BILINGUAL PUBLISHING CO.
Pioneer of Global Academics Since 1984

Journal of Zoological Research
https://ojs.bilpublishing.com/index.php/jzr

ARTICLE

Evaluation of the Therapeutic Potential of *Warburgia ugandensis, Prunus africana,* and *Piliostigma thonningii* against *Leishmania donovani in vitro* and in Balb/c Mice

Maria Divinah Mogaka[1*] Joshua M. Mutiso[1] John C. Macharia[2] Rebecca M. Ayako[1] Bernard Osero[3] Michael M. Gicheru[1]

1. Department of Zoological Sciences of the School of Pure and Applied Sciences, Kenyatta University, Kenya
2. Department of Tropical and Infectious Diseases, Institute of Primate Research, Kenya
3. Centre for Biotechnology Research and Development, Kenya Medical Research Institute, Kenya

ARTICLE INFO

Article history
Received: 27 July 2022
Revised: 9 August 2022
Accepted: 31 August 2022
Published Online: 29 September 2022

Keywords:
Promastigotes
Amastigotes
Toxicity
Parasite burden
IgG antibodies

ABSTRACT

Leishmaniasis is a zoonotic disease caused by protozoan parasites of the genus Leishmania. Conventional chemotherapy remains to be the most preferred measure against leishmaniasis despite being associated with high toxicity and relapse rates. They are also expensive and require hospitalization. Plant-based compounds provide a better treatment alternative because they are effective, cheap, and less associated with toxicity and resistance. This study examined the therapeutic potential of *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* against *Leishmania donovani* infection in BALB/c mice. Anti-promastigote and toxicity studies were evaluated by incubating the test compound with promastigotes and Vero cells, respectively. Serum was obtained from the mice for total immunoglobulin gamma (IgG) quantification. For in vivo studies, the mice were infected with virulent *Leishmania donovani* then treated with methanolic extracts of *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* and control drug, pentostam (sodium stibogluconate). Treatment with the plant extracts and standard drug resulted to significant reduction in parasite burden. Outcomes in the mice treated with plant extracts were comparable to those treated with pentostam ($P \geq 0.05$). In the promastigote assay, all the test compounds killed more than half of the promastigotes at the highest concentration (500 µg/mL). *Warburgia ugandensis, P. thonningii*, and *P. africana* reduced the number of promastigotes from 2.0×10^6 to 7.7×10^3, 72.0×10^3, and 5.0×10^3, respectively. Pentostam had the lowest IC50 (210 µg/mL), followed by *Warburgia ugandensis* (IC50 of 270 µg/mL). *Piliostigma thonningii* and *P. africana* were less toxic with IC50 of 720 µg/mL and 500 µg/mL, respectively. There was low production of IgG antibodies following treatment with the plant extracts and high levels in the untreated control.

**Corresponding Author:*
Maria Divinah Mogaka,
Department of Zoological Sciences of the School of Pure and Applied Sciences, Kenyatta University, Kenya;
Email: divinahmm@gmail.com

