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Research Article
Efficacy and Promising Ameliorative Effect of Crocin Against *Cryptosporidium parvum* with Colon Ultrastructure Examination in Male Rats

Bander Albogami

Department of Biology, College of Sciences, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia

Abstract

Background and Objective: The protozoan parasite (*Cryptosporidium parvum*) is known to parasitize humans, particularly children and individuals with compromised immune systems and is the cause of cryptosporidiosis. Water-soluble carotenoid crocin (CR) has a high capacity to scavenge free radicals and a variety of pharmacological effects, such as hypolipidemic and antioxidant effects. The purpose of the study was to assess the therapeutic efficacy of CR as a strong antioxidant agent as an alternative to anti-parasitic medications that may produce a number of undesired side effects. **Materials and Methods:** *Cryptosporidium parvum* oocysts were isolated from infected human tissue samples and analyzed using a modified Ziehl-Neelsen stain and Carbol fuchsin staining. The oocysts were diluted in distilled water and infected. Twenty one male albino rats were used in the study, with seven male rats per group. Three groups were created; I: Control (non-infected), II: Infected and untreated and III: Infected and treated with CR. Investigations focused on ultrastructural alterations in colon tissues and parasitological examination for oocysts in the stool. The study analyzed liver functions, lipid profiles and antioxidant parameters to predict heart or coronary diseases, including superoxide dismutase, GPx, CAT and non-enzymatic lipid peroxidation marker (MDA), using risk ratio calculations. **Results:** The treatment with CR led to a significant decrease in oocyst shedding and improved colon ultrastructural changes, hepatic biomarkers and lipid indices compared to the control group. Additionally, the number of oocysts decreased significantly and the lipid profile of the infected animals showed improvement, with a significant increase in antioxidant enzymes and a decline in MDA lipid peroxidation marker. **Conclusion:** Cryptosporidiosis in male albino rats caused a significant increase in oocyst shedding, changes in the structure of the colon, alterations of hepatic biomarkers and lipid indices. Meanwhile, treatment of the infected group with CR showed potential therapeutic effects as an antioxidant, hepatoprotective and antiparasitic agent.

Key words: Pharmacological actions, saffron, antioxidant activities, antiparasitic activity, anti-*Cryptosporidium*

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Corresponding Author: Bander Albogami, Department of Biology, College of Sciences, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

For many biological organs to generate energy, oxidation is a necessary process for many organisms. However, pollution and even pathogenic organisms, all produce an excessive number of free radicals, which are extremely harmful to human health. Free radicals can also start chain reactions. Antioxidant use has been the subject of extensive research to lessen oxidative damage to humans. A substantial amount of research indicates that natural extracts typically exhibit potent antioxidant properties, making them potential new sources of antioxidants[1,2].

Oxidative stress occurs in organs with high metabolic requirements[3]. Oxidative injury results from an imbalance between oxidants and antioxidants, which damages cells and tissues. In a rat model of *C. parvum* infection, oxidative stress has been shown to cause tissue damage[4,5]. Studies on humans and animals have shown that oxidative injury plays a marked role in the onset and disease progression. Antioxidants also offer significant defense against almost 50 harmful diseases[6]. Oxidative stress and the generation of free radicals are brought on by the acute infection's glucose metabolism, which powers parasite growth and energy production. But when the immune system is activated, defense mechanisms take over, using antioxidant enzymes to reduce free radical production[7].

As a first line of defense, superoxide dismutase (SOD) stops new radicals from forming and changes existing molecules into less dangerous ones. This happens when superoxide radicals are broken down into oxygen and Hydrogen Peroxide (H_2O_2), which help to neutralize and reduce harmful free radicals. To prevent oxidative damage and cell death, glutathione is essential[8,9]. These antioxidant enzymes are crucial in protecting the cell from oxidative stress and, ultimately, cell death by scavenging reactive oxygen species like superoxide and hydrogen peroxide[10-15].

The protozoan *Cryptosporidium* is the cause of the worldwide parasitic disease known as cryptosporidiosis[16]. The intestinal epithelium is infected by the apicomplexan parasite *Cryptosporidium*[17]. Inflammation of the digestive system brought on by these parasites is usually accompanied by noticeable changes in the gut's chemical composition and structure, as well as functional abnormalities. Scavenging enzymes like glutathione peroxidase, catalase and superoxide dismutase are part of the body's antioxidant defenses[18].

Currently, nitazoxanide is the recommended medication for treating cryptosporidiosis, as there is no available vaccine[19]. According to Albogami[20], nitazoxanide may cause some oxidative stress and certain active ingredients, such as ellagic acid, may have antiprotozoal, antiparasitic or antioxidant properties. Numerous studies were conducted to evaluate the effectiveness of traditional medicinal plants in treating cryptosporidiosis because there was a critical need to develop new anti-cryptosporidial agents[21].

