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Studying the Chemical Composition in Vitro Activity of *Cinnamomum zeylanicum* and *Eugenia caryophyllata* Essential Oils on *Leishmania major*

Oghlniaz. Jorjani[1], Mojtaba Raeisi[1*], Hajar Ziaei Hezarjaribi[2], Mina Soltani[3], Masoud Soosaraei[4]

[1]*Laboratory Science Research Center, Golestan University of Medical Sciences, Gorgan, Iran*

[2]*Department of Parasitology and Mycology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*

[3]*Department of Pathobiology, Faculty of Veterinary Sciences, Urmia University, Urmia, Iran*

[4]*Student Research Committee, Department of Parasitology and Mycology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran*

Corresponding author: Mojtaba Raeisi, Laboratory Science Research Center, Golestan University of Medical Sciences, Gorgan, Iran.

Abstract

Leishmaniasis is an important protozoan disease, which is still a widespread disease. In addition, avoiding drug resistance is extremely important, and herbal medicines may play a major role in reducing resistance to chemical drugs. This fact is considered by the World Health Organization. The main objective of this study was to assess the effects of *Cinnamomumzeylanicum (C. zeylanicum*) and *Eugenia caryophyllata*essential oilson *Leishmania major* under in vitro conditions. In the present study, the concentrations of 31.25, 62.50, 125, 250, 500, 1000 µg/mL of *C. zeylanicum*and *E. caryophyllata* extracts were assayed in vitro by MTT (3-(4,5-dimethylthiazol- 2yl)-2,5-diphenyltetrazolium bromide) against the promastigote forms of leishmania *major*. The results indicated that each medical herb was compared with other medical herbs and also with the results of the control groups. The results associated with promastigote assays showed that when the dose of both medical herbs increased, the promastigotes populations were reduced in comparison with the control group.

Key words: Leishmaniasis, promastigotes, *Cinnamomumzeylanicum*essential oil, *Eugenia caryophyllata*essential oil.

INTRODUCTION

Cutaneous leishmaniasis (CL) is a disease resulting from the species of the *Leishmania*spp including *L. major* and *L. tropica* [1] CL can be associated with significant morbidity and occasional deforming scars. *L. major* causes zoonotic cutaneous leishmaniasis (ZCL) in many rural areas of Iran, whereas *L.tropica* causes antroponitic cutaneous leishmaniasis (ACL).[2]Pentostam, pentavalentantimonateglucantime, amphotericin B, allopurinol and allopurinol riboside, paramomycin (minosidine) and aromatic diamidianes are the chosen drugs for all kinds of leishmaniasis in Iran and other parts of the world.[3] The use of these chemical compounds leads to problems such as relapse, drug resistance, adverse drug side effects, secondary bacterial infection, and high costs of treatment.[4]These compounds, chiefly meglumineantimoniate, are the principal line drugs for the treatment of all forms of leishmaniasis.[5] Based on a few studies carried out in recent years, approximately 10 to 15% of CL has not desirable response to meglumineantimoniate in Iran and seem indispensable to find new, safe and effective drugs for leishmaniasis.[6] It is necessary to develop novel, affordable, and accessible drugs with few side effects as the alternatives of the currently available chemical agents for leishmaniasis. Recently, superior aromatics plants have usually been used in folk medicine. The antimicrobial properties of these plants are well documented against parasites, fungi and, bacteria. [7] Most of the medical properties of these herbs are directly related to the essential oils produced by these plants. The extracts and essential oils of these plants are capable to control the microorganisms associated with food spoilage, skin diseases and dental caries. [8]

Cinnamomumzeylanicum(Cinnamon) primarily contains cinnamaldehyde, cinnamate, cinnamic acid, and numerous vital oils such as cinnamyl acetate, L-borneol, trans-cinnamaldehyde, α-thujene, L-bornyl acetate, b-caryophyllene, ugenol, α-cubebene, caryophyllene oxide, terpinolene, α-terpineol,and E-nerolidol. Antidiabetic, antioxidant, antimicrobial, anti-inflammatory and anticancer effects have been also reported for *Cinnamomumzeylanicum* [9].

