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Cytotoxicity and antiviral activities of *Haplophyllum tuberculatum* essential oils, pure compounds and their combinations against Coxsackievirus B3 and B4

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Dedicated to Professor Arnold Vlietinck on the occasion of his 80th birthday

Abstract

Haplophyllum tuberculatum is a plant commonly used in folk medicine to treat several diseases including vomiting, nausea, infections, rheumatism and gastric pains. In the current study, *H. tuberculatum* essential oils (EOs), hydrosols, pure compounds, *R*-(+)-limonene, *S*-(-)-limonene and 1-octanol, and their combinations, *R*-(+)-limonene/1-octanol and *S*-(-)-limonene/1-octanol, were screened for their cytotoxicity on HEp-2 cells after 24, 48 and 72h, and then tested for their activity against Coxsackievirus B3 and B4 (CV-B3 and CV-B4), at three different moments: addition of the plant compounds before, after or together with virus inoculation. Results showed that the samples were more cytotoxic after 72h than after 24h or 48h cell contact. However, the combinations *R*-(+)-limonene/1-octanol and *S*-(-)-limonene/1-octanol showed less effect on HEp-2 cells than pure *R*-(+)-limonene and *S*-(-)-limonene after 24h, 48h and 72h. 1-octanol exhibited the highest CC_{50} on HEp-2 cells after 24h (CC_{50}=93 µg/mL) and 48h (CC_{50}=83 µg/mL). The antiviral assays showed that the tested samples exhibited a potent inhibition of CV-B. IC_{50} values ranged from 0.66 µg/mL to 28.4 µg/mL. In addition, CV-B3 was more sensitive than CV-B4. Both CV-B strains are more inhibited when cells were pretreated with the plant compounds. The hydrosols have no effect, neither on HEp-2 cells nor on the virus. 1-octanol, *S*-(-) and *R*-(+)-limonene/1-octanol had important selectivity indexes over time. Although, EOs had potent antiviral activity, they can be considered for application in pretreatment of cells. However, 1-octanol and the combinations are within the safety limits, and thus, they can be used as an active natural antiviral agent for CV-B3 and CV-B4 inhibition.

Keywords: *Haplophyllum tuberculatum*, Rutaceae, Essential oils, Pure compounds, Coxsackievirus B, Cytotoxicity, Selectivity index

Introduction

Type B Coxsackieviruses B (CV-B) are single-stranded RNA viruses, encompassing six serotypes and belonging to the Enterovirus genus from the *Picornaviridae* family. They use the Coxsackievirus and adenovirus receptor (CAR) to infect a wide variety of human cells and, thereby, are involved in a large pathological spectrum, ranging from infraclinic or mild to severe acute or chronic infections. Indeed, CV-B are involved in common colds, meningitis, pleurodynia, myocarditis, pericarditis or dilated cardiomyopathy, as well as autoimmune diseases such as type 1 diabetes. CV-B are known to be essentially transmitted through the fecal-oral route, but vertical transmission was also documented and characterizes by a more severe and generalized pathology in fetuses and newborns [1-5].

Although CV-B are extensively studied to elucidate their structure, molecular biology and associated pathophysiological mechanisms, no specific inhibitor has yet been found and used in clinical practice. Thus, the improvement of working on the pure natural or synthetic compounds is increasing in order to identify suitable candidates as well as their putative targets in the virus life cycle [6,7].

Natural products from plants exhibit a huge range of chemical composition, and serve in various fields, such as the development of drugs, cosmetics, and bio-nutrients. Plant essential oils, which are considered enormous sources of bioactive components, showed several bioactivities, such us antioxidant, cytotoxic and anti-microbial ones [8,9].

H. tuberculatum Forss. (A. Juss), belonging to Rutaceae family, is a perennial species that colonized a wide range of soil varieties [10]. It is widespread in Mediterranean areas [11,12], in various African countries [13], and in some Middle East regions [14,15]. This plant is used in traditional medicine for several diseases, e.g. against vomiting, nausea, infections, constipation, rheumatism, stomach aches and pains [16]. Recent studies concentrated on the investigation of the medicinal plant *H. tuberculatum* [17,18]. For instance, the aerial part of this species inhibited the growth of *Echinochloa crus-galli* root and shoot, and decreased the germination of its seeds. The pathogenic bacteria *Pectobacterium carotovorum ss. Carotovorum*, *Rhizobium radiobacter* and *Ralstonia solanacerum* were sensitive for *H. tuberculatum* extract and its isolated pure compound, skimmianine.

According to our knowledge, this is the first report on the antiviral activity of its essential oils and their compounds. The aim of the present research is to investigate the essential oils (EOs) of *H. tuberculatum*, hydrosols (Hd), the pure compounds *R*-(+)-limonene, *S*-(-)-limonene and 1-octanol, as well as their combinations *R*-(+)-limonene/1-octanol and *S*-(-)-limonene/1-octanol, for their cytotoxicity on Human epithelial type 2 (HEp-2) cells and their antiviral activities against CV-B3 and B4. The occasional anti-CV-B activity were evaluated in three conditions by treating cells with samples before, together or after virus inoculation.

Results and discussion

The cytotoxicity assay results, summarized in Table 1, show that the CC_{50} varies depending on the tested sample. Moreover, the cell viability decreases over time. After 24h incubation, EO3 was more cytotoxic to Hep-2 cells (CC_{50}=23.3 µg/mL) than the other EOs, followed by *S*-(-)-limonene (CC_{50}=28.0 µg/mL). However, after 48h incubation, the three EOs revealed statistically the same effect on the cells.

The volatile oils were found to be more cytotoxic than the components. The three pure molecules showed a dose dependent effect, but the CC_{50} revealed that *S*-(-)-limonene (28.0 ; 26.0 ; 2.2 µg/mL, after 24, 48 and 72h, respectively) exhibited the highest cytotoxicity, followed by its enantiomer *R*-(+)-limonene (43.0 ; 36.2 ; 3.6 µg/mL). The pure 1-octanol has the lowest effect on HEp-2 cells after 24 and 48h (CC_{50}=93.0 and 83 µg/mL). The combinations *R*-(+)-limonene/1-octanol and *S*-(-)-limonene/1-octanol on the other hand indicated a markedly decreased impact on the cells compared to individual *R*-(+) or *S*-(-)-limonene. These combinations aimed to investigate possible synergistic effect between these compounds.

Our previous studies showed that *H. tuberculatum* EOs are rich in monoterpene hydrocarbons, followed by oxygenated monoterpenes and then, by non-terpene derivatives, Figure 1 [19]. Thus, the current cytotoxicity findings may be explained by the high oxygenated monoterpene amounts in EO1 (65.6%), EO2 (54.3%) and EO3 (46.0%), and their major compounds. This is in line with previous literature data showing that these phytochemicals possess an effect on HEp-2 cells [20,21]. For instance, elemene inhibited the growth of HEp-2 cells by decreasing the eukaryotic initiation factor 4E, the eukaryotic translation initiation factor 4 gamma, the basic fibroblast growth factor and the vascular endothelial growth factor [22]. HEp-2 cells growth was also inhibited by thymol (dose= 0.71–0.78 mM) [21]. Thymoquinone (22.9–34.6 µM) and carvone (0.47–0.62 mM) induced the apoptosis of these cells, by the depletion of glutathione and the activation of caspase 3, respectively [21,23].

