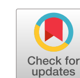
South African Journal of Botany 118 (2018) 52–57

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Cytotoxic and antiviral activities of the essential oils from Tunisian Fern, *Osmunda regalis*



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ARTICLE INFO

Article history:
Received 4 April 2018
Received in revised form 21 May 2018
Accepted 18 June 2018
Available online xxxx

Edited by AM Viljoen

Keywords:
Osmunda regalis
Fern
Essential oil
Hexahydrofarnesyl acetone
Antiviral
Cytotoxicity

ABSTRACT

This study was undertaken to assess the *in vitro* cytotoxic and antiviral activities of the essential oil (EO) from Tunisian fern, *Osmunda regalis*. The essential oil was obtained by hydrodistillation and its chemical composition was determined by gas chromatography and mass spectrometry (GC-FID and GC–MS) analyses that allowed detecting 85.35% of the components. The main compounds were hexahydrofarnesyl acetone (11.82%), 2,4-di-t-butylphenol (6.80%), and phytol (6.46%). Cytotoxicity of the essential oil was assessed on HEp-2 cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Antiviral activity was also evaluated *in vitro* against Coxsackievirus B4 (CV-B4), an enterovirus implicated in a variety of diseases such as myocarditis, type 1 diabetes and central nervous system diseases, by measuring cell viability following viral infection (using MTT) and appreciating the reduction of cytopathic effect (CPE). Hence, the 50% cytotoxic concentration (CC_{50}), 50% inhibitory concentration (IC_{50}) and selectivity index (SI) were determined. The essential oil turned out to be non-toxic against the tested cell line ($CC_{50} = 1772.41 \pm 0.95$) μg/mL, have a relevant anti-Coxsckievirus B4 activity ($IC_{50} = 2.24 \pm 0.99$) μg/mL and a high SI (789.66). Results presented here suggest that *O. regalis* EO is a potentially promising new source as active antiviral agent.

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1. Introduction

For thousands of years, humanity has used various plants to treat many devastating diseases and to relieve different sorts of suffering (Sadat-Hosseini et al., 2017). Essential oils, the secondary metabolites synthesized by medicinal and aromatic plants, have acquired a great renewed interest as a potential source of bioactive lead compounds for drug discovery. They are being studied for their possible use as an alternative for protection against cancer (Loizzo et al., 2007; Driss et al., 2016). A good example is that of the essential oils of Chinese propolis which have been cited to inhibit the proliferation of human colorectal cancer cells (Sena-Lopes et al., 2018). *Thymus vulgaris* essential oil inhibits human head and neck squamous cell carcinoma growth (Sertel et al., 2011). Moreover, the efficacy of many essential oils and their volatile constituents against a wide range of bacterial pathogens and viruses has been well documented and the stunning efficacy of almost of them against herpes lesions has been also demonstrated (Loizzo et al., 2008; Astani et al., 2010). In addition, many essential oils have been reported to possess a great antioxidant potential (Urbizu-González et al., 2017). *Osmunda regalis* L. known as royal fern (Magrini and Scoppola, 2012) is a member of the Osmundaceae family which is the most primitive fern comprising the genera: *Osmunda*, *Todea* and *Leptopteris*, with about 21 species (Moore et al., 2009). *O. regalis* is a cosmopolitan species, it is widely distributed throughout Southern Africa, America, Asia, New Zealand and Northern and Eastern Europe (Tian et al., 2008). In addition, *O. regalis* grows spontaneously in Tunisia, in humid slopes near water as a perennial plant with raised stems (20–35) cm. Previous phytochemical investigations of the surface lipids from the German *Osmunda regalis* fern, showed the presence of free fatty acids, such as linoleic and oleic acids (Gemmrich, 1977), alkanediols, ketoaldehydes and fatty acid esters (Jetter and Riederer, 1999).

