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RESEARCH ARTICLE

Potential effect of methanol leaf extracts from three *Podocarpus* species on experimental cryptosporidiosis

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ABSTRACT

Cryptosporidiosis causes diarrhea in both immunocompetent and immunocompromised individuals, with acute manifestations occurring particularly in children and the elderly. Up till now, there is no curative therapy for cryptosporidiosis, so discovery of new classes of drugs are of great importance. This study aimed to examine the effect of methanol leaves extracts of the three *Podocarpus* species; *P. macrophyllus* (Thunb.), *P. gracilior* (Pilg.) and *P. elongatus* (Aiton) L' Hér. ex Pers and their combination on *Cryptosporidium parvum (C. parvum)* in experimentally infected mice in comparison with the commercially used drug, Nitazoxanide. As well as spectrophotometric estimation of the total phenolic and flavonoid content of these extracts was done. Results revealed that treatment with these three *Podocarpus* extracts and their combination showed a significant reduction of the number of *C. parvum* oocyst shed in the stool of infected mice compared to infected control group and Nitazoxanide-infected treated group at $P < 0.001$. The combination of the three *Podocarpus* extracts was the most effective treatment showing the lowest number of oocysts shedding in comparison with other used extracts and Nitazoxanide. Histopathological inspection of sections from ilium and colon displayed signs of improvement after treatment with *P. macrophyllus* and *P. gracilior* extracts and more remarkable improvement when the three extracts were combined. It was concluded that the three *Podocarpus* species extracts used in this study had a promising anti-*Cryptosporidium* activity especially when they were combined.

Keywords: *Cryptosporidium parvum*; *Podocarpus*; Nitazoxanide; mice; colorimetric assay.

INTRODUCTION

Cryptosporidium parvum, a unicellular intestinal protozoan, infects the microvilli of the gastrointestinal tract in a wide range of animals (Spano *et al*., 1997). The higher load of oocysts is excreted in the environment by infected animals. The oocysts are extremely resistant to desiccation, disinfectants, and other environmental factors. The polluted environmental surroundings are considered a constant threat to healthy animals and humans. The clinical disease is characterized by mucous to hemorrhagic diarrhea, fatigue and fever. Infection is diagnosed by standard microscopic analysis of *Cryptosporidium* oocytes (Xiao *et al*., 1999).

C. parvum infection is resistant to many antimicrobic drugs and unlike many of its relatives (*Toxoplasma, Eimeria,* and *Plasmodium*), there is no curative treatment for cryptosporidiosis in immunocompetent adults and children, no certain treatment is indicated, ever since the infection is self-limiting. However, the disease may become chronic and threaten the life which could lead to death in some persons especially children, the elderly, and immunosuppressed patients, particularly AIDS patients (Obiad *et al*., 2012).

Medicinal plants are natural kind of therapy, and their importance is growing all over the world which makes no suspicion that plants have elements with curative importance (Kadir *et al*., 2008). *Podocarpus* is an evergreen woody plant which is known to have therapeutic properties for humans and animals (Kim *et al*., 2008).

Flavonoids are phytochemical compounds found in plants and their regular consumption is associated with decreased risk of several chronic diseases, such as cancer, cardiovascular disease and neurodegenerative disorders (Kozłowska, A. & Szostak-Węgierek, 2014). The main active nutraceutical components in plants are flavonoids which could act as powerful antioxidants and metal chelators. They also have antiviral, anticarcinogenic activities (Kuo *et al*., 2008), anti-inflammatory, hepatoprotective, antithrombotic, anti-allergic (Tapas *et al*., 2008). The biological and pharmacological activities of bioflavonoids of *Podocarpus* species are different. They include antibacterial, antifungal,

antiallergic, antiviral, antihepatotoxic, anticancer, and immune suppressive activities (Kim *et al*., 2008).

Phytochemical studies of *Podocarpus* species have guided to the isolation and elucidation of many flavonoids like apigenin, acacetin, catechin, kaempferol, luteolin, naringenin, naringin, orientin, quercetin, isoquercetin and vitexin 2”-o-b-d-(6”’-acetyl)-glucopyranoside (Kuo *et al*., 2008; Faiella *et al*., 2012; Kamal *et al*., 2012). Also, bioflavonoids of the amentoflavone and hinokiflavone types were identified e.g. II-4”, I-7-dimethoxyamentoflavone, isoginkgetin, sciadopitysin and podocarpus-flavone A (Yeh *et al*., 2012; Dávila *et al*., 2014).

Podocarpus species are used in traditional medicine in their native areas to treat several diseases including fevers, asthma, coughs, cholera, distemper, chest complaints, arthritis, rheumatism, painful joints and venereal diseases (Abdillahi *et al*., 2010). Reviewing the current literature, it was found that *Podocarpus* species under investigation are rich in flavonoids and other phenolic compounds, so it was important to throw light on the total phenolic and flavonoid content of the studied plants, which may find a relationship between the content of these biologically active constituents and the traditional use of the plant.

Recently, there has been renewed attention in the antimicrobic characteristics of *Podocarpus* and their phenolic compounds, but no work has been done on their antiparasitic activities. So, it was interested to investigate the effect of methanol leaves extracts of *Podocarpus macrophyllus* (Thunb.), *P. gracilior* (Pilg.) and *P. elongatus* (Aiton) L’ Hér. ex Pers. and their combination on experimental *Cryptosporidium* infection.

MATERIAL AND METHODS

Ethical consideration

The protocol of this study was approved by a scientific research ethics committee of TBRI and the Ethical Committee of Faculty of Medicine, Helwan University.

Plant material

Leaves of *P. macrophyllus* (Thunb.), *P. gracilior* (Pilg.) and *P. elongatus* (Aiton) L΄ Hér. ex Pers. were collected from El-Zohria Garden, Cairo, Egypt in January 2017. Identification of the plants was gently verified by Threase Labib head specialist of plant identification at El-Orman botanical garden, Cairo, Egypt. Voucher specimens No. (362014) have been banked in the herbarium of Faculty of Pharmacy, Cairo University.