DOI: https://doi.org/10.30564/jzr.v4i4.4926

1. Introduction

Leishmaniasis is a zoonotic disease caused by protozoan parasites of the genus *Leishmania* and spread by the bites of infected female sandflies of the genus *Phlebotomus*. More than 90 sand-fly species are proven or probable vectors worldwide [1]. The primary hosts are vertebrates, commonly hyraxes, canids, rodents, and humans. Leishmaniasis occurs in Africa, Asia, Europe, and South and Central America. In Kenya, leishmaniasis is endemic in Baringo, Mwingi, Machakos, West Pokot, and Elgeyo Marakwet [2]. There are 700,000 to 1 million new cases of leishmaniasis reported annually [1]. The disease presents itself in three forms: Cutaneous, Mucocutaneous, and Visceral leishmaniasis. The cutaneous form that is caused by *L. tropica, L. major,* and *L. braziliensis* presents with skin ulcers [3] while mucocutaneous form presents with ulcers of the skin, mouth, and nose. Cutaneous leishmaniasis cases every year are between 600,000 to 1 million [1]. The visceral form starts with skin ulcers and then later presents with fever, low red blood cells, enlarged spleen, and enlarged liver. Visceral leishmaniasis (VL) is fatal if left untreated and there are between 50,000 to 90,000 new cases of VL reported annually [1]. Current treatment approach is primarily based on high doses of pentavalent antimonial compounds, which have been associated with various draw backs and side effects [5]. Treatment is done over a long period of time (28 days), which ends up being costly to the patients. Furthermore, antimonial compounds are toxic in nature, and there is a tendency of the disease to relapse even after successful chemotherapy regime with these compounds [6]. Medicinal plants are economical, safe, with no problems of drug resistance. They are used by most communities worldwide as the best healing agents of parasitic diseases [7]. Recent advances in herbal medicine have listed plants and natural derived products that have shown some level of antileishmanial activity. *Warburgia ugandensis*, commonly known as the East African green heart is highly used in the African tropics and subtropics for treatment of malaria, cough, fever, and constipation, and among the Tugen tribe in Kenya, the bark is traditionally used to treat visceral leishmaniasis [8]. Previous studies have also shown good antileishmanial activities of *W. ugandensis* against *Leishmania major* [6,9]. *Piliostigma thonningii* has diverse chemical compounds, such as flavonoids, tannins, kaurane diterpenes, alkaloids, carbohydrates, saponins, terpenes, and volatile oils, which are responsible for its medicinal activities [10]. *Prunus africana*, the African cherry is used to treat asthma and chest complications [11]. However, there is limited information on the efficacy of *W. ugandensis, P. thonningii*, and *P. africana* against *Leishmania dovonavi* parasites. The objective of the present study was to evaluate the therapeutic potential of *W. ugandensis*, *P. africana*, and *P. thonningii* against *L. donovani.*

2. Materials and Methods

2.1 Experimental Design

The *in vitro* and *in vivo* studies were conducted with a comparative study design. The efficacy of the plant extracts against amastigotes was compared with that of sodium stibogluconate and a negative control. The efficacy against promastigotes and toxicity to Vero cells was evaluated by comparing the test samples to a control containing promastigotes and media only.

2.2 Ethical Permit

The research was approved by the Ethical review committee of Kenyatta University, Kenya.

2.3 Collection and Preparation of Plant Materials

Barks of *P. thonningii, W. ugandensis*, and *P. africana* were collected from various villages in Baringo County with the help of traditional healers and community members. Voucher specimens of the plant parts were taken to the East African Herbarium in Nairobi for identification and future reference. The barks of the plants were air-dried for 10 days in a shed and then ground into a powder using an electric mill. Methanolic extraction was done by soaking 500 g of the powder in absolute methanol for 72 hours before filtering, followed by solvent recovery under vacuum in a rotary evaporator, at 30 °C~ 35 °C. The extracts were packed into airtight storage bottles and stored under –20 °C.

2.4 Preparation of *Leishmania donovani* Parasites for Infection

The *L. donovani* strain NLB-065 obtained from the spleen of an infected patient and maintained by intracardiac hamster-hamster passage at the Institute of primate research was used in this study. A hamster splenic aspirate was cultured in Schneider's Drosophila insect medium supplemented with 20% fetal bovine serum and 100 µg/mL of Gentamicin at 25 °C till stationary phase [12]. Parasites harvested at the stationary phase of culture were centrifuged at 2500 rpm for 15 minutes at 4 °C and washed three times in sterile phosphate buffered saline (PBS) before being counted and used for the study [12].