As a medicinal plant, *O. regalis* is endowed of a great source of active ingredients useful to treat some diseases and could be considered of a highly efficient remedy. Moreover, it has been used in folk medicine for the treatment of some joint disorders, bone fractures, rheumatic and arthritic, arthrosis or back pain. *O. regalis* is antojil wine, which has been traditionally employed for muscle-skeletal disorders,

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https://doi.org/10.1016/j.sajb.2018.06.015
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traumatic injuries such as bruises, dislocations, or sprains. It is also used as tonic, against rickets, digestive and respiratory disorders (Molina et al., 2009). *O. regalis* has also been used in reproductive health of tribal women; so, it is an abortifacient. Leaves are mixed with thin cured for the birth control (Singh and Singh, 2012). This plant is cultivated as an ornament, its fibers are exploited for orchid-growing and the hairs of young leaves are used for textile production (Jetter and Riederer, 1999). On the basis of its potential pharmacological benefits, we report herein, the chemical composition of *O. regalis* essential oil, its toxicity towards HEp-2 cells and its effect on Coxsackie B viruses which is positive sense single-stranded RNA virus belonging to enterovirus genus and picornaviridae family and Coxsackie B viruses are associated with a variety of diseases including myocarditis (Huber, 2006), diabetes (Jaïdane and Hober, 2008) and central nervous system pathologies especially among new-born and infants (Michos et al., 2007; Kumar et al., 2012).

2. Materials and methods

2.1. Plant material and extraction of the essential oil

Aerial parts of *Osmunda regalis* L. was collected during mature stage from Northwest of Tunisia on June 2011. The identification of the plant material was performed by one of the authors (R.E.M). Some voucher specimens [PTER-OSM/01-Osm.r; 00017/2011] were deposited both in the local Herbaria of the Faculty of Pharmacy of Monastir and in the Faculty of Sciences of Bizerta, Jarzouna, Tunisia (Fig. 1). Collected material was air-dried at room temperature till totally dehydration. Then, a fine powder was obtained with a mean particle size of 1 mm. *O. regalis* essential oil was obtained by hydrodistillation in a Clevenger-type apparatus according to the procedure given by Okoh et al. (Okoh et al., 2011). The essential oil yields were calculated on a dry-weight basis (w/w).

2.2. GC-FID GC–MS analyses

GC analyses of the extracts were performed using a gas chromatograph (Agilent 7890A, Palo Alto, CA, USA), equipped with a 30 m × 0.25 mm i.d. with 0.25 μm stationary film thickness DB-5 capillary column (Agilent J&W) and a flame ionization detector (FID). The following temperature program was used: from 60 °C to 246 °C at rate of 3 °C min^{-1} and then held at 246 °C for 20 min (total analysis time 82 min). Other operating conditions are the following: carrier gas helium (purity ≥99.9999% – Air Liquide Italy); flow rate, 1.0 mL min^{-1}; injector temperature, 250 °C; detector temperature, 300 °C. Injection of 1 μL of diluted sample (1:100 in hexane, w/w) was performed with 1:10 split ratio, using an autosampler (Agilent, Model 7683B). GC–MS analyses were carried out using a gas chromatograph (Agilent 6890 N) equipped with a 30 m × 0.25 mm i.d. with 0.25 μm stationary film thickness HP-5 ms capillary column (Agilent J&W) coupled with a mass selective detector having an electron ionization device, EI, and a quadrupole analyzer (Agilent 5973). The temperature program was the same used for GC. Other chromatographic operating conditions are the following: carrier gas helium (purity ≥99.9999%); flow rate 1.0 mL min^{-1}; injector temperature, 250 °C. Injection of 1 μL of diluted sample (1:100 in hexane, w/w) was performed with 1:20 split ratio, using an autosampler (Agilent, Model 7683B). The MS conditions were as follows: MS transfer line temperature, 240 °C; EI ion source temperature, 200 °C with ionization energy of 70 eV; quadrupole temperature, 150 °C; scan rate, 3.2 scan s^{-1} at m/z scan range: (30 to 480). To handle and process chromatograms and mass spectra was used the software MSD ChemStation (Agilent, rev. E.01.00.237).

Constituents of the samples were identified by comparing: mass spectra fragmentation patterns with those of a computer library (Adams, 2007; Stein et al., 2008) and linear retention indices (RI) based on a homologous series of C8-C26 n-alkanes with those reported in literature (Stein et al., 2008). The Table 1 shows the chromatographic results, expressed as GC peak area percentages.

Fig. 1. *Osmunda regalis* L.