Preparation of methanol extract

Samples of *P. macrophyllus* (Thunb.), *P. gracilior* (Pilg.) and *P. elongatus* (Aiton) L΄ Hér. ex Pers. leaves were air dried independently in the shade, powdered, and kept in firmly closed amber colored glass bottle. Extraction of the dried leaves in methanol was done, sonicated in an ultrasound bath for one hour, kept overnight, filtered, and finally evaporated under reduced pressure (El-Hawary *et al*., 2015). Stock solutions (25 mg% in methanol) of plant extracts were prepared**.**

Quantitative estimation of total polyphenols and flavonoids using colorimetric assay

Estimation of total polyphenols and flavonoids was performed using UV-visible spectrophotometer (Sagar Scientific Works, Ambala cantt, India), Nicolet evolution 100 (Thermo) was used for measuring the absorbance at 765 nm (for polyphenols) and at 415 nm (for flavonoids) using the following authentic and reagents:

- Gallic acid was acquired from E- Merck, Darmstadt, Germany.
- Quercetin was acquired from Sigma-Aldrich, Hamburg, Germany.
- Sodium carbonate solution (74.2 g) was dissolved in 1000 mL distilled water (0.7 M solution) (Makky *et al*., 2012).
- Folin-Ciocalteu reagent was purchased from Sd fine Chem. Limited, Mumbai, India.
- Anhydrous aluminium chloride (2 g; Sigma-Aldrich, Hamburg, Germany) was cautiously dissolved in 100 mL of spectroscopic methanol (Aliyu *et al*., 2009).

Spectrophotometric determination of the total polyphenols

The adopted method was based on measuring the blue color intensity developed when phenolic compounds make a complex with Folin-Ciocalteau reagent (Sd fine Chem. Limited, Mumbai, India) according to the method shown by Aliyu *et al*. (2009) who used gallic acid as a standard. The total phenolics concentration was calculated as gallic acid equivalent (GAE µg/ µg sample) with referral to a pre-established calibration curve standard.

The preparation of the calibration curve was performed by using gallic acid as a standard through the following concentrations: 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, and130 µg/mL in methanol.

Folin-Ciocalteu reagent, diluted tenfold, (5 mL) was added to 1 ml of the methanol solution of each concentration of gallic acid and plant extract (250 µg/mL in methanol), and then 4 mL of (0.7 M) sodium carbonate was added. The contents were incubated at room temperature for 5 minutes and the absorbance of the resultant blue color was read at 765 nm. Three determinations were carried out for each concentration, and the average of the gotten absorbance was plotted versus the concentrations.

Spectrophotometric determination of the total flavonoids

The adopted method was based on measuring the developed color intensity when flavonoids make a complex with aluminum chloride according to the method shown by Makky *et al*. (2012). The flavonoids concentration was calculated as quercetin equivalent (QE µg/ µg sample) with reference to a pre-established calibration curve standard.

A calibration curve was prepared by using quercetin as a standard flavonoid through these concentrations (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 µg/mL), then one mL of each concentration was transferred to the test tubes and evaporated under reduced pressure.

A total of one mL of the stock solution of each plant extract (250 µg/mL) methanol was transferred to a test tube and evaporated under reduced pressure. Residue of each concentration and plant extract was mixed with 5 mL 2% aluminum chloride (w/w). The content was incubated for 30 minutes at room temperature and the absorbance was measured at 415 nm. Three determinations were carried out for each concentration, and the average of the gotten absorbance was plotted versus the concentrations.

Parasite

Stool samples were gathered from naturally infected calves from veterinary clinics in Faculty of Veterinary Medicine, Cairo University and identified by modified Ziehl Neelsen acid fast stain (Sigma-Aldrich, Hamburg, Germany).

The obtained positive stool samples were mixed with distilled water, and then filtered by using coarse sterile gauze. The homogenate was centrifuged at 2500×g for 5 min. The supernatant fluid was removed, and the sediment was

washed with 1 ml of phosphate buffer saline twice and centrifuged for two minutes at 13,000×g. After repetitive washing followed by centrifugation, fecal debris was totally removed (Lumb *et al*., 1993). *Cryptosporidium* oocysts were well-preserved in 2.5% potassium dichromate solution and kept at 4°C until the time of experimental infection (Khalifa *et al*., 2001).

Prior to mice infection, the preserved *C. parvum* oocysts were washed for three times in distilled water to get rid of the potassium dichromate, centrifuged at 1500×g for 10 min, and the oocysts were counted. Suspension containing the essential concentration for the infection (10^4 oocysts/ ml) was prepared by dilution of the oocysts in the suitable amount of distilled water (Gaafar *et al*., 2007).

Cryptosporidium oocysts which have been used in this study, were characterized at the molecular level using nested PCR and PCR-RFLP, and all the obtained parasites were *C. parvum* and these results have previously been published (Khater *et al*., 2017; Yousof *et al*., 2017).

Experimental animal

A batch of 56 male laboratory bred Swiss albino mice of CDI strain weighing 20–25 gram and aging 3 to 5 weeks, was used in the present study. Mice were saved in well-ventilated cages of the animal house, Theodor Bilharz Research Institute (TBRI). During the study, the animals were maintained on a standard diet containing 24% protein, 4% fat and about 5% fiber and water ad libitum. They were kept at room temperature and away from direct sunlight. The cages were cleaned twice a week to make sure good hygienic situation until required for use. The mice were allowed to adapt to the laboratory environment for one week before the experiment, and their stools were inspected daily by direct wet saline smear and iodine before and after concentrations then stained with the acid-fast stain to exclude the presence of any parasites. The experiment was conducted in agreement with internationally valid animal ethics guidelines.

Experimental design

The used Swiss albino mice were divided into seven groups, each of which consisted of eight mice as follows: normal control, infected control, infected treated with *Nitazoxanide*, infected treated with *P. macrophyllus* (Thunb.) extract, infected treated with *P. gracilior* (Pilg.) extract, infected treated with *P. elongatus* (Aiton) L′ Hér. ex Pers extract and infected treated with a combination of the three *Podocarpus* extracts at a ratio of 1:1:1.

Animal infection

Mice were infected orally by intragastric inoculation of 10^4 oocysts per mouse using esophageal tube. One week after inoculation, fecal pellets were collected from all mice individually and subjected to parasitological stool examination using modified Ziehl Neelsen acid fast stain to identify the oocysts and to ensure that all mice got the infection (Garcia, 2007). The mice were transported to new clean cages every other day throughout the experiment.

Drugs

Nitazoxanide (Nanazoxid®) 500 mg tablets were used and purchased from (Medizen pharmaceutical industries for Utopia pharmaceuticals, Al-Asher men Ramadan, Egypt). Tablets were crushed, liquefied in distilled water and provided orally to the mice using esophageal tube in a dose of 1000 mg/kg body weight (approximately 200 µL/mouse) for three consecutive days (Abd El-Aziz *et al*., 2014).

Podocarpus macrophyllus (Thunb.), *P. gracilior* (Pilg.) and *P. elongatus* (Aiton) L' Hér. ex Pers. methanol extracts of each plant or a combination of the three species extracts was dissolved in dimethyl sulfoxide (DMSO) and was given orally in a dose of 100 mg/kg/day for 7 consecutive days (Soufy *et al*., 2017).

