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Investigate the effect of *cocos* water on the viability and proliferation of *Leishmania donovani* promastigote

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Effect of *Cocos* water on the viability of the visceral leishmaniasis parasite was investigated by MTT assay after processed with different concentrations of *cocos* water, and compared to the effect of pentostam treatment at different times of 24 and 48 hours, the percentage viability of promastigotes will decrease with increasing concentrations, also show that the *cocos* water effect on the promastigtes growth ,initially the parasite was grown at a rate of 1×10^4 cells / mL in two groups and the parasites grow at low rate of division compared with NNN-media where it was in the peak of growth then the numbers of parasites began to gradually decrease with the time, until it became in 20 days with no notable growth has occurred, then juice color like red and purple stains and natural stains like red onion , *Crocus sativus*, red cherry as staning the parasites, showed that the red stain was the better one , while the *Crocus sativus* stain was the better in natural stains the results referred to the possibility of using for staining parasite and can be using it in research and in laboratories because it its more stable and cheaper.
Keywords: *Cocos* water, leishmania donovani, parasite, promastigote, proliferation, staining.

INTRODUCTION

Leishmaniasis is a vector-borne disease caused by protozoans of the Leishmania genus, transmitted through sand fly bites. Various clinical forms of the disease exist, including Visceral Leishmaniasis (VL), Cutaneous Leishmaniasis (CL), and Mucocutaneous Leishmaniasis (MCL) (Sastry and Bhat, 2018). Cocos nucifera (coconut) is a widely used fruit, known for its multifaceted properties, including antiparasitic and leishmanicidal activities, antimicrobial, and anti-inflammatory effects (Babita *et al.*, 2017). C. nucifera water, rich in phenolic compounds, has shown inhibitory effects against protozoan parasites (Friedman *et al.*, 2018).
In present study, Effect of *Cocos* water on the viability of the visceral leishmaniasis parasite was investigated by MTT assay after processed with different concentrations of *cocos* water.

MATERIALS AND METHODS

Cocos nucifera L obtained from the local markets and the fruits were washed well several times using tap water; then this was continued with distilled water to remove impurities and dust, and finally it was sterilized using absolute ethyl alcohol 100 ml, and drawing the water from the inside of the fruit, by making a hole in the upper functional pores with a sterile tool, then with drawing it by using syringe and placing the water in a sterile cup.

***HPLC analysis coconut water*:** HPLC analysis was performed by using Shimadzu 10AV-LC device equipped with Autosampler and coupled to a variable UV wave length detector used to detect the phenolic compounds like coumaric acid and salicylic acid in the coconut water *were* analyzed and the work was done in the Ministry of Science and Technology.

***Measured the effect of coconut water on the L.donovani promastigtes viability in vitro by using MTT assay*:** In this experiment, there were six groups, Each group contains six tubes, with one ml of SHE- medium where the steps were prepared according to the source (Ali *et al.*, 2020) and inoculated with *L.donovani* promastigotes (1×10^3 cells/mL),Then added to each group's tube from this material listed below:
Group 1: Added 1 mL (0.041 mg/mL) of pentostam.
Group (2,3,4, and 5): added 1mL with cocos water in different concentration (100%, 75%, 50%, 25%) respectively.
Group 6: left without addition and considered as a control group.
All groups were incubated at 26C° for 24 and 48 hours after each incubation period, put 100µL from each tube in the

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microplates well. MTT solution 50 µL added to the plates according to manufacturer's, and incubated the plate at 37°C for 3 hours. After incubation, 150µL of MTT solvent in each well was added to dissolve the MTT formazan which was produced and incubated for 30 min at room temperature Photometrically, the quantity of formazan generated by live cells per well was determined at 590 nm. From the OD values, the percentage of viability was determined using the following formula (Soflaei *et al*., 2014).

$$\text{Viable cells} = \frac{absorbance of treated cells}{absorbance of control cells} \times 100$$

***Statistical analysis*:** Statistical Package for Social Sciences (SPSS) version 21 is used to interpret the data. The information is given in the form of a mean, standard deviation. ANOVA was used to compare between tested mean data expressed as mean±SD. P- values of $p>0.05$ were considered statically non significant while $p\leq0.05$ considered significant results.