The cytotoxic effects of monoterpenes depend on the target cell line [24,25]. For instance, in our previous work [19], EO from *H. tuberculatum* leaves (CC_{50}=81.2; 80.6 µg/mL), from stems (CC_{50}=49.6; 82.6µg/mL), and from leaves+stems (CC_{50}=27.8; 79.1µg/mL), collected in 2013 and 2015, respectively, were found less cytotoxic on Chinese Hamster Ovary (CHO) cells than on the HEp-2 cells in the present study. In the same study, *R*-(+)-limonene (CC_{50}=29.65 µg/mL), 1-octanol (CC_{50}>100 µg/mL) and *S*-(-)-limonene (CC_{50}=75.9 µg/mL) showed ~~a~~ cytotoxicity on CHO [19], that is much lower than that found in our current investigation on HEp-2. Both studies demonstrated that limonene had a high cytotoxicity. A concentration (10%) of limonene in diet can inhibit the small G proteins in rat mammary carcinomas [26]. D-limonene may inhibit lung cancer growth by promoting autophagy and inducing apoptosis [27], arrest the cell cycle [28], remove the nuclear factor-kappa B pathway [29] and suppress the intracellular signaling pathway, phosphatidylinositol-3-kinase-AKT-mammalian target of rapamycin, in cancers [30]. At 0.098 and 0.150 µL/mL, limonene was cytotoxic for lung carcinoma A-549 and hepatocellular carcinomic human cell line HepG2 cell lines, respectively

[31]. Using *in vivo* models, it has been shown that D-limonene can inhibit the cancers of skin, breast, kidney and liver [32-35].

At 100µg/mL, piperitone had no activity on HEp-2 cells, while, it had low inhibition effects on the THP-1 (leukaemia) (11%), A-549 (lung) (27%) and IGR-OV-1 (ovary) (5%) cell lines [36]. Another study [37] showed that four piperitone carbazones were not cytotoxic on the human non cancer fibroblast WI38 and the CHO cell lines. Thus, piperitone, a major compound found in EO1, EO2 and EO3 of *H. tuberculatum*, was not correlated to cytotoxicity.

The compound 4-terpineol was cytotoxic after 24h incubation in human hepatocellular carcinomic (HepG2, IC_{50}= 0.06 g/L), epithelioid carcinomic (HeLa, IC_{50}=0.14g/L), human lymphoblastic leukemia T (Molt-4, IC_{50}=0.29 g/L), human chronic myelogenous leukemia (K562, IC_{50}=0.25 g/L) cell lines and an early B cell line from the bone marrow cells of a patient with acute myeloid leukemia (CTVR-1, IC_{50}=0.33 g/L) [38]. Several studies using *in vitro / in vivo* models showed that several monoterpene, such as α-pinene, β-pinene and camphene were cytotoxic on different cell lines [25,39].

The current study shows that cell viabilities decrease over time using EOs, pure compounds or their combinations. Literature proved that exposure time can reflect on the cell death [38].

Although no synergism was registered for the tested components, other EO-compound combinations may relatively explain this cytotoxicity. Literature revealed that the combination of 5% limonene and 4-hydroxyandrostrenedione (12.5 mg/kg) was active on N-nitroso-N-methylurea-induced rat mammary carcinomas [40]. When combined, thymoquinone and cisplatin, inhibited, for nearly 90%, the non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) cell lines growth. This combination was studied *in vivo* using a mouse xenograft model which revealed a significant reduction in the volume and weight of the tumor [41].

The potential antiviral impact of the EOs, their hydrosols, their pure compounds and the component combinations were studied in three conditions (“before”, “together with” and “after” virus inoculation) in order to assess the eventual preventive, inhibitory or curative potential of these samples on CV-B3 and 4. The results are presented in table 2.

In the condition “After”, EO3 (IC_{50}=3.6 µg/mL), 1-octanol (IC_{50}= 3.5 µg/mL) and the combination *R*-(+) and *S*-(-)-limonene/1-octanol (IC_{50}= 2.8; 3.5 µg/mL) showed a similar statistically strong effect on CV-B-3. However, only the compound combinations (IC_{50}= 2.2; 2.0 µg/mL) were effective against CV-B4. Pure *S*-(-)-limonene revealed the lowest antiviral

activity against both viruses compared to the rest of samples. Thus, in the condition “After”, CV-B3 was revealed to be more susceptible than CV-B4.

When pre-treating the HEp-2 cells with the tested samples, then 2h30 later, infecting them by CV-B, all samples expressed antiviral activity (IC_{50}<3 µg/mL) against both serotypes. However, *S*-(-)-limonene (IC_{50}= 0.7; 0.8 µg/mL) was the most active against CV-B3 and CV-B4. Moreover, both CV-B serotypes are also sensitive to the tested EO, the pure or combined compounds.

Simultaneous addition of sample and virus infection of HEp-2 cells, indicated that EO1 (IC_{50}= 2.5 µg/mL), EO3 (IC_{50}= 3.4 µg/mL), *R*-(+)-limonene (IC_{50}= 3.5 µg/mL), 1-octanol (IC_{50}= 3.7 µg/mL) and *R*-(+)-limonene/1-octanol (IC_{50}= 2.1 µg/mL) showed a statistically similar effect against CV-B-3. Both the compound combinations (IC_{50}= 1.8; 1.8 µg/mL) were the most active against CV-B4. In general, CV-B3 was more susceptible than CV-B4 when exposing cells simultaneously to both virus and sample.

Our experiments show that CV-B3 was more susceptible than CV-B4 in the “After” and “Together” conditions and that EOs activities may be attributed to both the pure and combined compounds.

The combination *R*-(+)-limonene/1-octanol was more active against CV-B4 when cells are treated and infected simultaneously. On the other hand, *S*-(-)-limonene/1-octanol has almost the same significant activity in all conditions and against both serotypes, except against CV-B3 in “After” and “Together” conditions. Thus, the *S*-(-)-limonene/1-octanol effect may explain in part the EOs activities. Hydrosols were inactive against CV-B3 and CV-B4, in any condition.