2.5 Cytotoxicity Assays

The toxicity study was done by culturing Vero cells in complete minimum essential medium (MEM). The Vero cells were then cultured in duplicate wells in a microtiter plate at 37 °C in 5% CO_2 for 24 hours for them to attach to the wells, after which the medium was aspirated off before 100 µL of the test compound was added and serially diluted across the wells [13]. The highest concentration of the test compounds was 1000 µg/mL. Further incubation at 37 °C was done for 48 hours. The controls used were Vero cells and medium, with no extract. Ten microliters of 3-(4,5-Dimethylimidazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added to each well and the cells were incubated for 3 hours until a purple precipitate was visible under a microscope. The medium together with MTT was aspirated, after which 100 µL of DMSO was added and plates shaken for 5 minutes. The absorbance was measured for each well at 562 nm using a microtiter plate reader. Cell viability (%) was calculated at each concentration using the following formula:

$$\mathrm{CV} = \frac{A_1 - B_1}{A_2}\, x\, 100.$$

where, CV is the Cell Viability (%), A_1 is the average absorbance in duplicate drug wells, A_2 is the average absorbance in control wells, and B_2 is the average blank wells.

2.6 Anti-promastigote Assays

Anti-promastigote assay was done as described by Mutiso et al. [12]. Briefly, stationary-phase promastigotes were counted and suspended in a concentration of 2.0×10^6 parasites/mL in culture medium. The tests were performed in 96-well microtiter plates maintained at 26 °C under 5% CO_2. Two hundred microliters of complete Schneider's Drosophila medium were put in the wells containing the maximum concentrations of the compounds and 100 µL in the next wells (2 to 12) and control; 2 µL of compound solutions of 20 mg/mL in distilled water was added to wells number 1 and diluted serially; the concentrations ranged from 500 µg/mL to 3.9 µg/mL. One hundred microliters of culture medium containing 2.0×10^6 stationary-phase *Leishmania donovani* parasites were added to each test well. Tests were performed in triplicates for each test compound concentration. Parasite observation and counting were done using a microscope. The untreated control group contained only media and promastigotes. The results were expressed as infection rates after 72 hours incubation period. The initial concentration for testing was 500 µg/mL.

2.7 IgG Quantification Assay

Total IgG antibodies were quantified using enzyme linked immunosorbent assay (ELISA). Polystyrene Micro-ELISA plates were coated overnight with 100 µL of *Leishmania donovani* soluble antigen at a concentration of 10 µg/mL, diluted in bicarbonate buffer (pH 9.6). After blocking nonspecific binding sites with 3% bovine serum albumin (BSA) in PBS/0.05% Tween 20 buffer (washing buffer) for 1 hour at 37 °C, plate was washed 4 times with washing buffer before the addition of 100 µL of the serum samples and incubation for 2 hours at 37 °C. The plate was washed 4 times as above and horse radish peroxidase-conjugated anti-IgG (Amersham) was added and incubated for 1 hour at 37 °C before addition of Tetramethylebenzidine (TMB) microwell peroxidase substrate and further incubation of the plate in the dark for 20 minutes. Optical densities were read at 630 nm in a micro-plate reader (Dynatech Laboratories).

2.8 Mice and Parasite Inoculation and Estimation of Parasite Burden

Inbred BALB/c mice, 6-8 weeks of age of both sexes were used for this study. The mice were infected intraperitoneally with 2×10^6 virulent *Leishmania donovani* parasites and kept for five weeks for establishment of visceral leishmaniasis. The mice were maintained in the rodent house throughout the experimental period and provided with feed and water daily. Infected mice were put into five groups and treated as follows: Group I, II and III were treated with plant extracts *P. thonningii, W. ugandensis*, and *P. africana*, respectively at a dose of 20 mg/kg body weight. Group IV mice were treated with the reference control drug sodium stibogluconate at a dose of 20 mg/kg body weight. Treatment with the plant extracts and control drugs commenced on the 36th day post infection were done intraperitoneally at a dose of 20 mg/kg body weight once daily for 18 days. Group V was the negative control, which was infected but not treated. On the last treatment day, the mice were sacrificed, and the spleen obtained for preparation of splenic impression smear for parasite burden quantification by microcopy. The amastigotes were counted against 1000 splenocytes and expressed as percentage parasite count.