2.3. Antiviral and cytotoxicity assays

2.3.1. Cell culture and virus preparation

HEp-2 cell line (Human epithelial cells) were used to propagate and titrate Coxsackie virus B4 (CV-B4), kindly provided by Prof. J. W. Yoon, Julia M.C. Farlane, Diabetes research center, Calgary, Alberta, Canada,

Table 1
Chemical compositions of *Osmunda regalis* L. essential oil (%).

Number	Compound	RI[a]	%RA[b]	Identification[c]
1	Camphor	1141	3.30	RI,MS
2	1-Dodecene	1187	0.71	RI,MS
3	Dodecane	1200	0.42	RI,MS
4	1-Tetradecene	1392	2.22	RI,MS
5	Tetradecane	1400	0.81	RI,MS
6	(E)-α-Ionone	1428	0.38	RI,MS
7	2,4-di-t-Butylphenol	1512	6.80	RI,MS
8	Dihydroactinidiolide	1525	0.86	RI,MS
9	1-Hexadecene	1588	4.12	RI,MS
10	Hexadecane	1600	0.97	RI,MS
11	Benzophenone	1626	0.52	RI,MS
12	1-Octadecene	1792	4.42	RI,MS
13	Octadecane	1800	1.37	RI,MS
14	Neophytadiene	1843	4.64	RI,MS
15	**Hexahydrofarnesyl acetone**	**1844**	**11.82**	**RI,MS**
16	Neophyltadiene isomer 1	1862	1.26	RI,MS
17	Neophytadiene isomer 2	1881	2.11	RI,MS
18	Nonadecane	1900	0.43	RI,MS
19	7,9-Di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione	1929	1.54	RI,MS
20	Isophytol	1949	0.84	RI,MS
21	1-Eicosene	1992	4.38	RI,MS
22	Eicosane	2000	0.50	RI,MS
23	γ-Palmitolactone	2105	1.74	RI,MS
24	Phytol	2111	6.46	RI,MS
25	Nonacosanal	2118	2.31	RI,MS
26	1-Docosene	2189	2.81	RI,MS
27	Docosane	2200	0.73	RI,MS
28	Tricosane	2300	2.33	RI,MS
29	1-Tetracosene	2396	1.89	RI,MS
30	Tetracosane	2400	0.87	RI,MS
31	Pentacosane	2500	1.97	RI,MS
32	1-Hexacosene	2593	0.97	RI,MS
33	Tretracosanal	2631	2.53	RI,MS
34	1-Heptacosene	2693	1.61	RI,MS
35	Heptacosane	2700	1.27	RI,MS
36	1-Hexacosanol	2798	0.81	RI,MS
37	Squalene	2823	1.11	RI,MS
38	Hexacosanal	2830	1.52	RI,MS
	Identified components (%)		85.35	
	Unidentified components (%)		14.65	

[a] Retention index relative to n-alkanes on DB-5 capillary column.
[b] Relative area (peak area relative to the total peak area).
[c] Identification: MS, comparison of mass spectra with MS libraries.

as $TCID_{50}$ (tissue culture infectious dose 50) according to the method of Reen and Muenc (Reed and Muench, 1938). HEp-2 cells were cultured in Eagle's essential medium (MEM, GIBCO, USA) supplemented with 10% heat inactivated fetal calf serum (FCS), 1% 2 mM L-Glutamine (Biowhittaker), 1% 50 μg/mL streptomycin, 1% 50 IU/L penicillin (GIBCO BRL), 1% non-essential amino acids (GIBCO BRL) and 1% (2.5 μg/mL) Fungizone (Amphoterin B, Apothecon).

2.3.2. Cell seeding and infection

The cytotoxicity effect of obtained essential oil was evaluated on HEp-2 cells using MTT assay. Only viable cells are capable of reducing the MTT salt to colored formazan. Cells were cultured in 96 well culture plates at a final concentration of 5×10^4/100 μL/ well for 24 h at 37 °C and 5% CO_2 in humidified atmosphere. Cells were washed 2 times with PBS before adding the compounds or the virus. In all of the experiments, cell control (cells that were not infected with the virus or treated with the compound) and virus control (cells that were infected only with the virus, but not treated with the compound in the antiviral assays) were taken into account.