Considering the synergistic effect, the antiviral activity was more interesting for both the compound combinations, *S*-(-)-limonene/1-octanol (IC_{50}= 3.5; 2.02 µg/mL) and *R*-(+)-limonene/1-octanol (IC_{50}= 2.8; 2.2 µg/mL), in the “After” condition against CV-B3 and CV-B4 (even though both serotypes were more susceptible to *S*-(-)-limonene/1-octanol), and against CV-B4 in the “Together” condition. These combination antiviral potentialities were better than that of each individual compound. However, limonene which area percentage represents 2.8%, 6.4% and 9.4% in EO1, EO2 and EO3, respectively, may interfere with other major compounds, such as *α*-pinene (2.1 %, 4.6 % and 7.2 %), *cis*-p-menth-2-en-1-ol (21.3 %, 15.9 %, 16.0 %), *trans*-p-menth-2-en-1-ol (18.8 %, 11.8 % and 12.6 %), *cis*-piperitol (5.6 %, 4.1 % and 3.1 %), 1-octyl acetate (9.9 %, 8.1%, 12.4%), piperitone (7.5 %, 6.8% and 5.2 %)

and isobornyl acetate (2.8 %, 9.8 % and 7.3 %). The combinations of major and minor compounds may explain the antiviral activity of the EOs.

A wide range of pure compounds found in volatile oils was screened against various target viruses. For instance, limonene inhibits human herpes simplex virus 1 (HSV1, with IC_{50}= 5.9µg/mL) [42], which is a similar effect as *R*- (+)-limonene against CV-B3 and CV-B4 in our "After" and "together" conditions, respectively. Moreover, *β*-pinene (IC_{50}= 3.5µg/mL) [42] and *p*-cymene (IC_{50}> 0.1 %) [43] showed also an anti-HSV1 activity. The antiviral activity of linalool was also studied against CV-B1 (IC_{50}=34.9 mg/L), enterovirus 71 (EV71) (IC_{50}= 42.2mg/L), HSV1 (IC_{50}> 200 mg/L) and HSV2 (IC_{50}> 200mg/L) [44]. The linalool antiviral effect variation depends on the tested virus, thus, it may partly explain the EOs effect.

Literature showed that some plant EOs can inhibit CVB [45]. As EOs are complex mixtures, the anti-CV-B activity can be due to the major or minor compounds or to their combination. In order to understand the mechanism how EOs and their compounds inhibit the replication of the virus, further studies were proceeded. Limonene, p-cymene and *β*-pinene showed that they act on the intercellular compartment by direct effect on free viruses [42,43].

Several studies are concentrated on the mechanism of pure compounds in infected cell lines. For instance, a recent research [7] studied the mechanism of action of menthol and capsaicin on CV-B infection using both a HeLa cell line and an *in-vivo* model. Results showed that CV-B triggers the fission of mitochondria and blocks the infection limits of the mitochondrial fission. The incubation of HeLa cells infected by CV-B at 39°C and at 25°C increased and decreased the infection and thus the virus replication, respectively. Menthol and cold (25°C) contributed to reduce infection and to decrease mitochondrial fission. The menthol lead to the stabilization of the mitochondrial antiviral signaling level.

The selectivity index (SI), defined as the ratio between the concentration causing 50% cytotoxicity (CC_{50}) and the concentration inhibiting 50% virus infection (IC_{50}) (SI = CC_{50} / IC_{50}), was calculated. The higher this index, the less harmful sample. An SI value higher than 4 is considered acceptable [46]. SI is a parameter necessary to select the tested samples for future use as drugs. Table 3 summarizes the results after 24, 48 and 72h. Suitable values were generally registered in the conditions "before". 1-octanol and the combinations *R*-(+)-limonene/1-octanol and *S*-(-)-limonene/1-octanol can be considered more selective against CV-B-3 and 4. The SI varied depending on the tested sample, the cell line and the virus particle

effects [47]. For instance, SI (RC-37 cells/ HSV-1) of limonene (10.2), β-pinene (24.3) [42] was reported.

Summarized, literature showed that essential oils, which are mixtures of chemical compounds, may quickly infiltrate in cells and inhibit virus replication by targeting vital pathways during its life cycle. Previous work proved that EO compounds can suppress CVB infection by blocking the cell autophagy, reduce the viral titers in pancreatic tissue and decrease the tissue damage and inflammation. Owing to the effect of EOs on CVB, the EOs from *H. tuberculatum*, as well as some pure and combined compounds were screened *in vitro* for their cytotoxicity in HEp-2 cells and for their anti-CV-B3 and B4 activities. The cytotoxicity was followed over time, at 24, 48 and 72h. Results showed that all samples were less cytotoxic after 24h of incubation than after 48 and 72h. The compound combinations and 1-octanol had lower effects on the cells at the three time points. The antiviral activity was investigated in three different experimental conditions: "Before", "Together" and "After" virus inoculation in order to evaluate any preventive, inhibition or curative effects of the EOs and their compounds. The compound combinations and 1-octanol were always most active on the virus inhibition. The SI was determined in all cases. The preventive condition (Before) showed an important SI compared to that of the other conditions, especially for the combinations compounds. In conclusion, our findings suggest that although interesting anti-CV-B3 and B4 activity of the EOs is seen, they only can be considered safe in "Before" conditions. The antiviral effects of 1-octanol and of the combinations *R*-(+)-limonene/1-octanol and *S*-(-)-limonene/1-octanol have, however, to be confirmed using an *in vivo* model. Thus further research is needed in order to proceed with the EOs or their pure compounds in the development of drugs with an antiviral effect.

Materials and methods

Plant material and extraction of essential oils

H. tuberculatum was collected from Médenine in South-Eastern Tunisia. Then, the leaves and stems were separated. The hydro-distillation of essential oils from each part and from the mixture was performed with a Clevenger apparatus [48]. The pure compounds *R*-(+)-limonene, *S*-(-)-limonene and 1-octanol were purchased. The essential oils and the hydrosols from leaves (EO1/Hd1), stems (EO2/Hd2) and leaves+stems (EO3/Hd3) and the three pure compounds were kept for the bio-assays.

Viruses and cells

Coxsackieviruses B3 (CV-B3 Nancy strain, American Type Culture Collection (ATCC)) and B4 CV-B4 E2 (kindly provided by Prof. J.W. Yoon, Julia M.C. Farlane, Diabetes Research Center, Calgary, Alberta, Canada) strains were propagated in Human epithelial type 2 (HEp-2) cells (Biowhittaker) in Eagle's Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% L-glutamine, 1% non-essential amino-acids, 50 µg/mL streptomycin, 50 U/mL penicillin and 0.05% (2.5 µg/mL) fungizone (Amphotericin B). Supernatants were collected three days post-infection and then clarified at 3000 g for 10 min. Virus titers were determined as the 50% tissue culture infectious dose on HEp-2 cells and stored in aliquots at -80°C until use [49].

Cytotoxicity determination using MTT assay

To evaluate the cytotoxicity of EOs, hydrosols, pure compounds and combinations of compounds, we used the colorimetric method with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). This method is based on the conversion of the yellow dye of MTT to the violet dye of formazan by mitochondrial and non-mitochondrial enzymes in actively growing cells [50,51]. HEp-2 cells were seeded at $5x10^4$ cells/well onto 96-well culture plates. Different concentrations of EOs, pure compounds and combination compounds (0,625-160 µg/mL), diluted in MEM with 2% FCS, were applied to cell layers in triplicate. Cells that were not treated with plant EOs or compounds served as controls. After incubation for 24 h, 48 h and 72 h at 37 °C and 5% CO_2, cells were washed by PBS and 50 µL of MTT were added. After 4 h of incubation, the reaction was stopped by adding dimethyl sulfoxide. The absorbance of the resulting formazan dye was measured on a spectrophotometer at a wavelength of 570 nm. The results were determined using the equation: (Atc/Ac)x100, Atc and Ac indicating the absorbance of the test compound and of the control, respectively. The Cytotoxic Concentration (CC_{50}), defined as the concentration (µg/mL) required for the reduction of cell viability by 50%, was determined for each of the plant EO and compounds.