***The effect of cocos water on the L.donovani promastigtes growth*:** The effect of *coco* water on the growth of *Leishmania* parasite was studied after it was added to Lock's solution in NNNmedia then cultured at 1×10^4 cells / ml promastigote, they calculated the numbers of parasite growth in NNNmedia for a period of 20 days.

***New stains*:**

- Industrial stain
- juice colors

Two Industrial powder juice coloring was used red and purple, where made in Iraq obtained from the local market,

1. Added 1.5 gm of the powder to 2 mL of distilled water,
2. Dissolved well
3. Filtered by using Whatman No. 1 filter paper.

***Natural stains*:**

A. ***Preparation red onion stains*:** The red onion waste was obtained from the local market.

1. They were thoroughly washed several times using tab water; to remove the impurities.
2. Then soak 100 gm of red onion waste in 30 ml of distilled water overnight,
3. Then boil left to cool after that filtered with Whatman No. 1 filter paper to form as the light red solution and put in the refrigerator until use.

B. ***Preparation Crocus sativus stain*:**

Stigma of Crocus sativus flowers obtained from local markets.

1. 50 gm was soaked in 20 mL of distilled water overnight
2. And then filtered by Whatman No. 1 filter paper to form as golden-yellow solution
3. Put in the refrigerator until use.

C. ***Preparation red cherry stains*:**

The red cherry was obtained from the local market.

1. The were thoroughly washed several times using tab water; to remove the impurities,
2. Then squeezed to get the juice
3. Filtered with Whatman No. 1 filter paper to form as red solution
4. Put in the refrigerator until use.

RESULTS AND DISCUSSION

***HPLC analysis*:** The chemical compounds present in the crude cold alcoholic extract of coconut water were identified by HPLC analysis. A total of 11 different phenolic compounds was identified and the chromatogram of the components with their peaks is shown in figure (4). The active principle component with higher peak area percentage were comaric acid 39.648% and the lower peak area percentage were salicylic acid 2.465%.

Table 1. The HPLC analysis of C. nucifera water for phenolic compounds.

peak	Compounds	R.T.min	Area	Height	Area %	Height%
1	Gallic acid	2.874	488425	76111	5.839	8.500
2	Caffic acid	3.060	978543	38081	11.699	4.336
3	Comaric acid	4.144	3316325	390010	39.648	43.383
4	Vallic acid	4.787	302620	23405	3.618	2.60
5	Benzoic acid	5.146	472611	65719	5.650	7.310
6	Ellagic acid	5.560	598633	83951	7.157	9.338
7	Syringic acid	5.771	237692	31376	2.842	3.490
8	Ferulic acid	6.708	719320	78645	8.600	8.581
9	Chloroge Nicole acid	7.296	716241	65423	8.570	7.094
10	Salicylic acid	8.614	206951	29057	2.465	2.233
11	Sinapic acid	9.139	29367	20976	3.497	2.894

Mahayothee *et al.* (2015) reported that Higher total phenolic content and antioxidant activity indices produced by mature coconuts, The two main phenolic compounds in the water were catechin and salicylic acid by a significant amount with maturity stage, in contrast to the meat, which contained gallic, caffeic, salicylic, and p-coumaric acid.

***The effect of cocus water on the L. donovani promastigtes viability in vitro by using MTT assay*:** The effects of four concentrations 100%, 75%, 50%,25% of coconut water on the viability of promastigotes in vitro were evaluated at after 24, 48 hour of exposure and compared it with pentostam and control groups. The results showed that, with increasing the coconut water concentrations, the percentage viability of promastigotes will decrease directly with times. The percentage of viability of promastigote after 24hr were 42%, 37%, 34% and 33%, in the groups treated with 100%, 75%, 50%, and 25% coconut water respectively, while in pentostam group was 52% and the control group was 100%

