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Potential Efficacy of *Salvia rosmarinus* Extracts as Anticancer, Anti-inflammatory and Antioxidant Characters

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Abstract

Salvia rosmarinus, which belongs to the Lamiaceae family, is recognized for its extract antioxidant, anti-inflammatory, and anti-cancer properties. The present work was focused on the anticancer and genotoxicity against prostate cancer (PC3), therefore, it was designed to evaluate plant characters considered as risk factors for cancer including antioxidant and inflammation properties by many procedures to assure the efficacy of plant crude extract and its fractions. The crude aqueous ethanolic extract and its fractions were analyzed for their phytochemical investigations using HPLC, GC/FID, and GC/MS. The biological evaluation was preceded on crude ethanolic extract as well as its petroleum ether and chloroform fractions. The fractions exhibited promising anti-proliferative (by MTT technique) and genotoxic effects (by Comet technique) on PC3, whereas chloroform and ethyl acetate (EtOAc) fractions showed strong inhibitory effects for cyclooxygenase-2 activity and produced superior antioxidant characters. The crude extract could be considered a rich phenolic source; hence, it contains rosmarinic acid, quercetin, and kaempferol. Fatty acid analysis (by GC) of petroleum ether fraction showed high unsaturated fatty acids, and the major fatty acid was linolenic acid. Omega fatty acids (ω,3:6:9) were detected at a high rate, and the essential oil analysis showed that *S. rosmarinus* could be considered a Bornanone chemotype, monoterpenes, and the major compounds are oxygenated monoterpenes. Therefore, *Salvia rosmarinus* extracts can be used for many purposes, such as in the food industry, the therapeutic sector, and the cosmetic industry.

Keywords: *Salvia Rosmarinus*; Antioxidant; Anti-inflammatory; Human prostate cancer cell line (PC3); Comet assay; Essential oil; Phenolics; Fatty acids.

1. Introduction

Rosmarinus officinalis, *Salvia rosmarinus* Schleid, and *Rosmarinus angustifolius* Mill are synonyms for rosemary [1, 2]. Rosemary is a valuable industrial crop that belongs to Lamiacaea family. It thrives primarily in Mediterranean nations [3, 4]. Its wild populations are largely found in the western Mediterranean basin. Domestication and breeding of rosemary result in more than 20 genotypes (varieties, cultivars, etc.), and it has been utilized for culinary, medicinal, and ornamental purposes since ancient times [5–7]. Ancient Egyptians used rosemary leaves at funerals to help Pharaohs find peace after death [1]. In the field of food science, it is recognized for its essential oil, which has strong antibacterial and antioxidant characteristics, allowing it to be used as a food preservative [6, 8–11]. As a preservative, rosemary extracts are said to have technological advantages as well as consumer benefits [12]. Therefore, rosemary oil is used to preserve practically all animal products, including pigs, cattle, lamb, poultry, and fish [7]. The plant is also utilized as a flavoring agent [13]. Ribeiro-Santos et al., [6] evaluated the nutritional values of *S. rosmarinus* and its bioactive component profiling and showed that it contains multiple vitamins (vitamin A, vitamin E, vitamin D, vitamin B1, vitamin B2, vitamin B3, vitamin B6, vitamin B9, vitamin B12, and ascorbic acid), minerals (Al, As, B, Ba, Bi, Ca, Cd, Co, Cu, Cr, Cu, Fe, K, Li, Mg, Mn, Na, Ni, P, Pb, Se, Sr, Ti, V, and Zn), and fatty acids (saturated, monounsaturated, and polyunsaturated). Furthermore, different types of phytochemicals are present in rosmarinus based on a variety of characteristics such as plant part, processing technique, and geographical origin [6, 14]. On other side, rosemary oil is widely employed not only in therapeutic sectors but also in the cosmetic business; bath essences, cologne waters, shampoo, hair toners and other cosmetic preparations [15, 16]. In addition, rosemary has been used in traditional medicine for centuries to treat a variety of ailments [17]. In addition, the plant's branches have been used to make herbal tea to benefit from its abortive, stomachic, carminative, cholagogue, and antispasmodic properties [18].

Also, they have antibacterial, antifungal, antidepressant, anti-diabetic, anticancer, antioxidant, anti-inflammatory, hepato-protective, and neuroprotective properties [6, 19, 20, 21]. Furthermore, it has been a pillar of many medical traditions over the

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years, particularly Ayurvedic, traditional Chinese, allopathic, and naturopathic medicine [22]. Currently, it is more relevant than ever and is the subject of extensive research, even in extraordinary situations like the most recent pandemic period [23–26]. The requirement to develop guidelines for a better knowledge of clinical indications, efficacy, and safety profiles is emerging [22, 27, 28], given its rise as a topic deserving of scientific research. Accordingly, the objective of this study was to evaluate the anticancer activity and genotoxicity of Salvia rosmarinus crude extract and its fractions on PC3 cell line. Estimation of antioxidant and anti-inflammatory capabilities, accompanied by chemical analyses that could explain the obtained efficacy, was also taken into consideration.

2. Materials and Methods

Chemicals

Ammonium thiocyanate was purchased from Merck (Frankfurter Str. 250 Darmstadt, Germany). Ferrous chloride, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric chloride ($FeCl_3$), trichloro acetic acid and potassium ferricyanide were purchased from Fine chem. Ltd company, India. RPMI-1640 medium, fetal bovine serum (FBS) and trypsin-EDTA were obtained from GIBCO™, Grand Island, New York, USA. Low melting agarose (LMA) and Normal standard agarose was purchased from Bio Basic Inc, Canada. All the other chemicals and kits were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents were HPLC grade from Merck.

Plant preparation and extraction

The fresh plant (rosemary) was acquired from the Botanical garden of Heliopolis University and was identified by professor Kamal Zayed, Faculty of Science, Cairo University, Egypt. The aerial parts were dried using the following procedure: first, they were subjected to a heat shock by placing them in an oven at a temperature of 60ºC for a duration of 90 seconds to stop any enzymatic activity [29, 30, 31]. Subsequently, the parts were dried in a shaded area at room temperature using a drying cabinet. Finally, the drying process was completed by placing the partially dried parts in an oven equipped with a fan at a temperature of 35ºC. The dried plants were pulverized into powders weighing 200g and subjected to extraction using maceration at room temperature. The extraction process involved the use of aqueous ethanol (70%), and this extraction was repeated three times for the same powder until complete extraction was achieved. The extract was concentrated through the application of reduced pressure using a rotary evaporator (Heidolph, Germany) at 35ºC until it was completely devoid of any solvent (10g). The concentrated crude extract was partitioned into two aliquots. One aliquot was transferred into a brown glass vial for subsequent biochemical analyses, while the other aliquot was subjected to successive fractionation using petroleum ether, chloroform, ethyl acetate, butanol, and getting the remaining aqueous fraction. In conclusion, a total of six extracts were acquired and subsequently concentrated. These extracts were then carefully stored in brown glass vials at a temperature of -20ºC until utilized for the evaluations.

Essential oil preparation

The dried aerial component (100g) was subjected to extraction using a typical Clevenger-type hydro-distillation apparatus, following the techniques outlined in the Egyptian Pharmacopoeia [32]. In this extraction procedure, the plant matrix, the aerial portions, was introduced into a round-bottomed flask with a capacity of 1 liter. The flask was then linked to Clevenger-type equipment, with 500ml of distilled water added to the flask. The process of heating was carried out utilizing a heat mantle for a duration of three hours. The extraction temperature was set to 100°C. The commencement of distillation time measurements occurred subsequent to the initiation of distillate droplet descent. The essential oil (HETV) that was obtained was subjected to a drying process using anhydrous sodium sulphate. It was then filtered, weighed, and stored in a brown glass vial at a temperature of 4°C until analysis.