Scarification

Scarification was done one week after termination of treatment on day 21 post-infection in infected groups treated with Podocarpus and on day 17 post-infection in the infected group treated with Nitazoxanide by intraperitoneal injection of an anticoagulant anesthetic solution (500 mg/kg thiopental and 100 units/ml heparin) (Liang *et al*., 1987).

Portions from ilium and colon were dissected from individual mice, fixed in 10% formalin, and embedded in paraffin for blind histological examination of 4 mm sections stained with hematoxylin and eosin (Sigma-Aldrich, Hamburg, Germany).

Assessment of anti-cryptosporidium activity of Podocarpus

Parasitological studies

After cessation of treatment, stool samples were collected (14^{th} and 21^{st} days post-infection) from Podocarpus treated groups and (10^{th} and 17^{th} days post-infection) in Nitazoxanide treated group. Samples have been weighted, dissolved in Formalin 7%, and passed through hygienic gauze. Then, 50 µl were taken from each sample using micropipette and stained with the modified Ziehl-Neelsen stain to count the number of *C. parvum* oocysts (Garcia, 2007). Examination of slides have been done by the oil immersion lens. Oocysts were calculated for each group by counting the number in ten microscopic fields, and the arithmetic mean of oocysts was calculated for each group to detect the number of oocysts/ gram of feces (Khalifa, 2016). The percentage of reduction in the number of the shed oocysts was then determined.

Histopathological studies

Sections from the ilium and colon of scarified mice from all groups were stained with hematoxylin and eosin to detect the histopathological changes happened due to *Cryptosporidium* infection and to evaluate the degree of healing of intestinal mucosa after drug administration (Bancroft & Stevens, 1990).

Toxicity Assessment and Determination of LD_{50}

A group of 18 adult healthy male Swiss albino mice of CDI strain weighing 20-25 gram were used to test the acute toxicity of the used plant extracts. They were subdivided into three subgroups of six mice each. Subgroups were orally treated with rising doses of 100, 500, 1000, 2000, 3000 and 4000 mg/kg plant extract dissolved in dimethyl sulfoxide (DMSO). The rates of mortality were recorded 24 hours post treatment. The death rate was used to judge toxicity.

Statistical analysis

All data were recorded, edited and entered using the SPSS version 24.0 software (IBM, Chicago, USA). The results were expressed as mean ± standard deviation (SD) of eight animals in each group. All data were were analyzed by an ANOVA followed by Student's t-test at 95% confidence level. Values of $P < 0.05$ were considered as statistically significant.

RESULTS

Results of quantitative determination of total polyphenols and flavonoids using colorimetric assay

The present study revealed that *P. gracilior* (Pilg.) leaves contain higher concentration of phenolic compounds (121±0.016 µg/µg) than both *P. macrophyllus* (Thunb.) (105±0.014 µg/µg) and *P. elongatus* (Aiton) L' Hér. ex Pers. which contains the lowest polyphenolic content (82±0.006 µg/µg). On the other hand, the flavonoid content of the leaves of *Podocarpus* species was very close to each other. The highest flavonoid content was that of *P. macrophyllus* (Thunb.) (70±0.004 µg/µg) followed by *P. elongatus* (Aiton) L' Hér. ex Pers. (65±0.007 µg/µg) and *P. gracilior* (Pilg.) (59±0.005µg/µg) (Table 1).

The absorbance of different concentrations of standard gallic acid and quercetin was also illustrated in Figures 1 and 2.

Parasitological results

Methanol leaves extracts of *P. elongatus* (Aiton) L' Hér. ex Pers., *P. gracilior* (Pilg.), *P. macrophyllus* (Thunb.) and the combination of the three extracts were effective in the treatment of the parasite with a reduction of 51%, 68%, 75% and 82%, respectively in oocyst shed in the stool of infected mice on 14th day post infection exceeding the effect of the nitazoxanide (52%), while the reduction percentage of the oocyst shed on 21st day PI was 58%, 72%, 79% and 91%, respectively. Groups treated with methanol leaves extracts of *P. macrophyllus* (Thunb.), *P. gracilior* (Pilg.) and the combination of the three extracts showed a significant difference in the number of oocysts shedding in the stool of infected mice from nitazoxanide infected treated group at $P < 0.001$. The combination of the three *Podocarpus* extracts was the most effective treatment of *Cryptosporidium parvum* showing the lowest number of oocyst shedding/ gm stool of the infected mice in comparison with other used extracts and nitazoxanide.

The effect of the methanol leaves extracts of *Podocarpus* and Nitazoxanide on *Cryptosporidium* oocysts shed was demonstrated in Table 2.

Histopathological results

(i) Histopathological picture of the ilium (Figure 3)

Histopathological examination of ileum tissues of infected control group revealed many pathological alterations in the form of shortening, broadening and distortion of many villi, epithelial desquamation and sloughing. Enterocytes display degeneration with vacuolation and fragmentation of the

Table 1. The total polyphenol content of the different extracts calculated as gallic acid equivalent (GAE) and the total flavonoid content of the different extracts calculated as quercetin equivalent (QE)

Podocarpus species extracts	Concentration (µg/µg sample extract)	
	Total polyphenol content as GAE	Total flavonoid content as QE
P. macrophyllus (Thunb.)	105 ± 0.014	70 ± 0.004
P. gracilior (Pilg.)	121 ± 0.016	59 ± 0.005
P. elongatus (Aiton) L' Hér. ex Pers.	82 ± 0.006	65 ± 0.007

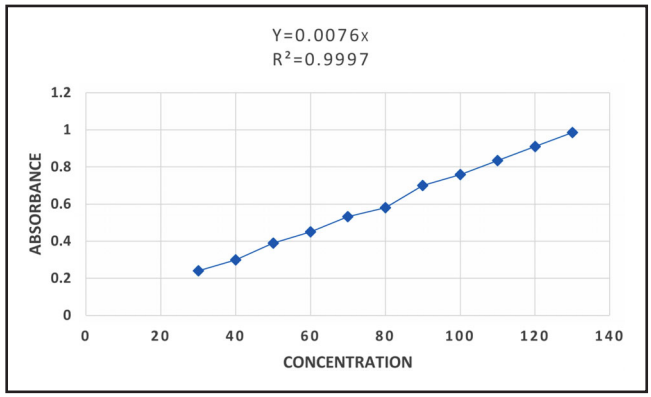

Figure 1. The calibration curve of gallic acid with different concentrations (µg/mL).