Antiviral activity

HEp-2 cells were seeded at $5x10^4$ cells/well in 96-well culture plates and incubated at 37 °C with 5% CO_2 until 90% of confluency. Cells were then treated with the plant EO or compounds and/or inoculated with the virus.

Cells were then inoculated with 100 $TCID_{50}$ of either CV-B3 or CV-B4; Treatment with non-cytotoxic concentrations (concentrations below the CC_{50}) of plant EO and compounds diluted in MEM with 2% FCS, was performed according to three different experimental conditions: before (cells pre-treated by the sample for 2h30, then infected by the corresponding virus), together

(cells treated by the sample and the corresponding virus together at the same time) or after (cells infected 2h30 by the corresponding virus, and then post-treated by adding the sample) virus inoculation. In all experiments, control cells (cells that were neither inoculated with the virus nor treated with the plant EO or compound), infected (cells that were only inoculated with the virus, but not treated with the plant EO or compound) and toxicity controls (cells that were only treated with the plant EO or compound, but not infected with the virus) were run simultaneously and used as a reference. The 96-well microtiter plates were incubated for 72 h at 37 °C and 5% CO_2, and then examined for the presence of cytopathic effects (CPE). The percentage of inhibition of the different concentrations of plant EO and compounds was determined using the equation: (Atv−Acv)/(Acd−Acv)×100; Atv indicates the absorbance of the test compounds and virus-infected cells. Acv and Acd indicates the absorbances of the virus control and the cell control, respectively. The concentration that reduces 50% of CPE (IC_{50}) was thus determined. The selectivity index (SI) was then calculated as the CC_{50}/IC_{50} ratio.

Statistical analysis

The biological tests were conducted in triplicate. The equations were calculated using Excel. The IC_{50} and the CC_{50} values Least Significant Differences were estimated by Graph-Pad Prism (Version 8.3.0). The confidence intervals (CI) of the IC_{50} and CC_{50} were determined. Duncan tests were effectuated with SPSS (Version 22) at the p=0.05 significance level.

Supplementing Information

Figure 2: Cytotoxicity of the EOs, pure compounds and their combinations in HEp-2 cells after 24, 48 and 72h of incubation (n=3). The error bars refer to the standard deviations.

Author Contributions

We declare that all authors contributed notably to this work. A Hamdi collected plant material and extracted essential oils; A Halouani fixed the protocol; A Hamdi and A Halouani tested the bio-activities; H Jaïdane controlled the experiments; I Aouf, J Viaene and B Marzouk partly contributed in resource, analysis and conception; J Kraiem, H Jaïdane and Y Vander Heyden supervised, reviewed and edited the manuscript.

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Conflict of Interest: we declare that there is no conflict of interest

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Figures legend:

Figure 1: Monoterpenes and non-terpene derivatives in the essential oils, EO1, EO2 and EO3 collected, in 2015

Figure 2: Cytotoxicity of the EOs, pure compounds and their combinations in HEp-2 cells after 24, 48 and 72h of incubation (n=3). The error bars refer to the standard deviations (see supporting information).

Table 1: Cytotoxicity evaluation of *H. tuberculatum* essential oil (EO), hydrosol (Hd), pure compounds and their combinations against HEp-2 cells

	CC_{50} µg/mL		
	24 h	48 h	72 h
EO1	31.7 ^{bc***}	16.9 ^{a**}	4.4 ^{bc*}
EO2	35.6 ^{c***}	15.7 ^{a**}	5.6 ^{c*}
EO3	23.2 ^{a***}	17.9 ^{a**}	4.7 ^{bc*}
R- (+)-limonene	42.7 ^{d***}	36.2 ^{c**}	3.6 ^{ab*}
S- (-)-limonene	28.1 ^{ab***}	26.0 ^{b**}	2.2 ^{a*}
1-octanol	92.9 ^{f***}	82.8 ^{e**}	18.4 ^{e*}
•*R*-(+)-limonene/1-octanol	70.4 ^{e***}	57.5 ^{d**}	36.2 ^{f*}
•*S*-(-)-limonene/1-octanol	71.5 ^{e***}	39.7 ^{c**}	12.2 ^{d*}
Hd1	>100	>100	>100
Hd2	>100	>100	>100
Hd3	>100	>100	>100

• Volume/Volume 1/1

The letters (a–f), within a column, indicate significant differences between the CC_{50} of essential oils, pure compounds and their combinations according to the Duncan test ($p < 0.05$).

The (*/**/***), within a row, indicate significant differences between the CC_{50} of a tested sample after 24, 28 and 72h of incubation, according to the Duncan test ($p < 0.05$)

Table 2: Antiviral activity IC_{50} (µg/mL) of essentials oil (EO), hydrosol (Hd), pure components and their combinations against CV-B3 and CV-B4

	Addition of sample relative to virus infection					
	Before		Together		After	
	CV-B3	**CV-B4**	**CV-B3**	**CV-B4**	**CV-B3**	**CV-B4**
EO1	2.1 ^{bcd*}	1.5 ^{bc*}	2.5 ^{a*}	7.0 ^{b**}	6.9 ^{b**}	7.5 ^{b**}
EO2	3.0 ^{d*}	2.5 ^{de*}	5.7 ^{b**}	18.1^{d****}	9.0 ^{c***}	24.7^{d*****}
EO3	1.7 ^{b*}	1.3 ^{b*}	3.4 ^{a**}	7.7 ^{b***}	3.6 ^{a**}	7.5 ^{b***}
***R*- (+)-limonene**	2.8 ^{cd*}	2.8 ^{e*}	3.5 ^{a*}	5.7 ^{b**}	5.4 ^{b**}	17.4 ^{c***}
***S*- (-)-limonene**	0.7 ^{a*}	0.8 ^{a*}	8.7 ^{c**}	12.5 ^{c***}	23.2 ^{d****}	28.4 ^{e*****}
1-octanol	1.5 ^{ab*}	1.7 ^{c*}	3.7 ^{a***}	7.8 ^{b****}	3.5 ^{a***}	4.8 ^{ab***}
• ***R*-(+)-limonene/1-octanol**	2.4 ^{bcd***}	2.4 ^{d***}	2.1 ^{a**}	1.8 ^{a*}	2.8 ^{a****}	2.2 ^{a**}
• ***S*-(-)-limonene/1-octanol**	1.9 ^{bc*}	1.8 ^{c*}	7.3 ^{c***}	1.8 ^{a*}	3.5 ^{a**}	2.0 ^{a*}
Hd1	>100	>100	>100	>100	>100	>100
Hd2	>100	>100	>100	>100	>100	>100
Hd3	>100	>100	>100	>100	>100	>100

• Volume/Volume 1/1

Before: cells pre-treated by the sample for 2h30, then infected by the virus (to evaluate any preventive effect).