2.3.3. Cytotoxicity assay

Cells were washed twice with PBS and inoculated with 200 μL of oil extract diluted in MEM supplemented with 2% FCS (two-fold dilutions, ranging from 2000 μg to 0.975 μg). Cells were allowed to grow for 72 h at 37 °C 5% CO_2. Then, medium was removed, cells were washed in PBS and 20 μL of MTT solution at 5 mg/ml (MTT, Sigma, Saint Louis, MO, USA) were added to each well. Plates were incubated for 4 h at 37 °C 5% CO_2 and the formed formazan crystals were solubilized in DMSO (Sigma, USA). The absorbance of formazan-generated dye was determined using a microplate reader (Thermo Fisher Scientific, USA) at 570 nm. The % of viability was calculated using the following formula:

$$(\%V) = \text{Absorbance of treated cells/Absorbance of control cells}$$

The viability of control cells was set to 100%, the CC_{50} value was derived from the corresponding dose-response curves as the concentration of the oil that reduced cell viability by 50%.

2.3.4. Antiviral activity assay

The assessment of the anti-CV-B4 activity of the *Osmunda*'s oil extract was based on the evaluation of the inhibition of virus induced cytopathogenicity and death in HEp-2 cells. For this purpose, cells were seeded into 96 well plates at a final concentration of 5×10^4/100 μL/well for 24 h at 37 °C and 5% CO_2. After 24 h, the medium was removed, cells were washed two times with PBS before adding 50 μL of oil extract diluted in MEM 2% FCS (two-fold dilutions, ranging from 500 μg to 0.975 μg). Shortly after, cells were inoculated with 50 μL of MEM 2% FCS containing 100 $TCID_{50}$ of CV-B4. Similarly, cells death induced by virus infection was assessed with MTT method. The virus inhibition percentages were measured using the following equation: T-VC/CC-VC, where T is the optical density (OD) of compound treated cells, VC is the OD of virus control and CC is the OD of cell control (Schmidtke et al., 2001). The IC_{50} value was calculated from the dose response curve generated from the data. The selectivity index (SI) was calculated as the CC_{50}/IC_{50} (Ellithey et al., 2014). Furthermore, cells were daily observed for the cytopathic effect under inverted microscope (TCM 400, Labomed, USA).

3. Results

3.1. Chemical composition of the obtained EO

The yield of *O. regalis* essential oil obtained by hydrodistillation of the dried material was 0.08% (w/w). The essential oil had a yellow color, with a strong perfumed odor. It was analyzed using GC-FID and GC–MS. The individual identified components, with their relative percentage, are given in Table 1. Thus, thirty-nine different components, representing about 85.35% of the total oil constituents, were identified on the basis of their mass spectra and retention indices (Fig. 2). The analyses revealed a complex mixture consisting mainly of hydrocarbons, terpenes, ketones, esters, alcohols and aldehydes. We noticed that 34.32% of the whole volatiles were terpenes, from which 20.7% are diterpenoids, while the non terpenic compounds (aldehydes, ketones, esters and hydrocarbons) were detected at a percentage of 51.03%. Seven major detected components were found to be