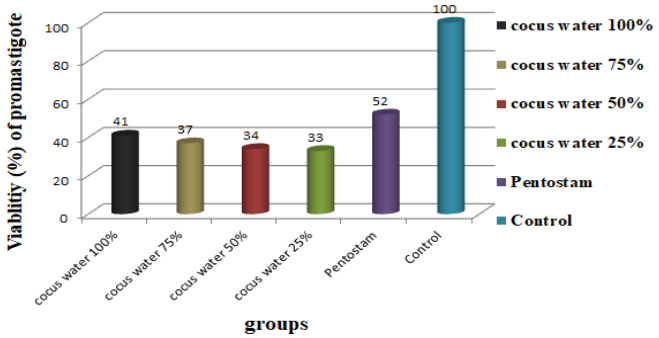

Figure 2. The viability percentage of Leishmania donovani promastigotes after exposure to different concentrations of cocos water and a pentostam drug by MTT assay after 24 hours.

After 48 hours, the percentage of viability decreased to 22%, 20%, 19%, and 18% in the groups treated with 100%, 75%, 50%, and 25% coconut water, respectively, compared to 15% in the pentostam group and 100% in the control group.

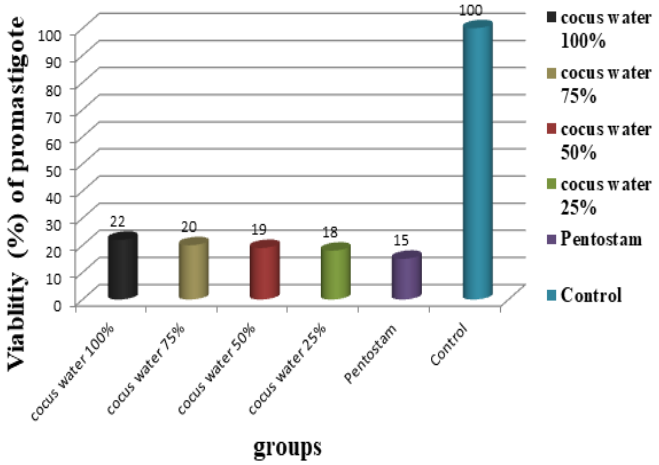

Figure 3. The viability percentage of Leishmania donovani promastigotes after exposure to different concentrations of cocos water and a pentostam drug by MTT assay after 48 hours.

***The effect of cocos water on the L.donovani promastigtes growth*:** The effect of *cocos* water on the *L.donovani* promastigtes growth when it added to lock the solution of NNN-media and compared with with lock solution only in NNN-media, initially the parasite was grown at a rate of 1 × 10^4 cells / mL in two groups. The results showed that the parasites grow at the rate of division was weak was 12×10^4 cells / mL compared with NNN-media was 23×10^4 cells / mL in the first two days ,until on the 8th day in the peak of growth , the numbers of parasite in media with cocos water was 29.1×10^4cells / mL compared with the NNNmedia was 68.2×10^4cells / mL, then the numbers of parasites began to gradually decrease with the time, until it became in 20th days the numbers of parasite in media with *cocos* water was zero cells / mL, while in the NNNmedia 4×10^4cells / mL, The results show an increase in the number of parasites on most days in NNNmedia. In comparison to *cocos* water media, where no notable growth has occurred.

***Detection of L. donovani by Different Stains*:** In this study new stains were used for the first time instead of standard giemsa stain include industrial stain like synthetic juice coloring and natural stain like Crocus sativus, red cherry and red onion would be useful for staining *leishmania*, because it's cheaper price and easy availability for detection of dead *L. donovani* promastigotes as follows;

***Giemsa stain*:** one of the most popular microscopic stains, commonly used in staning of *L. donovani* promastigotes and used as slandered stain to compare with new stains where the intensity of staining was 1.20 pg/ml with wavelength450nm as in Figure 5.