Eugenia caryophyllata (Cloves) is the aromatic flower buds of a tree belonging to the family *Myrtaceae*, *Syzygiumaromaticum*. The major vegetal sources of Clove are the phenolic compounds such as hidroxibenzoic acids, flavonoids, hidroxiphenylpropene and hidroxicinamic acids. The most important component of *Eugenia caryophyllata* is the phenyl-propene eugenol, since it has strong characteristic aroma. The major parts of the clove are consisted of eugenol that comprises 70 to 90% and the remaining part is consisted of dry weight.[10] Bacterial, yeast and molds growth could be inhibited by the application of clove essential oil.[11] Micro-organisms such as *Aspergillus sp., Bacillus sp., Alternariasp, Lactobacillus sp., Clostridium sp; Salmonella sp., Mucor sp., Fusarium sp., Penicillium sp.* could be inhibited by the clove essential oil.[12] The cloves are antiparasitic, antioxidant, anti-inflammatory, antithrombotic and antimutagenic. The essential oil extracted from the dried flower buds of clove is used for warts, parasites, scars and acne. Clove essential oil was used to dissolve eggs found in the intestines that had been left behind by worms. It is believed and assumed to be the only herb that destroys almost all parasite eggs effectively [8, 13, 14]. The present study aims to evaluate the in vitro antileishmanial activity

of *C. zeylanicum*and *E. caryophyllata* extract and compare its efficacy with a reference drug, namely Glucantime against *L. major*.

MATERIALS AND METHODS:

Preparation and analysis of *Cinnamomumzeylanicum*and *Eugenia caryophyllata*oil:

C. zeylanicum and *E. caryophyllata* oil were provided by Giahessance Industry Co., Ltd. Gorgan, Iran. The analysis of Cinnamon and Clove oils were conducted using an Agilent 6890N instrument equipped with an HP-5MS capillary column (30 × 0.25 mm ID × 0.25 mm film thickness). The carrier gas was helium with a flow rate of 1 ml/min. The column temperature was initially set at 50°C, and then gradually increased to 120°Cat a 2°C/min rate, held for 3 min at this temperature, and finally increased to 250°C and using an ionization energy of 70 eV. The compounds were identified by comparing their retention indices with those of authentic samples and mass spectral data available in the library (Wiley 2001 data software).

Parasite and culture of *Leishmaniamajor*:

The promastigotes of the Iranian strain of *L.major* (MRHO/IR/75/ER strain) were cultured in NNN, RPMI 1640 media supplemented with streptomycin, penicillin and 20% heat-inactivated fetal calf serum (FCS) at 25°C. Subsequently, the promastigotes from the third passage in the NNN medium were progressively adjusted to RPMI 1640 media, meglumineantimoniate and FCS.

Promastigote assay was performed using a previously described direct counting assay based on growth inhibition. The effects of the crude extracts were evaluated in 96-well microliter plates. The promastigotes were seeded at an initial concentration equivalent to that of the early log phase (2 × 105 promastigotes/mL).

Antileishmanial Activity Assays (MTT assay):

The antileishmanial activity of the *C. zeylanicum* and *E. caryophyllata* extract was evaluated in vitro against the promastigote forms of *L. major* using a MTT (3-4,5-dimethylthiazol-2yl;-2,5-diphenyltetrazolium bromide) based on the microassay as a marker of cell viability. A stock solution of MTT (Sigma Chemical Co., St. Louis, Mo.) was prepared by dissolving MTT in phosphate-buffered saline (PBS) at 5 mg/mL and storing in the dark at 4°C for 14 days before use. The growth, proliferation, and toxic effects of the drug were evaluated by the MTT test. In summary, ELISA plates were used to test MTT. In this method, the viability percent of promastigote parasites was assessed by the MTT colorimetric quantitative test. For the antileishmanial assays, 100 µL/well of the culture, which was limited to 2 ×105 cells/mL promastigotes was seeded in 96-well flat-bottom plates. Then, 10 µL/wells from various concentrations of *C. zeylanicum*and *E. caryophyllata*extract were added tothe repetitive wells and plates were incubated for 72 hours at 25±1°C. The first well of 96 wells was considered as a blank well, which contained only 100 µL culture medium without any plant extract, drug or parasite. At the end of the incubation, 10 µL of MTT was added to each well and plates were incubated for 4 hours at 25±1°C. Enzyme reaction was then stopped by the addition of 100 µL of 50% isopropanol and 10% sodium dodecyl sulfate. The plates were incubated for an additional period of 30 minutes under agitation at room temperature. Relative optical density (OD) was then measured at a wave length of 492 nm using a multi-well scanning spectrophotometer (ELISA reader). The background absorbance of multi-well plates was measured at 690 nm and subtracted from 570 nm measurement. The absorbance of the formazan produced by the action of the mitochondrial dehydrogenases of metabolically active cells is shown to correlate with the number of viable cells. All experiments were reiterated at least three times.

Statistical Analysis:

The univariate analysis of variance (Univariate ANOVA) and the students't-test, with the significance at P values of <0.05, were used to compare the antileishmanial activity of the *C. zeylanicum and E. caryophyllata* extract with the control group.

