Available Online at

http://www.ijcpa.in

April-June 2017

International Journal of CHEMICAL AND PHARMACEUTICAL ANALYSIS

eISSN: 2348-0726 ; pISSN : 2395-2466

DOI : http://dx.doi.org/10.21276/ijcpa

Research Article | Volume-4 | Issue-3 | Article ID: 1276

IN VITRO ACTIVITY OF WUSHWUSH GREEN TEA EXTRACTS AGAINST *LEISHMANIA* MAJOR PROMASTIGOTES

Alemu Tadesse[†], Ariaya Hymete [††], Adnan A, Bekhit[†††], Asrat Hailu[††††]

[1]Department of Pharmaceutical Chemistry and Pharmacognosy, School of Pharmacy, Addis Ababa University; Addis Ababa, Ethiopia.
[2]Department of Microbiology, Immunology and Parasitology, School of Medicine, Addis Ababa University; Addis Ababa, Ethiopia.

***Corresponding Author:** Email: donganegn@gmail.com*

Received: 16 March 2017 / Revised: 2 April 2017 / Accepted: 25 April 2017 / Available online : 29 May 2017

ABSTRACT

Objective: The aim of this study was to investigate the antileishmanial effect of the aqueous and methanolic extracts of Wushwush green tea samples against Leishmania major promastigotes. Methods: After evaporation and freeze-drying the extracts were dissolved in Dimethyl Sulfoxide (DMSO) 1% in PBS. The stock solutions of 128 mg/ml were prepared for the two solvents by dissolving 100 g of Wushwush green tea extract in culture media for anti-leishmanial assays. Clinical isolate of L. aethiopica strain (CL014/09) promastigotes were cultured in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum (HIFCS), 1% L-glutamine and 1% penicilline-streptomycin solution. Results: Aqueous and methanolic extracts of Wushwush green tea showed significant leishmanicidal activity against L. major promastigotes. Conclusion: Our study revealed a significant pharmacological activity against promastigotes of L. major and suggests that green tea extract has the potential of being used in leishmaniasis treatment in combination with the available antileishmanial drugs

Keywords – *Catechin;Polyphenols;Wushwush green tea;Camellia sinensis;Leishmaniasis*

1. INTRODUCTION

Tea is one of the most commonly consumed beverages throughout the world, next only to water [1,2] and well ahead of coffee, beer, wine and carbonated soft drinks [3]. Tea infusions, consumed by two thirds of the world's population are obtained from the leaves of the plant *Camellia sinensis* [4]. It can be categorized into three types, depending on the level of fermentation [5], i.e. green (unfermented), oolong (partially fermented) and black (fermented) tea. Another form of tea is white tea which is made from new growth buds and young leaves that have been steamed to inactivate polyphenol oxidation and then dried. The buds may be shielded from sun light to prevent formation of chlorophyll.

Of the 2.5 million metric tons of dried tea manufactured, only 20% is green tea and less than 2% is oolong tea [6].

Green tea is consumed as a popular beverage worldwide, particularly in Asian countries like China, Korea and Japan. There is hardly any other food or drink reported to have as many health benefits as green tea [7].The chemical composition of green tea varies with climate, season, horticultural practices and position of the leaf on the harvested shoot [8]. The major components of interest are the polyphenols.

The major polyphenols in green tea are flavonoids. The four major flavonoids in green tea are the catechins, epicatechin (EC), epigallocatechin (EGC), epicatechingallate (ECG) and epigallocatechingallate (EGCG).

Epigallocatechingallate is viewed as the most significant active component of green tea. The leaf bud and first leaves of green tea are richest in EGCG.

The usual concentration of total polyphenols in dried green tea leaves is, 8–12% [9- 11]. Other compounds of interest in dried green tea leave include gallic acid, quercetin,kaempferol, myricetin, caffeic acid and chlorogenic acid [9,12]. The secret of green tea lies in the fact that it is rich in polyphenols such as catechins and particularly EGCG. Green tea extracts contain antioxidant, anti-inflammatory and anti-carcinogenic activity that are potentially relevant to the prevention and treatment of various forms of cancer [13].