Together: cells subjected simultaneously to the sample and the virus (to evaluate any inhibition effect).

After: cells infected 2h30 by the virus, and then post-treated by adding the sample (to evaluate any curative effect).

The letters (a–e), within a column, indicate significant differences between the IC_{50} of essential oils, pure compounds and their combinations according to the Duncan test ($p < 0.05$).

The (*-*****), within a row, indicate significant differences between the IC_{50} of a tested sample against CV-B3 or CV-B4 in the three conditions (before, together and after) according to the Duncan test ($p < 0.05$).

Table 3: Selectivity index, HEp2/CV-B3 and HEp2/CV-B4, at 24, 48 and 72h

	HEp2(24h)/CV-B						**HEp2(48h)/CV-B**						**HEp2(72h)/CV-B**					
	Before		**Together**		**After**		**Before**		**Together**		**After**		**Before**		**Together**		**After**	
	CV-B3	**CV-B4**	**CV-B3**	**CV-B4**	**CV-B3**	**CV-B4**	**CV-B3**	**CV-B4**	**CV-B3**	**CV-B4**	**CV-B3**	**CV-B4**	**CV-B3**	**CV-B4**	**CV-B3**	**CV-B4**	**CV-B3**	**CV-B4**
EO1	15,3	20,8	12,8	4,5	4,6	4,3	8,2	11,1	6,8	2,4	2,5	2,3	2.1	2.8	1.8	0.6	0.6	0.6
EO2	12.0	14,2	6,2	2.0	4.0	1,4	5,3	6,2	2,8	0,9	1,8	0,6	1.9	2.2	1.0	0.3	0.6	0.2
EO3	13,9	18,0	7,0	3,0	6,5	3,1	10,7	13,9	5,4	2,3	5.0	2,4	2.8	3.6	1.4	0.6	1.3	0.6
***R*- (+)-limonene**	15,5	15,2	12,3	7,5	7,9	2,5	13,2	12,9	10,5	6,3	6,7	2,1	1.3	1.3	1.0	0.6	0.7	0.2
***S*- (-)-limonene**	42,8	35,3	3,2	2,3	1,2	1.0	39,7	32,7	3.0	2,1	1,1	0,9	3.3	2.8	0.3	0.9	0.1	0.1
1-octanol	63,3	56,1	25,0	12,0	26,9	19,4	56,4	50.0	22,3	10,6	24,0	17,2	12.5	11.1	5.0	2.4	5.3	3.9
***R*-(+)-limonene/1-octanol**	29,8	29,5	33,6	39,8	25,0	32,1	24,3	24,1	27,4	32,5	20,5	26,3	15.3	15.2	17.2	20.5	12.9	16.51
***S*-(-)-limonene/1-octanol**	37,1	40,0	9,8	39,7	20,3	35,4	20,6	22,2	5,4	22,0	11,3	19,7	6.3	6.8	1.7	6.8	3.5	6.1
Hd1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hd2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hd3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