Cell lines and treatment

Prostate cancer cells (PC3) was obtained from an American-type culture collection (ATCC; Manassas, United States). The cells were maintained in an RPMI-16 medium, supplemented with 10% FBS, penicillin (100IU/ ml), and streptomycin (100µg/ ml), in a 5% CO_2 incubator at 37ºC with 95% relative humidity. The cells were passaged every 3–4 days to maintain exponential growth. The plant extracts were dissolved in DMSO (<1%) as a 100mg/ ml stock solution and further diluted in culture medium before use to achieve final concentrations ranging from 1-100µg/ ml.

Methods

1. MTT assay applied on PC3 cell line

The cell viability of human prostate cancer (PC3) cells was evaluated by the mitochondrial-dependent reduction of yellow MTT [33]. Briefly, PC3 cells (5×10^3 cells/ well) were seeded into a 96-well plate and treated with seven concentrations of plant extracts (1.56, 3.125, 6.25, 12.5, 25, 50, and 100µg/ ml) for 48 h. Then, 20µL aliquots of MTT solution were added to each well and re-incubated for 4h at 37ºC. Next, the supernatant culture medium containing MTT was dumped off, and 200µL of DMSO was added to each well and left for 20 minutes to dissolve the formazan crystals. Then, the 96-well plate was shaken for 5 minutes to ensure a homogeneous dye in the solution. Finally, the optical density (OD) of each well was recorded by a microplate reader at a wavelength of 570nm. The percentage of cell viability was calculated as follows:

Cell viability (%) = [(OD of treated cells - OD of blank)/ (OD of control - OD of blank)]

Cytotoxic activity was expressed as IC_{50} (µg/ ml), which is the inhibitory concentration of the test sample that caused 50% inhibition of cell proliferation.

Doxorubicin (DOX) was used as a positive control and was prepared by the same procedures described above at the concentrations (0.039, 0.078, 0.156, 0.3125, 0.625, 1.25, 2.5, and 5µg/ ml).

2. Comet assay

PC3 cells (1× 106 / ml/ well) were seeded in 6-well plate with two concentrations of crude extract, pet. ether fraction or chloroform fraction of rosemary (half IC_{50} and IC_{50}) for 48h. The cells were treated with DMSO and doxorubicin (IC_{50}: 1.8µg/ ml) which were used as negative and positive control cells, respectively. The cells were collected for the examination of DNA damage using alkaline comet assay (pH > 13) as described previously elsewhere [34, 35]. The slides were stained with ethidium bromide and visualized by fluorescent microscope at ×400 magnification. The experiment was replicated three times for each concentration. Six hundred cells were analysed per concentration (200 cells for each experiment) using automatic comet score™ software (TriTek Corp, version 20.0, Sumerduck, VA 22742, United State). DNA damage was expressed as the percentage of DNA in the comet tail (% tail DNA), tail moment (TM), and Olive tail moment (OTM). The values of TM and OTM are measured in arbitrary units (AU).

3. Anti-inflammatory characters

The action of extracts as anti-inflammatory agents was studied using consecutive concentrations (25, 50, 100, 200 and 400µg/ ml) according to modified method of Larson *et al*. [36] against standard material, Celecoxib, at the same concentrations. The tested extracts against COX-1 showed low efficacy, therefore, we used ten times concentrations of COX-2 in this concern (100, 250, 500, 750 and 1000µg/ ml). The reaction was computed over 1 min at 502 nm using spectrophotometer (Jasco, serial No. C317961148, Japan).

4. Oxidative stress assessments

4.1. Scavenging properties

4.1.1. Superoxide radical scavenging ability

The superoxide anion (O^{2-}) scavenging capability of extracts was measured according to the method of Liu, *et al*. [37] using five consecutive concentrations (25, 50, 100, 200 and 400µg/ ml). The reaction mixture was read at 560nm and was implemented by the following formula;

The O^{2-} scavenging % = $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of the control, and A_1 was the absorbance of samples.

4.1.2. Nitric Oxide scavenging

The study compared extracts' ability to scavenge NO• radical with standard materials BHT and vit. C at five concentrations (25, 50, 100, 200, and 400µg/ ml) using Marcocci, *et al*. [38] method, with three replicates each, and measured absorbance at 540nm against the blank solution.

4.1.3. DPPH radical scavenging

Free radical scavenging ability of tested extracts were measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH•) according to Yamaguchi, *et al*. [39] method. Tested extract or standards at five concentrations (25, 50, 100, 200 and 400µg/ ml) were assessed. The absorbance was measured at 517nm. The test was applied with three replicates for each material. The DPPH• radical scavenging in the reaction medium was calculated from the following equation;

$$\text{DPPH• scavenging (\%)} = 100 - [(A_0-A_1)/A_0) \times 100]$$

Where A_0 was the absorbance of the control and A_1 was the absorbance of the sample.

4.1.4. ABTS, cation radical, scavenging (Total antioxidant capacity)

Total antioxidant capacity or ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) scavenging ability was applied according to Miller & Rice-Evans [40] and Arnao, *et al*., [41]. All materials were tested in triplicates at five concentrations (25, 50, 100, 200 and 400µg/ ml). The absorbance at 734nm was measured to represent the cation scavenging ability (total antioxidant capacity) and then was calculated as follows:

$$\text{ABTS scavenging ability percentage} = 1 - (A_{\text{sample}}/ A_{\text{control}}) \times 100$$

4.2. Reduction capability

The ability of plant extracts to reduce ferric ions in potassium hexacyanoferrate complex into ferrous ions were determined according to the method of Oyaizu, [42] at 25, 50, 100, 200 and 400µg/ ml methanol. The absorbance was measured at 700nm. vit. C and BHT were used as standards, higher absorbance of the reaction mixture indicated greater reducing power

4.3. Metal chelating effect

Ferrous ion chelation ability of extracts was preceded according to Dinis, *et al*., [43]. The absorbance of the solution was measured by spectrophotometer at 562nm. The percentage of ferrozine-Fe^{2+} complex formation inhibition was given by the formula:

$$\text{Inhibition (\%)} = [(A_0-A_1)/ A_0] \times 100$$

Where A_0 was the absorbance of the control, and A_1 was the absorbance of the sample and standards. The control contains $FeCl_2$ and ferrozine.

4.4. Scavenging of hydrogen peroxide

The ability of extracts and standards to scavenge hydrogen peroxide was determined according to the method of Ruch, *et al*., [44].

4.5. Lipid Peroxidation

The ability of extracts to inhibit Lipid Peroxides production from linoleic acid was determined according to Gülçin, *et al*. [45] in comparison with BHT and vit. C using ammonium thiocyanate medium. The peroxide level was determined for samples and standard at five concentrations (25, 50, 100, 200 and 400µg/ ml) in triplicates by reading daily of the absorbance at 500nm.

5. Phenolic compounds analysis by HPLC

The aqueous alcoholic extract of rosemary aerial parts was analyzed for phenolic compounds using HPLC technique. The condition of analysis was carried out using an Agilent 1260 series and separation Eclipse C18 column (4.6mm x 250mm i.d., 5µm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate 1ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0min (82% A); 0–5min (80% A); 5-8min (60% A); 8-12min (60% A); 12-15min (82% A); 15-16min (82% A) and 16-20 (82%A). The multi-wavelength detector was monitored at 280nm. The injection volume was 5µl and the column temperature was maintained at 40°C.

6. GC/FID analysis of fatty acids

Fatty acids of petroleum ether fraction were methylated using sodium methoxide [46] and determined using GC model 7890B from Agilent Technologies equipped with flame ionization detector at Central Laboratories Network, National Research Centre, Egypt. Separation was achieved using a Zebron ZB-FAME column (60m x 0.25mm internal diameter x 0.25µm film thickness). Analysis was carried out using hydrogen as the carrier gas at a flow rate of 1.8ml/ min at a split-1:50 mode, injection volume of 1µl and the following temperature program; 100°C for 3 min; rising at 2.5°C/ min to 240°C and held for 10min. The injector and detector (Flame Ionization Detector) were held at 250°C and 285°C, respectively. Fatty acids were identified by comparing their retention time (RT) with that of an authentic sample.

