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Biological Activity of *Rosmarinus officinalis* and *Acacia nilotica*: Induced Apoptosis and DNA Damage of A549 Lung Cancer Cell Line.

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ABSTRACT

Medicinal plants play an important role in our life. Therefore, this work aimed to study the antioxidant, anti-inflammatory and anticancer effects of the ethanolic extract of *Rosmarinus officinalis* (RO) and *Acacia nilotica* (AN). Screening for secondary metabolites including total phenols and alkaloids was investigated. The antioxidant activity was tested using DPPH method. Moreover, the anti-inflammatory activity of plants' extracts was studied using carrageenan as a positive control with 20mg/kg, 10mg/kg and 5mg/kg. The anticancer effect of these plants' extract was determined using Neutral Red assay; doxorubicin was used as a reference drug. The results showed antioxidant activity of RO and AN. In addition, RO had a higher antiinflammatory and anticancer activity. Tumor necrosis factor (TNF-α), Human 8-hydroxy-desoxyguanosine (8-OHdG) were determined in cancer cells. The data showed that RO had a higher effect than AN on both TNF-α and 8-OHdG concentrations in cancer cells.

Keywords: *Rosmarinus officinalis, Acacia nilotica*, A549, TNF-alpha, 8-OHdG

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INTRODUCTION

Reactive oxygen species (ROS) are generated by many redox processes that normally occur in metabolism of aerobic cells. ROS can attack important biological molecules, such as lipids, proteins, DNA, enzymes, and RNA. Thus, ROS are involved in a number of degenerative diseases such as cancer, cirrhosis, diabetes, and Alzheimer's reactive oxygen specie [1]. The presence of mediators and cellular effectors of inflammation it may be a great evidence of cancer. Inflammation is an initially badges of cancer the mediators and cellular effectors of inflammation are also involved in metastasis of malignant cells [2].

In 2012, WHO reported that 1.5 million deaths worldwide caused by lung cancer, which is one of the most common malignancies worldwide [3]. The researchers classified lung cancer into two types; non-small cell lung cancer which appeared in 85% of lung cancer and small cell lung cancer [4]. Medicinal plants play an important role in alternative medicine. These plants contain a complex mixture of phytochemical compounds, which can be extracted and entered the world of drug industry [5]. *Rosemarinus officinalis* L. (family Lamiaceae). It is cultivated in the Mediterranean region and is now widely spread in European countries. RO contains many active ingredients, which have much beneficial functionality like polyphenolics, including carnosic acid, carnosol, rosemarinic acid, ursolic acid, etc. [6]. Carsnol is active in anti-inflammation [7] and antimetastatic against malignant melanocytes [8]. Carnosic acid is the most potent antioxidant constituent, but the thermal-oxidation can destroy it [7].

Acacia nilotica is the most significant genus of the subfamily Mimosoideae[9]. *Acacia nilotica* contains many of secondary metabolites such as terpenoids, tannins, alkaloids saponins and glycosids [10]. The oxidative stress causes DNA damage, which leads to mutations that lead to several diseases, 8-hydroxyguanine is considered as the major product that release when DNA was damaged [11].

TNF- alpha is a kind of cytokines, which is a proinflammatory cytokine that induces conflicting pro-apoptotic and anti-apoptic signals whose relative strengths determine the scope of cell death [12]. Nicholson and Thomberry [13] showed that TNF-α induces the activation of caspase 8 and 3 however, TNF-α activates cell survival by activation NF-KB. Therefore, the current study was designed to investigate the antioxidant, anti-inflammatory and anticancer activities of tested extracts. In addition, phytochemical screenings of tested extracts were studied.

MATERIAL AND METHODS

Plant materials

Rosmarinus officinalis and *Acacia nilotica* were collected from local market and was authenticated by the horticulture department, faculty of Agriculture, Cairo University. The plants were grained till it was fine powder.

Chemicals:

All chemicals and reagents were of the highest analytical grade. The lung cancer cell lines A549 was obtained from the cell culture laboratory, Cairo University Research Park, faculty of agriculture, Cairo University.