2.9 Data Analysis

The data collected on in vivo and in vitro parasite burden and absorbance were analyzed using GraphPad prism software. In vitro experiments were conducted in tripli-

cates while in vivo experiments involved 6 mice per treatment group. Differences between groups were analyzed by one-way ANOVA. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1 Cytotoxicity Assay

Drug cytotoxicity was measured by the death of Vero cells after 72 hours of incubation with the test drugs. The standard drug, Pentostam was the most toxic drug with a lower IC50 of 210 µg/mL. The concentration of *Warburgia ugandensis* required to kill 50% of the Vero cells was 270 µg/mL, which was lower compared to the other plant extracts. *Piliostigma thonningii* and *Prunus africana* killed 50% of the cells at a concentration of 720 µg/mL and 500 µg/mL, respectively (Table 1). There was a significant difference in cell viability between the wells containing *P. thonningii* and *Prunus africana* when compared to pentostam (P<0.05). Cell viability in the wells containing *W. ugandensis* not significantly different from pentostam (P>0.05).

Table 1. IC50 of the test drugs against promastigotes

Compound	IC50 (µg/mL)
P.thonningii	720 µg/mL
P.africana	500 µg/mL
W. ugandensis	270 µg/mL
Pentostam	210 µg/mL

3.2 Efficacy of *W. ugandensis, P. thonningii* and *P. africana* on *L. donovani* Promastigotes

There was a decrease in the number of promastigotes with an increase in the drug concentration. The efficacy of the plant extracts against promastigotes is indicated in Figure 1. The parasite count after incubation with 500 µg/mL of *P. africana* was 5.0×10^3, which shows a more than half decrease from the initial concentration (2.0×10^6). *Warburgia ugandensis* was the second most effective plant against promastigotes at a high concentration, since it reduced the number of promastigotes from the initial concentration of 2.0×10^6 to 7.7×10^3. *Piliostigma thonningii* was the least effective compared to all the test compounds because it killed the least number of promastigotes at the highest concentration. It reduced the parasite number from 2.0×10^6 to 72.0×10^3. However, at the highest concentration, all the test compounds killed more than half of the promastigotes. At 500 µg/mL, data analysis indicated a significant difference between the test compounds and the untreated control group, which had only the promastigotes in media (p<0.0001).

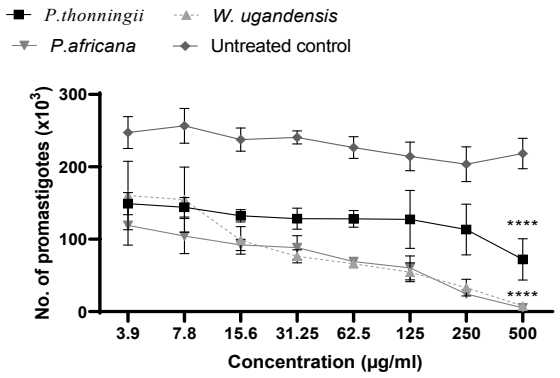

Figure 1. Number of promastigotes in *P. thonningii, W. ugandensis*, and *Prunus africana* treated *L. donovani* cultures.

3.3 Efficacy of *W. ugandensis, P. thonningii* and *P. africana* on *L. donovani* Amastigotes in Balb/c Mice

The group treated with *W. ugandensis* had the lowest number of amastigotes at 13% when counted against 1000 spleen cell nuclei followed by the group treated with sodium stibogluconate, which had a 14.2% parasite count; this data is presented in Figure 2. The percentage parasite counts in the mice treated with *P. thonningii* and *P. africana* were 23.2% and 30.4%, respectively. The outcome of treatments with *P. thonningii*, *W. ugandensis*, *Prunus africana*, and Pentostam were comparable (P≥0.05). All mice treated with both plant extracts and the untreated control drug showed significant differences (p<0.001). (*P ≤ 0.05, **P ≤ 0.01)

