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Study on the Therapeutic Effect of Coconut Oil Extracts as An Alternative Medicinal Plant in *Cryptosporidium* Infected Mice

Cryptosporidium ile Enfekte Farelerde Alternatif Bir Tıbbi Bitki Olarak Hindistan Cevizi Yağı Ekstraktlarının Terapötik Etkisi Üzerine Çalışma

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ABSTRACT

Objective: Cryptosporidiosis caused by *Cryptosporidium* sp. is a globally spreading disease. Nowadays, new researches are moving towards an effective treatment without side effects, especially for young and immune-compromised patients. The current study was designed to evaluate the therapeutic effect of the coconut oil extracts as an alternative medicinal plant in *Cryptosporidium* infected immunocompromised mice.

Methods: Sixty white albino mice were classified into six groups; Group I: Infected with *Cryptosporidium* oocysts treated with Nitazoxanide, Group II: Infected with *Cryptosporidium* oocysts and treated with coconut water extract, Group III: Infected with *Cryptosporidium* oocysts and treated with coconut Hexan extract, Group IV: Infected with *Cryptosporidium* oocysts and treated with coconut ethanol extract, Group V: Positive control, Group VI: Negative control. Stool samples were collected and examined; histopathological and immune-histochemical assessment using anti caspase-3 and anti CDX2 monoclonal antibodies were performed.

Results: Coconut oil extracts results revealed a significant decrease of oocyst count, correlated with an amelioration of histopathological and confirmed by immunohistochemical changes in ileal tissue.

Conclusion: The present study has opened fresh avenues for development of natural therapy like coconut oil extracts, which have a potential therapeutic efficacy against Cryptosporidiosis. That was confirmed by different methodologies, parasitological examination, histopathological examination, and immunohistochemical assays. It paves the way for being a promising anti-parasitic agent for infection eradication. However, further studies are still required to gain more knowledge about different coconut extracts in order to reach the best treatment efficacy.

Keywords: *Cryptosporidium* sp., coconut oil, caspase-3, CDX2

ÖZ

Amaç: *Cryptosporidium parvum* (*C. parvum*), Cryptosporidiosis hastalığına neden olan ve tüm dünyada yaygın olarak bulunan protozoan bir parazittir. Günümüzde yeni araştırmalar özellikle genç ve bağışıklık sistemi baskılanmış hastalarda yan etkisi olmayan etkili tedaviye doğru ilerlemektedir. Bu nedenle, mevcut çalışma, kriptosporidium ile enfekte olmuş bağışıklığı baskılanmış farelerde alternatif bir tıbbi bitki olarak hindistan cevizi yağı ekstraktlarının terapötik etkisini değerlendirmek için tasarlanmıştır.

Yöntemler: Altmış beyaz albino fare, altı gruba ayrıldı; Grup I: Nitazoksanid ile tedavi edilen *C. parvum* ookistleri ile enfekte. Grup II: *C. parvum* ookistleri ile enfekte ve hindistan cevizi suyu özü ile tedavi edildi. Grup III: *C. parvum* ookistleri ile enfekte edilmiş ve hindistan cevizi heksan özü ile tedavi edilmiştir. Grup IV: *C. parvum* ookistleri ile enfekte edilmiş ve hindistan cevizi etanol özü ile tedavi edilmiştir. Grup V: Pozitif kontrol. Grup VI: Negatif kontrol. Dışkı örnekleri toplandı ve incelendi, anti-kaspaz-3 ve anti-CDX2 monoklonal antikorları kullanılarak histopatolojik ve immünohistokimyasal değerlendirme yapıldı.

Bulgular: Hindistan cevizi yağı ekstraktlarının sonuçları, histopatolojik iyileşme ile ilişkili ve ileal dokudaki immünohistokimyasal değişikliklerle doğrulanan ookist sayısında önemli bir azalma olduğunu ortaya koydu.