7. GC/MS analysis of essential oil

GC/MS analysis of the essential oil was carried out using an HP5890 Series II Gas Chromatograph, HP 5972 Mass Selective Detector and Agilent 6890 Series Autosampler (Agilent Technologies, USA). A Supelco MDN-5S 30m × 0.25mm capillary column with a 0.5µm film thickness was used with helium as the carrier gas at a flow rate of 1.0ml/ min. The GC oven temperature was programmed at an initial temperature of 40°C for 5 minutes, then heated up to 140°C at 5°C/ min and held at 140°C for 5min, then heated to 280°C at 9°C/ min and held for five additional minutes. Injector and detector temperatures were set at 250°C. Mass spectrometry was run in the electron impact mode (EI) at 70 eV. The chemical compounds were identified by comparing their respective retention time and mass spectra with the published data from NIST and WILLY library of the GC/MS system.

Statistical analysis

The arithmetic mean and standard error were calculated. Data of comet assay were checked for normality and the homogeneity of the variance using the Kolmogorov–Smirnov and Levene tests, respectively. Multiple comparisons between averages of different concentrations of groups (n= 3 replicates) in antioxidant and anti-inflammatory assessments as well as the differences among groups in comet assay with normal distribution were analyzed via one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test. Results were regarded as significant when $P \leq 0.05$. Data were entered into the Statistical Package for Social Sciences (SPSS ver. 25, Armonk, New York: IBM Corp).

Ethical approval

The Medical Research Ethics Committee, National Research Centre, Egypt, approved the two following studies under registration No 19/295 as part of the project entitled "Discovery of new pharmaceutical raw material for treating prostate diseases".

Results

1- Cytotoxic efficacy of rosemary aerial part extracts against PC3

In-vitro anticancer ability of rosemary extract and fractions was investigated against PC3 cells using MTT assay within 48h the data are given in Table (1).

Data in the table clearly show that the essential oil, ethyl acetate, butanol, and the remaining aqueous fraction of rosemary have limited cytotoxic activity on PC3 cells, hence the IC_{50} values exceeding 100µg/ ml. In contrast, Pet. ether and chloroform extracts exhibited significant IC_{50} values (41.87 and 59.10µg/ml, respectively). Additionally, the crude extract showed moderate IC_{50} value (94.40µg/ ml). Based on these findings, it can be inferred that the pet. ether and chloroformfractions derived from rosemary aerial parts exhibited promising cytotoxicity compared to the other tested materials which could be organized as following order: rosemary pet. ether extract > rosemary chloroform extract > rosemary crude extract.

2- Comet assay in PC3 cells

Table 2 and Figure (1) present the impact of rosemary extracts on the induction of DNA breakage in PC3 cells.Results obtained showed that the different concentrations of RO-EtOH and its fractions resulted in a considerable increase in comet tail production. The treatment with IC_{50} concentrations of RO-EtOH resulted in the highest percentage of tail DNA. Treatment with PEF (½IC_{50}) and positive control resulted in similar values in the TM, OTM. In comparison to the positive control, RO-EtOH and CHF (IC_{50}) exhibited greater efficacy in generating DNA in PC3 cells.

Table (1): Cytotoxic effect of rosemary extracts on independent human prostate cancer PC3 cells

Sample No.	Sample name	IC_{50} (µg/ml)
1	Crude extract	94.40
2	Pet. ether fraction	41.87
3	Chloroform fraction	59.10
4	Ethyl acetate fraction	>100
5	Butanol fraction	>100
6	Remained aqueous fraction	>100
7	Essential oil	>100
8	Positive control (DOX)	1.8

IC_{50}: Inhibitory concentration of the sample which causes the death of 50% of cells in 48hr. Data were calculated by fitting the dose response of the concentration-%viability to non-linear regression model on GraphPad Prism software (version 6.0, San Diego, USA).

Table (2): Comet assay findings after treatment with rosemary crude extract and its fractions 48h later in PC3 cells

Treatment	Conc. (µg/ml)	Tail DNA (%)	TM (A.U)	OTM (A.U)
Negative Control	----	6.36 ± 0.11^{a}	0.50 ± 0.06^{a}	1.29 ± 0.10^{a}
Positive Control (DOX)	IC_{50}	15.52 ± 1.04^{bc}	3.73 ± 1.19^{bc}	4.11 ± 0.63^{bc}
Rosemary ethanol crude extract (RO-EtOH)	½ IC_{50}	18.11 ± 0.61^{de}	7.12 ± 0.11^{d}	6.04 ± 0.04^{d}
	IC_{50}	28.88 ± 0.67^{f}	18.34 ± 0.54^{f}	12.15 ± 0.30^{f}
Petroleum ether fraction (PEF)	½ IC_{50}	13.34 ± 0.03^{b}	3.70 ± 0.23^{bc}	3.72 ± 0.11^{bc}
	IC_{50}	16.87 ± 0.08^{cd}	5.17 ± 0.03c	4.68 ± 0.02c
Chloroform fraction (CHF)	½ IC_{50}	14.38 ± 0.74^{bc}	2.97 ± 0.48^{b}	3.26 ± 0.30^{b}
	IC_{50}	20.07 ± 1.99^{e}	10.09 ± 1.12^{e}	7.20 ± 0.64^{e}

Data expressed as mean % ± standard error (n=3). Six hundred cells per each treatment were analyzed using automatic comet score™ software. In each column, the means with the similar superscript letters have no statistical significance (P>0.05), while the means with dissimilar superscript letters have statistical significance (ANOVA, Tukey's test, P<0.05)

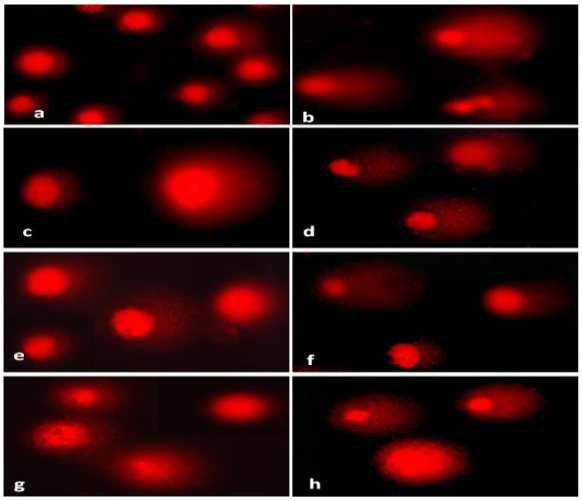

Fig. (1): Photomicrographs of alkaline comet assay showing different pattern of comet tail formation in PC3 cells: (a) Negative control cells; (b) Positive control cells (DOX); (c, d) Cells treated with RO-EtOH; (e, f) Cells treated with PEF; (g, h) Cells treated with CHF (original magnification 400×)

3- Anti-inflammatory efficacy

It is evident from obtained results for COX-2 that all the assessed materials showed concentration dependent inhibitory action. Concerning the IC_{50} values of tested materials, the ethyl acetate and butanol fractions produced the same inhibitory action on COX-2 and they were the most promising materials followed with chloroform extract and the remained aqueous fractions. The crude extract and all the five mentioned materials have IC_{50} values less than the reference drug (Fig. 2). On the contrary, petroleum ether fraction exhibited the greatest IC_{50} value. It is worth mentioning that crude extract, chloroform fraction, and reference material presented a close inhibitory percentage at 400µg/ ml with an insignificant difference.

The evaluated materials showed weak inhibitory action on COX-1 at the assessed concentrations of COX-2 (25, 50, 100, 200 and 400µg/ml). Therefore, they were reassessed using high concentrations represented ten times of test concentration for COX-2 (100, 250, 500, 750 and 1000 µg/ml). The most inhibitory action on COX-1 was recorded for rosemary crude extract

which means it is less safe on COX-1 as compared to reference drug. The reference drug, remained aqueous fraction and ethyl acetate exhibited the second inhibitory level which was near to chloroform fraction. Additionally, Butanol fraction has highest IC_{50} values on COX-1 and is safe (Fig. 3). It is evident from the obtained results that rosemary extracts produced selective inhibitory action against COX-2 with protecting effect on COX-1 and all of tested materials presented better or nearer data to reference drug.