Since leishmanicidal activity of Wushwush green tea has not been evaluated so far, the present study was conducted to investigate the *in-vitro* antileishmanial activity of this plant. Leishmaniasis is a vector born disease caused by protozoan parasites of the genus *Leishmania*. Motile form of the parasite (promastigote) is transmitted to humans by the bite of infected hematophagous female sand flies (subfamily *Phlebotominae*). Human leishmaniasis is found in South America, North America, Asia, Europe and Africa and is endemic to the tropical and sub-tropical regions.

Globally, it is believed that there are approximately 12 million cases of leishmaniasis, with about 1.5 million to 2 million new cases of cutaneous leishmaniasis (CL) and 500,000 new cases of visceral leishmaniasis (VL) occurs every year. Leishmaniasis is reported endemic in 88 countries with sixty two countries having endemic VL, and the geographical distribution of this disease is expanding in several areas of the world [14]. Visceral leishmaniasis is caused by two leishmanial species; *L. donovani*or *L. infantum*, depending on the geographical area and the infected host status. *L. infantum* infects mostly children and immuno-compromised individuals, whereas *L. donovani* infects people of all age groups.

Cutaneous leishmaniasis (CL) is caused by *L. major, L. tropica* or *L. aethiopica*.

In Ethiopia, CL manifests in three forms: localized, diffused and mucosal. CL in Ethiopia principally caused by *L. aethiopica* and rarely by *L. tropica* and *L. major*. The diffuse and mucosal forms are often continuation of the self-healing CL caused by *L. aethiopica* [15].

Leishmaniasis is characterized by both diversity and complexity. Depending on the strain (s) of the parasite involved in pathogenesis and the immune response established by the host it can cause clinical symptoms that range from mild self-limiting cutaneous lesion to fatal visceral disease [16].

2. MATERIALS AND METHODS

2.1 Plant Materials

Wushwush green tea leaf samples were collected from Wushwush tea plantation which was found in South Nations Nationalities and Peoples Region, Kaffa Zone whose plantation was located at 460 km south west of Addis Ababa. The leaves were collected in July 2009 and dried at room temperature.

2.2 *Leishmania* Culture Medium

RPMI-1640 and medium-199 (both from Gibco, Invitrogen Co., UK), fetal calf serum, nutrient agar, penicillin-streptomycin solution, D(+)-glucose, calcium chloride dihydrate, potassium chloride, disodiun hydrogen phosphate, sodium bicarbonate and potassium dihydrogen phosphate (all from Sigma Chem. Co., St. Louis, USA), sodium chloride (CarboErbaReagenti, Imbalati, Italy) were used to make complete culture mediums.

2.3 *Leishmania* Parasite Culture

Leishmania aethiopica (promastigote stages) were obtained from Leishmaniasis Diagnosis and Research Laboratory at the Department of Microbiology, Immunology and Parasitology, Addis Ababa University. Prior to *in vitro* assays, the final promastigote cultures were

expanded in RPMI 1640 complete medium and brought to stationary phase in 25 cm^3 culture flask (Corning®) with continuous monitoring for 3-4 days under inverted microscope.

2.4 Preparation of Green Tea Extracts

The ground material (100 g) was soaked in absolute methanol for 24 hrs. The extract was filtered, dried with Na_2SO4 and the solvent removed under vacuum in a rotary evaporator at 30°C-35°C. The aqueous extract was prepared by pouring boiling distilled water onto the dry leaves (100gm), followed by stirring with a magnetic stirrer for 20 minutes and additional steeping for 24hrs at room temperature. The extracts were first filtered through nylon sieve, followed by vacuum filtration using Whatman No.54 filter paper.