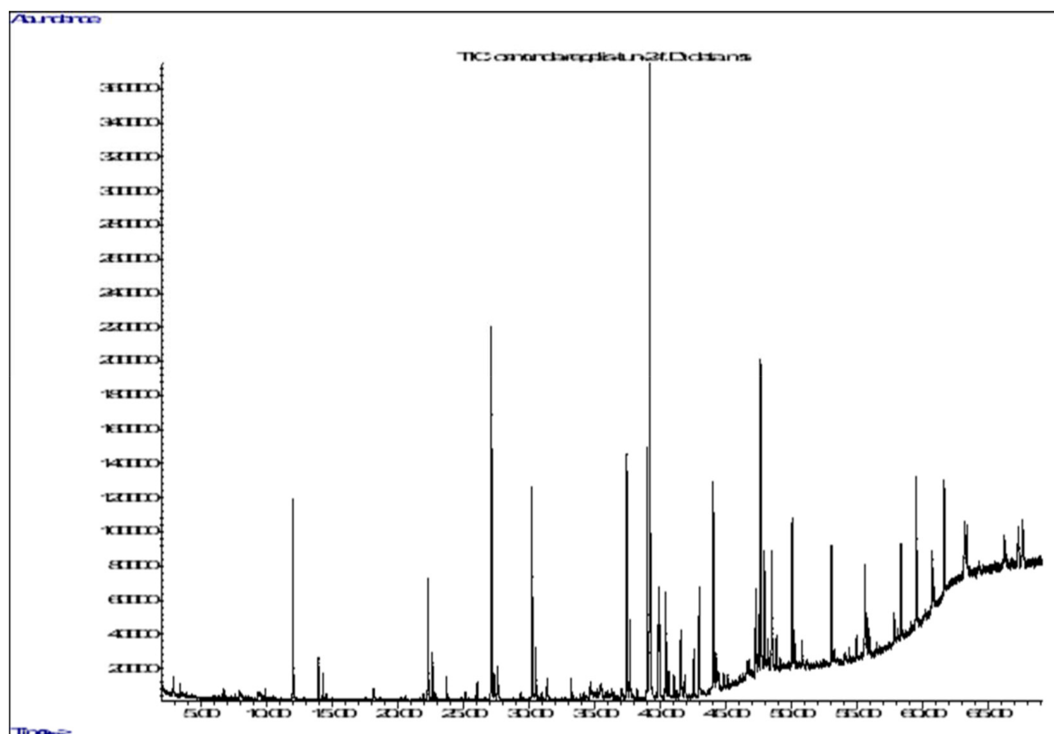

Fig. 2. GC chromatogram of the essential oil of *Osmunda regalis* L.

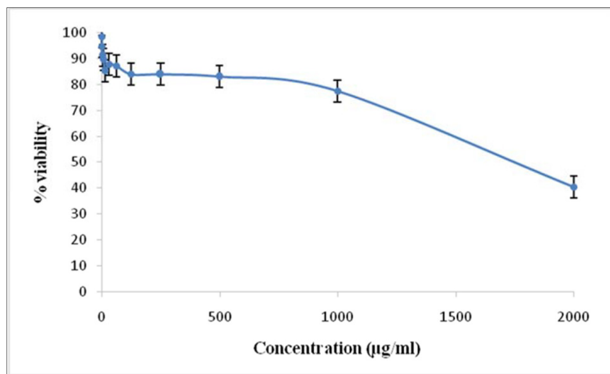

Fig. 3. Cytotoxicity of *Osmunda regalis* L. essential oil.

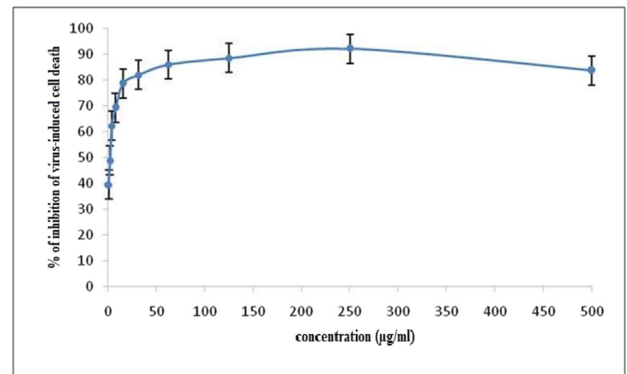

Fig. 6. Antiviral activity of *Osmunda regalis* L. essential oil.

hexahydrofarnesyl acetone (11.82%), 2,4-di-t-butylphenol (6.80%), phytol (6.46%), neophytadiene (4.64%), 1-octadecene (4.42%), 1-eicosene (4.38%), and 1-hexadecene (4.12%).

3.2. Cell viability test

In order to assess the potential application of antiviral activity and cytotoxicity of the essential oil samples of *O. regalis* was evaluated *in vitro* using the MTT assay against Hep-2 cells. As mentioned in materials and methods section, the EO was tested in a range between (0.97 and 2000) μg/mL. The CC_{50} was found to be (1772.41 ± 0.96) μg/mL. Moreover, the effective minimal concentration CC_{80} of essential oil should be under 500 μg/mL for the antiviral assay (Zandi et al., 2011) (Figs. 3 and 4).