To evaluate the selectivity of *Salvia rosmarinus* extracts towards COX-2, the inhibitory action percentage of materials against COX-2 was divided into the inhibitory action percentage of same extract on COX-1 at the same concentration and the obtained values were shown in Fig. (4). It is clearly observed that most of the tested materials showed inverted selectivity effect towards COX-2. This means that increasing of extract concentration decreases the selectivity. On the other hand, crude extract and chloroform fraction exhibited high selectivity to COX-2 as compared to reference drug whereas, ethyl acetate and butanol fractions produced near selectivity levels. Concomitantly, the chloroform fraction showed the greatest selectivity action towards COX-2 and insignificant differences for celecoxib and aqueous fraction (at 25µg/ ml), butanol fraction and crude extract (at 100µg/ ml) and butanol fraction + chloroform fraction (at 400µg/ ml)

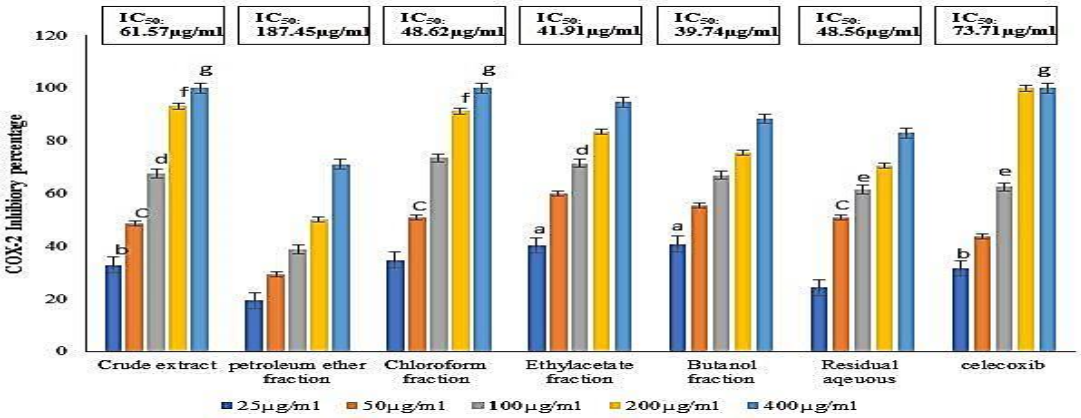

Fig. (2): Cyclooxygenase-2 inhibitory efficacy of *salvia rosmarinus* and reference Drug Celecoxib.

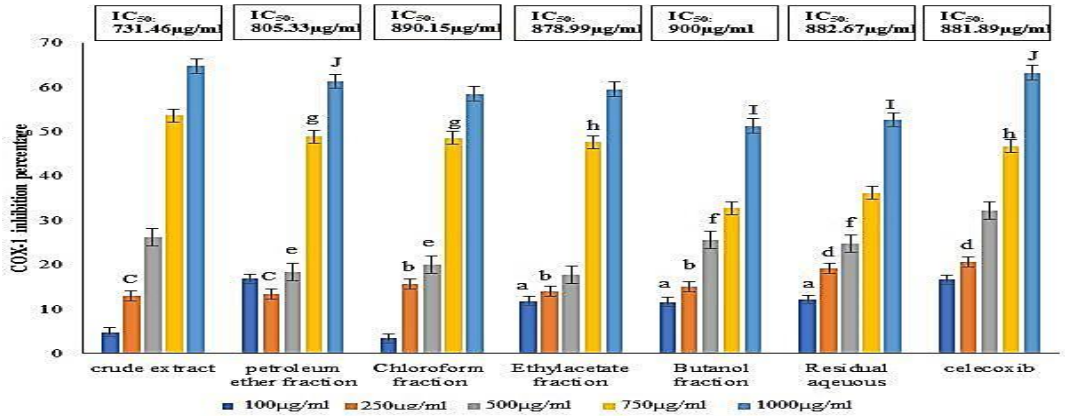

Fig. (3): Cyclooxygenase-1 inhibitory efficacy of *Salvia rosmarinus* and reference drug.

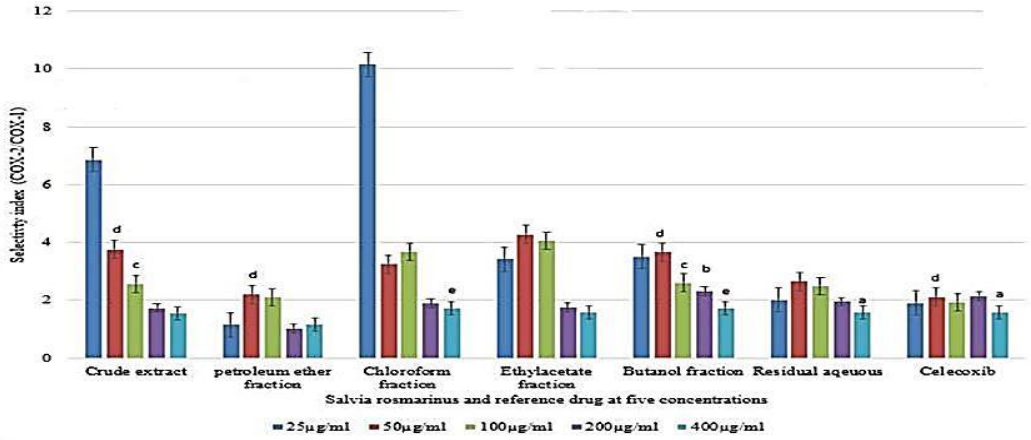

Fig. (4): Selectivity index of *Salvia rosmarinus* towards COX-2 and reference drug

4- Antioxidant properties

4.1- Scavenging properties

4.1.1- Superoxide radical scavenging ability

There are many types of radicals that are produced usually in the human physiological system through lots of biological processes. In the present work, crude extract and its fractions were evaluated against many radical types. Concerning IC_{50} values, results presented in Fig. (5) prove the great efficacy of crude extract on quenching the superoxide radicals hence, the IC_{50} values were low compared to the reference materials and other assessed fractions. Ethyl acetate fraction showed nearly the same scavenging efficacy of BHT and vit. C whereas, aqueous, butanol and chloroform fractions gave moderate scavenging effect. On the other hand, petroleum ether fraction presented the lowest effect in this concern.

4.1.2- Nitric oxide scavenging ability

Nitric oxide radical generated by sodium nitroprusside at physiological pH was found to be inhibited by tested plant extracts and the effect was concentration dependent. Results presented in Fig. (6) showed that the crude extract gave the same NO scavenging ability of BHT and vit. C whereas, the butanol and ethyl acetate fractions produced moderate efficacy. On the other hand, petroleum ether and aqueous fractions exhibited less efficacy as compared to references. Chloroform fraction presented the lowest effect in this concern. Generally, it could be concluded scavenging property that rosemary raw material as crude extract has promising scavenging activity against reactive nitrogen species.

4.1.3. DPPH radical scavenging

It is clear from the obtained results that rosemary materials and extracts have scavenging radical scavenging capacity. The alcoholic extract, ethyl acetate and butanol fractions have the same scavenging ability at 1000µg/ ml. Ethyl acetate fraction produced closest radical scavenging property to crude extract at all tested concentrations (Fig. 7). The calculated IC_{50} values refer to 1) the similar effect of ethyl acetate fraction and vit. C, 2) potent effect of crude extract, butanol fraction and remained aqueous fraction and 3) chloroform and petroleum ether fraction producing the least radical scavenging efficacy by presenting the greatest IC_{50} values. Butanol fraction came at the second level of activity followed with remained aqueous fraction> chloroform > petroleum ether fraction.

In conclusion, ethyl acetate fraction and crude extract was the promising as radical scavenger followed with butanol fraction and then chloroform and finally, petroleum ether.