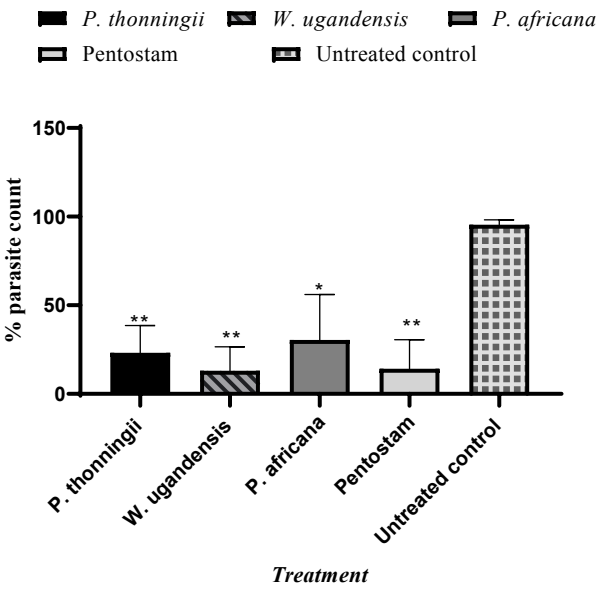

Figure 2. Estimation of infection rates among the different treatment groups.

3.3 Antileishmanial Antibody Responses

Treatment with *W. ugandensis* induced the lowest production of IgG antibodies as indicated by low mean optical density (0.1268) while *Prunus africana* induced the highest level of IgG (OD = 0.1356) among the treatment groups as shown in Figure 3. There was high production of IgG antibodies in the untreated mice as indicated by a higher optical density of 0.2379. However, there was no significant difference in the amount of IgG produced among the treatment groups, including the standard drug, Pentostam (P>0.05). There was a significant difference in the production of IgG in mice treated with *Prunus africana* and *W. ugandensis* when compared with the untreated control (P=0.0002).

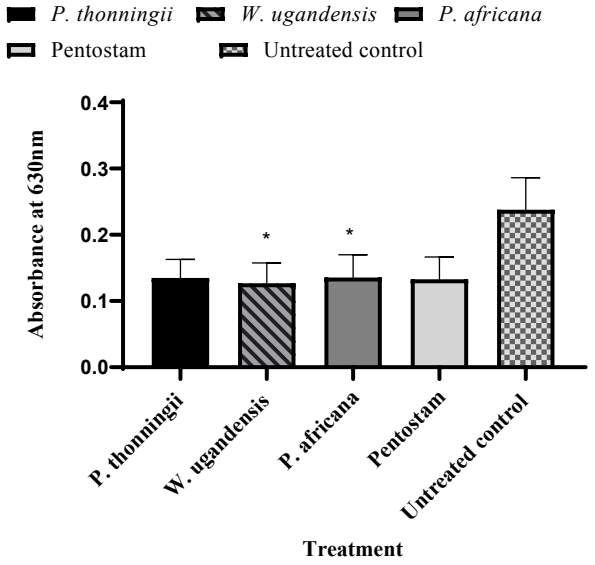

Figure 3. IgG production in *L. donovani* infected mice following treatment with *P. thonningii, W. ugandensis,* and *Prunus africana.*

