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In vitro Evaluation of Antimicrobial and Cytotoxic Activities of *Rosmarinus officinalis* L. (Lamiaceae) Essential Oil Cultivated from South-West Tunisia

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ABSTRACT

Rosmarinus officinalis (Lamiaceae), commonly known as rosemary and iklil, is often used by North African populations for the treatment of several inflammatory and infectious diseases. This study aimed to investigate the antimicrobial and cytotoxic properties of essential oil extracted from the seeds and leaves of *R. officinalis*. Antimicrobial activity assays involved the determination of inhibition zones and the minimum inhibitory concentration with regards to sixteen pathogenic microbial strains, using disc diffusion and minimum inhibitory concentration methods. The oil showed excellent activity against *Staphylococcus aureus*, followed by *Staphylococcus epidermidis* and *Staphylococcus aureus* 25923, with strong inhibition zones of 38.00, 29.40 and 26.00 mm, respectively. Cytotoxicity assays involved the application of an MTT testing method against HeLa cell lines. The results yielded high IC_{50} value values of up to 26,77 µg/ml. overall, the findings provided strong support for the strong candidacy of this plant for potential future application, particularly in the food and pharmaceutical industries, as a safe and cost-effective natural additive to substitute toxic synthetic food additives.

INTRODUCTION

Aromatic plants, herbs and spices are natural resources that have often been employed to produce highly valued extracts and essential oils for various food, cosmetics, nutritional and pharmaceutical industries (Dulger and Gonuz, 2004; Wagensteen *et al.*, 2004; Edeoga *et al.*, 2005). Although their healing properties and health-promoting effects have not yet been fully elucidate, several traditional herbal medicinal products have long been used to heal and cure diseases and to improve health (Foye, 1995). Moreover, several troublesome problems associated with the current application of a number of antimicrobial drugs for the treatment of inflammatory and infectious diseases have recently revived the search for natural substances and compounds with antimicrobial properties (Jain *et al.*, 2010), including medicinal plants (Bauer *et al.*, 1966). In fact, several antimicrobial substances have been produced from plant and herbal sources, including aromatic plants. A number of plant products, namely extracts and essential oils, have been screened for their antimicrobial activities and the results indicated that the plant kingdom is a rich source of biologically active compounds that can be used for the production of new antimicrobial agents against various antibiotic resistant strains (Afolayan, 2003; Zarai *et al.*, 2011).

Of particular relevance to this continuous search for biologically active natural compounds, the Rosemary (*Rosmarinus officinalis* L.), an evergreen plant belonging to the Lamiaceae family of herbs and spontaneously growing in the Mediterranean region, has long been used to prevent the oxidation of fats and oils in various food and cosmetic products. Owing to its desirable flavor and antimicrobial and antioxidant activities, this plant has been widely employed as a spice and flavoring agent in the food processing and pharmaceutical industries (Oluwatuyi *et al.*, 2004;

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Rezzoug *et al*., 2005; Moghtader and Afzali, 2009 ; Kadri *et al*., 2011). It has also been commonly used in ethno-medicine as a general stimulant for the enhancement of blood circulation as well as for the treatment of rheumatic pains, hyperglycemia and skin diseases (Hamedo and Abdelmigid, 2009). The essential oil of *R. officinalis*, commonly known as rosemary oil, has often been reported to inhibit osteoclast activity and to increase bone density *in vitro* (Putnam *et al.*, 2007).

Its cytotoxic activity has also been demonstrated in the literature (Khafagi *et al.*, 2000; El-Meleigy *et al.*, 2010). In spite of the wide flow of data on essential oils of *R. officinalis*, little work has been performed on the *R. officinalis* grown in the South-West of Tunisia.

Considering the potential new opportunities that the latter might open, the present study is the first attempt, to investigate and report on the antimicrobial (pathogenic microorganisms) and cytotoxic (HeLa cell lines) activities of essential oils of *R. officinalis* grown at the South-West of Tunisia.