3.3. Antiviral activity

Osmunda regalis essential oil showed a significant antiviral effect against Coxsackievirus B4 as shown in (Figs. 5 and 6), the addition of the volatile oil has efficiently reduced the cytopathic effect induced by viral infection and that inhibition of cytopathic effect was proportional to the concentrations of EO applied. Cell death ensuing viral infection in the presence and absence of OE at different concentrations was evaluated using MTT. Furthermore, the percentage of inhibition of cell death caused by viral infection was determined and the IC_{50} value was found to be (2.24 ± 0.99) μg/mL (Fig. 6). The selectivity index (SI) was calculated from the ratio (CC_{50}/IC_{50}), it was high (789.84) (Table 2).Given the interesting SI recorded, the *Osmunda*'s EO has a very important poten-

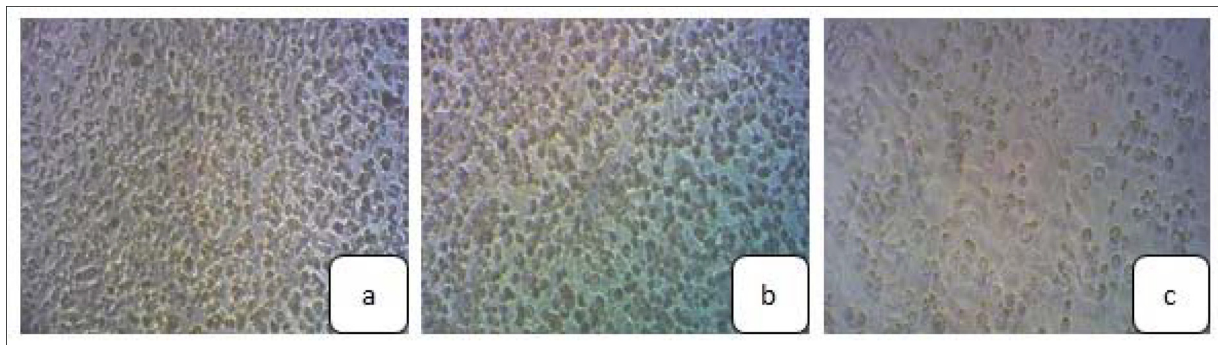

Fig. 4. Morphological changes of treated HEp-2 cells for a 72 h treatment with oil extract of *Osmunda regalis* at different concentrations detected by inverted microscope (magnification 200×). a: Cytotoxicity (cell shrinking and loss of inter-cell connections) observed in cells treated with 2 $mg.mL^{-1}$ of EO. b: Reduced cytotoxicity at the concentration of 1 $mg.mL^{-1}$. C: No cytotoxic effect was observed at the cc of 0.5 $mg.mL^{-1}$.

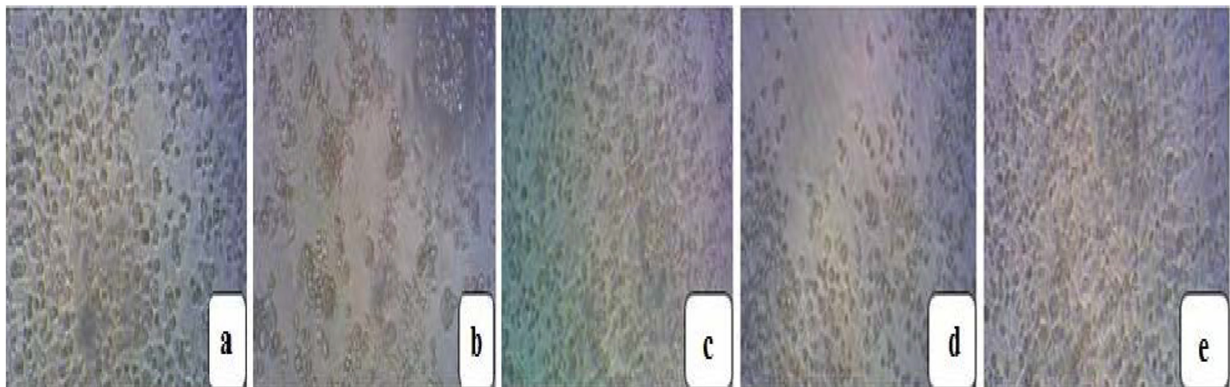

Fig. 5. Inhibition of development of CV-B4 cytopathic effect in HEp-2 cells (magnification 200×). a: cell control (not treated). b: virus control with induced cytopathic effect featured by cellular detachment and cell shrinking. c: Significant inhibition of the cytopathic effect by extract at the cc of 500$μg.mL^{-1}$. d: Inhibition of the cytopathic effect by extract at the cc of 250$μg.mL^{-1}$. e: Absence of the inhibition of virus cytopathic effect at the cc of 0.975$μg.mL^{-1}$.