4.1.4. ABTS, cation radical, scavenging (Total antioxidant capacity)

Total antioxidant capacity of rosemary extract and fractions were assessed, and their results were presented in Fig. (8). The antioxidant capacity of rosemary extracts was increased with concentration. The arrangement of cation radical scavenging efficacy capacity of investigated rosemary extracts was the same as DPPH radical scavenging. Ethyl acetate fraction presented the highest antioxidant capacity at all concentrations.

In addition, the ethyl acetate fraction exhibited the same efficacy of standards (vit. C and BHT) by presenting close IC_{50} values followed with crude extract. In parallel, butanol fraction and aqueous fraction have close IC_{50} values. On the contrary, petroleum ether and chloroform fractions showed moderate cation radicals scavenging activity as IC_{50} values.

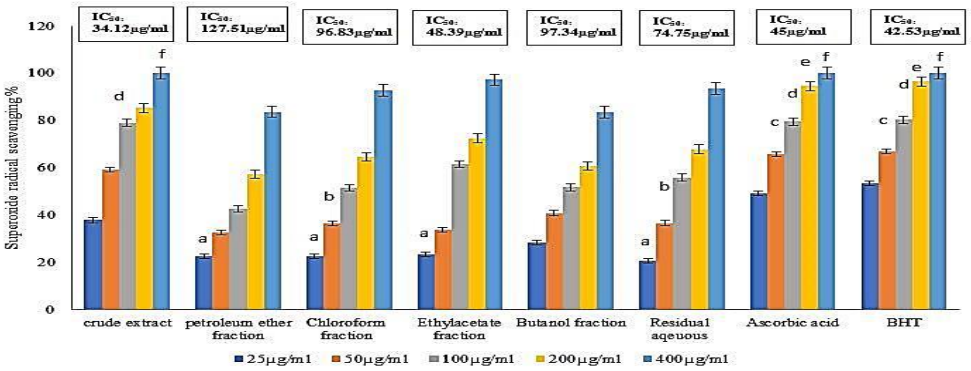

Fig. (5): Superoxide radical scavenging efficacy of *Salvia rosmarinus* and standards.

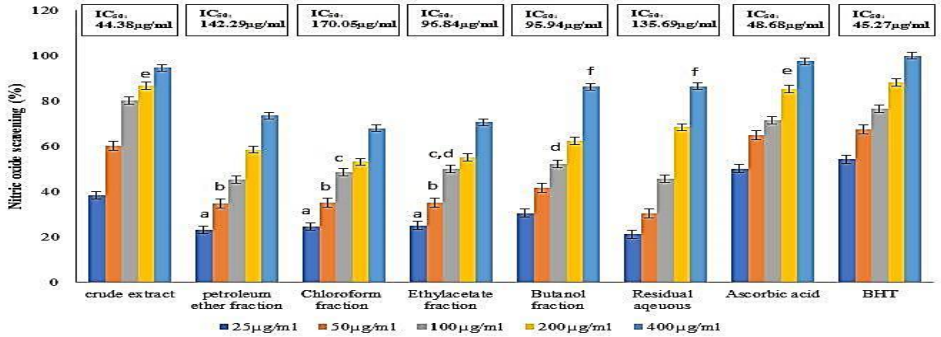

Fig. (6): Nitric oxide scavenging efficacy of *Rosmarinus officinalis* and standards.

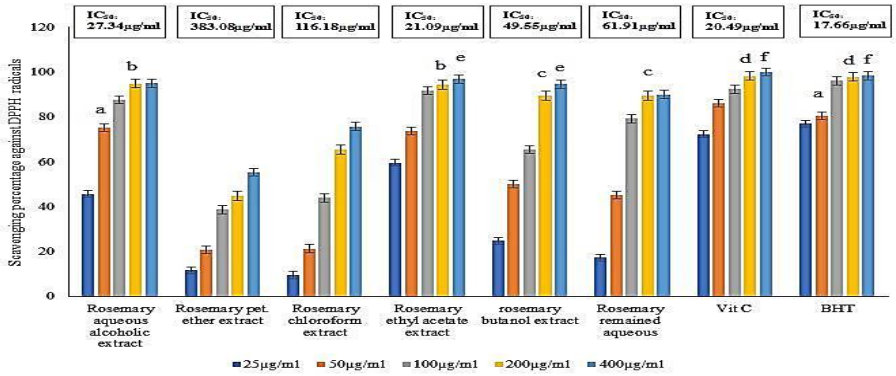

Fig. (7): Radical scavenging property of rosemary successive extracts and standards (vit. C and BHT) at five concentrations.

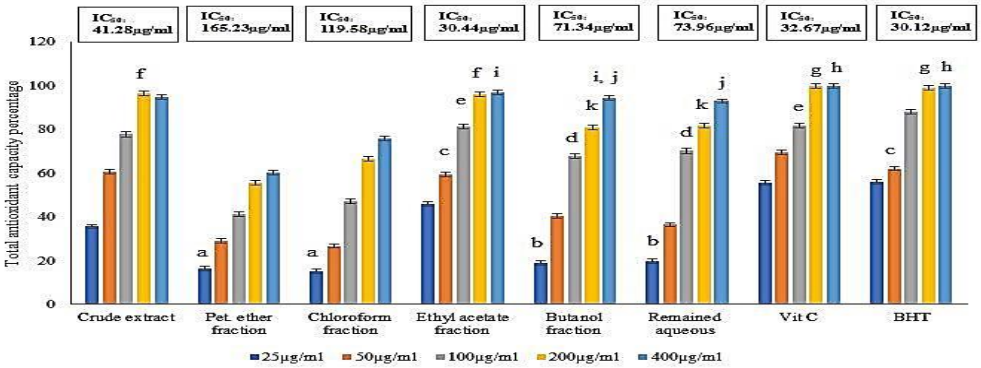

Fig. (8): Total antioxidant capacity (cation radical scavenging %) of rosemary crude, successive extracts and standards (vit. C and BHT) at five concentrations

4.2- Reducing power:

The reduction capability of investigated plant extracts was evaluated in medium by reduction of ferric ions into ferrous ions. The obtained results showed that reduction property of evaluated extracts was increased and the effect was concentration dependent. The ethyl acetate extract showed similar results to crude extract with insignificant difference at all concentrations (Fig. 9).

In summary, ethyl acetate fraction and butanol fraction produced promising reduction capability followed with crude extract and aqueous fraction then chloroform fraction whereas the least mean values were recorded for petroleum ether.

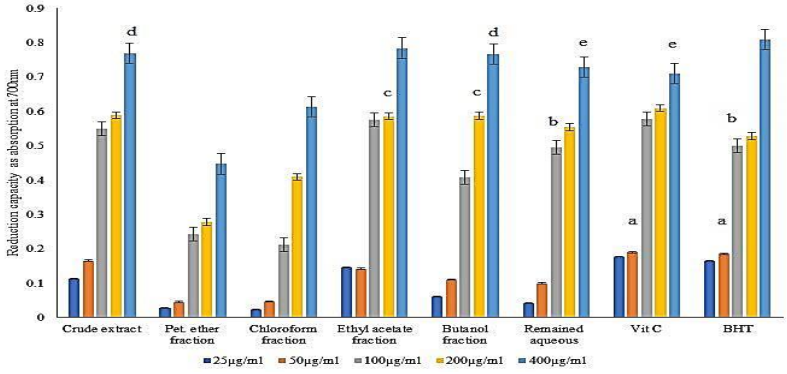

Fig. (9): Reduction capability of rosemary successive extracts and standards (vit. C and BHT) at five concentrations.

4.3- Metal ion chelating effect:

Metal ion chelation character of investigated extracts showed similar trend of scavenging ability and all extracts showed chelating effects with different effective levels. The ethyl acetate fraction exhibited the greatest chelating effect at all tested concentrations. Concerning the IC_{50} values, ethyl acetate showed the nearest value to vit. C and BHT followed with crude extract whereas, butanol fraction and aqueous fraction produced moderate efficacy with near IC_{50} values. It was also observed that the chelating effect increased by concentration (Fig. 10).