The filtrate was freeze-dried, weighed and stored at -20°C until used. Then extracts were dissolved in Dimethyl Sulfoxide (DMSO) 1% in PBS.

2.5 Preparation of Parasite Culture

Clinical isolate of *L. aethiopica* strain (CL014/09) promastigotes were cultured in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum (HIFCS), 1% L-glutamine and 1% penicilline-streptomycin solution [17].

2.6 Preparation of Stock and Working Solutions of Tea Extracts

Stock solutions of 128 mg/ml were prepared for by dissolving 100g of Wushwush green tea methanol and water extracts in culture media for anti-leishmanial assay. The stock solutions were re-sterilized by filtering through 0.22 µm filter in the laminar flow hood. If some components of the extracts were not easily soluble in water or media, they were first dissolved in 1% dimethyl sulfoxide (DMSO) to avoid solvent carryover. All prepared extract solutions were stored at 4^0C and retrieved only during use. Assays for the two test samples were carried out separately.

Then stocks were diluted to 800 µl using complete RPMI (440 ml RPMI + 50 ml HIFCS + 5 ml 100 IU penicillin/ml-100 µg/ml streptomycin solution) and 5 ml L-glutamine) to obtain aliquots of 64 mg/ml.

Six dilutions (2, 4, 8, 16, 32, 64mg/ml) were made from the methanol and water water to get different concentrations. 100 µl of the above serial dilutions of extracts were added to 96-well cell culture plates containing 100 µl of *L. major* promastigotes (1 × 106 parasites/ml) in the stationary growth phase and it was incubated for use in three successive times (24, 48, and 72 hrs) at 21 ºC. 1% DMSO and 100 µl of 43 mg/ml Glucantime were added to separate wells containing *L. Major* Promastigotes to serve as negative and positive controls respectively. All tests were performed in triplicate.

The number of promastigotes in each concentration was calculated using a hemocytometer slide for counting the live and non-living promastigotes in each well.

3. RESULTS AND DISCUSSION

In this study, aqueous and methanolic extracts of Wushwush green tea showed significant leishmanicidal activity against *L. major* promastigotes with increasing concentration. At higher concentrations the mean live promastigotes for both aqueous and methanolic extracts were decreased to zero percent. The extract was diluted with DMSO 1% in PBS buffer.

In comparison with glucantime the mean live promastigotes in 16 mg/ml concentration of aqueous extracts of green tea was almost the same as 43 mg/ml glucantime (Table 1). But at higher concentrations the aqueous green tea extracts as indicated in Table 1 were more effective than glucantime.

As it was seen from the table the aqueous extract is more active compared to the methanolic and this is an advantage as tea is prepared in water.

Table 1 demonstrates mean live promastigotes in different concentrations of green tea extract, DMSO 1% in PBS buffer and RPMI1640 according to intervals and in comparison to positive control. Clearly there was an increase in anti-leishmanial activity of both aqueous and methanolic extracts with increasing concentrations.

The activity of Wushwush green tea extracts against *L. major* showed that the plants contained some pharmacologically active substances that could prevent growth and proliferation of *L. major* promastigotes. Other studies have revealed that flavonoids like catechins have antimicrobial properties against fungi, Gram-positive and Gram-negative bacteria [18]. The ant-leishmanial activity observed in this study against *L. major* strains could be due to the ability of flavonoids to form complexes with the parasite cell wall, affecting cell-linked processes and inhibiting the action of DNA polymerase thereby inhibiting its growth. Flavonoids are also known to inhibit cell enzyme activities [18].

The antileishmanial activity of Wushwush green tea observed in this study may also be due to the presence of catechins which exhibit antibacterial activity by inhibiting the action of the DNA polymerase. The leishmanial inhibitory activity could also be attributed to the tannins which are found in large quantities in green tea. The tannins could be disrupting the cell membranes of the *L. major*, hence their inhibitory activities.