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Türkiye PARAZİTOLOJİ Dergisi

Sonuç: Bu çalışma, farklı metodolojiler parazitolojik inceleme, histopatolojik inceleme ve immünohistokimyasal testler ile onaylanan Cryptosporidiosis'e karşı potansiyel bir terapötik etkinliğe sahip hindistan cevizi yağı özleri gibi doğal terapinin geliştirilmesi için yeni yollar açmıştır ve bu da umut verici bir anti-parazitik ajan olmanın yolunu açmaktadır (enfeksiyon eradikasyonu). Bununla birlikte, en iyi tedavi etkinliğine ulaşmak için farklı hindistan cevizi özleri hakkında daha fazla bilgi vermek için daha fazla çalışmaya ihtiyaç vardır.
Anahtar Kelimeler: *Cryptosporidium* sp., hindistan cevizi yağı, kaspaz-3, CDX2

INTRODUCTION

Cryptosporidium sp. are a group of zoonotic protozoan parasites that infect a wide variety of vertebrate hosts. Mostly it causes asymptomatic infection or diarrhea in patients with strong immune system, but it can cause severe and chronic diarrhea, pancreatic, biliary and respiratory tract infections and even death in immunocompromized (1).

Nitazoxanide is shown to treat diarrhea, which is caused by *Cryptosporidium*, but it has a limited efficacy in immunocompromised or malnourished patients and not licensed for infants younger than 1 year of age (2). Hence, development of new effective drugs for cryptosporidiosis presents a pressing need.

Coconut oil is a natural oil that consists of 92% saturated acids, with a main and most important structure, with many beneficial antimicrobial, antioxidant, and anti-inflammatory properties known as Lauric acid (3,4). Many researches regarding its antimicrobial activity first shown by John Kabara in the 1970s. He found that it had broad-spectrum antimicrobial activities including antibacterial, antiviral and antifungal (5).

In the field of parasitology, Lauric acid proved to be safe, easy to use with excellent tolerability against giardiasis when combined with metronidazole. It has also proven to be a prophylactic measure, especially in cases of traveler's diarrhea. Besides, Lauric acid may have an immune-stimulant effect and so offers a good solution for immune-compromised patients (6).

Long time ago, it was found that *Cryptosporidium* was causing death of epithelial cells in the intestine in the form of villous structure changes with apoptotic epithelial cells found in the intestine. It was stated an important relationship between epithelial apoptosis and cryptosporidiosis pathogenesis (7).

During apoptosis, Caspase-3 is one of the cysteine proteases that are responsible for the morphological changes within the cells. It is considered as a meeting point of the two main apoptotic pathways and cleaves most of the cellular substrates in the apoptotic process (8). Therefore, measurement of Caspase-3 activity is considered as an important reliable determinant of apoptosis (9). Many researches stated that it is an independent prognostic factor for tumors of the digestive system. It is considered an perfect indicator for measuring apoptosis in cancers especially colorectal carcinomas (10).

CDX2 is a homeobox domain-containing transcription factor. It has a valuable role in regulation of the proliferation and differentiation of intestinal cells. CDX2 expression is indicative of intestinal differentiation, as it is expressed inside the nuclei of intestinal epithelial cells starting from the duodenum reaching the rectum. So, It has an important benefit in determining the origin of metastatic tumors (11,12).

By immunohistochemistry, CDX2 is expressed uniformly in most of the colorectal and duodenal adenocarcinoma. unlike carcinomas of the genitourinary, breast, lung, and head and neck (13,14).

The aim of this study was exploring the effect of coconut oil extracts against cryptosporidiosis in infected mice and evaluating it's protective and curative properties using parasitological, histopathological, and IHC anti caspase-3 and anti CDX2 monoclonal antibodies.

METHODS

Oil Preparation

Mature coconuts (Cocos nucifera L.) were purchased from a market at Giza governorate in Egypt. Coconuts were manually dehusked and cracked to collect coconut water, which was carefully filtered using a filter membrane and concentrated using a rotary evaporator (Rotatory evaporator, Buchi, Switzerland) under pressure to obtain a concentrated water extract. Whereas, the coconut pulp was crushed using electric mill to small pieces. 200 g of crushed coconut pulp were soaked in 800 mL of n-hexane and left for one 2 days at room temperature with shaking from time to time followed by filtration using filter paper (Whatmann No.1). Then the filtrate was concentrated under reduced pressure using rotary evaporator at 40 ºC, this process was repeated 3 times to give 10 g dry n-hexane extract. The residue after extraction with n-hexane was soaked in 800 mL 70% EtOH for 2 days at room temperature, then filtered using filter paper. The filtrate was concentrated under reduced pressure using rotary evaporator at 40 ºC (repeated 3 times) to yield 15 g dry 70% EtOH extract. After complete dryness, all extracts were kept in brown dry plastic vials for biological experiments.