Preparation of plant extracts:

100gm of each plant were extracted with 1.5-2 L of ethanol (Sigma-Aldrich). This process was done for 3-4 times, the extracts was filtered and evaporated with rotary evaporator.

Quantitative analysis of secondary metabolites

Total phenols

Total phenol content of plants was determined by the folin-Ciocalteu method [14]. An aliquot of 0.1g ethanol extracts was dissolved in 1ml water, 2ml of sodium carbonate 2% and 0.1ml of Folin-Ciocalteu reagent

50%. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 750nm. Ferulic acid was used for preparation of standard curve.

Total alkaloids

Total alkaloids content was performed according to the procedure of Harborn [15]. Five gram of the grained plants and 200ml of 20% acetic acid in ethanol were added and covered to stand for 4 hr. these were filtered and the extracts were concentrated using water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until complete the precipitation. Precipitates were collected and washed with diluted ammonium hydroxide and then filtered. The residues were dried and weighed.

Determination of antioxidant potential:

2,2-Diphenyl-1-picrylhydrzyl (DPPH) assay was performed according to the procedure of Brand-Williams et al. [16]. Different concentrations were prepared (1000,750, 500, 250, 100, 50 and 25 µg/ml). Sample (50µl) was mixed with 450µl of tris HCl pH 7 and 1ml of DPPH then they incubated at 37°C for 30 min. After that absorbance was measured at 517 nm. Quricetin used as standard. Inhibition % was calculated according to following formula: Inhibition %= [(absorbance of DPPH-absorbance of sample) /absorbance of DPPH] *100.

Determination of anti-inflammatory effect

The present study was done on seventy male adult albino rats with average body weight 130-150gm (Animal house of National Organization for Drug Control and Research, Egypt). They were maintained on standard pellet diet and tap water ad libitum and were kept in polycarbonate clean cages under a 12 h. light/dark cycle and room temperature 22-24°C. Rats were acclimatized for two week prior to experimental use. The Ethics Committee, NODCAR, approved this study.

The rats were fasted before the study for 19 hours then they were divided into eight groups each one contained five rats. The thickness of back paw of each animal was determined using vernier calipers. Six groups were given with one oral dose of 20, 10 or 5 mg/kg of each ethanolic extracts. The two other groups one worked as positive group was taken carrageenan (1%) of each rat, the other group was taken indomethacin (1%). One hour after these administrations, each rat was received in its left back paw a subplanter injection of 1% carrageenan suspension (0.1ml per animal). The thickness of the back paw of each rat was measured at 1, 2, 3 and 4 hours after the injection of carrageenan. The thickness of inflammatory was calculated by subtracting the thickness of foot before inflammation from that of the inflamed foot at the different time intervals and after treatment [17].

Anticancer activity:

Determination of cell viability

Neutral Red assay was used to assess the cytotoxicity of *Rosmarinus officinalis* and *Acacia nilotica* against A549 cell line [18].

Determination of TNF-α

TNF-α of lung cancer cell line was determined by ELISA KIT (eBioscience-North America). A standard curve is prepared from 7 human TNF-alpha standard dilutions.

Determination of Human 8-hydroxy -desoxyguanosine (8-OHdG)

8-OHdG of lung cancer cell line was determined by ELISA KIT (Sun Long Biotech. Co. LTD, China). The concentration of 8-OHdG in the samples was calculated by comparing the OD of the samples to the standard curve .

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Statistical analysis

The values were expressed as the mean±SD for the eight rats in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the statistical package for social sciences (SPSS) software package for Windows (version 13.0). Post hoc testing was performed for intergroup comparisons using the least significant difference (LSD) test. A value corresponding to $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Quantitative phytochemical screening

Total phenolic contents:

The total phenolic contents of four tested ethanolic extracts were determined. All results are expressed as µg/ml ferulic acid equivalents Fig. (1). RO showed a higher content of total phenols than AN. Phenolic compounds such as tannins, flavonoids and phenolic acids are substantial to the antioxidant activity of plants. There is a strong relationship between them and diverse biological activities of plants, such as antimutagenicity, anticancer, antiaging [19] and may be related to their antioxidant activity [20]. Folin-Ciocalteu assay depends on the basic mechanism of redox reaction. The antioxidant activity of phenolic compounds comes from their redox properties that can absorb and neutralize free radicals [21,22].