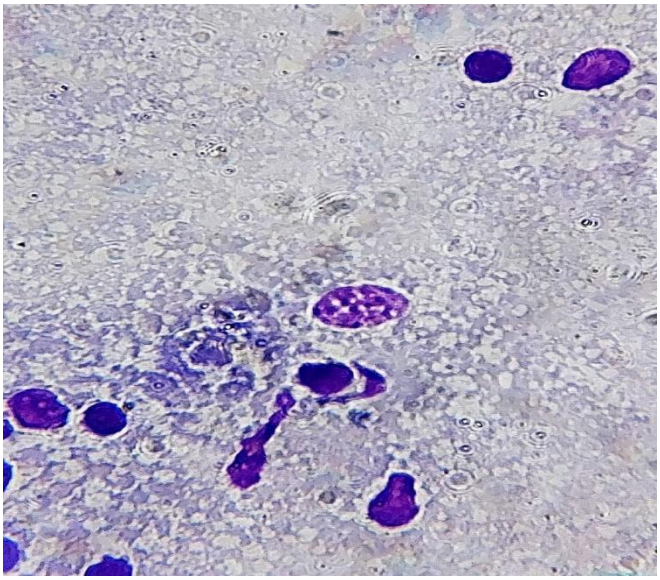

Figure 5. *L. donovani* promastigotes stained with giemsa stain and took on a blue color (100 X).

***Natural stain*:** Two plants extract Red onion and *Crocus sativus* had been used for the first time to stain L. *donovani* promastigotes as follows:

***Red onion stain*:** The aqueous extract for Red onion stain appeared the *L. donovani* promastigotes with yellow color where the intensity of staining was 1.32 pg/ml with wavelength450nm as in Figure 5.

***Crocus sativus stain*:** An aqueous extract of stigma *Crocus sativus* appeared the promastigotes with yellow color as where the intensity of staining was 1.56 pg/ml with wavelength450nm in Figure 6.

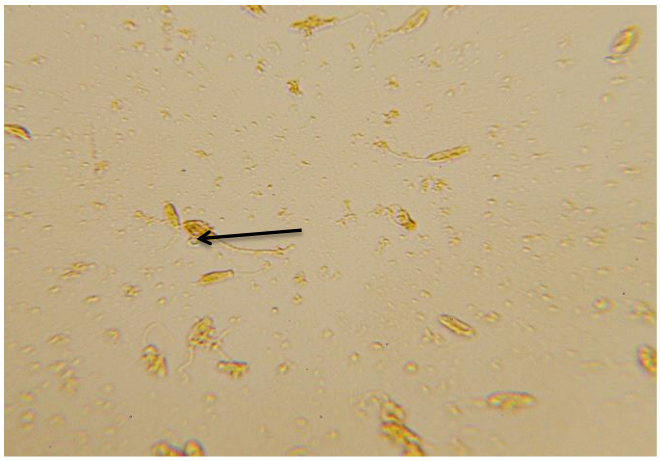

Figure 6. *L. donovani* promastigotes stained with red onion stain and took on a yellow color (100 X).

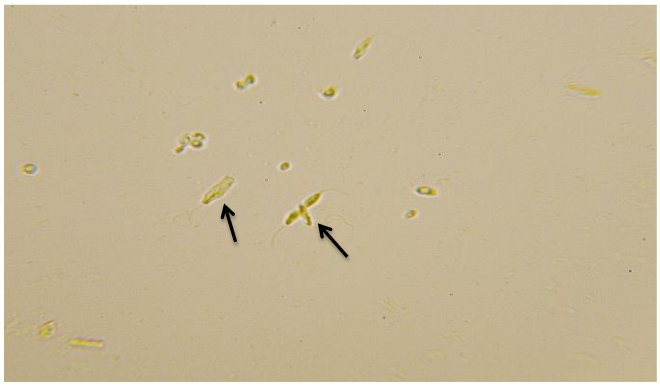

Figure 7. Microscopic appearance of *L. donovani* stained with *Crocus sativus* appeared as transparent gold yellow (100 X).

***Red cherry stain*:** The aqueous extract for Red cherry stain appeared the *L. donovani* promastigotes with light green color as where the intensity of staining was 1.31 pg/ml with wavelength 450nm in Figure 7.

