Effects of Phenolic Compound on the Viability of *Leishmania tropica* promastigotes: A comparative *in vitro* study

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الخلاصة

تهدف الدراسة الحالي لتقييم فاعلية (caffeic acid)علـى حيويـة الطـور الأمـامي السـوط
(promastigote)للـشمانيا الجلديـة خـارج الجـسم الـحأظمي. رت النـت ـائج بـ أنلل ـ
caffeic acid تأثيـر واضـح على حيوية هذا الطور باستخدام تراكيز مختلفة، حيثيۇ ۇدي الـى
نقص ان فـ ي عـ دد الـ promastigote-الحيۃ قريجياً بزيـ ادة التركيۇز. و أنۇع دل الم ـ وتكـ ـان
(24×10⁴ ، 30×10⁴، 38×10⁴، 44×10⁴ و 53×10⁴) promastigotes ل مليلتـ ر
عذ د التركيۇز (10, 15, 20, 25, 30 mg/ml)علـى التـ والبجـ د ثلاثـ ة ايـ ام مـ ن الحـضن.
يستدل من هذه النتائج بأن caffeic acid يمتلك تأثير قاتل على حيوية الط ور الأمـامي السـوط
لطفيلي اللشمانيا الجلدية خارج الجسم الحي. " P < 0.01 "

Abstract

This *in vitro* study aimed to evaluate the effectiveness of Caffeic acid against the viability of *Leishmania tropica* promastigotes in vitro. The result showed that Caffeic acid had a fatal effect on the viability of *Leishmania tropica* promastigotes with the use of different concentrations leading to a gradual decreasing of living promastigotes number with the increase of Caffeic acid concentration. The mean parasiticidal effect of Caffeic acid was ($24×10^4$, $30×10^4$, $38×10^4$, $44×10^4$, $53×10^4$) promastigote /ml at concentrations (10, 15, 20, 25, 30) mg/ml respectively after three days of incubation. It can be concluded that Caffeic acid posses a cidal effect on the *Leishmania tropica* promastigotes *in vitro*." P < 0.01 ".

Introduction

Leishmaniasis is a disease caused by obligate intracellular, kinetoplastid protozoa of the genus *Leishmania* (Trypanosomatidae) [1]. *Leishmania* has two stages in its life cycle amastigote stage in animal host and promastigote stage in vector [2]. Natural transmission may be zoonotic or anthroponotic, and it is usually by the bite of a phlebotomine

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sand fly species (order Diptera, family: Psychodidae; subfamily Phlebotominae) of the genera *Phlebotomus* (Old World) and *Lutzomyia* (New World) [3, 4]. The disease manifest itself in variety of clinical forms, ranging from self-healing cutaneous lesion to the more serious, potentially fatal visceralizing form, and include the metastasize muco-cutaneous form and the post kala-azar dermal Leishmaniasis [5]. Leishmaniasis is one of the leading causes of morbidity and mortality and it has been found to be a major global health problem [6]. It is endemic in 88 countries, with an estimated yearly incidence of 1-1.5 million cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis, three hundred and fifty million people are estimated to be at risk, and there is an overall prevalence of 12 million cases [7]. Cutaneous and muco-cutaneous leishmaniasis are more prevalent in Afganistan, Saudi Arabia and some Latin American countries [6].

Proven therapies against human leishmaniasis include pentavalent antimonials (sodium stibogluconate and meglumine antimonite), amphotericin B, pentamidine, miltefosine and paromomycin [8, 9]. These drugs are unsatisfactory because of their limited efficacy, frequent side effects and increasing drug resistance [10]. Development of resistance by the parasites have been reported [11, 12, 13], so that new therapies are needed to supplement or replace currently available therapies. A search for a new active compound with potential Leishmanicidal property remains essential for the development of a new antileishmanial therapy. Extracts from medicinal plants are being widely tested for Leishmanicidal activity [14]. Caffiec acid is naturally phenolic compound and member of flavonoids presents in coffee, olive oil, white wine, cabbage etc [15]. Caffiec acid is 3, 4-dihydroxy cinnamic acid which is derived from shikimic acid and similar to cinnamic acid. CA and its derivatives, ethyl ester (CAEE) and phenethyl ester (CAPE) act carcinogenic inhibitors and they also show anti-oxidant (radical-scavenging) activity *in vitro* study [16]. Caffeic acid has antimicrobial activity that inhibits the radial growth of *Ganoderma Boninense* [17]. This study aimed to determine the effect of caffeic acid on the viability of *Leishmania tropica* promastigote *in vitro*.

Materials and Methods

Organisms Used in the study:

Leishmania tropica isolate (MHOM/IQ/1992/MRC3) was obtained from research unit in the College of Medicine, AL-Nahrain University, Baghdad. It was isolated from infected human. The strain was diagnosed by isoenzyme assay and cultivated on biphasic culture medium at 25 ºC.