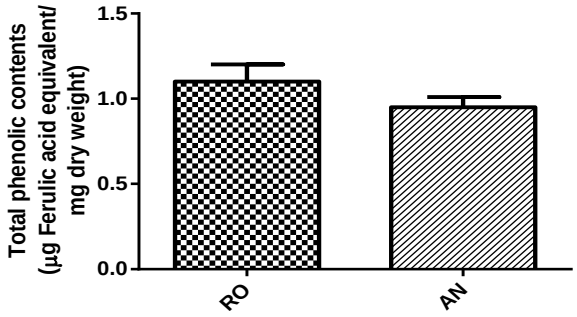

Fig. 1. Total phenol contents of *Rosmarinus officinalis* (RO) and *Acacia nilotica* (AN). Results are expressed as mg ferulic equivalent/ml dry weight. Values are expressed as means±SD (n=3).

Total alkaloid contents

Total alkaloid contents of tested extracts were evaluated (Fig. 2).AN showed a higher alkaloid content than RO. Alkaloids play an important role in chemotherapeutic drugs such as camptothecin, a famous topoisomerase I (TopI) inhibitor [23]. Evodiamine alkaloid has anticancer activity [24], antiproliferating effects in tumor cell [25]. It inhibits angiogenesis [26]. It also induces caspase dependent and caspase independent apoptosis in human leukemic U937 cells [27].

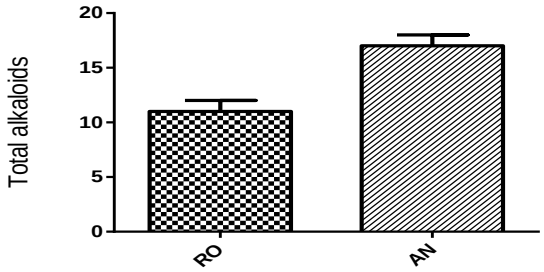

Fig. 2. Total alkaloid contents of *Rosmarinus officinalis* (RO) and *Acacia nilotica* (AN). Values are expressed as means±SD (n=3).

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Antioxidant activity

Table 1 illustrates the percentage inhibition of tested plants compared with qurecetin. There are different methods were used to determine the antioxidant activity. These methods depend on different mechanisms of the oxidant defense system that is scavenging active oxygen species and hydroxyl radicals, inhibiting of lipid peroxidation or chelating of metal ions [28]. The free radical of DPPH contains an free electron which is responsible for the deep purple color when DPPH accept an electron donated by an antioxidant compound, it is decolorized which can be quantitatively measured from the changes in absorbance [29]. Higher antioxidant activity was appeared in *RO* antioxidant activity. The antioxidant activity of RO may be due to the presence of carnosol and carnosic acid which may play an important role in scavenging of peroxyl radicals [**30**].

Table 1. Free radical scavenging activity of tested plants measured by DPPH assay.

Plant extract	Inhibition %						
	25 µg/ml	**50 µg/ml**	**100 µg/ml**	**250 µg/ml**	**500 µg/ml**	**750 µg/ml**	**1000 µg/ml**
Acacia nolitca	35.7±2.1	44.7±0.4	67.6±0.6	70.2±0.2	71.6±0.8	75.9±0.4	76±0.1
Rosmarinus officinals	27.3±0.6	34±0.4	40 ± 0.1	58.7±0.7	64.4±1.4	74.4±0.3	76±0.3
Qurecetin	78.9±4	78.9±5.4	80.7±0.7	81±1.8	81.3±0.9	81.8±1.4	82.5±1.3

Values are expressed as mean ±SD (n=3). Antioxidant activity was determined using DPPH assay.