RESULT AND DISCUSSION

Chemical composition of the essential oils:

The yield of the *C. zeylanicum* and *E. caryophyllata* oils based on the dry weight of the *Cinnamon* barks and clove buds were determined 1% and 0.5% respectively. As can be observed in Tables 1 and 2, 29 and 10 components were identified in the *C. zeylanicum* and *E. caryophyllata* essential oils representing 98.12% and 99.64% of the total oils, respectively. The major component of *C. zeylanicum* oil was (*E*)-Cinnamaldehyde (83.47%), while in *E. caryophyllata* oil, Eugenol was the major with 78.54%.

Table 1. The analysis of the chemical components of *E. caryophillata* essential oil

Chemical Tests			
No	**Components**	**KI**	**%**
1	Methyl salicylate	1205	0.09
2	Chavicol	1267	0.12
3	Eugenol	1370	78.54
4	α-Copaene	1381	0.09
5	E-Caryophyllene	1427	5.24
6	α-Humulene	1464	0.65
7	Eugenol acetate	1530	13.94
8	Carydan-8-ol	1590	0.11
9	Carryophyllene oxide	1595	0.78
10	CitronellylPentanoate	1625	0.08
	Total Identified		99.64

Table 2. The analysis of the chemical components of *C. zeylanicum* essential oil.

Chemical Tests			
No	**Components**	**KI**	**%**
1	α-Pinene	935	0.16
2	Benzaldehyde	976	0.33
3	1,8-Cineole	1037	0.11
4	Borneol	1183	0.17
5	(*Z*)-Cinnamaldehyde	1236	0.58
6	(*E*)- Cinnamaldehyde	1297	83.47
7	Cyclosativene	1375	0.23
8	α-Copaene	1381	2.57
9	Sativene	1398	0.24
10	Coumarin	1462	0.17
11	Allo-Aromadendrene	1483	0.55
12	Ar-Curcumene	1488	0.42
13	α-Muurolene	1507	1.97
14	β-Bisabolene	1514	0.27
15	γ-Cadinene	1523	0.22
16	δ-Cadinene	1526	1.55
17	*trans*-Calamene	1533	1.01
18	*trans*-Cadina-1(2),4-diene	1546	0.18
19	(*E*)-o-Melthoxycinnamaldeyde	1551	1.57
20	Caryophyllenyl alcohol	1590	0.18
21	Caryophyllene oxide	1595	0.12
22	Gleenol	1598	0.13
23	Isolongifolan-7-α-ol	1624	0.14
24	1,10-di-epi-cubenol	1641	0.36
25	epi-α-Muurolol	1659	0.62
26	α-Muurolol	1661	0.28
27	α-Cadinol	1670	0.14
28	Cadalene	1689	0.20
29	epi-α-Bisabolol	1699	0.18
	Total Identified		98.12

Table 3. Effect of different concentrations of *C. zeylanicum*extract on the number of promastigotes of *L. major*.

Treatment / Times	Control	Glu	1000 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml	62.50 µg/ml	31.25 µg/ml
30 min	320.8^{A}	86.5^{B}	112.01^{C}	154.35^{D}	195.54^{E}	214.62^{F}	261.13^{G}	294.06^{H}
60 min	334.66^{A}	73.6^{B}	82.43^{B}	139.84^{C}	172.36^{D}	221.09^{E}	258.55^{F}	276.28^{G}
120 min	327.73^{A}	80.05^{B}	102.22^{C}	174.09^{D}	183.95^{D}	217.85^{E}	259.84^{F}	285.17^{G}

Different letters in the same raw indicate significant differences ($p<0.05$).

Table 4. Effect of different concentrations of *E. caryophillata* extract on the number of promastigotes of *L. major*.

Treatment / Times	Control	Glu	1000 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml	62.50 µg/ml	31.25 µg/ml
30 min	321.08^{A}	89.6^{B}	168.51^{C}	274.02^{D}	280.5^{D}	286.28^{D}	287.48^{D}	306.21^{E}
60 min	332.94^{A}	93.4^{B}	145.27^{C}	253.33^{D}	268.8^{E}	271.35^{E}	279.15^{E}	294.64^{F}
120 min	327.01^{A}	91.5^{B}	156.89^{C}	263.67^{D}	274.65^{D}	278.81^{D}	283.31^{D}	300.42^{E}

Different letters in the same raw indicate significant differences ($p<0.05$).