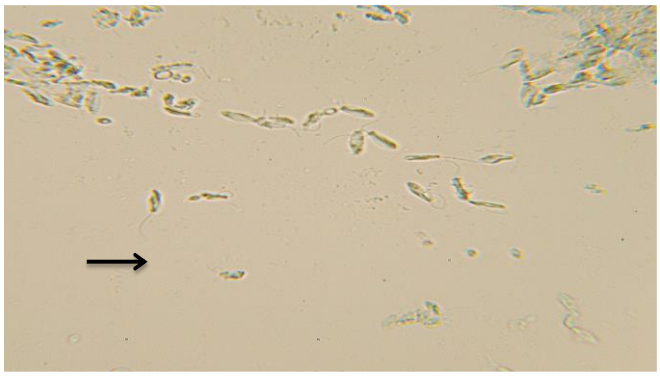

Figure 8. Microscopic appearance of *L. donovani* stained with red cherry appeared as light green (100 X).

***Industrial stain*:** Two Industrial juice color was used in the current study to stain the promastigotes of the *L. donovani* as the following:

***juice colors*:** Two Industrial juice color is used in this study red and purple stain, the promastigotes of *L. donovani* acquired pink color when stained with purple at 1.33 pg/ml intensity (a) and the red one when stained with dark red color at 1.54 pg/ml intensity (b) with wavelength450nm as in the Figure 8.

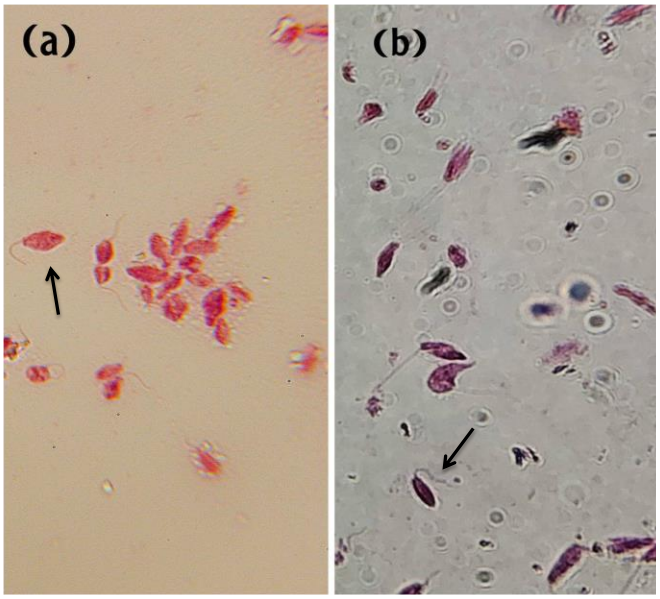

Figure 9. Microscopic appearance of *L. donovani* stained with industrial red and purple stain appeared and (a) the red with dark red color (b). the purple promastigotes with pink color (100X).

DISCUSSION

***The effect of cocos water on the L.donovani promastigtes viability in vitro by using MTT assay*:** Friedman *et al*. (2018) reported that *C.nucifera* water, which richly contains phenolic compounds where it has been amongst other natural compounds to have inhibitory effects against protozoan parasites, Where the phenolic compounds that elicited significant anti-leishmanial activity such as rosmarinic acid and Apigenin which affected parasites' growth for their ability to better scavenge iron from the parasite and hence the more pronounced growth inhibitory effect, where it have many function as antioxidants by chelation of metal ions and removal of free radical (Antwi *et al*., 2019; Ogungbe *et al*., 2014). Also, other studies found that kefir grains can affect entameba histolytica and *leishmania donovani* in the mice and in vitro (Mohammed *et al*., 2016; Ali *et al*., 2020)

***The effect of cocos water on the L.donovani promastigtes growth*:** These results support earlier research that

demonstrated that coconut water can affect parasite viability due to the contains large quantities of phenolic compound were elicited a significant anti-leishmanial activity (Antwi *et al.,* 2019) and the compounds found in the leaves of *C. nucifera* are (flavones, isoorientin, apigenin, vitexin, isovitexin, and luteolin) are antimalarial activity against the malaria caused by parasite *P. falciparum* and inhibited parasitic growth (Nicole *et al*., 2019).