Oocyst Preparation

Fecal samples of mice that have *Cryptosporidium* positive infection was examined and oocysts were confirmed by staining with MZN Stain. Samples were irrigated by 10 mL saline and then strained; followed by centrifugation of the suspension was centrifuged at 3000 g/10 minutes. This washing step was recurred two times with removing the supernatant. The sediment re-suspended with Sheather's solution and let aside for 10 minutes. Again the supernatant was raised and washed with saline twice. Finally, the oocysts were counted in the deposit using hemocytometer to calculate the required concentration for infection. The infection dose was 10^3 *Cryptosporidium* oocysts, dissolved in 200 µL of PBS.

Animal Groups

Sixty healthy laboratory-bred male, white Albino mice of CDI strain, about 6-8 weeks old, 25-30 gram, white Albino mice of CDI strain (obtained from Theodor Bilharz Animal house). Mice were

maintained in standard environmental conditions at temperature (24 °C), relative humidity (50%) with a 12:12 light: Dark cycle, and fed a standard commercial diet and water, away from direct sunlight ensuring good sanitary condition.

Immunosuppression: Using nasogastric feeding tube, mice were given administered with 0.25 ug/g/day of dexamethasone sodium phosphate dexazone) daily for two weeks. This step was done before oral inoculation with *Cryptosporidium* oocysts.

Mice were divided into six groups containing ten mice each:

Group I (GI): Mice infected with *Cryptosporidium* oocysts with a dose of 103 oocysts/0.2 mL/mouse orally and treated with Nitazoxanide 65 mg daily orally by using nasogastric feeding tube every day for 7 days post infection. The doses were calculated by extrapolation of human therapeutic doses to animal doses.

Group II (GII): Infected with *Cryptosporidium* oocysts with a dose of 10^3 oocysts/0.2 mL/mouse orally and treated by water extract of coconut a dose of 0.2 Mg/g.

Group III (GIII): Infected with *Cryptosporidium* oocysts with a dose of 10^3 oocysts/0.2 mL/mouse orally treated by hexan extract of coconut oil with a dose of 0.2 Mg/g.

Group IV (GIV): Infected with *Cryptosporidium* oocysts with a dose of 10^3 oocysts/0.2 mL/mouse orally and treated by ethanol extract of coconut oil with a dose of 0.2 Mg/g.

Group V (GV): Infected with *Cryptosporidium* oocysts with a dose of 10^3 oocysts/0.2 mL/mouse orally (positive control).

Group VI (GVI): Non-infected (negative control).

Evaluation of the Experimental Drug Treatment

1- Parasitological examination: At the 7th day after starting treatment administration, samples were collected from each group and subjected to parasitological examination after staining by modified Ziehl-Neelsen stain. Compound microscope was used to confirm mice infection and to calculate the numbers of *Cryptosporidium* oocysts in each sample.

Oocyst count in stool was completed in all groups except GVI, followed by sacrifice of the tested mice by cervical dislocation and for GVI it was done at the same time as previous groups.

2- Histopathological examination: Seven days post infection, sacrificing of all mice was done. The ileocecal region was obtained and fixed in 10% buffered formalin solution then embedded in paraffin wax blocks. Sections of 4 µm thickness were rehydrated and stained with (H&E). All slides were examined by a pathologist which was blinded to the experimental design to assess the pathological changes.

3- Immunohistochemical study: De-paraffinization and rehydration of ileocecal paraffin sections were done. Obtaining of antigen was made by microwaving the sections in citrate buffer, pH 6.0. 3% hydrogen peroxide methanol were added to block endogenous peroxidase and incubated overnight at 4 °C in humid chamber with the primary antibodies: Anti-caspase-3 antibody (31A1067): (sc-56053), or for two hours with anti CDX2 (catalog: M3636; monoclonal; host: Mouse) antibody in a dilution of 1:100-1:400, followed by adding secondary antibody (Biotin-streptavidin link) (DAKO). After that, 3,3'diaminobenzidine tetrahydrochloride (DAB) substrate chromogen solution (Universal Detection Kit, Dako Envision, Denmark) was added to localize antigen. Followed by counterstaining with hematoxylin, dehydration in alcohol and mounting was done.