4.4- Hydrogen peroxide scavenging capability

The results presented in Fig. (11) showed that crude extract of rosemary exhibited promising hydrogen peroxide scavenging potency. The IC_{50} value was very similar to standards whereas, ethyl acetate and butanol fraction came at the second level of

efficacy followed by aqueous fraction and chloroform. In contrast, petroleum ether gave less efficacy for hydrogen peroxide scavenging capability.

4.5- Inhibition of Lipid peroxides production

The inhibition of lipid peroxides production was evaluated by rosemary crude extract and its fractions using linoleic assay in thiocyanate medium. The obtained results indicated that crude extract, chloroform fraction and ethyl acetate fraction have promising inhibitor activities. Whereas butanol and aqueous fractions gave moderate inhibitory action while petroleum ether showed low inhibitory action with high IC_{50}. Furthermore, the inhibitory action was considerably concentration dependent (Fig. 12).

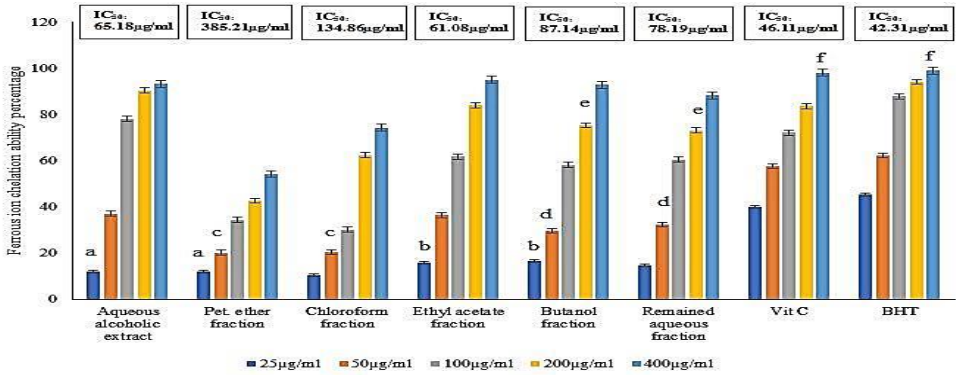

Fig.(10):Ion chelating property of rosemary successive extracts and standards (vit. C and BHT) at five concentrations.

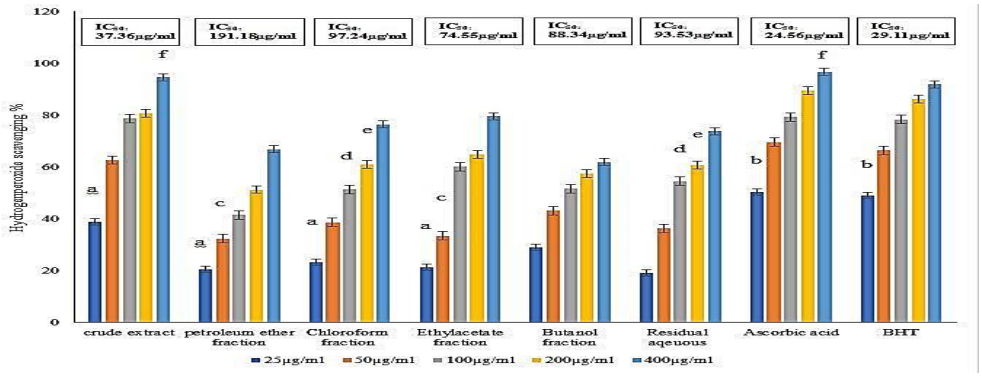

Fig. (11): Hydrogen peroxide scavenging efficacy of *Salvia rosmarinus* and standards.

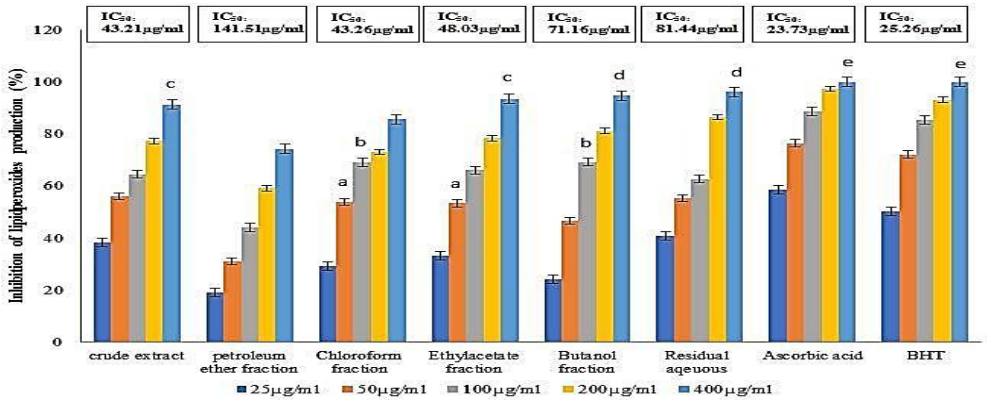

Fig. (12). Inhibition of Lipid peroxide production by *Salvia rosmarinus* and standards.

5- HPLC analysis of phenolics in aerial parts aqueous alcoholic extract

Considering the promising efficacies of aqueous alcoholic extract, it was incorporated into analysis using HPLC to assess its phenolic profile and results were mentioned in Fig. (13). It is evident from results that aqueous alcoholic extract contains high content of phenolic acids. The crude extract of rosemary constitutes phenolic acid in a concentration of 10573.60µg/ g. The major phenolic compound is rosmarinic acid (9619.85µg/ g) and the other major phenolic acid followed with Protocatechuic acid (260.45µg/ g), caffeic acid (230.87µg/ g), p-coumaric acid (130µg/ g) and then chlorogenic acid (115.35µg/ g). Furthermore, the extract contains quercetin (27.49µg/ g) and kaempferol (39.01µg/ g).

6- GC analysis of pet. ether methylated of fraction:

The methylated aerial parts pet. ether fraction was prepared and was analyzed using GC instrument, the obtained results was presented in Fig (14). It is evident from the obtained results that this extract could be considered as unsaturated fatty acids rich extract as the major fatty acids are the unsaturated acids representing 54.18% whereas the saturated fatty acids represented 45.82%. The major unsaturated fatty acids are linolenic acid (27.86%), oleic acid (12.90%) and linoleic acid (8.46%) whereas major saturated fatty acids are palmitic (28.95%) and stearic (4.27%).

It is evident from the presented data that the saponifiable matter prepared from the nonpolar extract, pet.ether contains omega-3 fatty acid; α-linolenic acid, with promising percentage, omega-6 fatty acid;linoleic acid and Homo-γ-linolenic acid, as well as omega- 9;Oleic acid. These plausible fatty acids constituted in saponifiable matter in a ratio 2.79:0.474:1.29(ω-3: ω-6:ω-9).

7- GC/MS analysis of essential oil from aerial part

Essential oil of Rosemary aerial parts was extracted by hydro-distillation (2.80%) and was analyzed using GC/MS and then was identified by comparing results of them with those of authentic. It is occurred from the essential oil analysis that *Salvia rosmarinus* considered in this study is bornanone chemotype (Fig. 15).

It could be concluded from the obtained results that essential oil is solely mono terpenes, and they are fourteen compounds. It constitutes eight oxygenated monoterpenes as major compounds (74.45%) as well as six mono-terpene hydrocarbons (25.55%) presented in less percentage than oxygenated. The major compounds are 2-bornanone (45.61%) followed with eucalyptol (17.82%) and then verbanone (17.82%). The major hydrocarbons are α-pinene (16.22%) and camphene (5.07%) (Fig. 16). The oxygenated compounds are classified into alcohols (22.14%) and the major alcohol is eucalyptol (17.82%), one aldehyde (α-campholenal; 0.53%) and ketones which are 2-bornanone (45.61%) and verbenone (6.18%). On the other hand, many minor compounds are detected; β-Pinene (1.66%), ɣ-Terpinene (1.13%), endo-Borneol (0.96%), α-Terpinolene (0.83%), Terpinen-4-ol (0.77%), linaloo(0.75%), β-Myrcene (0.64%) and α-Campholenal (0.53%), Fig. (16).

