different Rosmarinus officinalis essential oils revealed that there were variations in components percentage of the essential oil, these results agreed with previous reports on the composition of essential oil from Rosemary plant which stated that essential oil components differences according to the cultivars and fertilization [29]**.**

Regarding the effect of fertilization treatments on volatile oil components, it could be concluded that, the maximum α-Pinene, camphor, 1,8 cineole, linalool, limonene and rosmarinic acid content (3.17, 8.56, 15.90, 16.11, 6.08 and 38.67 % respectively) were resulted in VO3 group while the lowest content of those components was recorded with VO1 group.

These results coincided with those of [30] on *Thymus vulgaris* who found that the application of fertiliztion increased the percentages of P-cymene, thymol and carvacrol in thyme volatile oil, [31] on *Carum carvi* indicated that limonene and carvone content of essential oil percentages were increased as a result of biofertilization , [32] on rosemary plants represented that application of compost with biofertilizer inoculation (namely: *Azotobacter chrocooccum* and *Bacillus megaterium)* resulted in an increment in essential oil yield and components (1,8-Cineol, Camphor, a Pienene, Bornyl acetate and Boreol) in comparison with the recommended dose of chemical fertilization as (control) and [33] on Lemongrass *(Cymbopogon flexuosus* Nees) cited that application of Humic acids with concern of plant spacing increased citral content in oil.

Rosmarinus officinalis (RA) essential oil was described to possess antimicrobial activity against wild strains of Bacillus subtilis, Micrococcus luteus and Escherichia coli [34], Pseudomonas aeruginosa, Staphylococcus aureus, Shigella sp., and Enterobacter [35]. Further, it was reported that RA oil possessed bactericidal activity against acne causing pathogens such as S. aureus, Staphylococcus epidermidis, and Propionibacterium acne through its membrane-damaging effect [36].

In the current study the highest antibacterial activity which was obtained from VO3 may be attributed to the high content of α-Pinene, Camphor, 1,8 Cineole and limonene, these findings in accordance with results of [37, 38], they attributed the antimicrobial property of the essential oil to the presence of α-pinene, 1,8 cineole, camphor, verbenone and limonene.

However, they indicated that, limonene was being the most effective essential oil compound, followed by camphor and verbenone. Similarly, [39] reported that, although α-pinene and 1, 8 cineole contents of rosemary essential oil were high, the inhibitory effect against bacteria may be related also to the content of camphor.

Interestingly, Rosmarinus officinalis essential oil is also capable of preventing lipid peroxidation, a destructive process that is caused by oxidative stress [40]**.** In addition to reducing the amount of reactive species in the body, rosemary has been found to increase the activity of antioxidant enzymes [41]. All these effects augment the body's defense against harmful reactive species and oxidative damage

In the present study, antioxidant activity resulted from VO3 was the highest, whilst the lowest antioxidant activity was gained from VO1, get along with this, the maximum rosmarinic acid content was acquired from VO3 treatment, at the same time the minimum rosmarinic acid content was gained from VO1, get along with this phenolic diterpenes such as rosemarinic acid had been identified as the strongest antioxidant present in rosemary essential oil [42-44]**.**

Concerning antitumor and antidiabetic activity, the promising activity of VO3 may be attributed to the highest content of the major compound (rosmarinic acid), rosmarinic acid have been shown to induce apoptosis within these cancer cells, possibly through the production of nitric oxide [17]. Rosmarinic acid appeared to be the strongest promoter of apoptosis [45]. Rosemary extract also has intriguinganti- tumorigenic activity. Not

surprisingly, multiple studies have identified rosmarinic acid as a promising anti diabetic agent [46-47].

Despite these encouraging findings, more research on the VO3 mechanisms of rosemary's biological activities is needed before it is consistently used to treat human disease.

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