Regarding the negative control group, all steps were done in the above-mentioned sequences but non-immune immunoglobulin G were added (IgG; DAKO, Glostrup, Copenhagen, Denmark).

Instead of the primary antibodies.

4- Interpretation of immunostaining and scoring analysis: Immunohistochemical analysis were blind-quantified by two pathologists. The sections were examined by using light microscope (Scope A1, Axio, Zeiss, Germany). Photomicrographs were taken using a microscope-camera (AxioCam, MRc5, Zeiss, Germany).

Positive CDX2 was mentioned when nuclei of epithelial cells took brown color, while positivity for Caspase-3 was mentioned when the cytoplasm of epithelial cells took brown color. Calculation of the percentage of positive cells in 10 HPF, follwed by grading the intensity of the color from 1 to 3.

Ethical consideration: According to the NIH guidelines for animal experimentation, animals were reared and sacrificed. According to the valid International Guidelines for animal experimentation, animal protocols were conducted and approved by the Ethical Committee at Theodor Bilharz Research Institute (TBRI).

Statistical Analysis

Using Statistical Package for Social Sciences, Windows version 22., Student's (t)-test and analysis of variance test were used to evaluate the possible dscrepancy among the study groups. P-value <0.05 was considered significant.

RESULTS

1. Parasitological Examination of Stool of Different Study Groups

GI, GII, GIII and GIV showed highly significant statistical difference between mean oocysts count when compared with GV (p-value <0.001). GII showed the best response with GII showed the best response with severe decrease of the mean count of oocysts (Figure 1, Table 1).

2. Histopathological Examination of Sections from Ileocaecal Regions of the Studied Groups

The present study revealed mild amelioration of the histopathological changes following infection including partial villous atrophy with moderate diminution in ratio of villous height to crypt length. A little bit inflammatory infiltration was found in the lamina propria (Figure 2, 3).

GII revealed significant improvement of the histopathological changes with no oocysts found (Figure 4, 5). GIII showing many adherent and separate cryptospores, villous broadening, moderate mucosal cellular infiltration and focal ulceration (Figure 6, 7). GIV showed nearly the same histopathological features compared to GIII but with less ulcerations (Figure 8, 9). So, we found that coconut extract in water (GII) gave the best response compared to the positive control GV (Figure 10, 11) and the negative control GVI (Figure 12, 13).

C- Immunohistochemical results (IHC)

In positive control group, Caspase-3, which is a marker for apoptosis, was detected by finding discrete cytoplasmic positive cells in the intestinal crypt for Caspase. This increased apoptotic activity is due to the oncogenic effect of cryptosporidiosis with nearly negative staining by CDX2 (Figure 14). In negative control group Caspase-3 is nearly negative with remarkable nuclear positivity for CDX2 (Figure 15).

Sections in intestine of group infected then treated with coconut oil extract showing focal negative staining by CDX2 (Figure 16). Thiös could be due to the oncogenic effect of cryptosporidiosis, that could not be -at least- partially antagonized by the coconut extract. Also show few positive cells stained by Caspase-3. Being compared to the positive control group, there is decrease in the apoptotic index after treatment with coconut extract (Figure 17).

Table 1. Analysis of different groups using post-hoc test

		Least significance difference	p-value
PC	**Coconut ethanol**	**126.6**	**<0.001 HS**
	Coconut H_2O	**153**	**<0.001 HS**
	Coconut hexane	**111.4**	**<0.001 HS**
	Nitazoxanide	**105.8**	**<0.001 HS**
Coconut ethanol	**PC**	**-126.6**	**<0.001 HS**
	Coconut H_2O	26.4	0.061 NS
	Coconut hexane	-15.2	0.266 NS
	Nitazoxanide	-20.8	0.133 NS
Coconut H_2O	**PC**	**-153**	**<0.001 HS**
	Coconut ethanol	-26.4	0.061 NS
	Coconut hexane	**-41.6**	**0.005 S**
	Nitazoxanide	**-47.2**	**0.002 S**
Coconut hexane	**PC**	**-111.4**	**<0.001 HS**
	Coconut ethanol	15.2	0.266 NS
	Coconut H_2O	**41.6**	**0.005 S**
	Nitazoxanide	-5.6	0.678 NS
Nitazoxanide	**PC**	**-105.8**	**<0.001 HS**
	Coconut ethanol	20.8	0.133 NS
	Coconut H_2O	**47.2**	**0.002 S**
	Coconut hexane	5.6	0.678 NS