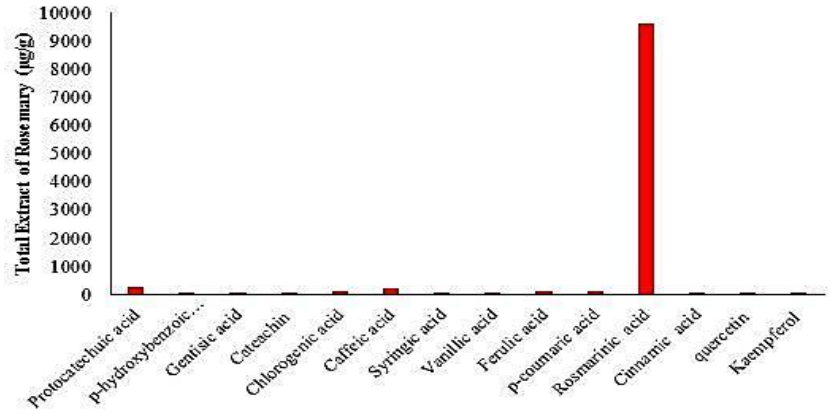

Fig. (13). HPLC analysis of phenolics in rosemary aerial parts aqueous alcoholic extract

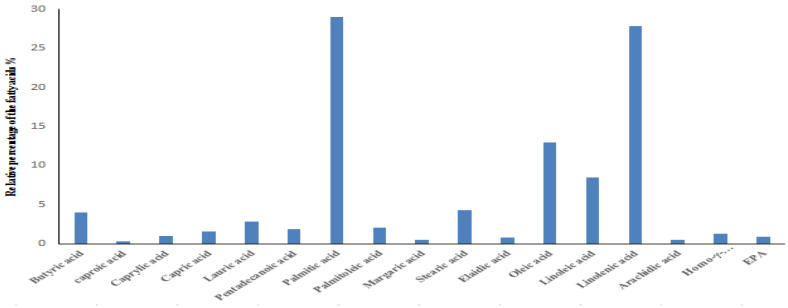

Fig. (14). GC analysis of petroleum ether methylated of rosemary

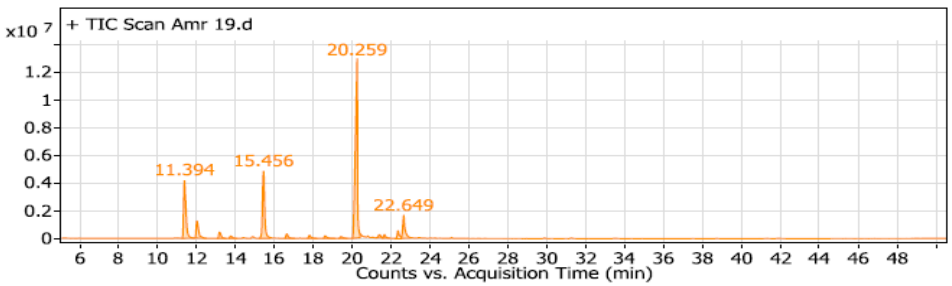

Fig. (15). Essential oil analysis chromatogram of rosemary aerial parts

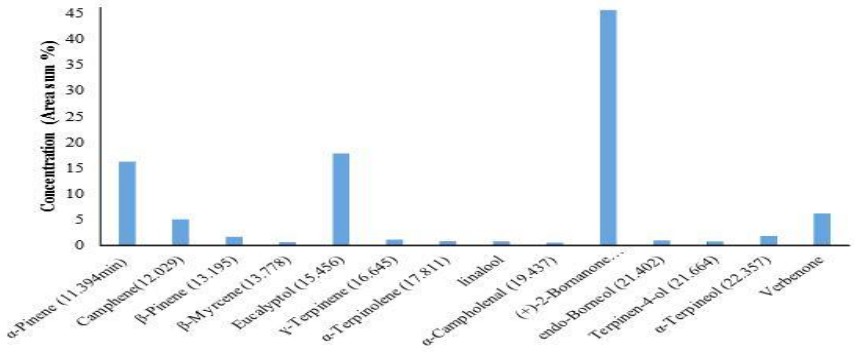

Fig. (16). Essential oil composition of rosemary aerial parts

Discussion

Salvia rosmarinus is widely used for many purposes as food, cosmetic, pharmaceutical industries like creams, perfumes, lotions, soaps and as a fragrance. On the other hand, its leaves have been used in the preparation of soft drinks, beverages of alcohol and different sauces [47]. *Salvia rosmarinus* considered in this study was cultivated in Egypt under organic conditions. *S. rosmarinus* is widely used for many purposes in medicinal applications and food industries applications. Aqueous alcoholic extract of rosemary was prepared and was then fractionated into five fractions; pet. ether, chloroform, ethyl acetate, n. butanol and remained aqueous fractions as well as the crude extract. On the other hand, essential oil was extracted and analyzed; phenolics and fatty acids profile were identified. In addition, antioxidant, anti-inflammatory and antiproliferative properties of the mentioned crude extract and fractions as well as genotoxicity of promising anti-proliferative materials, the obtained results proved the promising antioxidant and anti-inflammatory characters of rosemary extracts as well promising anti-proliferative efficacy for pet. ether and chloroform fractions and potent efficacy for crude extract on PC3 which showed distinctive properties as genotoxic agents on PC3 cell lines[48].

Genotoxic anticancer activity of RO-EtOH and its fractions toward PC3 cells was examined by alkaline comet assay. This assay detects DNA strand breaks (single and double) and bass loss in the individual cells [49]. Our experiments exhibited that incubation of PC3 cells with two concentrations of RO-EtOH, CHF, and PEF for 48 h considerably increased DNA breakage. Similar findings showed that RO-MeOH of leaves induced comet tail formation and apoptosis in human melanoma cell lines [50, 51]. These results indicate that phytochemical constituents of RO-EtOH and its fractions interact with DNA directly or indirectly manner. In this context, spectroscopic studies showed that rosemary extract and its phytoconstituents (e.g., carnosic acid, carnosol, and rosmanol) may interact with DNA via various mechanisms such as intercalation, groove binding, and external binding [52]. Rosemary enhances nitrosative stress, which in turn is implicated in DNA strand breaks, followed by increased poly ADP ribose polymerase (PARP) cleavage, which is an indicator of genomic instability and apoptosis in many cancer cell lines [53, 54, 55, 56]. These mentioned characteristics are due to the phytochemicals constituted in rosemary extracts. Overproduction of reactive nitrogen species is called nitrosative stress which may occur when the generation of reactive nitrogen species in a system exceeds the system's ability to neutralize and eliminate them. Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal function [57].

Recently, the biological and medical sciences are invaded by a modern concept is named oxidative stress. The cell is not able to control the overproduction of reactive oxygen species (ROS), including superoxide anions (O^{2-}), hydroxyl radical (OH^{-}), and nitric oxide (NO). These ROS are generated normally by biological burning in the respiration system. Oxidative stress is not a disease but is potentially implicated in several diseases as a trigger or correlating with complexity through their development [58]. To maintain the biological system healthy, a balance between anti-oxidation and oxidation status is urgent. Therefore, the consumption of enough antioxidants compounds to prohibit or decrease oxidative stress statue is targeted.