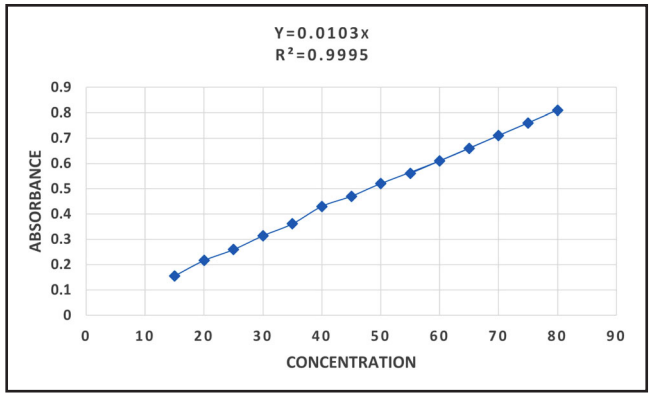

Figure 2. The calibration curve of quercetin with different concentrations (µg/mL).

Table 2. Number of oocysts shed per gm stool of infected mice treated with the three *Podocarpus* extracts compared to Nitazoxanide just after cessation of treatment and one week later

Animal groups	Number of oocyst shedding/ gm stool × 10^3 just after cessation of treatment (Mean ± SD)	% Reduction	Number of oocyst shedding/ gm stool × 10^3 one week after cessation of treatment (Mean ± SD)	% Reduction
Infected control	196.6 ± 11.74	–	202.6 ± 8.29	–
Nitazoxanide	94.8 ± 6.02*	52%	93.2 ± 4.02*	54%
P. elongatus	97 ± 13.96*	51%	85.4 ± 6.11*	58%
P. gracilior	62 ± 5.79*#	68%	56.8 ± 7.85*#	72%
P. macrophyllus	48.8 ± 7.60*#	75%	42.2 ± 8.53*#	79%
P. elongates + P. macrophyllus + P. gracilior	35.6 ± 7.37*#	82%	18.2 ± 5.26*#	91%

Data are presented as mean ± SD (n = 8 in each group).
(*) Significantly different from infected group at $P < 0.001$.
(#) Significantly different from Nitazoxanide infected treated group at $P < 0.001$.

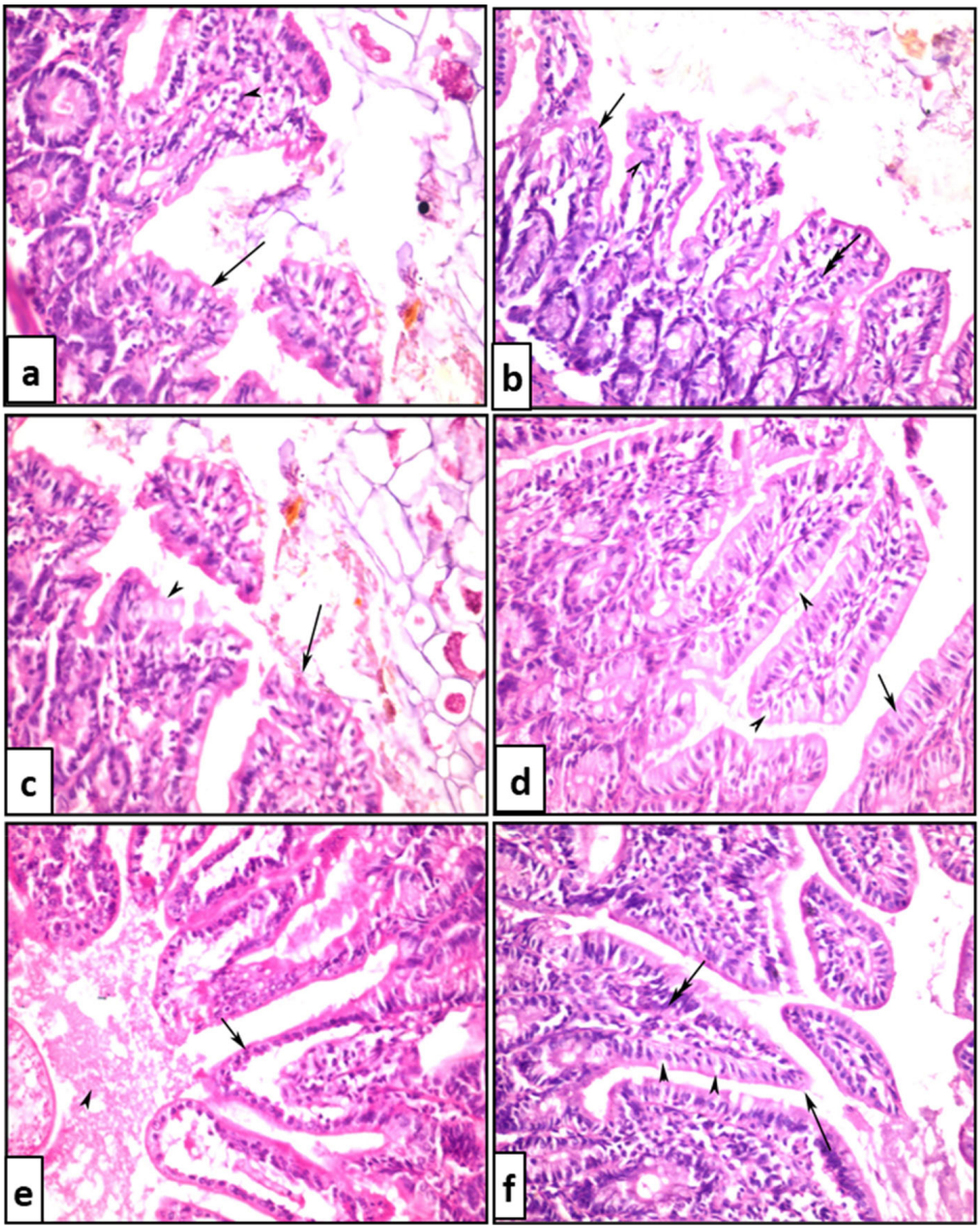

Figure 3. Photomicrographs of iliac tissues from (a) infected untreated control group showing many enterocytes with severe degenerative changes (arrow) and core of inflammatory infiltrate (arrow head), (b) infected treated with Nitazoxanide showing short villi (arrow), enterocytes with moderate degenerative changes (arrow head) and a core with cellular infiltrate (double arrow), (c) infected treated with *P. elongatus* (Aiton) L' Hér. ex Pers. showing short villi (arrow), denuded tips of villi (arrow head), (d) infected treated with *P. gracilior* (Pilg.) showing long villi (arrow), intact enterocytes (arrow head), (e) infected treated with *P. macrophyllus* (Thunb.) showing long villi with denuded enterocytes (arrow) and edema (arrow head) (f) infected treated with combination of the three extracts long villi (arrow) with intact enterocytes (arrow head) and goblet cell (double arrow) (H&E) (× 400).