S: p-value <0.05 is considered significant.
NS: p-value >0.05 is considered non-significant.
HS: p-value <0.001 is considered highly significant

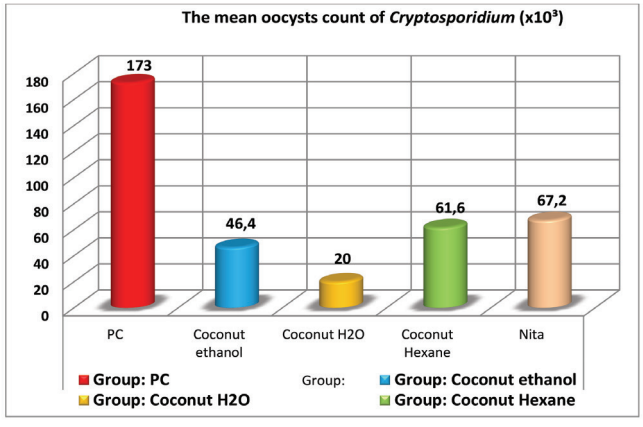


Figure 1. Chart showing comparison between groups as regard the mean oocysts count of *Cryptosporidium*

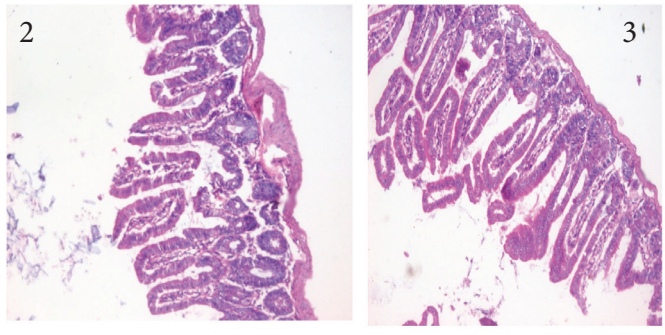
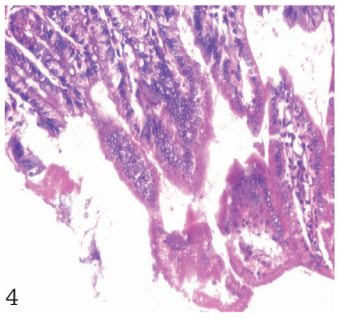


Figure 2, 3. Section of small intestine in GI showing mild blunting of the villi with moderate lowering in villous height to crypt length. slight inflammatory cells appear in lamina propria (H&E stain x200)



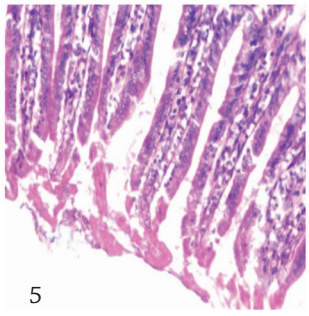
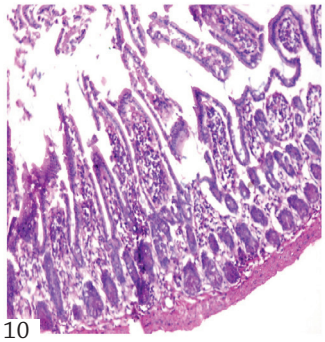


Figure 4, 5. GII group revealed milder inflammation and mild villous broadening compared to GI, minimal surface erosions with normal brush border and goblet cells and slight inflammatory cells in lamina propria without *Cryptosporidium* oocysts (H&E stain x200)



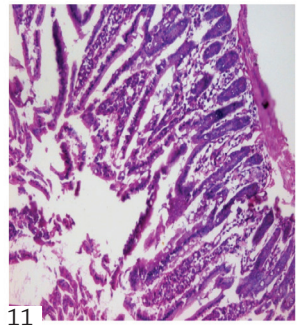
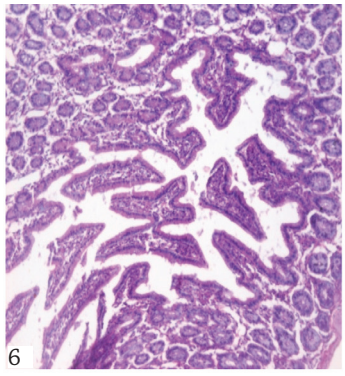