***HPLC analysis cocos water*:** Mahayothee *et al.* (2015) reported that total phenolic content and antioxidant activity indices increased as the coconut matured, Catechin and salicylic acid were the major phenolic compounds found in the water, while gallic, caffeic, salicylic, and p-coumaric acids were found in the meat. significantly with maturity stage. Additionally, Seto *et al*. (1997) exhibited HPLC chromatograms of a liquid extract of C. nucifera, and the findings indicated the presence of many phenolic compounds, including epicatechin and its derivatives, catechin, caffeoylquinic acid and chlorogenic acid. Falih *et al*. (2022) found that the *Capparies* silver nanoparticles (AgNPs) synthesis from the leaves of the *Capparis spinosa* plant effects on leishmania donovani in the liver of mice infected with leishmania parasites. Ghadi *et al*. (2018) found that the *fusarium* silver nanoparticles have a significant effect in reducing the multiplication of the *leishmania donovani* promastigote within infected macrophage cells compared with pentostam and control group. Abd *et al*. (2018) Also CdO NPs had antiparasitic effect on viability rate of *Leishmania donovani in vivo* and *in vitro*.

***Detection of L. donovani By Different Stains*:** An aqueous onion tunic (Allium cepa) extract was used by Kumar and Mandal (2022) to create natural stains ,Where Platyhelminthes parasites showed good staining qualities, from aqueous onion tunic extract which in the near future, will be environmentally acceptable, inexpensive, and non-hazardous herbal extract stains and might be an excellent alternative to currently available, risky, and conventional stain . Another study was done by Marhaba and Haniloo (2018) used an aqueous extract of *Allium cepa* skin, Juglans regia husk and *Rubia tinctorum* roots for staining some trematode worm proglottids such as *Fasciola spp*. and D. dendriticum, cestodes Moniezia spp. proglottids, the results showed the aqueous extracts of *Allium cepa* skin appeared dark red, aqueous extracts of *Juglans regia* husk appeared brown, and *Rubia tinctorum* root extract appeared brown to yellowish brown. Nurlaelasari *et al*. (2023) show on his study to utilize *Biancaea Spain* and *Crocus sativus* as a natural dye for *Fasciola gigantica*, were the extracts utilized to stain adult *F. gigantica*, The results showed that structures and internal organs were pigmented successfully such as oral sucker, ventral sucker, uterine, eggs inside the uterine. These methods are eco-friendly and safe since the herbs do not have carcinogenic effects. Mohammed *et al*. (2016) found that the use of various synthetic food colors such as green, red, blue, and yellow to stain the *leishmania* promastigote, showed that green, red, and blue stains were the best at staining parasites, while yellow stain was the least effective, where in another study Saeed *et al.* (2021) used industrial food colors stain were used such as red, green, blue and natural stain were used like *Rhus coriaria*, *Carthmuaus tinctorius*, *Crocus sativus* for staining *Echinococcus granulosus* the results showed that, the aqueous extract of industrial stain (food color) showed better quality than natural stains can be use as viability detection stain, the red stain was the better one and can be used as differential stain, while the *Crocus sativus* stain was the better natural stains and would be useful for staining *leishmania*, because it's cheaper price and easy availability.

***Conclusion*:** Based on the findings, it can be concluded that;

1. when used an aqueous extract of industrial stains and natural stain as detection stain, the red stain was the better one, while the *Crocus sativus* stain was the better in natural stains and both are environmentally acceptable, inexpensive, and non-hazardous herbal extract stains and might be an excellent alternative to currently available, risky, and conventional stain.
2. HPLC analysis show that coconut water is rich with mineral and phenolic compound that effect on the viability of *Leishmania* parasite and can be considered as a new antileishmanial agent.
3. coconut water effect on growth of *L.donovani* promastigote, which have leishmanicidal activitiy, and affects parasite proliferation *invitro* and which stimulate immunity to increase IFN- γ and IL-10 production is indicative of their impact on immune response *in vivo*.

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