MATERIALS AND METHODS

Chemicals, reagents and plant material

Chemicals and reagents were purchased from Prolabo (Paris, France) and Pharmacia (Uppsala, Swedeen). Aerial parts of *R. officinalis* were collected from a local area at Mount Sidi Aich, Gafsa, South-west Tunisia, between February and March 2009. The plant materials were confirmed by a senior A. Bekir. Voucher specimens were deposited at ISET, Sfax, Tunisia (Department of Process Engineering) as Bekir 29.

Distillation of essential oil and GC/MS analysis conditions

Fresh aerial parts of *R. officinalis* (300 g) were hydrodistilled using a Clevenger-type apparatus for 4 h to recover essential oils. The distilled essential oils were dried over anhydrous sodium sulfate, filtered and stored at +4°C.

They were then analyzed using an Agilent-Technologies 6890 N Network GC system equipped with a flame ionization detector and HP-5MS capillary column (30 m x 0.25 mm, film thickness 0.25 µm; Agilent-Technologies, Little Falls, CA, USA) (Zarai *et al.*, 2011).

Microbial strain

The antimicrobial activity of *R. officinalis* essential oil was assayed individually against sixteen human pathogenic microbial strains.

The microorganisms consisted of twelve species of bacteria, namely *Staphylococcus aureus* 1327, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Staphylococcus aureus* 25923, *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa* 27853, *Klebsiella pneumoniae* WHO24, *Escherchia coli* 25922 and four species of fungi, namely Botrytis cinerea, *Fusarium solani*, *Penicillium digitatum* and *Aspergillus niger* were used in this study.

Agar diffusion method

The agar diffusion method was used for the determination of antibacterial activities of *Rosmarinus officinalis* essential oil according to the method described by Vanden Berghe and Vlietinck, 1991). Prior to analysis, the essential oil was dissolved in absolute ethanol to create final concentration of 0.10 mg/ml and sterilized by filtration trough 0.22 µm Nylon membrane filter. Different concentrations of the *R. officinalis* essential oil were used to set a correlation between oil activity and its dose (Zarai *et al.*, 2011). All experiments were performed in triplicates.

Determination of the minimal inhibitory concentration (MIC)

The Minimal Inhibitory Concentration (MIC) was obtained by a broth microdilution method (Wade *et al.*, 2001) testing, which was based on reference method M38-P recommended by the NCCLS (Zarai *et al.*, 2011). Experimental values represent the average of triplicates.

Antibacterial assay disc-diffusion method

All tests were performed in MHB supplemented with ethanol 5% (May *et al.*, 2000; Ferreira *et al.*, 2006). Bacterial strains were cultured overnight in MHB at 37°C. Tubes of MHB containing various concentrations of essential oil were inoculated with 10 µl bacterial inoculums adjusted to 10^6 CFU/ml. They were incubated under shaking conditions (100-120 rpm) at 37°C for 24h (Saidana *et al.*, 2008). Control tubes without tested samples were simultaneously assayed. The assays were performed in triplicate.

Antifungal assay disc-diffusion method

The biological activity against yeasts was determined by employing disc agar diffusion method using Sabouraud Dextrose agar (Hamza *et al.*, 2006). The *Rosmarinus officinalis* essential oil was deposited on sterile paper discs (6 mm diameter) which were subsequently placed in the centre of the inoculated Petri dishes. After an incubation period of the 24h at 30°C, the inhibitory activity was compared to that of commercial cycloheximide at a concentration of 1 mg/ml.

Cell lines and culture condition

HeLa cells (cervical cancer line, adherent) were used to investigate the cytotoxicity effect of essential oil. This cell lines were grown in RPMI 1640 media (Gibco) supplemented with 10% (v/v) foetal calf serum (FCS) and 2 mM L-glutamin in tissue culture flasks (Nunc). The media were changed twice a week and kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

MTT test

The proliferation rates of HeLa cells after treatment with essential oil were determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The yellow compound MTT was reduced by mitochondrial dehydrogenases to the water-insoluble blue compound formazan, depending on the viability of cells (Mosmann, 1983, Zarai *et al.*, 2011)

RESULTS AND DISCUSSION

Antimicrobial assays

Several microorganisms that cause harm to human health may exhibit drug resistance due to inadequate use of antibiotics. Recent research has, accordingly, focused on the search for new alternative substances from natural sources, including essential oils from various aromatic plants. The present study was undertaken to quantitatively and qualitatively evaluate the antimicrobial activity of essential oils of *R. officinalis* grown at the South-West of Tunisia against sixteen microorganisms.