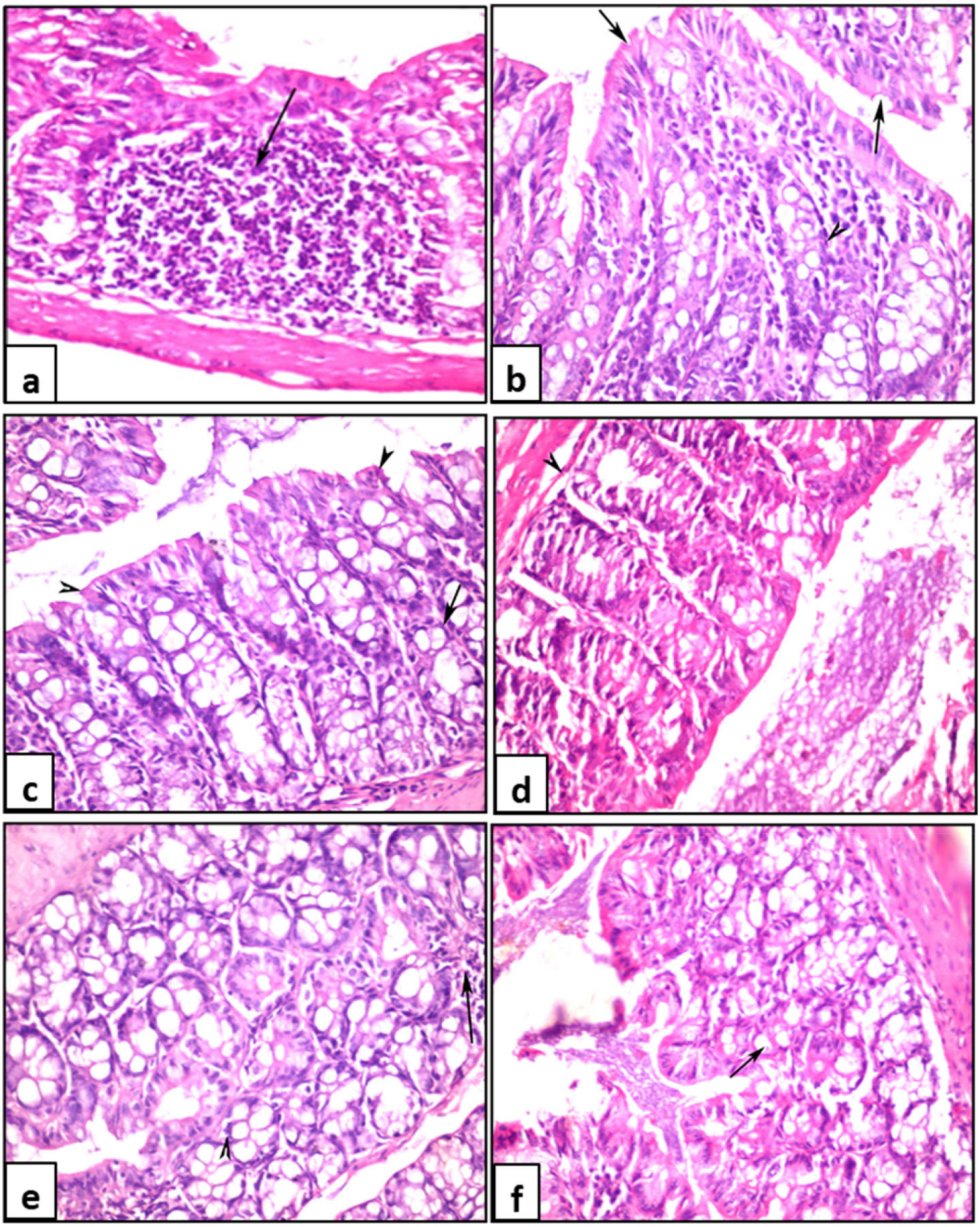

Figure 4. Photomicrographs of colonic tissues from (a) infected untreated control group showing mass of inflammatory cells in lamina propria (arrow), (b) infected treated with Nitazoxanide, (c) infected treated with *P. elongatus* (Aiton) L' Hér. ex Pers. showing denuded absorptive colon cells (arrows) and degenerative change in some absorptive cells (arrows head), (d) infected treated with *P. gracilior* (Pilg.) showing degenerative changes in epithelial columnar cells lined colonic crypts (arrow), (e) infected treated with *P. macrophyllus* (Thunb.) showing foci of inflammatory cells (arrow) and degenerative changes of few absorptive cells, (f) infected treated with combination of the three extracts showing intact absorptive cells of colon (arrow) (H&E) (× 400).

nuclei. Focal loss of brush border, together with severe loss of goblet cells. No sign of improvement due to administration of Nitazoxanide and *P. elongatus* (Aiton) L' Hér. ex Pers., where most obvious lesions still observed in the form of short villi, enterocytes with degenerative changes besides severe denuding in brush border of villi and loss of goblet cells. Signs of improvement were recorded after administration of *P. gracilior* (Pilg.) mainly due to presence of many long villi, goblet cells retain normal distribution, however sub-epithelial edema spaces under the tip of villi, inflammatory cellular infiltration together with mild loss of brush border were found where short villi core filled with inflammatory cells were observed. The pathological alterations observed after administration of *P. macrophyllus* (Thunb.) and the combination of the three extracts were significantly ameliorated, where the villi and their columnar epithelium were almost similar to the normal histological profile.

(ii) Histopathological picture of the colon (Figure 4)

Histopathological examination of colonic tissues of infected control group revealed evidence of degenerative changes in epithelial columnar cells lined colonic crypts with severe depletion of goblet cells and a mass of inflammatory cells in the lamina propria could be seen. Less curative impact was detected in groups of mice treated with Nitazoxanide, *P. elongatus* (Aiton) L' Hér. ex Pers. and *P. gracilior* (Pilg.) where depletion of goblet cells and focal inflammatory aggregates together with degenerative changes in colonic absorptive epithelial cells. In groups of mice treated with *P. macrophyllus* (Thunb.) and a combination of the three extracts, the histological inspection displayed signs of improvement, in form of goblet cells with normal distribution, intact colonic epithelial cells, and reduction of inflammatory reaction.