In all concentrations, the effect of *C. zeylanicum*and*E. caryophyllata* is time-depended. In other words, at all times, living promastigotes were decreased in different tubes in comparison to the past time. In addition, the effect of *C. zeylanicum*and*E. caryophyllata* on *L. major* was significantly different from the negative control ($P<0.001$). As can be seen in Tables 3 and 4,*C. zeylanicum*and*E. caryophyllata*with concentration of 1000µg/ml had strong antileishmanial activity. It was determined that the antileishmanial effect of 1000µg/ml of *C. zeylanicum*at 60 min was significantly similar to Glutamine as a conventional drug ($p>0.05$). Extracts with higher concentration had more effect on promastigote population ($P<0.001$). Our results indicated that similar

concentrations of the extracts of *C. zeylanicum* were more effective than those of *E. caryophyllata*.

Leishmaniasis has become one of the major health issues in Iran. Chemotherapy is somewhat painful and ineffective. In this regard, people use medicinal herbs drugs in the local market as a treatment to cure their wounds. There is an inclusive, inexpensive and lack of effective chemotherapeutic agents for the treatment of leishmaniasis. Although trivalent antimonial drugs such as pentavalent antimonial and potassium antimonyl tartrate are the first-line treatment for leishmaniosis, with pentamidine and amphotericin B used as alternative drugs, all of these have serious side effects and resistance has become a severe problem. Therefore, new drugs are urgently required. Natural products have potential in the search of new and selective agents for the treatment of important tropical diseases caused by protozoans [15].

The results of the GC/MS analysis of the *C. zeylanicum*and *E. caryophyllata* essential oils showed that cinnamaldehyde and Eugenol were the major components of test essential oils. According to previous studies, the major component of the *C. zeylanicum* essential oil was reported as cinnamaldehyde in the range from 44% to 97%. [16, 17] It has been reported that the major component of clove essential oil was Eugenol [18, 19]. These findings are in agreement with the results of our study. It should be considered that The difference in the composition of various essential oils may be due to many factors such as genotype, environmental and climate conditions, geographic location, cultivation and harvest time, vegetative stage and growing season, plant maturity, method of distillation and storage conditions [20].

In this report, a relevant viability test (MTT) was used to investigate the killing effect of plant extract on the *Leishmania major* promastigotes in vitro. The side effects of the current treatment cause researchers to search for new therapeutic agents. Many studies were not conducted in the field of medicinal plants for anti-leishmanial effects. Our study observation is that the extracts of *C. zeylanicum*and *E. caryophyllata* were effective concerning *L. major*promastigotes in vitro.

In earlier studies, it has been shown that *Artemisia species, Allium sativum* and *Achilleamillefolium* were the most effective plants that could kill 100% of promastigotes after different times. The results of the present study indicated that *C. zeylanicum*and *E. caryophyllata* killed more population of promastigotes after 120 minutes in the concentration of 1000 µg/ml. Our results showed that there was no significant difference between plants extract and glucantime ($P>0.05$) in MTT test. In this study, the results showed that with increase in concentration, the killing effects of plants on promastigotes will be increased, and relative optical density will be decreased. The decrease of formazan is a reason for OD decrease, which is produced by the action of the mitochondrial dehydrogenases of metabolically active cells and is shown to relate to the number of viable cells [21-24].

Therapeutic assessments for medicinal herbs are essential owing to the therapeutic use and growing interest in the alternative therapies of natural products. Natural products can be lead compounds, allowing the rational design and planning of new drugs, biomimetic synthesis development, and discovery of new therapeutic properties not yet attributed to the known compounds [25]. Natural products have made, and continue to make, an important contribution to this area of therapeutics. Probably, their future potential will be even more promising.

This activity represents an exciting advance in search of novel anti-leishmania agents from natural sources, since a significant and important effect against the promastigote form of the protozoan was exerted. Although these herbs showed significant activity against *L. major* promastigotes in vitro, in vivo studies and further synthesis are indicated to validate these results [26]. Our results reveal a novel pharmacological activity of *C. zeylanicum*and *E. caryophyllata* against *L. major* and suggest that these extracts have the potential of being used as a topical application in anti-leishmanial effects. Further investigations are essential to provide extra clinical evidence for the traditional uses of this spice against cancer and inflammatory, cardio protective, and neurological disorders.

CONCLUSION

By considering an increase in drug resistance against leishmania, it is possible to use the different extracts of *C. zeylanicum*and*E. caryophyllata*instead of chemical drugs. The use of different concentrations of the essential oil of *C. zeylanicum* controlled *L. major* more effectively by increasing the extract concentration. Future research will assess the effect of this extract on the amastigote form of *L. major* in *in vivo* study.

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