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Investigation of the Essential Oil Constituents of Pimenta racemosa Aerial Parts and Evaluation of Its Antiviral Activity against Hsv 1 and 2

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ORIGINAL STUDY

Investigation of the Essential Oil Constituents of *Pimenta racemosa* Aerial Parts and Evaluation of its Antiviral Activity Against Herpes Simplex Virus Type 1 and 2

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Abstract

Pimenta racemosa* is a well-known plant used in traditional medicine to treat several ailments. Current pharmaceutical industries rely heavily on the discovery of secondary metabolites found in plants to aid in the development of novel drugs with potential biological activity. This study aimed to investigate the essential oil constituents of *Pimenta racemose* (Mill.) J. W. Moore aerial parts (leaves and small branches) and evaluate their antiviral activity. The essential oil was prepared by conventional hydro distillation method. The prepared oil was analyzed by GC/MS. Identification of the essential oil constituents was achieved by comparing retention indices (RI) relative to standard *n*-alkanes (C_8−C_{28}); and their MS to NIST and WILEY mass spectral library database. 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was applied to detect the cytotoxic properties of the prepared oil against Vero cell line. A cytopathic effect inhibition assay was applied to test the antiviral properties. Forty-five compounds were identified. Eugenol (34.85%), *β*-pinene (18.29%), linalool (13.64%), and limonene (11.92%) were the predominant components. The essential oil showed good antiviral activity against herpes simplex type 1 virus (HSV-1), while it showed Moderate antiviral activity against herpes simplex type 2 virus (HSV-2). Finally, this study was conducted that eugenol was the main component of the essential oil of *P. racemosa*, the essential oil showed antiviral activity against herpes simplex type 1 and 2.

Keywords: Antiviral, Cytotoxic, Essential oil, Eugenol, *Pimenta racemosa*

1. Introduction

Natural products may be used as an alternative therapy to conventional drugs because of their relative safety, efficacy, and protective effects, Current pharmaceutical industries rely heavily on the discovery of secondary metabolites found in plants to aid in the development of novel drugs with potential biological activity (Soliman et al., 2023). Plant derived-essential oils (EOs) one of the most important secondary plant metabolites, have several biological activities; additionally, they are important in cosmetic and pharmaceutical industries (Al-Gendy et al., 2017). There are many plant families rich in essential oils such as apiaceae, asteraceae, fabaceae, myrtaceae, and lamiaceae (Ebadollahi & Jalali Sendi, 2015).

Family Myrtaceae comprises about 150 genera and over 5650 species (Grattapaglia et al., 2012). It includes important genera such as *Eucalyptus, Pimenta, Psidium, Syzygium, Eugenia,* and *Callistemon*. Several members of this family are used in folk medicine, mainly as antidiarrheal, antimicrobial, antioxidant, antiseptic, antirheumatic, and anti-inflammatory agents (Ebadollahi, 2013).

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Genus *Pimenta* comprises 15 species and more, it is native to the Caribbean region and Central America, and distributed in India, China, Middle East. The species of this genus are widely utilized in the production of condiments, flavors, perfumes, and cosmetics. Moreover, *Pimenta* species are applied traditionally in the treatment of fever, rheumatism, toothache, abdominal pain, pneumonia, colds, diarrhea, and inflammation. Previous studies reported many pharmacological activities for different *Pimenta* species including anticancer, antidermatophytic, antihemorrhagic bleeding, anti-inflammatory, antimicrobial, antinociceptive, antioxidant, antipyretic, hypoglycemic, hypotensive, and insect-repellent activity (Contreras-Moreno, 2018).

Pimenta racemosa (*P. racemose*) (Myrtaceae) is commonly known as the West Indian Bay tree, bay rum tree, and climate. It is native to the Carbbean region, but it is also cultivated in many warm parts of the world. It is a large tree up to 7 m high (Moharram et al., 2018).

There are many studies on *P. racemosa* EOs extracted from leaves and flowers, and few reports about EOs extracted from aerial parts (Abaul et al., 1995). These studies showed that EO of *P. racemosa* is characterized by the presence of high content of Eugenol (Abaul et al., 1995; Alitonou et al., 2012; Ayoub et al., 2022), also several biological activities were reported for the oil such as antimicrobial, antioxidant (Abaul et al., 1995; Al-Gendy et al., 2017), Anti-helicobacter pylori (Abaul et al., 1995), cytotoxic (Al-Gendy et al., 2017; Youssef et al., 2021) and insecticidal (Brito et al., 2021) activities.