Anti-inflammatory activity

The results exerted the anti-inflammatory activity of ethanolic extract of RO and AN (Fig. 3 and 4). indomethacin was used as positive control. These results are in agreement with Elena *et al*. [31] who found that RO has a high anti-inflammatory effect due to presence of a high quantity of carnosic acid and carnosol. Carnosol reduces the pro-inflammatory leukotrienes in intact polymorphonuclear leukocytes, inhibit 5-lipoxygenase, antagonize the intracellular Ca^{2+} mobilization and inhibit the secretion of leukocyte elastase [32]**.**

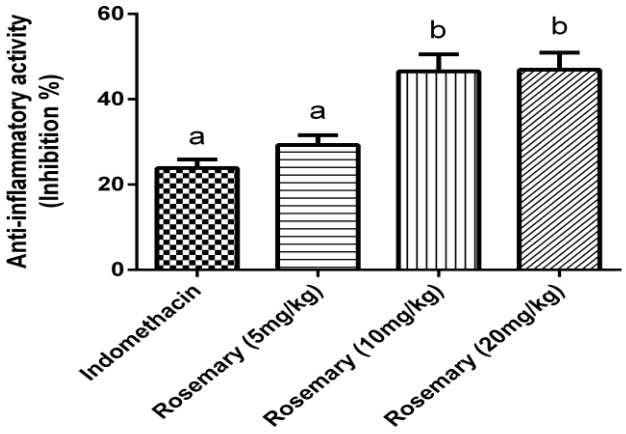

Fig. 3. Anti-inflammatory *effects of Rosmarinus officinals* ethanolic extract with different concentrations on carrageenan-induced hind paw edema in rats. Values are expressed as means±SD (n=3). Values not sharing the same superscript letters were significantly different (*P*<0.01).

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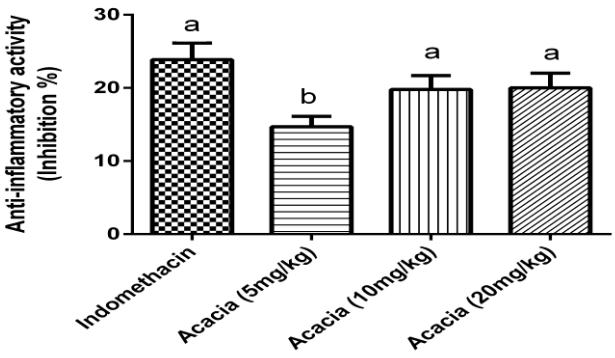

Fig. 4. Anti-inflammatory effects of *Acacia nolitca*ethanolic extract on carrageenan-induced hind paw edema in rats. Values are expressed as means±SD (n=3). Values not sharing the same superscript letters were significantly different (*P*<0.01).

Anticancer activity

Neutral Red assay was used for the evaluation of anticancer activity of ethanolic extracts of RO and AN against A549 lung cancer cell line. RO recorded a higher anticancer activity (Fig. 7) whereas AN has a lower anticancer activity (Fig. 8). RO has many active compounds, which were investigated and shown an anticancer effect. Carnosol and carnosic acid were shown a cytotoxic activity against breast cancer cell line (MCF-7). Carnosol promotes G2 cell cycle arrest in prostatic cancer cells (PC3), which lead to cell death [33]. Khan *et al*. [34] reported that carnosol plays an important role in modulate multiple signaling pathways of cell cycle proteins like PI3K/AKT and apoptotic related proteins. In addition, Carnosic acid preserves from the liver carcinogen aflatoxin A [35].