Figure 10, 11. Section of small intestine in GV (positive control) showing many cryptospores, distortion of villi, ulceration and dense cellular infiltration (H&E stain x200)



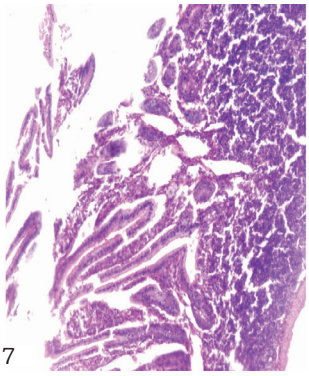
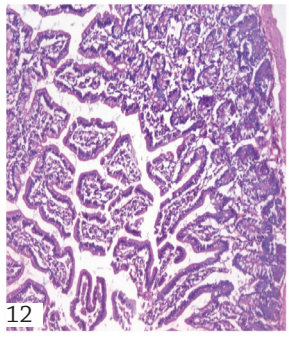


Figure 6, 7. GIII group revealed many adherent and separate cryptospores, villous broadening, moderate mucosal mononuclear inflammatory cells, focal ulceration (H&E stain x200)



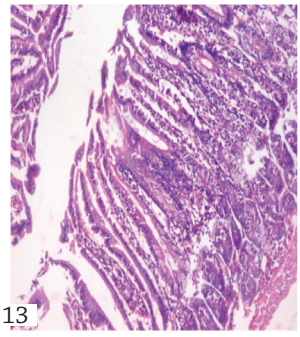
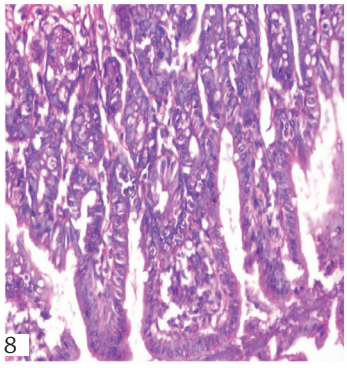


Figure 12, 13. Section of small intestine in GVI (negative control) showing normal architecture of villi with average length and width with intact brush border (H &E stain x200)



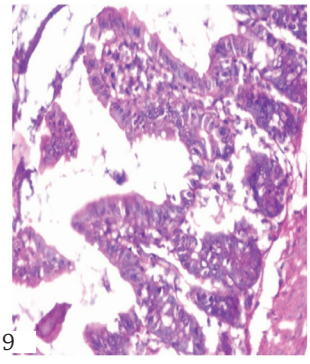


Figure 8, 9. Section of small intestine in GIV showing nearly same features compared to group GIII but with less ulcerations (H&E stain x200)

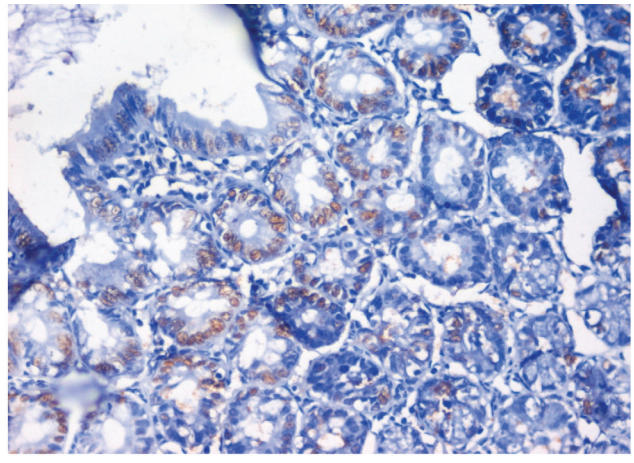


Figure 14. Negative control group revealed obvious positivity for CDX2 in the nuclei (Brownish color) (X400)