Previous studies suggested that there was a considerable variation in biochemical profiles and yield of oil from different agro-climatic regions. There are chemotypes of the plant and analysis of the EOs of each type showed differences concerning the chemical composition and physical characteristics. For example: in Guadeloupe, three kinds of plants that have the botanical name of *P. racemosa* var. *racemosa* (P.Miller) J. W. Moore (Myrtaceae) can be present. They differ from one another by the smell of their leaves: smells like those of clove, lemon, and anise respectively. Two chemotypes, major amounts of phenolic ethers (eugenol, chavicol, estragole, and methyl eugenol) were observed whereas the third one contained acyclic oxygenated monoterpenes such as geranial and neral (Brito et al., 2021).

Herpes simplex virus-1 and herpes simplex virus-2 HSV-1 and HSV-2 are extremely common among people everywhere. HSV-2 causes genital herpes, a sexually transmitted disease, whereas HSV-1 is primarily responsible for HSV-induced lesions in the oral cavity and epidermis (Reichling, 2022). Both viruses create a latent infection in the lumbosacral sensory ganglia after the initial infection. Acyclovir-sensitive and acyclovir-resistant HSV-1 and/or HSV-2 viral replication cycles have been inhibited by several EOs and EOs constituents over the past 10 years.

Till now no studies reported the antiviral activity of the essential oil isolated from *P. racemosa;* so, this study aimed to investigate the composition of EO of *P. racemosa* aerial parts cultivated in Egypt and evaluate its antiviral activity against HSV. Also the study aimed to find a way to discover more alternative therapies for controlling herpes infections.

2. Material and methods

2.1. Plant material

The aerial parts of *P. racemosa*, were collected during July 2022, from Giza Zoo, Egypt. Voucher specimen N0. (16*Pra*3/2022) was kept in the herbarium in the Department of Pharmacognosy, Faculty of Pharmacy, Helwan University, Cairo, Egypt. The plant was identified by Dr. Therese Labib, Consultant of Botanical Gardens and Plant Taxonomy, Ministry of Agriculture.

2.2. Preparation of essential oil

P. racemosa fresh aerial parts (250 g) were suspended in 500 ml distilled water and subjected to hydrodistillation using a Clevenger-type apparatus for 4 h. The essential oil layer was separated from the aqueous layer. Then, the oil was dried over anhydrous sodium sulfate, and stored in sealed vials at −2 °C until analysis using GC/MS (Ibrahim et al., 2020). The method was repeated three times.

2.3. GC/MS analysis and identification of components

GC/MS analysis was performed using Shimadzu GCMS-QP2020 (Tokyo, Japan) and Shimadzu HS-20. The GC was equipped with Rtx-1MS fused bonded column (30 m × 0.25 mm i.d. x 0.25 μm film thickness) (Restek, USA) and a split–splitless injector. The initial column temperature was kept at 45 °C for 2 min (isothermal) and programmed to 300 °C at a rate of 5 °C/min and kept constant at 300 °C for 5 min (isothermal). Injector temperature was 250 °C. The Helium carrier gas flow rate was 1.41 ml/min. All the mass spectra were recorded applying the following conditions: (equipment

current) filament emission current, 60 mA; ionization voltage, 70 eV; ion source, 200 °C. Diluted samples (1% v/v) were injected with split mode (split ratio 1 : 15). The MS data was operated as follows: Ion source and interface temperatures are 200 and 280 °C respectively, electron ionization mode is 70 eV, and the scanning range is 35−500 amu.

Identification of the essential oils components was done by comparing retention indices (RI) of relative to standard n-alkanes (C_8−C_{28}); and their MS to NIST and WILEY mass spectral library database; (similarity index >90%) (Adams, 2005). The retention indices were obtained using GC/MS solution program. Quantification was based on the relative area percentage calculated from the GC detector response without using correction factors. Standards were used for the identification of major constituents.