The results shown in Table-1 indicate that the essential oil inhibited the growth of the assayed bacterial strains, producing inhibition zones with diameters ranging from 10.2 to 38 mm for Gram (+) bacteria and from 6.20 to 12.80 mm for Gram (-), depending on the susceptibility of the strain. The highest inhibitory zone observed for Gram (+) bacteria was attained against *Staphylococcus aureus* (38.00 mm), followed by *Staphylococcus epidermidis* (29.4 mm), *Staphylococcus aureus* 25923 (26.00 mm) and *Bacillus subtilis* (20.40 mm). Moderate antimicrobial activities were noted for *Enterobacter cloacae* (16.60 mm), *Micrococcus luteus* (12.00 mm) and *Enterococcus faecalis* (11.80 mm). In the case of Gram (-) bacteria, on the other hand, moderate antimicrobial activities in the order of 12.8 mm and 12.20 mm was observed against *Klebsielle pneumoniae* WHO24 and *Salmonella*, respectively. The inhibition zone for ampicillin (10 µg/disc), used as positive controls for bacteria, was recorded in the range of 20-26 mm.

The antifungal activities of the aerial part of *R. officinalis* essential oil obtained by the disc diffusion method are shown in Table-1. The findings demonstrate that the essential oil was able to inhibit the growth of *Fusarium solani* and *Penicillium digitatum* with inhibition zones of 16 mm and 10.4 mm, respectively. It was, however, noted to exhibit low antifungal activity against *Botrytis cinerea* and *Aspergillus niger*. The inhibitory effects of the oil with regards to the growth of fungal strains were lower when compared to ampicillin.

The MIC and IC_{50} values of the aerial part of *R. officinalis* oil are listed in Table-1. The findings revealed that the oil exerted various levels of antimicrobial activity against the different pathogenic microorganisms under investigation. While the MIC and IC_{50} values were noted to range from 50 µg/ml to 150 µg/ml and from 110 µg/ml to 270 µg/ml for Gram (+) bacteria, they were noted to range from 50 µg/ml to 150 µg/ml and from 110 µg/ml to 270 µg/ml for Gram (-) bacteria, respectively. The MIC and IC_{50} values recorded for fungi were observed to range between 90 µg/ml and 180 µg/ml and between 140 µg/ml and 250 µg/ml, respectively.

Oil composition analyses indicated that the antimicrobial activity of the essential oil was strictly related to its chemical composition (Kadri *et al*., 2011). A GC-MS analysis previously performed on the aerial part of *R. officinalis* essential oil using capillary columns led the identification of fifteen compounds accounting for 99.42% of the oil with a yield of 0.48%. The oil was reported to contain a complex mixture of 77.32% of monoterpene and 22.10% of sesquiterpene with new chemotype as 1,8-cineol and trans-caryophyllene. The major constituent was 1,8-cineol (35.32%), followed by trans-caryophyllene (14.47%), borneol (9.37%), camphor (8.97%), α-pinene (7.90%) and α-thujene (6.42%).

Compared to the standards, the essential oil was noted to exhibit high inhibitory activities against three pathogenic bacteria (Table-1). The antimicrobial properties of essential oils from aerial part of *R. officinallis* are, in part, presumably related to their high contents in 1.8-cineole, α-pinene, borneol and camphor. These compounds were previously reported to display marked antimicrobial effects (Mourey and Canillac, 2002; Gachkara *et al*., 2007; Okoh *et al*., 2010). The major component of this oil, 1.8-cineole, was also previously described to display antimicrobial activity against various bacterial and fungal strains (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus typhi, Staphylococcus aureus, Staphylococcus intermedius and Bacillus subtilis*) (Djenane *et al*., 2011). It is reported to increase fungal cell permeability and membrane fluidity and to inhibit medium acidification.