4. Discussion

Treatment of leishmaniasis is primarily based on antimonial compounds. However, their mode of action is poorly understood and associated with serious side effects, which sometimes force patients to desert treatment [14]. Furthermore, chemotherapy is expensive and therefore, the residents of Baringo County prefer traditional medicinal plants as alternative treatment of leishmaniasis. Medicinal plants are economical, safe, and with no problems of drug resistance. Hence, most communities worldwide use various parts of plants to treat different types of diseases, including those caused by protozoans. The present study investigated the leishmanial activity of three plants against *Leishmania donovani* amastigotes and promastigotes. Based on the results, *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* are promising compounds that can be used as alternative therapies for leishmaniasis. These findings are supported by a study conducted by Githinji and colleagues [7], which indicated that the bark of *Warburgia ugandensis* has significant antileishmanial activity. The authors attribute the biological effects of the plant on the alkaloid metabolites present in the bark such as tannins, mannitol, and waburganal [7]. *Warburgia ugandensis* also contains an alkaloid called muzigadial, which has trypanocidal activities against *Trypanosoma brucei* [7]. Trypanosome and leishmania parasites are kinetoplastids with similar structural and biochemicals features and are therefore likely to have similar responses to the same ethno-botanicals. Phytochemical studies conducted on *Piliostigma thonningii* reveal that it has various chemical compounds including flavonoids, tannins, alkaloids, volatile oils, and saponins, which confers it the vast activities as a medicinal plant [8]. Antileishmanial activity of *Prunus africana* has not been evaluated before, however, it has been shown to have antibacterial, antifungal, and anticancer activities [15].

Prunus africana, *Warburgia ugandensis*, and *Piliostigma thonningii* are safe to use since they have less impact on human Vero cells. Other studies found the extracts of *Piliostigma thonningii* to be safe when administered orally [16]. *Warburgia ugandensis* and *Prunus africana* have been proven to be nontoxic even at a concentration that is higher than 250 µg/mL [9]; the authors recommend the plants as safe remedies for leishmaniasis.

Though the production of IgG antibodies in the treatment groups was little, their presence indicates disease progression. The role of antibodies in leishmaniasis is not clear and there has been contradicting conclusions made by various researchers. High levels of anti-Leishmania IgG are indicative of active disease and correlate to high parasitemia [17]. Other studies show that IgG antibodies do not protect against Leishmaniasis infections but rather contribute to disease progression [18]. Hence, IgG activity cannot be linked to the reduced parasitemia in the mice treated with the plant extracts.

5. Conclusions

The results of the present study show that *Warburgia ugandensis*, *Prunus africana*, and *Piliostigma thonningii* have antileishmanial activities. The results also show that both plants are not toxic to host cells. However, more studies should be done to determine the active components that have antileishmanial activity in *P. africana.*

Acknowledgment

The authors acknowledge Lucy Makau of Kenyatta university for providing technical assistance during data

collection and analysis.

Conflict of Interest

There are no competing interests whatsoever.

References

[1] World health organization (WHO), 2022. Leishmaniasis. Available at: https://www.who.int/news-room/fact-sheets/detail/leishmaniasis.

[2] Ouma, F.F., Mulambalah, C.S., 2021. Persistence and changing distribution of leishmaniases in Kenya require a paradigm shift. Journal of Parasitology Research. 1-6.
DOI: https://doi.org/10.1155/2021/9989581

[3] Ali Al-Hajj, M.M., Al-Shamahy, H.A., Alkhatib, B.Y., et al., 2018. In vitro anti-leishmanial activity against cutaneous leishmania parasites and preliminary phytochemical analysis of four yemeni medicinal plants. Universal Journal of Pharmaceutical Research.
DOI: https://doi.org/10.22270/ujpr.v3i4.183

[4] Mann, S., Frasca, K., Scherrer, S., et al., 2021. A review of leishmaniasis: Current knowledge and future directions. Current Tropical Medicine Reports. 8(2), 121-132.
DOI: https://doi.org/10.1007/s40475-021-00232-7

[5] Moraes Neto, R.N., Setúbal, R.F., Higino, T.M., et al., 2019. Asteraceae plants as sources of compounds against leishmaniasis and Chagas disease. Frontiers in Pharmacology. 10.
DOI: https://doi.org/10.3389/fphar.2019.00477

[6] Selvapandiyan, A., Croft, S.L., Rijal, S., et al., 2019. Innovations for the elimination and control of visceral leishmaniasis. PLOS Neglected Tropical Diseases. 13(9), e0007616.
DOI: https://doi.org/10.1371/journal.pntd.0007616