2.4. Antiviral activity

2.4.1. Cell culture and virus propagation

Vero cells (derived from the kidney of African green monkey) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The Vero cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, HEPES buffer and 50 μg/ml gentamycin. All cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and were subcultured two times a week (Vijayan et al., 2004). The cytopathogenic HSV-1 or HSV-2 virus was propagated and assayed in confluent Vero cells (Randazzo et al., 2018). Infectious viruses were enumerated by determining the 50% tissue culture infectious dose (TCID50) with eight wells per dilution and 20 μl of inoculum per well using the Spearman-Karber method (Pintó et al., 1994).

2.4.2. Cytotoxicity evaluation

The Vero cell lines in the cytotoxicity assay were seeded in 96-well plates at a cell concentration of 2×10^5 cells/ml in 100 μl of the growth medium. Fresh medium containing different concentrations of the tested sample was added after 24 h of seeding. Serial two-fold dilutions of the tested compound (started from 3000 μg/ml to 2 μg/ml) were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, Jersey, NJ, USA) using a multichannel pipette. The microtiter plates were incubated at 37 °C in a humidified incubator with 5% CO_2 for a period of 48 h. Three wells were used for each concentration of the tested sample. Control cells were incubated without test samples and with or without dimethyl sulfoxide (DMSO). The small percentage of DMSO present in the wells (maximal 0.1%) was not found to affect the experiment.

After the end of the incubation period, the viable cell yield was determined by a 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) colorimetric method (Mosmann, 1983). In brief, the media was removed from the 96 well plates and replaced with 100 μl of fresh culture medium without phenol red then 10 μl of the 12 mM MTT stock solution (5 mg of MTT in 1 ml of phosphate-buffered saline (PBS)) were added to each well including the untreated controls. The 96 well plates were then incubated at 37 °C and 5% CO_2 for 4 h. An 85 μl aliquot of the media was removed from the wells, and 50 μl of DMSO was added to each well and mixed thoroughly with the pipette and incubated at 37 °C for 10 min. Then, the OD (optical density) was measured at 590 nm (Vijayan et al., 2004) with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [(ODt/ODc)] × 100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relation between surviving cells and tested compound concentration was plotted to get the survival curve of the Vero cell line after treatment with the specified compound. The 50% cytotoxic concentration (CC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each concentration. Using Graphpad Prism software (San Diego, CA. USA). The maximum nontoxic concentration [MNTC] of each compound was also determined and was used for further biological studies.

2.5. Evaluation of the antiviral activity

The antiviral screening was performed using a cytopathic effect inhibition assay at the Regional Center for Mycology and Biotechnology (RCMB, Al-Azhar University, Cairo, Egypt). This assay was selected to show specific inhibition of a biological function, that is, a cytopathic effect in susceptible mammalian cells measured by MTT method (Al-Salahi et al., 2015; Hu & Hsiung, 1989). In brief, monolayers of (2×10^5 cells/ml) Vero cells adhering at the bottom of the wells in a 96-well microtiter plate were incubated for 24 h at 37 °C in a humidified incubator with 5% CO_2. The plates were washed with fresh DMEM and challenged with 10^4 doses of HSV-1 or 2, and then the cultures were simultaneously treated with the tested compound

conc. in a fresh maintenance medium; following this, they were incubated at 37 °C for 48 h. Infection controls, as well as an untreated Vero cell control were made in the absence of tested compounds. Six wells were used for each concentration of the tested compound. Antiviral activity was determined by the inhibition of the cytopathic effect compared with a control, that is, the protection offered by the tested compound to the cells was calculated.

Three independent experiments were assessed, each containing four replicates per treatment. Acyclovir was used as a positive control in this assay system. After the incubation period, the viability of the cells was determined by MTT assay as described before in the cytotoxicity section (Mosmann, 1983).

The viral inhibition rate was calculated as follows: $[(A - B)/(C - B)] \times 100\%$ where A, B and C indicate the absorbance of the tested compounds with virus-infected cells, the absorbance of the virus control, and the absorbance of the cell control, respectively.

2.6. Statistical analysis

Data was analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test. The experimental results were expressed as a mean,±SD. The difference between groups were considered significant when P less than 0.001. All analyses were performed using GraphPad Prism software (San Diego, CA. USA) software.