Acute toxicity and determination of LD_{50}

No dead animals (zero % mortality) were observed 24 h post treatment with rising doses of plant extracts starting from 100 mg/ kg to 4000 mg/kg, LD_{50} >4000 mg/ kg.

DISCUSSION

Plants provide a source of chemically diverse constituents, that may serve as novel replacements for the development of efficient antimicrobial agents to reverse the present threat caused by those pathogenic microorganisms. As a result, some medicinal plants containing active ingredients against viruses, bacteria, fungi and parasites have been recognized (Maregesi *et al*., 2008).

Polyphenols are phytochemical compounds found in plants that are believed to have important health benefits. Some evidence exist that polyphenols help in prevention of health danger. In the present study, the methanol leaves´ extracts of *P. macrophyllus* (Thunb.), *P. gracilior* (Pilg.) showed a statistically significant reduction in the oocysts number shedding in the stool of infected mice when compared to infected control group and nitazoxanide infected treated group at P< 0.001.

The combination of the three *Podocarpus* extracts was the most effective treatment showing the lowest number of oocyst shedding/ gm stool of the infected mice in comparison with other used extracts and nitazoxanide, this may attribute to the synergistic effect of the three extracts. The high concentration of flavonoids and other polyphenolic compounds in the methanol extracts of *Podocarpus* species leaves may be the cause of their biological action against *C. parvum*. Similarly, Anthony *et al*. (2007) found that the polyphenol rich blueberry extract could affect, modify the morphology, and reduce the viability of *C. parvum* in a dose dependent manner.

In the present study, Nitazoxanide showed the least reduction percentage of oocysts shed compared to other plant extracts and these results agreed with Shoultz *et al*. (2016) whose found that Nitazoxanide, the only drug that is now approved by the Food and Drug Administration (FDA) for treatment of *Cryptosporidium* infection, has cure rates of only 56% in undernourished children with a disadvantage of being not effective in immunodeficient individuals and undernourished children. Similarly, Taha *et al*. (2017) revealed a reduction percentage of *Cryptosporidium* oocyst shed on the 21st day post infection by 52.7% following Nitazoxanide treatment.

The effect of some plants extracts on *C. parvum* was also studied, for example pine bark extract (Pycnogenol) (Kim & Healey, 2001), the watery and methanol extracts of *Coriandrum sativum, Curcuma longa* and *Viscum album* (Obiad *et al*., 2012). Regarding the effect of different *Podocarpus* species, Sindiga *et al*. (1995) reported that *P. gracilior* and *P. milanjianus* both known as Podo used in traditional African medicine for treatment of stomachache and cattle infections.

Abdillahi *et al*. (2008) stated that *Podocarpus* species used in traditional medicine, for example a decoction of the fruit serves as a tonic for cleaning the kidneys, lungs, and stomach. In Ethiopia, *Podocarpus falcatus* oils are used to cure gonorrhea. It was also found that the crude extracts of four South African *Podocarpus* species viz; *Podocarpus elongatus, P. falcatus, P. henkelii and P. latifolius* showed a broad-spectrum antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Candida albicans*.

Abdillahi *et al*. (2010) further reported the use of many *Podocarpus* species in traditional medicine for example, the bark of *P. nagi* was used traditionally in Ayurvedic medicine as an antiseptic, astringent and carminative and has evidenced to be useful in the treatment of fevers, asthma, and coughs. As well as the stem bark of *P. macrophyllus* was used in the treatment of worms and blood disorders in Ayurvedic medicine. Other plants of the *Podocarpus* genus have been reported to be febrifuge, anti-inflammatory, and expectorant. Majority of the individuals in the developing countries use various kinds of this herbal concoction in diarrhea and pain and it could be concluded that *Podocarpus* species has antiparasitic activity.

Also, Bagla *et al*. (2014) revealed that the methanol extract of *Podocarpus henkelii* was active against *Stapz*tracts of the leaves of *P. gracilior* showed high efficacy against Gram negative bacteria (*E. coli*) and Gram-positive bacteria (*S. aureus*) and were proved to show antioxidant scavenging affinity against DPPH.

The methanol extract of leaf of *Podocarpus neriifolius* D. Don showed *in vivo* peripheral analgesic and antidiarrheal activities in Swiss Albino mice as it revealed a dose dependent inhibition of Castor oil- induced diarrhea with 43.77% and 56.23% inhibition of feces at 200 and 400 mg/kg body weight, respectively **(**Razan *et al*., 2016).

Cryptosporidium parvum infection causes inflammatory injury of the small intestine, with impairment of absorption and secretion enhancement stimulating diarrheal disease. So that restoring the small intestinal villi integrity is considered a significant issue in cryptosporidiosis treatment that will significantly improve the patient's condition (Checkley *et al*., 2014).

In this study, histopathological examination of intestinal sections from ilium and colon of infected control group revealed many pathological alterations and evidence of degenerative changes. These findings were matched with

those stated by Waters & Harp, (1996); Abu El Ezz *et al*. (2011); Al-Mathal & Alsalem, (2012); Gaafar (2012); Al-Warid *et al*. (2013); and Taha *et al*. (2017) whose revealed different histopathological changes which range from partial to complete villous atrophy and inflammatory infiltration due to *Cryptosporydium parvum* infection. The pathological alterations observed after administration of *Podocarpus* species particularly when the three extracts were combined showed significant amelioration and improvement, where the villi and their columnar epithelium were almost similar to the normal histological profile.

Interestingly, no sign of improvement due to administration of Nitazoxanide was observed in the present study, where most obvious histopathological lesions were detected. Similarly, Amadi *et al*. (2009) found that Nitazoxanide didn't offer any clinical enhancement of the symptoms in Zambian children with HIV-related immunosuppression. In the same context, Taha *et al*. (2017) found that Nitazoxanide treatment didn't display any improvement in the histopathological changes after *Cryptosporidium* infection in the form of persistent disturbed villous architecture, edema, and infiltration of the lamina propria with inflammatory cells.

The pathological alterations observed after administration of *Podocarpus* species particularly when the three extracts were combined showed significant amelioration and improvement, where the villi and their columnar epithelium were almost similar to the normal histological profile.

It was concluded that using a combination of the methanol leaves extracts of *P. macrophyllus* (Thunb.), *P. gracilior* (Pilg.), *P. elongatus* (Aiton) L' Hér. ex Pers. showed the best results in the treatment of *Cryptosporidium parvum* when compared with Nitazoxanide or each extract alone. The high efficacy of the extracts may return to their high phenolic and flavonoid contents. Also, this improvement candidates these extracts to be safe and effective drugs in treatment of cryptosprodiosis in the future.