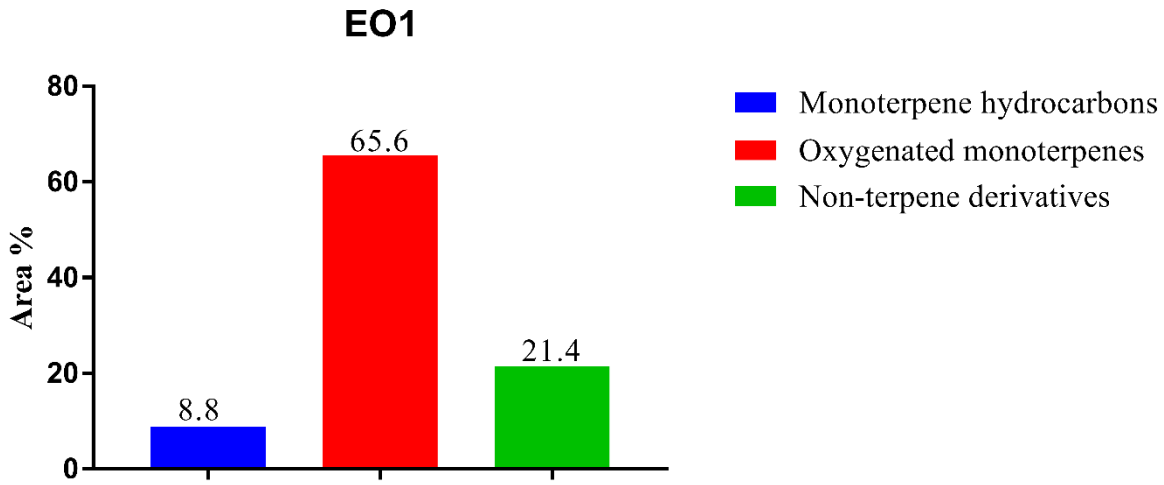

Figure 1. Monoterpenes and non-terpene derivatives in the EO1

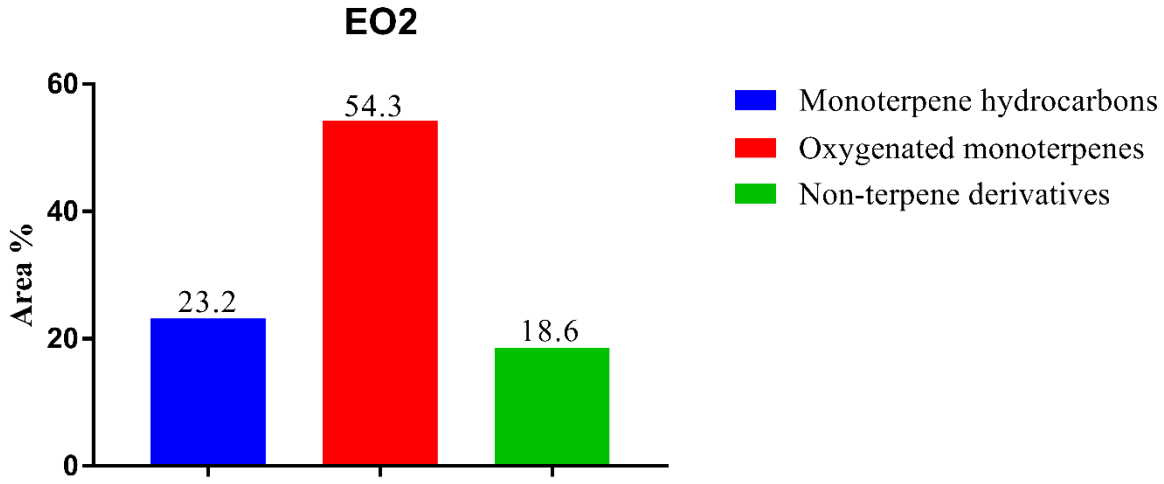

Figure 1. Monoterpenes and non-terpene derivatives in the EO2

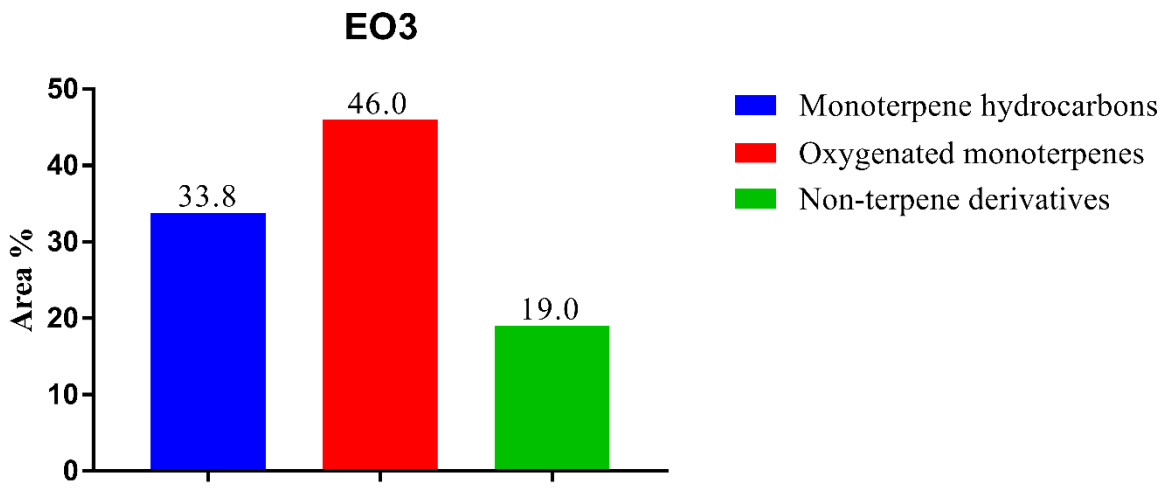

Figure 1. Monoterpenes and non-terpene derivatives in the EO3

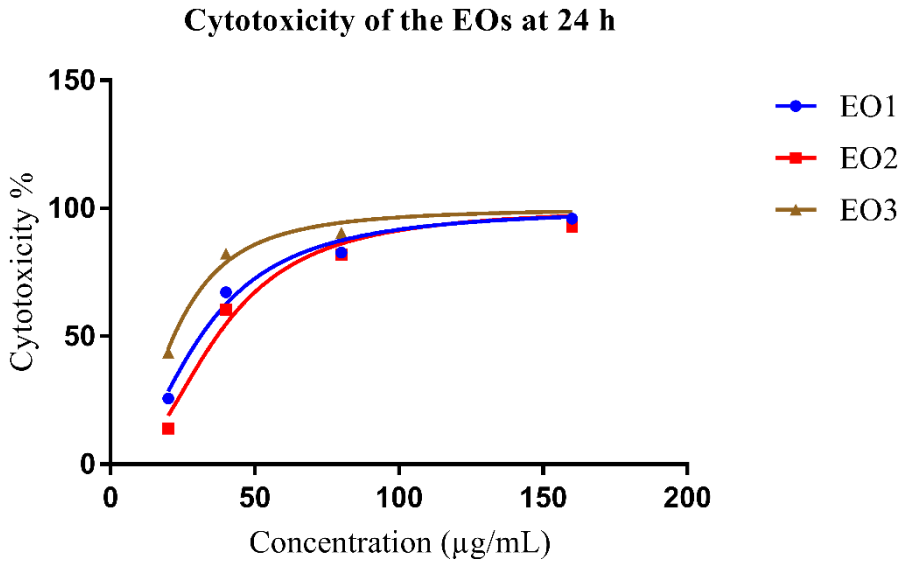

Figure 2. Cytotoxicity of EO at 24h