3. Results

3.1. Essential oil composition

The results of the volatile oil analysis are compiled in Table 1. The oil yielded by Hydro distillation was 2 ml representing (0.008 v/w), and a total of 45 compounds were identified representing 98.9% of the total oil. Eugenol (34.85%), β-pinene (18.29%), linalool (13.64%), and limonene (11.92%) were the major identified components. Eugenol and linalool were the major oxygenated monoterpene which represents (48.49%) of the total oxygenated monoterpene (52.05%). β-pinene (18.29%) and limonene (11.92%) were the major nonoxygenated monoterpene hydrocarbon which represents (30.21%).

3.2. Antiviral activity

3.2.1. Evaluation of cytotoxicity against VERO cell line

MTT assay was applied for the evaluation; results are represented by Table 2 and Fig. 1. The Cytotoxic activity against Mammalian cells (Vero) cells was detected under these experimental conditions with 50% cell cytotoxic concentration (CC_{50}) = 11.83 ± 0.49 μg/ml.

Table 1. Chemical composition of P. racemosa *aerial parts essential oil.*

Compound	Rt	KI ^{cal}	KI ^{Ref}	% composition
(E)- 3-Hexen-1-ol	5.150	832	833	0.01
3-Thujene (Origanene)	7.390	917	917	0.08
1R-α-Pinene	7.570	923	923	0.67
1-Octen-3-ol	8.695	961	961	1.07
β-Pinene	9.315	981	981	**18.29**
α-Phellandrene	9.595	990	990	2.36
3-Carene	9.815	998	998	0.03
α-Terpinene	9.955	1002	1002	0.26
β-Cymene	10.050	1005	1004	1.76
β-Phellandrene	10.280	1013	1014	1.62
Limonene	10.380	1016	1016	**11.92**
Trans-β-Ocimene	10.600	1023	1024	0.11
cis-β −Ocimene	10.930	1033	1033	1.07
γ-Terpinene	11.215	1042	1046	0.41
1-Octanol	11.475	1051	1051	0.10
Linalool oxide	11.935	1065	1065	0.05
Terpinolene	12.130	1072	1072	0.63
Linalool	12.435	1081	1081	**13.64**
Amyl vinyl carbonyl acetate	12.735	1091	1092	0.09
Terpinen-4-ol	14.635	1153	1153	1.87
Methyl salicylate	14.840	1159	1160	0.08
α-Terpineol	14.965	1163	1163	0.54
Capric aldehyde	15.425	1178	1179	0.35
Trans-Piperitol	15.520	1181	1181	0.04

(*continued on next page*)

Table 1. (continued)

Compound	Rt	KI ^{cal}	KI ^{Ref}	% composition
Nerol (cis geraniol)	16.175	1203	1205	0.06
Neral	16.280	1206	1208	0.06
4-(2-propenyl)-Phenol	16.735	1222	1219	5.60
Geraniol	16.950	1229	1229	0.17
Citral	17.145	1236	1239	0.07
Caprinic alcohol	17.565	1250	1254	0.04
Eugenol	19.725	1326	1329	**34.85**
Nerol acetate	20.020	1336	1336	0.07
Cyclohexasiloxane, dodecamethyl-	20.205	1343	1342	0.02
Copaene	20.795	1364	1364	0.05
Lauryl aldehyde	21.255	1381	1384	0.03
Caryophyllene	21.880	1404	1404.3	0.17
α-Caryophyllene	22.735	1437	1437	0.10
γ-Muurolene	23.300	1458	1459	0.11
Germacrene D	23.405	1462	1462	0.05
β-Bisabolene	23.725	1475	1476	0.06
trans-β-farnesen	23.895	1481	1476	0.20
Calamenene A	24.300	1497	1497	0.02
Caryophyllene oxide	25.725	1568	1568	0.07
Junenol	26.660	1611	1616	0.04
Di-epi-1,10-cubenol	26.870	1618	1613	0.06
Total identified		98.9%		
Oxygenated monoterpenes		52.05%		
Nonoxygenated monoterpenes		39.06%		
Oxygenated sesquiterpenes		0.17%		
Nonoxygenated sesquiterpenes		0.71%		
Others		6.91%		

KI ^{cal}, calculated kovats indices; KI ^{ref}, experimental Kovats Indices; Rt, retention time.