Table 2
The CC_{50}, IC_{50}, CC_{80}, and IC_{80} values of *Osmunda regalis* L. essential oil.

SI	IC_{50} (μg/mL)	CC_{50} (μg/mL)	CC_{80} (μg/mL)	IC_{80} (μg/mL)
789.84	2.244 ± 0.90	1772.41 ± 0.55	737.93 ± 0.48	21.77 ± 0.27

tial to be used as a substitute phytomedication or in association with antiviral agents employed to remedy enterovirus infections.

4. Discussion

The essential oil chemical composition of the medicinal fern *O. regalis*, has been firstly analyzed in this study. Thirty nine components representing 85.35% of *O. regalis* essential oil were identified on the basis of GC/FID and GC/MS analysis. The volatile oil from *O. regalis* is mainly composed of carbonylic compounds, monoterpene hydrocarbons, oxygenated monoterpenes, alkylated phenol, terpenoid ketones and diterpene alcohol. The diterpenoid hexahydrofarnesyl acetone HHA (*6,10,14*-trimethylpentadecan-2-one), identified as the chemotype of *O. regalis* essential oil, has been found, previously, to be a major component in tribial fragrances male orchid bees, *Euglossa* spp. (Eltz et al., 2010). Moreover, it is recognized for its antimicrobial activity (Christos, 2007). According to the literature, diterpenoids components are abundant in many medicinal ferns, they are mostly found in *Petris* species (Ho et al., 2010). According to the literature, there is no report on the antiviral effects of *O. regalis* essential oil. However, the methanolic extract of *O. japonica* has been described for its antiviral effects against Herpes simplex virus type HSV-1 and HSV-2 (Baskaran et al., 2018). No antiviral medications are currently approved for the treatment of enterovirus infections and usually ribavirin, a broad spectrum antiviral drug, is prescribed in case of such infections. In this context, we tried to evaluate the antiviral activity of *Osmunda regalis* essential oil on the enterovirus CV-B4. We started by assessing the safety of the volatile oil by testing its cytotoxicity on HEp-2 cells and it turned out to be non-toxic according to criteria fixed by Prayong et al., 2008 (CC_{50} > 1000 μg/ml). Afterwards, anti-CV-B4 activity of the tested oil was evaluated and it exhibited a relevant activity against CV-B4 with an IC_{50} bellow 100 μg/ml according to criteria indicated by Cos et al., 2006. Most importantly, the selectivity index was high (789.84) which bring together efficacy and safety. It is generally considered that a drug has a good safety profile if its SI exceeds the value of 10 (Tamargo et al., 2015). Interestingly, the EO from *O. regalis* represents a promising source as an anti-enteroviral agent. Otherwise, further studies are required to clarify if this activity is related essentially to the major constituents or if it is the result of combination or synergistic effects between all organic volatiles. Mecanism/s underlying the observed anti-CVB4 activity needs also to be explored.

5. Conclusion

This study is the first report on the chemical composition, cytotoxic and antiviral activities of the essential oil from royal fern (*Osmunda regalis* L., Osmundaceae) essential oil. Our results demonstrated that the essential oil has non cytotoxic effects on HEp-2 cell lines and possess an effective antiviral activity against the human Coxsackievirus-B (CV-B4). This makes the plant a promising source for bioactive compounds such as hexahydrofarnesyl acetone, 2,4-di-t-butylphenol (a very common artificial antioxidant agent) and phytol which may play an important role as antiviral agents. However, we cannot, for the moment, discount the possibility that the observed bioactivity of the essential oil of the Tunisian *O. regalis* can be due to synergistic effects between the major and minor compounds.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

The authors wish to express their sincere thanks to S.H. and R.E.M. for planning the work. R.E.M for providing the plant. S.H. and K.F. for preparing the essential oil. S.H. and S.B. for writing the paper. H.J for performing the cytotoxicity and antiviral tests. D.F. A.P and S.P. for analyzing the chemical composition of the essential oil. All authors read and approved the final manuscript.

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