Methods

Preparation of Media:

1. Biphasic media:

It's composed of two phases, one is a solid phase and the other is liquid phase. This media is used for cultivation and continuation of promastigotes stage of leishmania. It was used for the first time by Nove and Mac Neal in 1904 these phases are:

A. Solid Phase: it was prepared by kagan and Norman [18].

B. Liquid phase (lock's solution): it was prepared by Dawson *et al* [19].

2. RPMI 1640 (Roswell park memorial institute medium)

This media was prepared by adding 10.4 gram of powder media in 900 ml of distill water and 100 ml of preheated fetal calf serum (55˚C for 50 minute) then added 1 ml of previously prepared antibiotic solution and the pH was justified to 7.2. Sterilization has been done by Nalgen filter of 0.22 micrometer then distributed 5 ml of media in sterile tube of 10 ml size and incubated in 37 ˚C for 24 hour to avoid the contamination tubes and the sterile tubes put in 4 ˚C till use [20].

2.5 Growth curve:

Promastigotes were cultured in RPMI-1640 with 10% fetal calf serum medium and incubated in 26˚C. Counting the promastigote each 24 hours to determine logarithmic phase. The promastigote increased in number in the first, second, third days and reach the peak in the fourth day, the fourth day represent the logarithmic phase (Figure1).
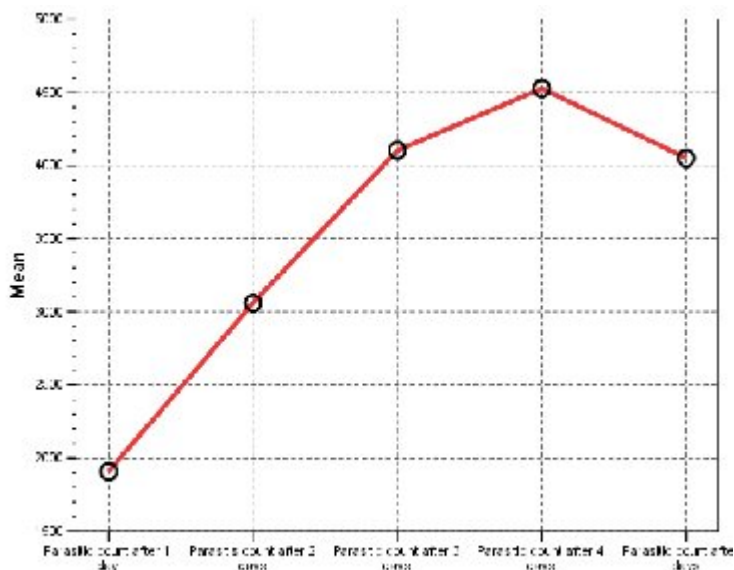
Figure 1: line graph showing the mean count of parasites in culture within 5 days after Initial inoculation

Preparation of samples:

Assays on *L. tropica* promastigote were performed as follows:

Promastigote were cultured in RPMI-1640 medium with 10% fetal calf serum and incubated in 26ºC, the parasite with a density of $(1\times10^6$ promastigote/ml) were then added to each well of a 96-microtiter plate (100µL). Different concentrations of agent were added on the parasite in each well (50µL). Further two controls samples were used included: growth medium without agent (100% viability) and growth medium containing studied the anti-Leishmanial drug (Sodium stibogluconate) (Pentostam®) (20µg/ml) [21]. The plate was incubated in 26˚C for 72 hours. Growth was measured in each well through counting the promastigotes after (72hours) by the conventional slide chamber method [22].

Preparation of Caffiec acid:

The solvent (Acetone):

A 1000 ml bottle of acetone solution (100%) was purchased from (Medex Company, patch no: (200-662-2). The solution of acetone (50%) prepared by adding acetone: water (50:50 v/v). This solution was used as a solvent to the tested chemical agent [17].

Caffeic acid: A 10 gram crude powder of caffeic acid was purchased from Sigma Aldrich Company (Batch no. 21909058). Using acetone 50% (as a solvent) and stirrer to prepare five different concentration of caffeic acid (10, 15, 20, 25, 30) mg/ml.

Results
The effect of increasing dosage

The parasiticidal effect is the rate of parasitic killing after adding a certain dose of a specific experimental drug. It is calculated as the number of dead parasites/Total number of parasites (dead+alife) multiplied by 100. This index ranges between a minimum of zero to a maximum of 100. The higher the index the stronger is the effect.

Caffeic acid:

As shown in table (1.1), after adding 10 mg of Caffeic acid the parasiticidal effect is significantly increased by a mean of $24×10^4$ promastigote/ml compared to baseline controls. After each successive increase in dosage the parasiticidal effect is significantly increased to reach a maximum increase by a mean of $53×10^4$ promastigote/ml compared to baseline control. The Cohen's d effect size is increased from 4.4 after 10 mg dosage to reach a maximum of 10.8 after the highest dose of 30 mg. The dose of added Caffeic acid showed a statistically significant positive strong linear correlation (r=0.93) with parasiticidal effect. The regression model explaining the effect of Caffeic acid dosage was statistically significant and able to explain 87% of variation in the dependent variable (parasiticidal effect). For each one mg increase in Caffeic acid dosage the parasiticidal effect is expected to significantly increase by a mean of 1.69.