Table 2. The Cytotoxic activity (Viability %) of P. racemosa *essential oil against Mammalian cells from African green monkey kidney (Vero).*

Sample conc. (μg/ml)	Viability %	Inhibitory %	S.D. (±)
500	0.85	99.15	0.21
250	2.79	97.21	0.37
125	7.06	92.94	0.28
62.5	13.48	86.52	0.66
31.25	28.69	71.31	0.75
15.6	43.27	56.73	1.41
7.8	57.12	42.88	2.64
3.9	68.49	31.51	1.73
2	80.61	19.39	0.63
1	88.52	11.48	0.46
0.5	93.47	6.53	0.31
0.25	95.08	4.92	0.64
0	100	0	

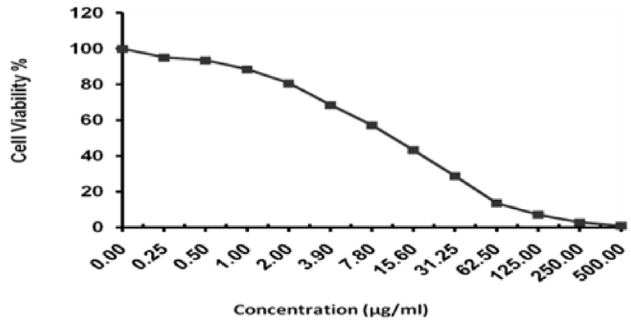

Fig. 1. Cytotoxic activity of P. racemosa essential oil on VERO cell line.

3.2.2. Antiviral activity of the essential oil against HSV-1 and 2

Results of antiviral activity against both HSV-1 and HSV-2 are compiled in Table 3. The oil sample showed good activity against HSV-1 and moderate activity against HSV-2 as compared with standard acyclovir. The essential oil showed good antiviral activity against HSV-1 with CC_{50}, $EC_{50,}$ SI (11.83, 0.34, 34.79 μg/ml), while it showed moderate antiviral activity against HSV-2 with CC_{50}, $EC_{50,}$ SI (11.83, 7.26, 1.63 μg/ml).

Selectivity index (SI) can be defined as the ratio of the toxic concentration of a sample against its effective bioactive concentration. The ideal drug should have a relatively high toxic concentration but with a very low active concentration the antiviral activity may be attributed to the EOs (Eugenol, Terpinen-4-ol, Terpinolene, α-Terpineol, Methyl salicylate and Caryophyllene).

4. Discussion

The plant derived essential oils are complex mixtures of lipophilic, low-molecular, aromatic and volatile chemicals; including monoterpenes, sesquiterpenes, and phenylpropanes, as well as their oxygenated derivatives (alcohols, aldehydes, esters,

Table 3. The antiviral effects of P. racemosa *essential oil against (herpes simplex type 1 virus and herpes simplex type 2 virus) when tested at maximum non-cytotoxic conc. (MNCC).*

Sample	MNCC (μg/ml)	Antiviral effect on HSV-1 (%) tested at MNCC	Antiviral effect on HSV-1 (Qualitative)*	Antiviral efficiency		
				EC_{50}	CC_{50}	SI
Oil	0.5	56.19 ± 3.57	+++	0.34 ± 0.12	11.83 ± 0.49	34.79
Acyclovir	20	95.73 ± 3.15	++++	1.65 ± 0.43	112.89 ± 3.17	68.41
Sample	MNCC (μg/ml)	Antiviral effect on HSV-2 (%) tested at MNCC	Antiviral effect on HSV-2 (Qualitative)*	EC_{50}	CC_{50}	SI
Oil	0.5	27.56 ± 1.22	++	7.26 ± 0.95	11.83 ± 0.49	1.63
Acyclovir	20	87.69 ± 2.73	++++	3.74 ± 0.62	112.89 ± 3.17	30.18

*Where (−): No antiviral activity.
(+): weak antiviral activity (1-<25%).
(++): moderate antiviral activity (25-<50%).
(+++): good antiviral activity (50-<75%).
(++++): excellent antiviral activity (75−100%).

ketones, phenols, and oxides). EOs and many of their individual constituents have been evaluated biologically in numerous in-vitro and in-vivo studies. These therapeutic potentials include antibacterial, antifungal, antiviral (Reichling, 2010), antioxidant (Juergens et al., 2003), immunomodulatory (Asif et al., 2020), anti-inflammatory (Paul et al., 2020), and wound healing effects (Khezri et al., 2019). The EO constituents can reach the systemic body circulation because of their low molecular weight and fat solubility, which enable them to pass through skin, mucosa, and cell membranes (Salehi et al., 2019).