Enzymatic and non-enzymatic natural antioxidants play a regulatory role in oxidative stress modulation in physiological system by controlling the reactive oxygen species content including free radicals and many other processes in human body. Furthermore, disruption in these systems could affect the balance of equilibrating reactions inducing increment in oxidative molecules that harm human body and participating in the initiation and progression of many oxidative stress related diseases like neurodegenerative diseases [59], inflammatory disorders [60], cardiovascular diseases [59, 61], cancers [62] …etc. Antioxidant materials, natural or synthetic, play an urgent role in neutralizing free radicals which could prevent the corrosive effect of them on the human body. Phytochemicals extracted from plants have been used as a source of antioxidants and other beneficial pharmacological actions all over the world [63]. The chemical analysis of rosemary showed presence of rosmarinic acid, protocatechuic acid, caffeic acid, p-coumaric acid, kaempferol and quercetin. Rosmarinic acid (a-o-caffeoyl-3,4-dihydroxyphenyl lactic acid) was documented as promising antioxidant agent in in-vitro and in-vivo studies. In in-vitro study, it quenched DPPH radicals and inhibit generation of reactive oxygen species in HepG2 cells [64] and enhanced non-enzymatic antioxidant system, glutathione mechanism, with prevention of lipid peroxides accumulation that ameliorated renal

function and maintaining fertility in intoxicated rats [65]. Furthermore, many pharmacological studies conducted on rosmarinic acid referred to its promising role as anti-inflammatory agent as it inhibited the production of IL-6 [66] and ameliorating the production of Il-4, IL-5 and IL-13 in ear skin lesions [67]. In addition, RA was reported for anticancer efficacy by many mechanisms like decreasing viability of breast cancer stem-like cells by inhibition of their migration, induction of apoptosis, downregulation of B-cell lymphoma- 2 (Bcl^{-2}) expression & upregulation of Bax expression [68], inhibition of tumor growth by regulating the secretion of inflammation and angiogenesis cytokines (IL-1β, IL-6, tumor necrosis factor-α (TNF-α), vascular endothelial growth factor, and transforming growth factor-β) & suppressing NF-κB p65 expression in the microenvironment [69]. Also, RA also showed an anti-tumor effect on 7,12-dimethylbenz(a)anthracene induced skin carcinogenesis [70], a cytotoxic effect on ARH-77 human (multiple myeloma) cells [71], prostate cancer cells [72], and human Hep-G2 liver carcinoma cells [73], and an inhibitory effect on the metastatic properties of colorectal cancer cells [74].

Protocatechuic acid (PA) found in many plants as rosemary proved as useful treatment and/or prophylaxis for a large number of various ailments associated with oxidative stress damage in multiple body systems in vitro and in vivo. It has been documented as antioxidant activity, antiulcer, antidiabetic, antiaging, anticancer, anti-inflammatory, antiviral, antibacterial, antifibrotic, antiatherosclerosis, analgesic and hepatoprotective [75]. In invitro assessments, PA showed radical scavenging characters on many radicals like DPPH, ABTS, superoxide anion and hydroxy radicals via donating hydrogen atom or electron and it presented chelation effect towards cupric and ferric ions with promising reducing power concentration dependently [75]. In addition, PA exhibited chemopreventive efficacy by inhibiting chemical carcinogenesis (in-vitro) and antiproliferative efficacy against different cells which in turn regard its quenching ability towards varied radical types and up regulation of antioxidant enzymes as well as its potential role in phase-I and phase-2 of carcinogen metabolism as it may prohibit the coupling between carcinogen and DNA molecule and therefore prevents mutation and consequently neoplastic transformation. It is worth mentioning that PA has the capability to inhibit inducible nitric oxide synthase, cyclooxygenases and influence cell cycle regulating proteins included in oncogenesis, therefore, it has cancer chemopreventive characters [76].

The other compound detected in rosemary is p-coumaric acid (CA), the most popular isomer of coumaric acid derivatives, has characteristic antioxidant property regarding it capability to decrease oxidative stress through many mechanisms including scavenging characters against radicals [77] as well as presence of the phenyl hydroxy group which able to introduce electron or hydrogen [78]. Consequently, it showed protection capacity against oxidative stress on endothelial cell cultures [79] and alleviated oxidative stress provoked by hydrogen peroxide in the same cells [80]. It prohibited atherosclerosis by oxidation inhibition of low-density lipoprotein (LDL) [81] and cholesterol oxidation with reduction of lipid peroxide concentration [82, 83]. These antioxidant characters were also occurred in in-vivo models as it induced enzymatic and nonenzymatic system in rats [84]. Therefore, p-CA could be a promising candidate in drug schemes in treating several diseases, particularly characterized by oxidative stress. On the other hand, inflammatory conditions controlling was reported for p-CA which could potentially participate in treating certain diseases relevant to inflammatory response like cancer, Alzheimer's, heart diseases….etc [85]. It controlled the cytokine production through the inducible nitric oxide synthase inhibitory action [86]. Furthermore, it was reported as anticancer by different mechanisms including antioxidant pathway [86], changing gene expression that affects cell cycle progression in cancer cells as it impairs G2/M phase and produces antiproliferative action [87]. Additionally, it reduced neuroblastoma cells viability by reduction of cancer cell migration and adhesion that helping in the reduction of cancer spreading in stem cells [87-89] and it showed antiproliferative efficacy on many cancer cell lines as A549 cells [90], MCF7 [91] and HPG2 [90]. It was also documented as inhibitor for AKT and ERK signalling pathways on ECV304 cell line which in turn hinder cell proliferation [92].

Other compounds detected in rosemary in this study have been proved for many characters that support the evaluated properties of rosemary extracts in this study. Fatty acid profile of rosemary petroleum ether extract showed presence of Omega-3, -6 and -9 fatty acids. These fatty acids were reported as 1) antioxidant, it significantly scavenged DPPH radicals and hydrogen peroxide [93], 2) anti-inflammation action by inhibition of many issues involved in inflammation like expression of adhesion molecule, leucocyte-endothelial adhesive interactions, leucocyte chemotaxis, prostaglandins and leukotrienes production from omega-6 fatty acids [94, 95] and reactivity of T cells [96]. 3) Another mechanism of ω-3 anti-inflammatory action is alteration of phospholipids fatty acids composition of cell membrane [97], inhibition of nuclear factor kappa-B transcription [98] which reduces expression of pro-inflammatory and activates anti-inflammatory transcription gene expression (NR1C3) like peroxisome proliferator activated receptor G [99], therefore, many studies support ω-3 and ω-3 fatty acids role in treating rheumatoid arthritis [100, 99] anticancer property as it exhibited induction of apoptosis in human cancer cell lines; colon cancer, gastric cancer, hepatocellular carcinoma, pancreatic cancer, in a dose and time dependent manner through the induction of apoptosis by regulation of caspase pathway [101].

It is commonly known that essential oil components of rosemary are varied according to its chemotype occurs when aromatic plants grow at different ecological system that refers to different climatic and soil conditions [102, 103]. It is evident from the explained results of this study that the evaluated rosemary is bornanone chemotype. Many studies performed the analysis of rosemary essential oil [104, 105, 106] and they explained that the major compounds are monoterpenes and the oxygenated compounds constitutes approximately 75% but they are different in compounds and their percentage according to the growing conditions. In addition, essential oil of rosemary was reported for many activities. It showed radical scavenging capability

against ABTS and DPPH and inhibited lipid peroxidation as well as promising chelating character on iron ions [107]. These results agree with Nawaf *et al*. [108] who concluded that essential oil of rosemary contained oxygenated monoterpenes and monoterpene hydrocarbons exhibited antiradical scavenging activity in DPPH assay as well as promising cytotoxicity against many cell lines; skin cancer, cervical adenocarcinoma, hepatocellular carcinoma, colorectal adenocarcinoma, breast cancer and human epithelial kidney cells.

It is evident from the previous explanation that *Salvia rosmarinus* extracts may exerted their antioxidant, anti-inflammatory and anticancer efficacy on human prostate cancer cell line due to the presence of many phytochemical groups as phenolic acids, flavonoids, omega fatty acids and monoterpenes in essential oil. These results support incorporation of promising extracts into advanced studies on related diseases.

Conclusion

Extracts of *Salvia rosmarinus* have anti-oxidative stress and anti-inflammatory characters. The crude extract and ethyl acetate fraction showed superior antioxidant properties and inhibitory action on COX-2 which support incorporation of the promising materials into more advanced studies on relevant diseases. Additionally, *S. rosmarinus* in this study is bornanone chemotype, furthermore, extract could be considered as rich in phenolics and the major compound is rosmarinic acid. On the other hand, pet. ether fraction is rich in omega fatty acids.

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Declarations

Conflict of interest: The authors declare no conflict of interest.

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