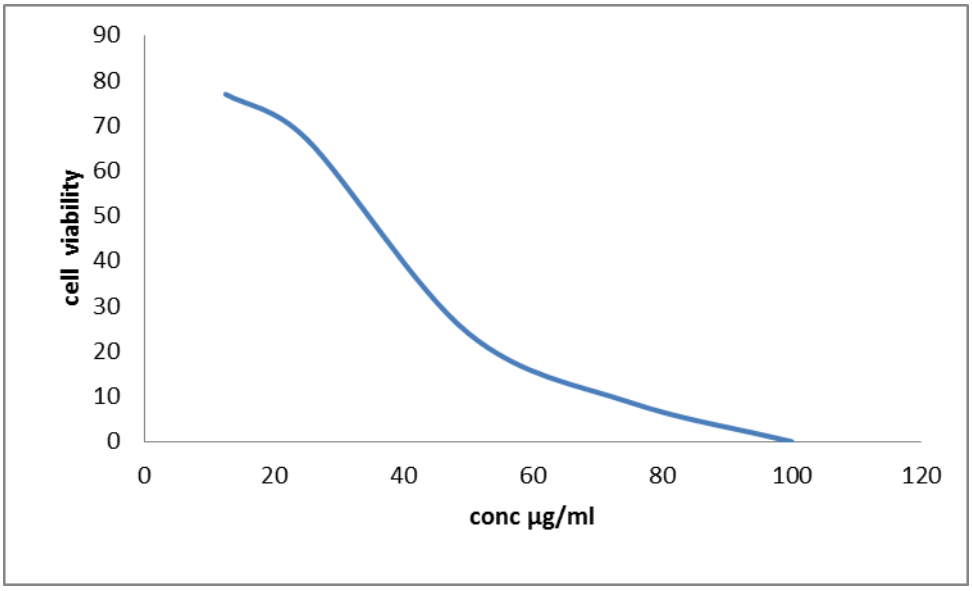

Fig. 5. Dose response relation between *Rosmarinus officinals* for cytotoxic effect against lung cancer A549 cell (n=3).

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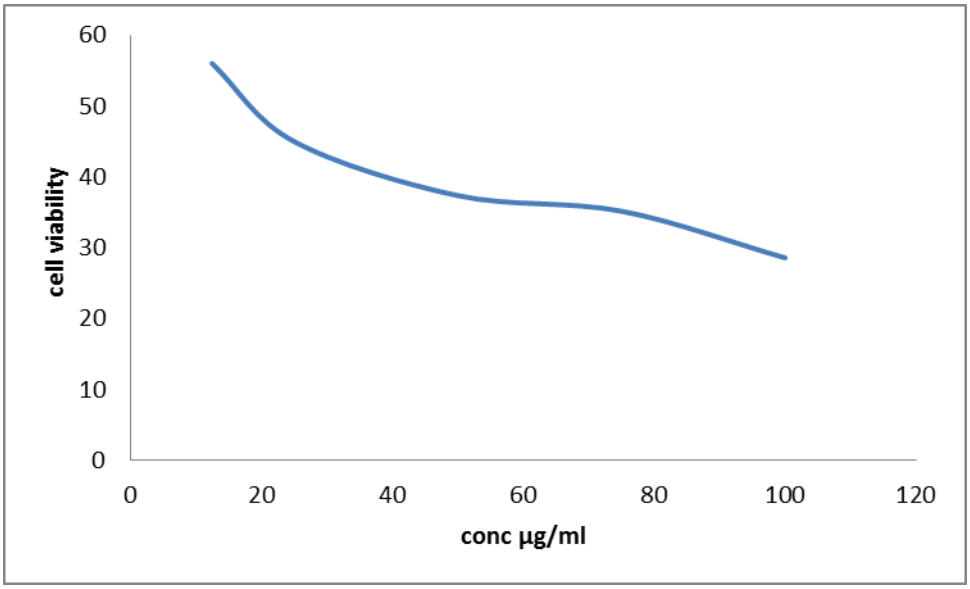

Fig 6. Dose response relation between for Acacia nolitca cytotoxic effect against lung cancer A549 cell.

Determination of 8-hydroxy-desoxyguanosine

The effect of both RO and AN ethanolic extracts on 8-OHdG of A549 cell line was analyzed. The data showed that 8-OHdG was significantly weaker in cancer cells (354±3.84pg/ml). The concentrations of 8-OHdG were increased with RO, AN, doxorubicin treatment (2096±6.18, 1389±10.19, 2281.97±8.14 pg/ml, respectively). In the present study, treatment of lung cancer with tested extracts exhibited stronger expression of the oxidative stress marker 8-OHdG. ROS-derived oxidative stress may be one of the key factors in the pathogenesis of lung cancer