HSV-1 and 2 are extremely common among people everywhere. HSV-2 causes genital herpes, a sexually transmitted disease, whereas HSV-1 is primarily responsible for HSV-induced lesions in the oral cavity and epidermis (Khezri et al., 2019). Both viruses create a latent infection in the lumbosacral sensory ganglia after the initial infection. Acyclovir-sensitive and acyclovir-resistant HSV-1 and/or HSV-2 viral replication cycles have been inhibited by several EOs and EO compounds over the past ten years; through direct interaction with the infectious virions outside of the host cells, EO_S produce their most anti-HSV actions and so stop the viruses from attaching to the cells or from penetrating the cells. This mode of operation differs significantly from the impact of synthetic medicines (such as acyclovir and pencyclovir), which solely target the intracellular replication stage of viruses (Khezri et al., 2019). According to previous research, EOs primarily targets the lipid membrane of viruses. Before being exposed to host cells, cell-free virions lost their lipid sheath when they were kept at room temperature for a 1 h incubation period with oregano essential oil. As a result, these virions lost their ability to attach to the host cells and were no longer infectious.

The current study reported that eugenol was the main component of the EO distilled from *P. racemosa* aerial parts, which was consistence with the previously reported data (Abaul et al., 1995; Alitonou et al., 2012; Salehi et al., 2019; Brito et al., 2021). The other major constituents were β-pinene, linalool and limonene respectively. The research literature revealed the presence of chavicol, eucalyptol, and β-myrcene, these components were absent in the present study. Not only the date of collection but also the place where the plant was collected is affecting the yield of EOs (Alitonou et al., 2012).

Eugenol exerts a virucidal effect on HSV-1, by disabling the viral lipidic envelope. On the other hand, some studies have shown that eugenol induced glutathione S-transferase (GST) in rat liver in vivo have reported an antiviral activity of glutathione, inhibiting the invitro replication of HSV-1. Thus, the overall antiviral activity of eugenol could be also due to an indirect effect of its reported modulatory influences on cellular GST activities and acid-soluble sulfhydryl levels (Benencia & Courreges, 2000).

There are few studies performed on the essential oil prepared from the flowers of *P. racemose* in Egypt, 52 components were identified, including 3.14% monoterpenes, 94.85% oxygenated monoterpenes, and 0.42% oxygenated sesquiterpenes where 1,8-Cineole (75.4%) and linalool (9.08%) represented the major constituents (Khezri et al., 2019).

The EO showed good antiviral activity against HSV-1 with CC_{50}, $EC_{50,}$ and SI (11.83, 0.34, 34.79 μg/ml), while it showed moderate antiviral activity against HSV-2 with CC_{50}, $EC_{50,}$ and SI (11.83, 7.26, 1.63 μg/ml).

The antiviral activity of the prepared essential oil may be attributed to (Eugenol, Terpinen-4-ol, Terpinolene, α-Terpineol, Methyl salicylate and Caryophyllene), which was matched with the data in the literature review (Benencia & Courreges, 2000). Based on the results, we recommend further clinical studies

concerning the application of *P. racemose* EO in preparations for the treatment of herpes infections.

4.1. Conclusion

Natural products may be used as alternative and adjuvant therapy to conventional drugs because of their relative safety, efficacy and protective effects. The result of this study was conducted that eugenol was the main component of the essential oil of *P. racemosa*, Eugenol is well known for its antiviral properties, so we deemed of interest to investigate the antiviral activity of the oil seeking new adjuvant and complementary herbal medications for infectious diseases. The essential oil showed good antiviral activity against HSV-1, while it showed Moderate antiviral activity against HSV-2.

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Authors' contributions

All the participant researchers contributed to this work. All authors read and approved the final manuscript.

Conflicts of interest

The authors declared no conflict of interest.

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