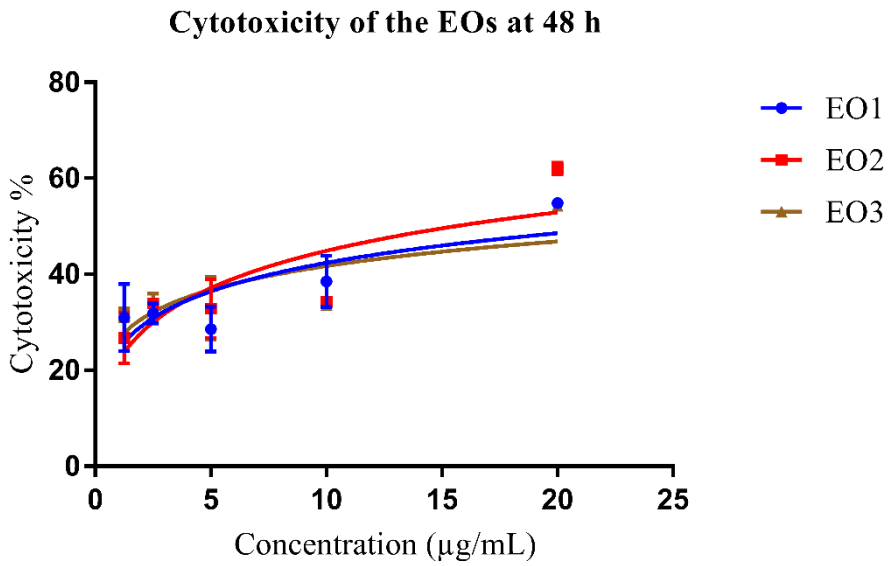

Figure 2. Cytotoxicity of the EOs at 48h

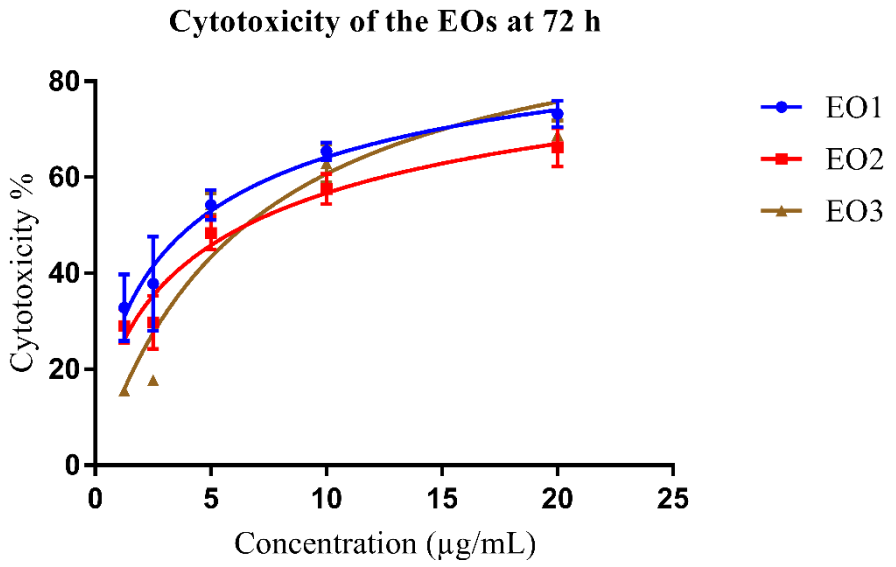

Figure 2. Cytotoxicity of the EOs at 72h

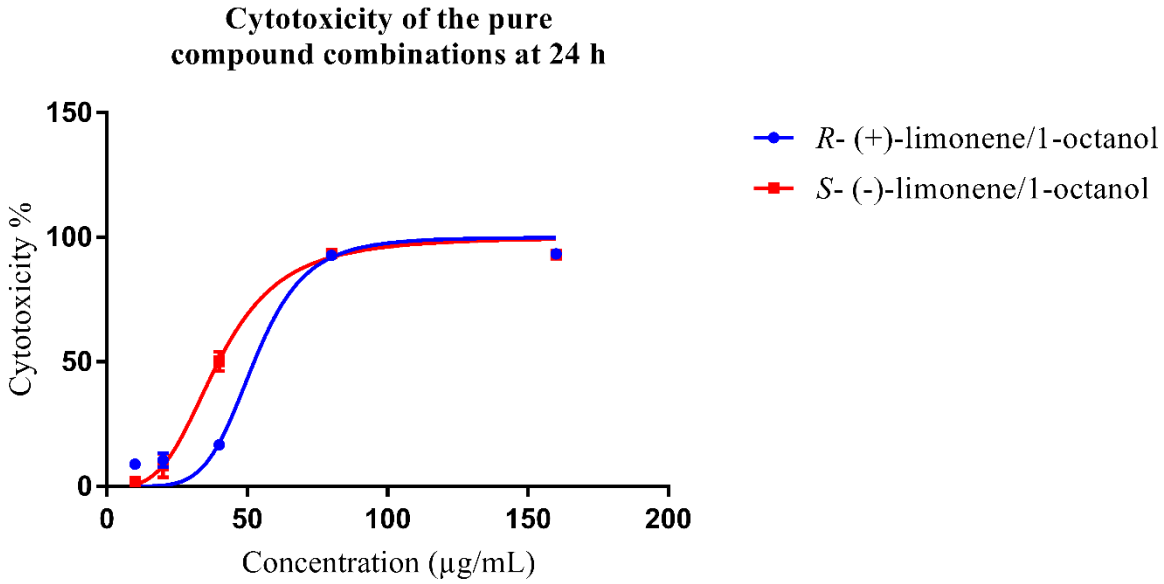

Figure 2. Cytotoxicity of the pure compound combinations at 24h

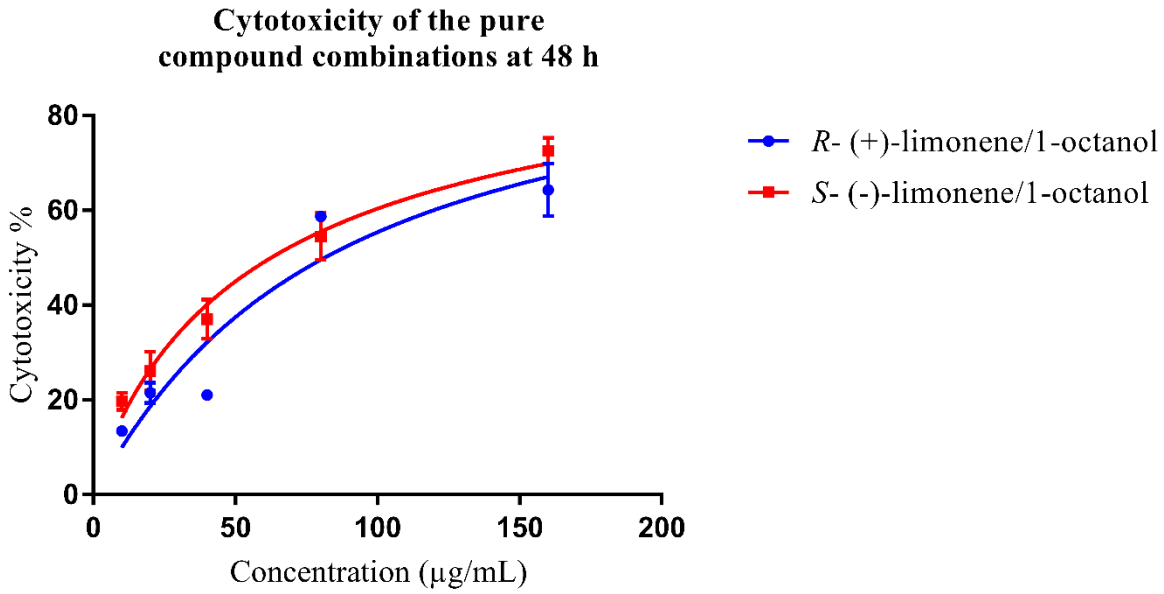

Figure 2. Cytotoxicity of the pure compound combinations at 48h