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Conflict of Interest

The authors declare that they have no conflict of interests.

REFERENCES

Abd El-Aziz, T.M., El-Beih, N.M., Soufy, H., Nasr, S.M., Khalil, F.A.M. & Sharaf, M. (2014). Effect of Egyptian propolis on lipid profile and oxidative status in comparison with nitazoxanide in immunosuppressed rats infected with *Cryptosporidium* spp. *Global Veterinaria* **13**: 17-27.

Abdillahi, H.S., Stafford, G.I., Finnie, J.F. & Van Staden, J. (2008). Antimicrobial activity of South African Podocarpus species. *Journal of Ethnopharmacology* **119**: 191-194. https://doi.org/10.1016/j.jep.2008.06.023

Abdillahi, H.S., Stafford, G.I., Finnie, J.F. & Van Staden, J. (2010). Ethnobotany, phytochemistry and pharmacology of *Podocarpus sensu latissimo* (s.l.). *South African Journal of Botany* **76**: 1-24. https://doi.org/10.1016/j.sajb.2009.09.002

Abu El-Ezz, N.M.T., Khalil F.A.M. & Shaapan, R.M. (2011). Therapeutic effect of onion (allium cepa) and cinnamon (cinnamomum zeylanicum) oils on cryptosporidiosis in experimentally infected mice. *Global Veterinaria* **13**: 179-183.

Aliyu, A.B., Ibrahim, M.A., Musa, A.M., Ibrahim, H., Abdulkadir, I.E. & Oyewale, A.O. (2009). Evaluation of antioxidant activity of leave extract of Bauhinia rufescens Lam. (Caesalpiniaceae). *Journal of Medicinal Plants Research* **3**: 563-567.

Al-Mathal, E.A. & Alsalem, M.A. (2012). Pomegranate (Punica granatum) peel is effective in a murine model of experimental *Cryptosporidium parvum*. *Experimental Parasitology* **131**: 350-357. https://doi.org/10.1016/j.exppara.2012.04.021

Al-Warid, H.S., AL-Saqur, I.M. & Mahmood, S.H. (2013). Histopathological changes in mice infected with *Cryptosporidium* spp. *International Journal of Pharmacy and Biological Sciences* **3**: 220-227.

Amadi, B., Mwiya, M., Sianongo, S., Payne, L., Watuka, A., Katubulushi, M. & Kelly, P. (2009). High dose prolonged treatment with nitazoxanide is not effective for cryptosporidiosis in HIV positive Zambian children, A randomised controlled trial. *BMC Infectious Diseases* **9**: 195. https://doi.org/10.1186/1471-2334-9-195

Anthony, J.P., Fyfe, L., Stewart, D., McDougall, G.J. & Smith, H.V. (2007). The effect of blueberry extracts on *Giardia duodenalis* viability and spontaneous excystation of *Cryptosporidium parvum* oocysts, *in vitro*. *Methods* **42**: 339-48. https://doi.org/10.1016/j.ymeth.2007.02.011

Bagla, V.P., McGaw, L.J., E Elgorashi, E.E. & Eloff, J.N. (2014). Antimicrobial activity, toxicity and selectivity index of two biflavonoids and a flavone isolated from Podocarpus henkelii. *BMC Complementary and Alternative Medicine* **14**: 383. https://doi.org/10.1186/1472-6882-14-383

Bancroft, I.D. & Stevens, A. (1990). Theory and Practice of Histological Techniques. 3rd edition. Edinburgh: Churchill Livingston, pp. 255.

Benamrouz, S., Conseil, V., Chabé, M., Praet, M., Audebert, C., Blervaque, R., Guyot, K., Gazzola, S., Mouray, A., Chassat, T. *et al.* (2014). *Cryptosporidium parvum*-induced ileo-caecal adenocarcinoma and Wnt signaling in a mouse model. *Disease Models & Mechanisms* **7**: 693-700. https://doi.org/10.1242/dmm.013292

Checkley, W., White, J., Jaganath, D., Arrowood, M.J., Chalmers, R.M., Chen, X-M., Fayer, R., Griffiths, J.K., Guerrant, R.L., Hedstrom, L. *et al*. (2014). A review of the global burden, novel diagnostics, therapeutics and vaccine targets for *cryptosporidium. Lancet* **15**: 85-94. https://doi.org/10.1016/s1473-3099(14)70772-8

Dávila, M., Stenier, O. & Hinojosa, N. (2014). Secondary metabolites from *Podocarpus parlatorei* Pilger. *Revista Boliviana de Química* **31**: 21-27.

El-Hawary, S., Taha, K., Kirollos, F., Dahab, A., Saleh, N. & El-Mahis, A. (2015). Molecular identification, GC/MS and antimicrobial activity of the essential oils and extracts of three *Podocarpus* species. *International Journal of Pharmacognosy and Phytochemistry* **30**: 1360-1369.

Faiella, L., Temraz, A., Siciliano, T., Tommasi, N. & Braca, A. (2012). Terpenoids from the leaves of *Podocarpus gracilior. Phytochemistry Letters* **5**: 297-300. https://doi.org/10.1016/j.phytol.2012.02.005

Gaafar, M.R. (2007). Effect of solar disinfection on viability of intestinal protozoa in drinking water. *Journal of the Egyptian Society of Parasitology* **37**: 65- 86.

Gaafar, M.R. (2012). Efficacy of *Allium sativum* (garlic) against experimental cryptosporidiosis. *Alexandria Journal of Medicine* **48**: 59-66. https://doi.org/10.1016/j.ajme.2011.12.003

Garcia, L.S. (2007). Diagnostic Medical Parasitology. 5th edition. Washington DC: ASM press, pp. 771-812.

Kadir, M.A., Al-Alousi, T.I. & AI-Sawah, D.A. (2008). Comparison between the efficacy of different medical herbs on *Cryptosporidium* spp. *Journal of the Faculty of Medicine – University of Baghdad* **50**: 68-76.

Kamal, A., Abdelhady, M., Elmorsy. E., Mady, M., Abdelkhalik, S. (2012). Phytochemical and biological investigation of leaf extracts of *Podocarpus gracilior* and Ruprechtia polystachya resulted in isolation of novel polyphenolic compound. *Life Science Journal* **9**: 1126-1135.

Khalifa, A.M., El Temsahy, M.M. & Abou El Naga, I.F. (2001). Effect of ozone on the viability of some protozoa in drinking water. *Journal of the Egyptian Society of Parasitology* **31**: 603- 616.

Khalifa, E.A. (2016). Probiotics as a promising treatment of experimental cryptosporidiosis in an immuno suppressed mouse model. *International Journal of Current Microbiology and Applied Sciences* **5**: 97-106.