Moreover, essential oils containing terpenes with aromatic rings and phenolic hydroxyl groups were previously shown to be able to form hydrogen bonds with the active sites of the target enzymes, although other active terpenes, as well as alcohols, aldehydes and esters, can contribute to the overall antimicrobial effect of essential oils that are consistent with the ones presented in the current work (Belletti *et al.*, 2004). The specific action of terpenes are thought to induce alterations in cell permeability by inserting lipid bilayers between the fatty acyl chains that make up the membrane, thus disrupting lipid packing and causing changes to membrane properties and functions (Sikkema *et al.*, 1995; Carson *et al.*, 2002). Monoterpene hydrocarbons (α-pinene and β-pinene) are also chemicals whose strong antimicrobial potentials are well documented (Alireza, 2012).

The fact that the antimicrobial activity recorded for the essential oil was more pronounced against Gram-positive than against Gram-negative bacteria could be attributed to the presence of terpene constituents at high percentages, though the mechanism of action of this class of compounds against Gram-negative bacteria is not yet fully understood. It could also be related to the absence of an outer phospholipidic membrane which, in Gram-positive bacteria, restricts the diffusion of hydrophobic compounds through its lopopolysaccharide covering by causing the leakage of vital intracellular constituents and the impairment of the bacterial enzyme systems (Singh *et al.*, 2002; Zarai *et al*., 2011).The components occurring in lower amounts may also contribute to the antimicrobial activity of the essential oils, presumably involving some type of synergism with other active compounds (Marino *et al*., 2001).

Table. 1: Antibacterial and antifungal activity of the essential oil of *R. officinalis* using agar disc diffusion, IC_{50} and minimal inhibition concentration (MIC).

Strains	DD[a]	IC_{50}[b]	MIC[c]	DD[d]
Bacterial strains Gram (+)				
Staphylococcus aureus	38.0±0.5	190±5	100.00	20±0.5
Staphylococcus epidermidis	29.4±0.7	110±6	090.00	26±0.5
Micrococcus luteus	12.0±0.5	120±5	060.00	20±1.5
Enterococcus faecalis	11.8±1.2	140±1	080.00	25±1.0
Enterobacter cloacae	16.6±0.7	161±90	070.00	21±1.4
Staphylococcus aureus 25923	26.0±1.1	270±80	150.00	24±0.5
Bacillus subtilis	20.4±1.3	130±1	090.00	26±0.6
Bacillus cereus	10.2±0.6	113±2	050.00	21±1.0
Bacterial strains Gram (-)				
Pseudomonas aeruginosa 27853	06.2±0.5	470±15	300.00	21±0.5
Klebsielle pneumoniae WHO24	12.8±0.6	330±10	282.00	20±1.0
Escherchia coli 25922	08.2±0.4	452±8	320.00	21±0.9
Salmonella	12.2±0.7	130±5	040.00	22±0.8
Fungal strains				
Botrytis cinerea	06.2±0.5	190±12	100.00	29±1.0
Fusarium solani	16.4±0.5	220±20	090.00	28±0.6
Penicillium digitatum	10.3±0.4	250±16	0120.00	21±0.9
Aspergillus niger	04.3±0.3	140±18	180.00	30±0.5

Results are means of three different experiments,

[a] DD: Disc Diameter of inhibition (halo size) in (mm) , E.oil 100 µg/disc,

[b] MIC: minimum inhibitory concentration (µg/ml),

[c] IC_{50}: 50% inhibition concentration (µg/ml),

[d] DD: Disc Diameter of inhibition zone of ampicillin (10 µg/disc) and cycloheximide (10 µg/disc), were used as positive controls for bacteria and fungi, respectively,

NS: not sensitive.

Table. 2: Cytotoxic activity of *R. officinalis* essential oil determined by the MTT assay.