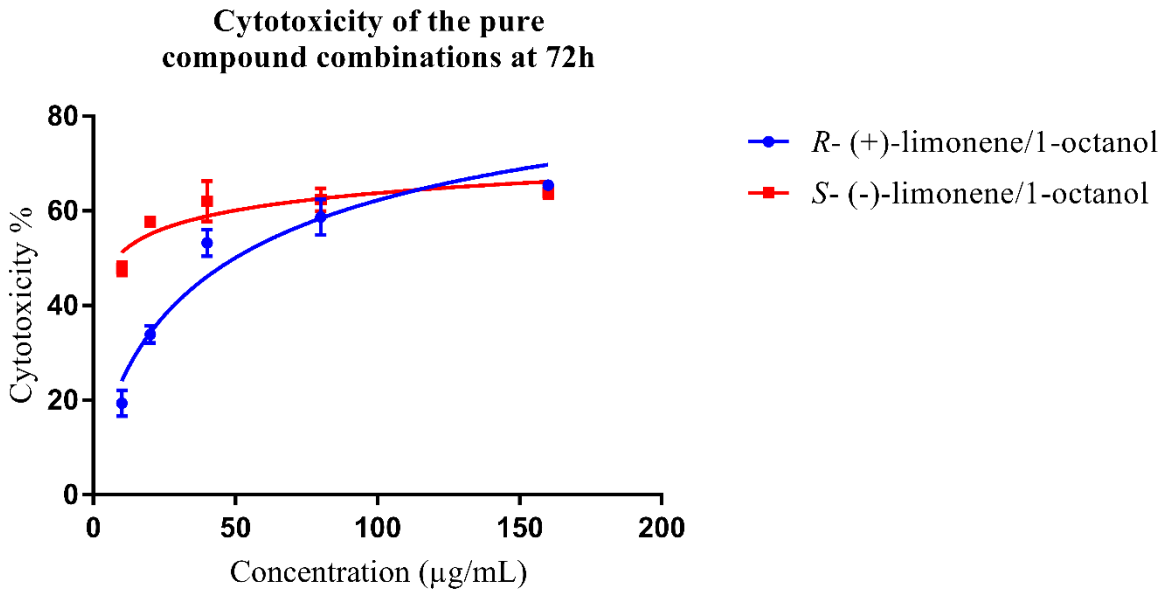

Figure 2. Cytotoxicity of the pure compound combinations at 72h

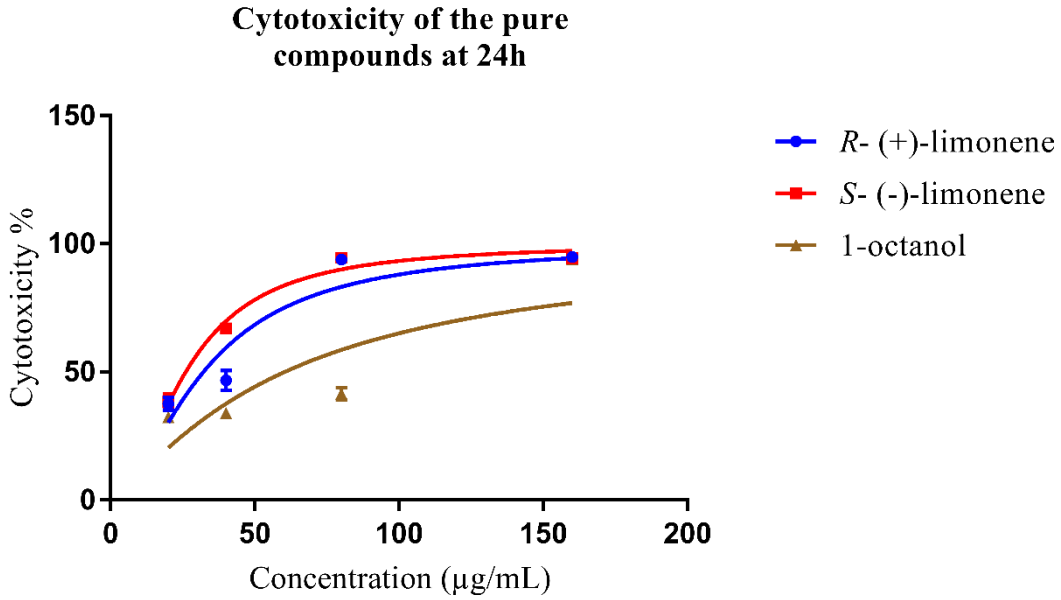

Figure 2. Cytotoxicity of the pure compounds at 24h

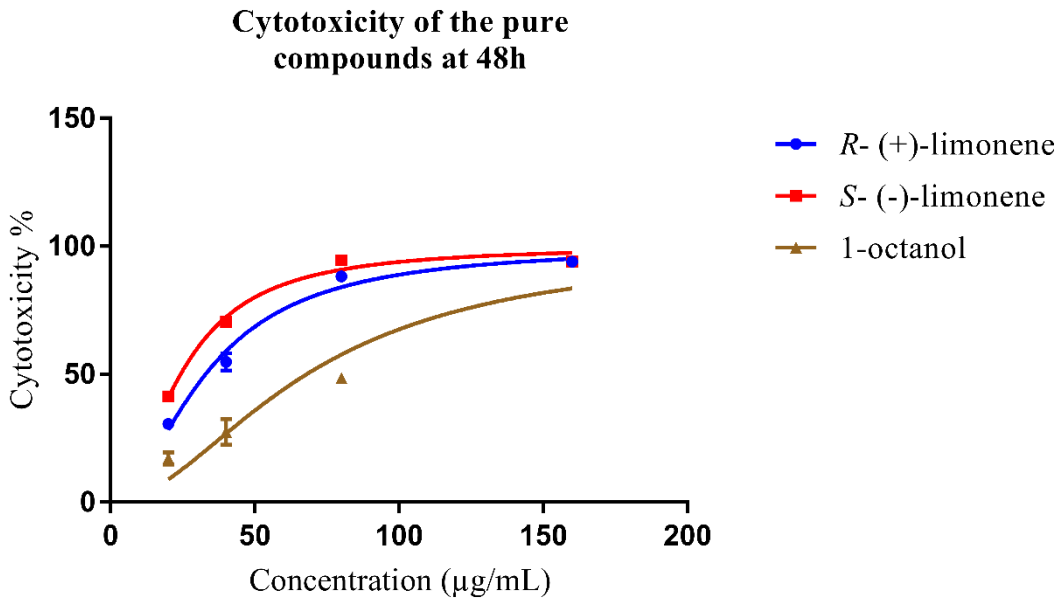

Figure 2. Cytotoxicity of the pure compounds at 48h

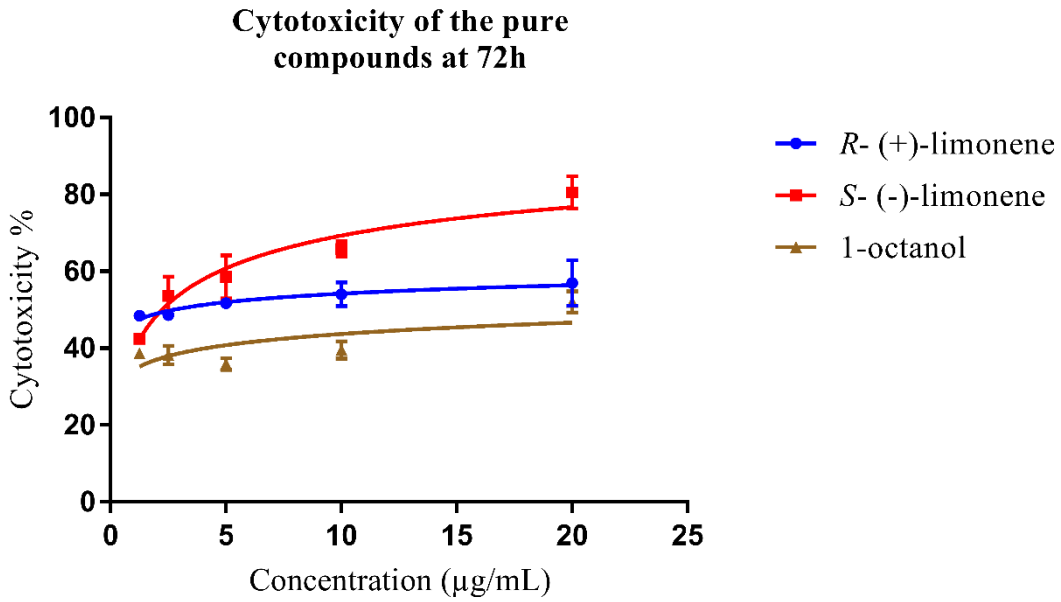

Figure 2. Cytotoxicity of the pure compounds at 72h