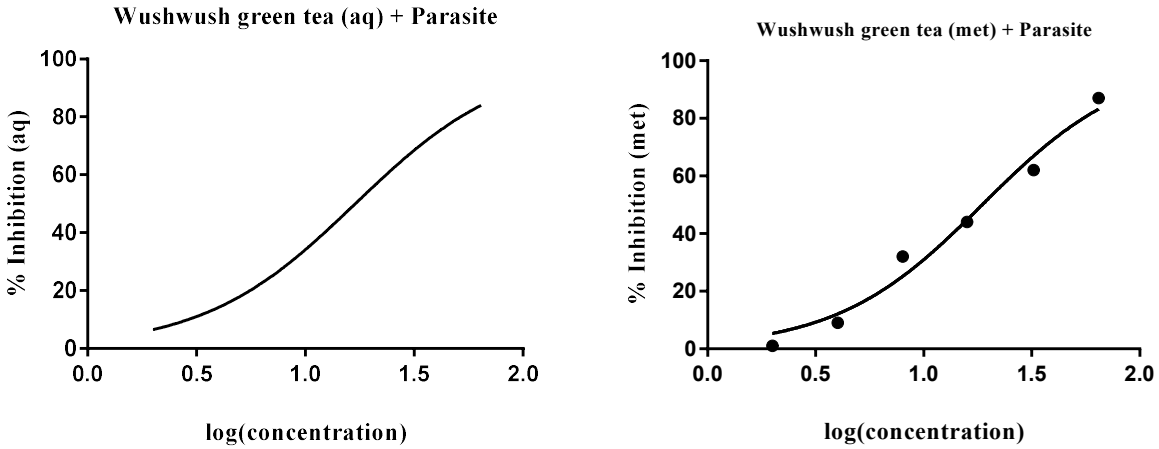

Fig. 1: Effect of Wushwush green tea aqueous and methanol extract on *Leishmania* parasite

Table 1: Mean live promastigotes in different concentrations and times.

Culture medium and extracts		Mean (%) of live promastigotes according to hours			
		Initial treatment	24 h	48 h	72 h
RPMI1640		100	100	100	100
DMSO 1% in PBS buffer		100	100	100	100
43mg/ml Glucantime		100	54 ± 1	20± 3	7 ± 1
2mg/ml extract	Aqueous	100	97 ± 2	87± 1	64 ± 2
	Methanolic	100	99 ± 1	90± 2	69± 2
4mg/ml extract	Aqueous	100	89± 1	81± 2	54 ± 3
	Methanolic	100	91± 2	89 +3	58 ± 1
8mg/ml extract	Aqueous	100	65± 1	51 ± 2	36 ± 2
	Methanolic	100	68± 2	57 ± 2	38 ± 2
16mg/ml extract	Aqueous	100	53± 2	21± 2	6 ± 1
	Methanolic	100	56± 3	24± 1	9± 1
32mg/ml extract	Aqueous	100	36± 2	11 ± 2	0
	Methanolic	100	38± 1	17± 3	4± 1
64mg/ml extract	Aqueous	100	12± 1	0	0
	Methanolic	100	13± 2	2± 2	0

4. CONCLUSION

Aqueous and methanollic extracts of Wushwush green tea had shown inhibitory effect on the *Leishmania* parasite when it was added to the parasite containing 96-well cell culture plates as observed fluorometrically. There is a direct relation between the concentration of WGT (aqueous and methanolic) extracts and its percent inhibition.

The inhibitory activity of WGT may be attributed to the presence of major catechins such as EC, EGC, EGCG and ECG which are very important in determining health potential and the chemical properties of tea. It is therefore possible that if fortified and/or used together with other drugs, the extracts could provide additive or synergistic effects in the control of resistant strains of *Leishmania* parasite.

Finally this study revealed significant pharmacological activity against promastigotes of *L. major* and suggests that green tea extract has the potential of being used in the management of *leishmaniasis* but more studies are needed to find out its activity against amastigote and appropriate route of administration.

5. CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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