Finally, it seems that there is increased ROS production in lung cancer cell line after treatments in vitro, leading to inhibition proliferation of lung cancer. From our data, we observed high hydroxyl radical-derived oxidative stress and high antioxidant activity in treated lung cancer. In addition, inhibition of repair enzyme function would also lead to low expression of 8-OHdG in treated tumor cells via acceleration of cleavage and secretion of 8-OHdG from cells. In line with this, notable over expression of the cleaving enzyme of 8-OHdG, human 8-oxoguanine glycosylase (hOGG1), in breast cancer compared with pre-malignant breast lesions [36]. The high immunohistochemical expression of 8-OHdG and elevated concentrations of serum 8-OHdG correlated to poor survival and worse differentiation in ovarian carcinoma [37].

Determination of TNF-alpha

The effect of both RO and AN ethanolic extracts on TNF-α concentration of A549 cell line was determined. The data showed that TNF-α was significantly weaker in cancer cells (322± 7.5pg/ml) while its concentration increase with RO, AN, doxorubicin treatment (457±10, 364.83±21.5, 395.5±15 pg/ml, respectively). In the present study, treatment of lung cancer with RO and AN ethanolic extracts exhibited stronger expression of TNF-α.

The TNF-α super family is a group of cytokines with important functions in immunity, inflammation, differentiation, control of cell proliferation and apoptosis. TNF family members exert their biological effects through the TNF (TNFR) superfamily of cell surface two receptors TNF receptor type 1 (TNFR-1), which is found in most cells in the body, and TNF receptor type 2 (TNFR-2), which is mainly expressed on hematopoietic cells [38]. TNF-α can activate pathways leading to three different cellular responses: cell survival and proliferation, transcription of pro-inflammatory genes, and cell death [39].

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Table 2. Effect of *Rosmarinus officinalis* and *Acacia nilotica* on TNF-α and 8-OHdG compared to Doxorubicin as a positive control.

	TNF-α (pg/ml)	8-OHdG (pg/ml)
Rosmarinus officinalis	457 ± 10	2096±6.18
Acacia nilotica	364.83 ± 21.5	1389±10.19
Doxorubicin (Positive control)	395.50 ± 15	2281.97±8.14
Negative control	322±7.5	354±3.84

Values are expressed as mean ±SD (n=3).

The majority of studies that focus on the role of TNF-α in cancer biology have been mainly in the context of soluble TNF-α. As described in previous sections, TNF-α is initially expressed as a membrane bound protein with various biological functions. However, our knowledge of membrane TNF-α's role in modulating tumor inflammatory cells and its effect on tumor growth is limited. There have been very few studies that suggest membrane TNF-α has a role in tumor biology. Some TNFRSF members, such as Fas and TNF-R1, contain an intracellular DD and are known as death receptors [40]. Fas is an effective prototypical cell-killing receptor. The intracellular DD of Fas directly recruits a DD-containing protein known as Fas-associated DD (FADD) via DD–DD interactions [41]. FADD also contains a death-effector domain (DED), which further recruits the DED-containing pro-caspase-8 or pro-caspase-10 to elicit caspase activation and apoptosis [42,43,44].

DR4 and DR5 also recruit FADD and caspase-8 or caspase-10, similar to Fas [45]. TNF-R1-like death receptors, on the other hand, possess the intrinsic capability of both cell-death and cell-survival induction. The underlying mechanism for this duality lies on the recruitment of a multifunctional protein, TNF receptor-associated DD (TRADD), via DD: DD domain interactions, by TNF-R1 [46]. TRADD recruits TRAF2 and FADD [46,47], leading to both survival and death signaling in a "cellular context"-dependent manner. Finally, the results showed that RO has a higher effect than AN on both TNF-α and 8-OHdG protein expression in comparing with doxorubicin and negative control.

In conclusion RO and AN, were the plants under investigation. They are full of secondary metabolites which play an important role in there medicinal effect. RO and AN recorded the highest anticancer effect. The study suggested that both RO and AN may can induce apoptosis in lung cancer cell line, this confirming by increase the concentration of TNF-α and 8-OHdG in treated cancer cells. In addition, they have higher antioxidant and anti-inflammatory effect in compare with reference compounds.

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