Oil (µg/ml)	Mean OD_{570} (nm)	% Viable HeLa cell line
0	0.971	100.00
3.90	0.747	76.93
7.81	0.684	70.44
15.63	0.565	58.19
31.25	0.473	48.71
62.50	0.337	34.70
125	0.248	25.54
250	0.194	09.68
500	0.047	04.91
1000	0.014	01.41
1500	0.001	00.11

Cytotoxicity assays

Several essential oils and their constituents display a number of cytotoxic properties that have often been linked to anticarcinogenic activity, which makes them promising potential candidates for application as antitumor agents. In the present work, the aerial part of *R. officinalis* essential oil was submitted, at various concentrations, to *in vitro* cytotoxicity bioassays against HeLa cell lines using the MTT assay based on cell viability. The results presented in Table-2 revealed that *R. officinalis* essential oil displayed dose-dependent inhibition effects on human cell growth. While at a concentration of 7.81 µg/ml the essential oil inhibited the growth of HeLa cells by about 30%, at a concentration starting from 1000 µg/ml it completely blocked the proliferation of HeLa cell lines. Cytotoxicity was expressed as the concentration of oil inhibiting cell growth by 50% (IC_{50}).

As proposed by previous studies (Sylvestre *et al.*, 2006b) that performed the cytotoxic effect of essential oils, IC_{50} values between 10–50 µg/ml represent a strong cytotoxic activity. Moreover, IC_{50} values between 50–100, 100-200, and 200- 300/ µl indicate moderate, weak, and very weak cytotoxic properties, respectively. Furthermore IC_{50} value of *R. officinalis* essential oil was 26.77µg/ml, which represents a higher cytotoxic activity. This result is in agreement with previous reports emphasizing on the strong candidacy of this oil for potential application as a cancer therapeutic agent (Wang *et al.*, 2012).

The cytotoxic activity of the essential oil of the *R. officinalis* leaves may be attributed to specific components of the oil. Some compounds found in the *R. officinalis* leaf essential oil have previously been tested for cytotoxic properties. The cytotoxicities exhibited by α-pinene and β-caryophyllene on a number of cell lines were described to be comparable to those of anticancer agents, such as Paclitaxel and Mitomycin-C. While α-pinene was reported to exhibit in vitro cytotoxicity to HEP G2 human hepatocellur carcinoma cells (Setzer *et al.*, 2006), β -caryophyllene was reported to be cytotoxic to MCF-7, MDA-MB-468 and UACC-257 cancer cell lines. Other studies previously reported that α--humulene (Sylvestre *et al.*, 2005a), geraniol and farnesol (Burke *et al.*, 1997) were active against tumor cell lines.

Furthermore, several terpenes are known for their antitumor attributes. A number of studies previously reported that the volatile sesquiterpene hydrocarbons α-humulene, *β*-caryophyllene and α-caryophyllene isolated from the family Rutaceae were active against human alveolar basal epithelial cells (A-549), colon carcinoma cells (DLD-1) and human prostate adenocarcinoma (LNCaP) cell lines.

They were also described to possess anti-proliferative abilities towards myeloid leukemia (K562) cells. Other hydrophobic compounds could easily cross and/or interact with the membrane to cause a loss of structural integrity. This increased permeability of protons and ions could result in cell death (Sikkema *et al.*, 1995). The abundance of these components in the essential oil, which contains a complex mixture of mono and sesquiterpenes, could presumably account for the cytotoxic activity of the *R. officinalis* essential oil, which might explain the synergism of active compounds with the other minor components involved in the process (Shunying *et al*., 2005).

CONCLUSION

The findings presented in the current work indicate that the essential oil of *R. officinalis* exhibited attractive antimicrobial activities. The latter were more pronounced against Gram-positive than against Gram-negative bacteria, with the strongest inhibitory effect being observed against *Staphylococcus* strains. The results provided evidence in support of the usefulness of this oil for the treatment of various infectious diseases caused by bacteria and fungi. The oil also showed moderate *in-vitro* cytotoxicity against HeLa cell lines. The findings presented in this study suggest that this oil has a number of promising properties and attributes that make it a potential strong candidate for application, particularly in the food and pharmaceutical industries, as a safe and cost-effective natural additive to substitute toxic synthetic food additives.

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