[7] Badirzadeh, A., Heidari-Kharaji, M., Fallah-Omrani, V., et al., 2020. Antileishmanial activity of *urtica dioica* extract against zoonotic cutaneous leishmaniasis. PLOS Neglected Tropical Diseases. 14(1), e0007843.
DOI: https://doi.org/10.1371/journal.pntd.0007843

[8] Ngure, P., Ng'ang'a, Z., Kimutai, A., et al., 2014. Immunostimulatory responses to crude extracts of *Warburgia ugandensis* (Sprague) subsp *ugandensis* (canellaceae) by Balb/C mice infected with leishmania major. Pan African Medical Journal. 17.
DOI: https://doi.org/10.11604/pamj.supp.2014.17.1.3638

[9] Githinji, E., Irungu, L., Tonui, W., et al., 2010. In vitro effects of *Warburgia ugandensis*, *Psiadia punctulata* and *Chasmanthera dependens* on Leishmania major promastigotes. African Journal of Traditional, Complementary and Alternative Medicines. 7(3).
DOI: https://doi.org/10.4314/ajtcam.v7i3.54791

[10] Afolayan, M., Srivedavyasasri, R., Asekun, O.T., et al., 2018. Phytochemical study of *Piliostigma thonningii*, a medicinal plant grown in Nigeria. Medicinal Chemistry Research. 27(10), 2325-2330.
DOI: https://doi.org/10.1007/s00044-018-2238-1

[11] Karani, L., Tolo, F., Karanja, S., et al., 2013. Safety of *Prunus africana* and *Warburgia ugandensis* in asthma treatment. South African Journal of Botany. 88, 183-190.
DOI: https://doi.org/10.1016/j.sajb.2013.07.007

[12] Mutiso, J.M., Macharia, J.C., Barasa, M., et al., 2011. In vitro and in vivo antileishmanial efficacy of a combination therapy of diminazene and artesunate against leishmania donovani in BALB/C mice. Revista do Instituto de Medicina Tropical de São Paulo. 53(3), 129-132.
DOI: https://doi.org/10.1590/s0036-46652011000300003

[13] Jumba, B.N., Anjili, C.O., Makwali, J., et al., 2015. Evaluation of leishmanicidal activity and cytotoxicity of ricinus communis and Azadirachta indica extracts from western Kenya: In vitro and in vivo assays. BMC Research Notes. 8(1).
DOI: https://doi.org/10.1186/s13104-015-1605-y

[14] Marango, S.N., Khayeka-Wandabwa, C., Makwali, J.A., et al., 2017. Experimental therapeutic assays of Tephrosia vogelii against leishmania major infection in murine model: In vitro and in vivo. BMC Research Notes. 10(1).
DOI: https://doi.org/10.1186/s13104-017-3022-x

[15] Komakech, R., Kang, Y., 2019. Ethnopharmacological potential of African cherry (*Prunus africana*). Journal of Herbal Medicine. 17-18, 100283.
DOI: https://doi.org/10.1016/j.hermed.2019.100283

[16] Olela, B., Mbaria, J., Wachira, T., et al., 2020. Acute oral toxicity and anti-inflammatory and analgesic effects of aqueous and Methanolic stem bark extracts of *Piliostigma thonningii* (Schumach.). Evidence-Based Complementary and Alternative Medicine. 1-10.
DOI: https://doi.org/10.1155/2020/5651390

[17] Magalhães, A., Carvalho, L.P., Costa, R., et al., 2021. Anti-leishmania IgG is a marker of disseminated leishmaniasis caused by *Leishmania braziliensis*. International Journal of Infectious Diseases. 106, 83-90.
DOI: https://doi.org/10.1016/j.ijid.2021.02.016

[18] De Lima, C.M., Magalhães, A.S., Costa, R., et al., 2021. High anti-leishmania IgG antibody levels are associated with severity of mucosal leishmaniasis. Frontiers in Cellular and Infection Microbiology. 11.
DOI: https://doi.org/10.3389/fcimb.2021.652956