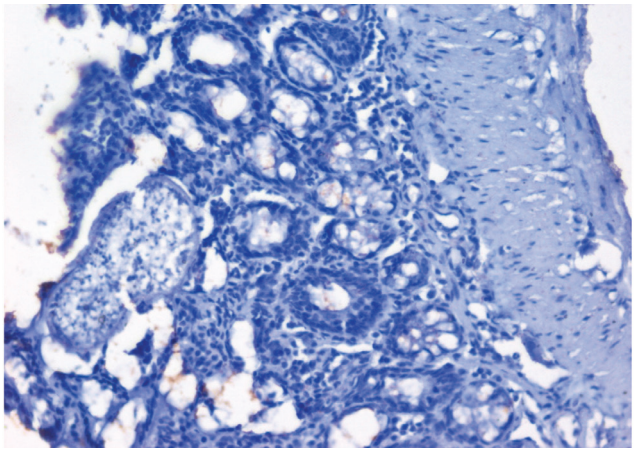


Figure 15. Sections in intestine of group (*C. parvum* infected for 60 days then treated with coconut) showing focal negative staining by CDX2 (X400)

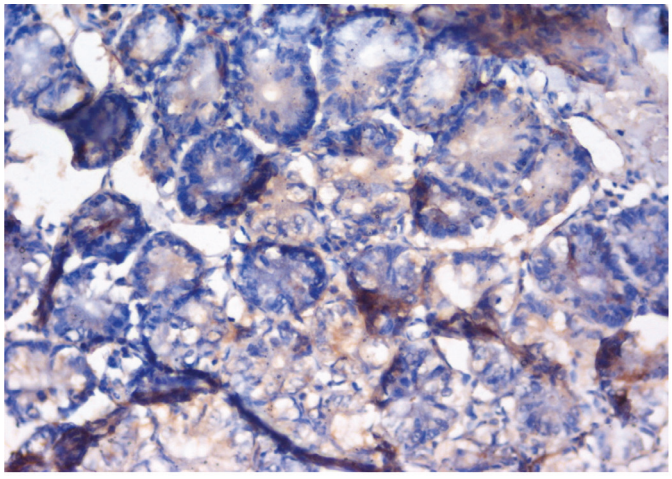


Figure 16. Sections in intestine of (positive control) revealed obvious brownish discoloration in the cytoplasm in the crypts of the intestine indicating Caspase 3 (x400)

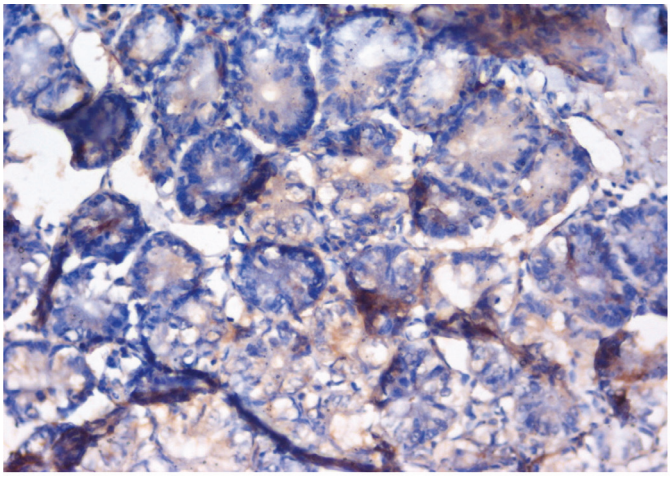


Figure 17. Sections in intestine of Group (Cryptosporidium infected for 2 months and cured by coconut) showing few positive cells stained by Caspase 3 (X400)

DISCUSSION

"Back to nature" is one of the newly notions used in the field of general health. Since the early days of human civilization, using medications from plant origin has been started. In the last three decades, the search for using herbal medicines increased especially with the rise in the resistance of human pathogens to usual treatment (15).

Coconut oil is a natural oil chemically composed of triglyceride compounds that contain large amounts of saturated medium chain fatty acids. Among which is Lauric acid (C12:0) which is the major fatty acid in coconut oil accounting for around 50% of the total fatty acids (3). It has beneficial effects, such as antimicrobial, anti-inflammatory, immunostimulant, and antioxidant actions (16).

In this study, highly statistically significant discrepancy in the *Cryptosporidium* oocysts count was found among nitazoxanide, positive control, coconut ethanol, coconut hexane and coconut H_2O groups with the lowest count was shown in coconut H_2O (20 oocysts/0.001 g feces). These results are congruent with those reported by several authors working on other protozoa. Hassan et al. (17) reported highly significant reduction of concentration of *blastocystis* cells after administration of monolaurin ($p<0.01$). These results were convenient with Rayan et al. (18) who confirmed that Lauric acid has an anti-*Giardia* effect comparing to metronidazole.