Table 1.1: The changes in mean Parasiticidal effect (rate of Parasitic killing) after increasing dosage of Caffeic acid ($\times 10^4$) promastigote/ml compared to baseline control.

Caffeic acid	At base line	After 10 mg dose	Change after 10 mg dose compared to baseline	After 15 mg dose	Change after 15 mg dose compared to baseline	After 20 mg dose	Change after 20 mg dose compared to baseline	After 25 mg dose	Change after 25 mg dose compared to baseline	after 30 mg dose	Change after 30 mgdose compared to baseline
Mean	5	29	24	35	30	43	38	49	44	58	53
SE	0.51	1.26	1.6	1.2	1.23	1.6	1.75	1.27	1.3	1.33	1.59
N	24	24	24	24	24	24	24	24	24	24	24
Cohen's d (effect size) for changes after each dose compared to baseline			4.4		6.7		6.6		9.4		10.8
P (paired t-test) =	0.001 <		<0.001		<0.001		<0.001		<0.001		<0.001

r (dosage x rate of parasitic killing) =0.93   P<0.001
R^2=0.87
P (Simple Linear Regression Model) < 0.001
Regression coefficient for dosage = 1.69

Sodium stibogluconate:

As shown in table (1.2), after adding 20 µg of Sodium stibogluconate the parasiticidal effect is significantly increased by a mean of 79 compared to baseline controls. The Cohen's d effect size reach 26.3 after 20µg dose.

Table 1.2: The changes in mean Parasiticidal effect (rate of Parasitic killing) after adding 20 µg of Sodium stibogluconate ($\times 10^4$) promastigote/ml compared to baseline control.

Sodium stibogluconate	At baseline	After 20 µg dose	Change after 20 µg dose compared to baseline
Mean	8	87	79
SE	0.65	0.55	0.91
N	24	24	24
Cohen's d (effect size) for changes after each dose compared to baseline =			26.3
P (paired t-test) =			0.001<

Discussion

Leishmaniases is a group of disease caused by Leishmania species, the disease is considered as a major public health problem in 82 countries in the world causing morbidity and mortality [23].

There is a general lack of effective and in expensive chemotherapeutic agents for the treatment of leishmaniasis. Although trivalent antimonial like potassium antimonyl tartrate and pentavalent antimonial drugs are the first-line treatment for this disease, with amphotericin B and pentamidine being used as alternative drugs, all of these have serious side effects and resistance has become a challeng problem. Therefore, new drugs are urgently required, natural products have potential in the search for new and selective agents for the treatment of important tropical diseases caused by protozoans [24].

In the vast areas of the world, modern drugs are simply not available or if they are available they often prove to be too expensive. The majority of drugs active against infectious agents are in fact derived from natural products [25]. Different plants of medicinal value are used traditionally worldwide for the treatment of leishmaniasis [26]. In the literature there are several reports on the activity of a variety of crude natural extracts, especially from plants collected in tropical zones against *leishmania* species [27, 28, 29, 30].

The effect of phenolic compounds on *Leishmania* parasite viability is not yeat known, and, to date, no other out comes studies have been announced.

In this study we observed that the tested agent (Caffeic acid) have different parasiticidal effect depending on the difference in concentration after duration 72 hours. The parasiticidal effect is tested according to five different concentrations (10, 15, 20, 25, 30mg/ml). This study demonstrates that Caffeic acid (CA) has a strong parasiticidal activity depending on the variation in concentration after duration of 72 hours. This study showed the mean of parasiticidal effect of caffeic acid at 10mg/ml is (24×10^4) promastigote/ml as compared to baseline control. After each successive increase in concentration the parasiticidal effect is increased to reach a mean (30×10^4) promastigote/ml in a concentration 15mg/ml. The parasiticidal effect more increased when adding (20, 25, 30mg/ml) to reach a mean $(38 \times 10^4, 44 \times 10^4, 53 \times 10^4)$

promastigote/ml respectively. These observations are in consistence with the study of Chong *et al*, reported that caffeic acid inhibits the radial growth of *Ganoderma Boninense* [17]. Another study by Ismail and Pierson investigated the effect of CA against *Clostridium botulinum*, they state that CA reduces the viability of Clostridium spores [31]. The antimicrobial effect of CA may be attributed to the interaction with metal ions such as iron which is essential for microbial growth [32]. Further more CA could cause change in the structure of cytoplasmic proteins that inhibit cell division [33]. In another report, CA was also found inhibiting the growth of sweet potato pathogenic fungi, inhibitory activity reported and suggests high periderm CA levels contribute to the storage root defense chemistry of some sweet potato genotypes [34].

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