Khater, M., El-Sayed, S., Yousof, H., Mahmoud, S., El-Dib, N. & El-Badry, A. (2017). Anti-Cryptosporidium efficacy of Olea europaea and Actinidia deliciosa in a neonatal mouse model. *Kasr Al Ainy Medical Journal* **23**: 32-37.

Kim, H., Park, H., Son, K., Chang, H. & Kang, S. (2008). Biochemical Pharmacology of Biflavonoids: Implications for Anti-inflammatory Action. *Archives of Pharmacal Research* **31**: 265-273. https://doi.org/10.1007/s12272-001-1151-3

Kim, H.C. & Healey, J.M. (2001). Effects of pine bark extract administered to immunosuppressed adult mice infected with *Cryptosporidium parvum*. *American Journal of Chinese Medicine* **29**: 469-475. https://doi.org/10.1142/s0192415x01000484

Kozłowska, A. & Szostak-Węgierek, D. (2014). Flavonoids – food sources and health benefits. *Roczniki Państwowego Zakładu Higieny* **65**: 79-85.

Kuo, Y., Hwang, S., Wu, M.D., Liao, C.C., Liang, Y., Kuo, Y.H. & Ho, H.O. (2008). Cytotoxic Constituents from *Podocarpus fasciculus*. *Chemical and Pharmaceutical Bulletin* **56**: 585-588. https://doi.org/10.1248/cpb.56.585

Liang, Y.S., Bruce, J.I. & Boyd, D.A. (1987). Laboratory cultivation of schistosome vector snails and maintenance of schistosome life cycles. *Proceeding of the first Sino-American Symposium* **1**: 34-48.

Lumb, R., Swift, J., James, C., Papanaoum, K. & Mukherjee, T. (1993). Identification of the microsporidian parasite, Enterocytozoon bieneusi in faecal samples and intestinal biopsies from an AIDS patient. *International Journal for Parasitology* **23**: 793-801. https://doi.org/10.1016/0020-7519(93)90077-c

Makky, E.A., Mashitah, M.Y. & Ibrahim, M.M. (2012). Impact of medicinal plants phytocomponents against antibiotic resistant bacteria. *Journal of Chemical and Pharmaceutical Research* **4**: 881-893.

Maregesi, S.M., Pieters, L., Ngassapa, O.D., Apers, S., Vingerhoets, R., Cos, P., Berghe, D.A. & Vlietinck, A.J. (2008). Screening of some Tanzanian medicinal plants from Bunda district for antibacterial, antifungal and antiviral activities. *Journal of Ethnopharmacology* **119**: 58-66. https://doi.org/10.1016/j.jep.200805.033

Obiad, H.M., Al-Alousi, T.I. & Al-Jboori, A.H. (2012). The *in vivo* effect of some medicinal plant extracts on *Cryptosporidium* parasite. *Journal of University of Anbar for Pure Science*. **6**: 2012.

Razan R., Rahman M., Tahia F., Hossain K. & Rashid M. (2016). Analgesic and antidiarrheal activities of leaf of *Podocarpus neriifolius* D. Don. *Bangladesh Pharmaceutical Journal* **19**: 215-218. https://doi.org/10.3329/bpj.v19i2.29283

Shoultz, D.A., De-Hostos, E.L. & Choy, R.K.M. (2016). Addressing Cryptosporidium Infection among Young Children in Low-Income Settings: The Crucial Role of New and Existing Drugs for Reducing Morbidity and Mortality. *PLoS Neglected Tropical Diseases* **10**: e0004242. https://doi.org/10.1371%2Fjournal.pntd.0004242

Sindiga, I., Nyaigotti-Chacha, C. & Kanunah, M. (1995). Traditional Medicine in Africa. Nairobi: East African Educational Publishers Ltd Brick court, pp. 5-16.

Soufy, H., El-Beih, N.M., Nasr, S.M., Abd El-Aziz, T.H., Khalil, F.A.M., Ahmed, Y.F. & Abou Zeina, H.A.A. (2017). Effect of Egyptian propolis on cryptosporidiosis in immuno-suppressed rats with special emphasis on oocysts shedding, leukogram, protein profile and ileum histo-pathology. *Asian Pacific Journal of Tropical Medicine* **10:** 253-262. https://doi.org/10.1016/j.apjtm.2017.03.004

Spano, F., Putignani, L., McLauchlin, J., Casemore, D. & Crisanti, A. (1997). PCR-RFLP analysis of the Cryptosporidium oocyst wall protein (cowp) gene discriminates between *C. wrairi* and *C. parvum*, and between *C. parvum* isolates of human and animal origin. *FEMS Microbiology Letters* **150**: 209-217. https://doi.org/10.1016/s0378-1097(97)00115-8

Taha, N.M., Yousof H.S. & EL-Sayed S.H. (2017). Atorvastatin repurposing for the treatment of cryptosporidiosis in experimentally immunosuppressed mice. *Experimental Parasitology* **181**: 57-69. https://doi.org/10.1016/j.exppara.2017.07.010

Tapas, A.R., Sakarkar, D.M. & Kakde, R.B. (2008). Flavonoids as nutraceuticals: A review. *Tropical Journal of Pharmaceutical Research* **7**: 1089-1099. https://doi.org/10.4314/tjpr.v7i3.14693

Waters, W.R. & Harp, J.A. (1996). *Cryptosporidium parvum* infection in T-cell receptor (TCR)-alpha- and TCR-delta-deficient mice. *Infection and Immunity* **64**: 1854-1857. https://doi.org/10.1128/iai.64.5.1854-1857.1996

Xiao, L., Morgan, U., Limor, J., Escalante, A., Arrowood, M., Shulaw, W., Thompson, R. & Fayer R. (1999). A Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. *Applied and Environmental Microbiology* **65**: 3386-3391. https://doi.org/10.1128/aem.65.8.3386-3391.1999

Yeh, P., Shieh, Y., Hsu, L., Yang Kuo, L., Lin, J., Liaw, C. & Kuo, Y. (2012). Naturally occurring cytotoxic [32'→83"]-Biflavonoids from *Podocarpus nakaii*. *Journal of Traditional and Complementary Medicine* **2**: 220-226. https://doi.org/10.1016/s2225-4110(16)30103-1

Yousof, H., Khater, M., El-Sayed, S. & El-Badry, A. (2017). Diagnostic biomarkers in murine Cryptosporidiosis: dose- and age-related infection. *Journal of Parasitic Diseases* **41**: 831-836. https://doi.org/10.1007/s12639-017-0898-2