These results are compatible with Aly et al. (6) who found found improvement of the therapeutic effect against giardiasis when combined Lauric acid with metronidazole. This agrees with Helmy, who declared decrease in *Giardia* cysts and trophozoites outcomes using both Lauric acid with metronidazole (98.83 to 96.95%) (19). Moreover, Fahmy et al. (20) found same results in *E. histolytica* by high reduction in trophozoite and cyst (90.12%, 92.56%, respectively).

In this study, the experimentally-treated mice groups with coconut water extract kept their normal appearance with varying degrees of histopathological corrections in comparison with the positive control with promising results in treatment of cryptosporidiosis (Figure 4, 5). This coincides with Helmy, who found, by histopathological examination and electron microscopic examination, a complete healing of intestinal mucosa after the combined treatment of metronidazole and Lauric acid for treatment of giardiasis (19).

In birds, Hafeez et al. (21) recorded that inclusion of 2% coconut oil as a supplement improved growth performance and villus length was significantly improved ($p<0.01$) in broiler chicks exposed to experimentally induced coccidiosis caused by *Eimeria*.

Owing to the importance of coconut oil, not only as anti-parasitic, it was included in studies of the pandemic COVID-19 as antiviral and anti-inflammatory. Angeles-Agdeppa et al. (22) evaluated it's effect of on cases of COVID-19 in Philippines and found more rapid relief from symptoms with significant decline in the CRP levels.

It is noteworthy that coconut oil can modulate the cellular immunity and can be a potential alternative to antibiotics, as it has a broad-spectrum activity as an antibacterial and antiviral (23,24).

Aforethought, many studies investigated the appearance of Caspase-3 in ileal epithelial cells of infected mice and it's important role of apoptosis of epithelial and stromal cells during the course

of Cryptosporidiosis. Activation of Caspase-3 is considered to compel the cell toward irreversible apoptotic death (25).

In the present study, IHC results of Caspase-3 showed, before treatment with coconut oil, sections of intestine of positive control group showed scattered cytoplasmic positive cells in the intestinal crypt for Caspase-3. This is due to the increased apoptotic activity due to oncogenic effect of cryptosporidiosis. This is reconcilable with previous studies, which found that Caspase-dependent apoptosis was increased by *C. parvum in vitro* and *in vivo* infecion (26,27).

Following treatment with coconut oil in the present study, few positive cells stained by Caspase-3 were found. This indicate that there is decrease in the apoptotic index after treatment with coconut oil with promising results in treatment of cryptosporidiosis.

In contrast, Samaka et al. (28) stated that there was no significant association between dual treatment of infected mice with phenyl vinyl sulfone and nitazoxanide and Caspase-3 expression. This disparity may be because of the rabbit polyclonal anti Caspase-3 used in contrast to the monoclonal antibody used in our study, which is more specific, precise and showed the characteristic apoptotic nuclear changes (28).

It is worth noting that Caspase-3 was evaluated in tumors as a marker of tumorigenesis by Noble et al. (29), who found diffuse Caspase-3 expression in cases of colorectal carcinoma and declared the higher the caspase-3 levels, the higher the apoptotic rate.

CDX2 is essential for intestinal development and differentiation as it is a marker normally expressed in colonic mucosa and decreased in cases of tumorigenesis (30). In the present study, CDX2 of negative control group showed remarkable nuclear positivity for CDX2 and following infection with cryptosporidium then treatment with coconut, the result was focal negative staining by CDX2, this could be due to the oncogenic effect of cryptosporidiosis, that could not be -at least- partially antagonized by the coconut extract.

Previously, CDX2 was evaluated in all stages of colorectal cancer. It's absence was found in advanced tumor grade and is related to poor outcome and metastasis (31-33).

CONCLUSION

It could be concluded that our study has opened fresh avenues and paves the way for development of natural therapy like coconut oil extracts which have potential therapeutic efficacy against Cryptosporidiosis confirmed by parasitological, histopathological, and immunohistochemical assays that exemplify for being a promising anti-parasitic agent against *Cryptosporidium*. However, further studies are still required to give more knowledge about different coconut extracts in order to reach the best treatment efficacy.

* Ethics

Ethics Committee Approval: According to the valid International Guidelines for animal experimentation, animal protocols were conducted and approved by the Ethical Committee at Theodor Bilharz Research Institute (TBRI).

Informed Consent: N/A.

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