Crocin, a water-soluble carotenoid containing the chemical moieties crocetin and gentiobiose, is one of the extract components of saffron. Crocin has been studied and the results indicate that it has potent free-radical scavenging activity and a wide range of pharmacological effects, such as hypolipidemic, antiatherosclerotic, antioxidant and anticancer effects. Its ability to scavenge free radicals has been linked to its therapeutic effects[22]. Therefore, the present study was carried out to investigate both the antioxidant and the hepatoprotective effects of CR against experimental cryptosporidiosis in male rats infected with *Cryptosporidium parvum*.

MATERIALS AND METHODS

Study area and duration: This study was carried out in animal laboratories. The first step of infection based on the ZU-IACUC approval committee was carried out in the Animals Laboratory for the duration, beginning on 1 July, 2023, following this, the infected animals were treated with CR. The study was conducted over 20 consecutive days; the end of the experiment and specimen collection was on 23 July, 2023.

Following a high level of technical preservation of colon tissues in glutaraldehyde and additional chemical processing steps, the preserved specimens of colon tissues were subjected to a second investigation step, during which resin capsules were investigated and captured at an electron microscope (JEOL 100s Microscopy, Tokyo, Japan) Unit at Taif University in Saudi Arabia. In the meantime, risk ratios were calculated and the biochemical analyses of hepatic markers and antioxidant enzymes were carried out in "Animal Laboratory No. 1", with code number ID "38101", at the Department of Biology, College of Sciences, Taif University, Saudi Arabia, following sample preservation with liquid nitrogen and a high-cold refrigerator. All experimental, ultrastructural, biochemical and statistical analysis were completed by August 31, 2023, after the end of the analysis period.

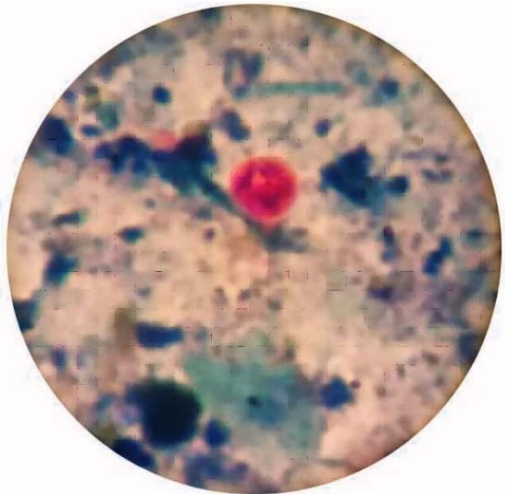

Fig. 1: *Cryptosporidium parvum* oocysts stained with modified Ziehl-Neelsen stain

Ethical consideration: This study was carried out in compliance with the Guide for the Care and Use of Laboratory Animals in accordance with following the other ethical animal approval of Zagazig University animal ethical committee, under the approval ethical number (ZU-IACUC/1/F/103/2023). The study duration was daily for successive 20 days.

Oocysts preparation and isolation: The Oocysts of *Cryptosporidium parvum* were obtained from samples of naturally infected humans. Oocysts were concentrated by H_2O/EtOH technique and identified by Carbol fuchsin staining as part of the modified Ziehl-Neelsen stain[23] method as shown in (Fig. 1) for two infected samples, then identified by Henriksen and Pohlenz[24]. Oocysts were used for infection for two days after being diluted in distilled water to obtain 10^6 oocysts/mL, after being washed three times in PBS and counted using a hemocytometer (Marienfeld, Germany)[23,25].

Experimental animals: Twenty one laboratory-bred male albino rats (n = 21) were used in this study were 7 weeks old and weighed between 100 and 120 g. By analyzing their stools using the modified Ziehl-Neelsen technique[24] and the ether concentration method[3], it was possible to conclude that none of them had any parasitic infections.

Calculation of the sample's size: The sample size was calculated by the "G-Power" program, justified via using of 7 male rats per group. The 21 male rats were divided into 3 groups. Group I: Control non infected (-Ve control), Group II: Infected and non-treated group (+Ve control) and Group III: Infected and treated group with CR (Fig. 1). Each rat in 2nd and 3rd groups were orally infected with *C. parvum* oocyst inoculum.

Compound used: The CR was obtained from Sigma Aldrich Company, Sigma, St. Louis, Missouri, USA in high purity and analytical grade. The CR was highly soluble in normal physiological saline solution (0.9%). The CR was given orally at a dose (100 mg/kg)[26].

Infection: All the male rats in the studied infected groups were confirmed the infected after collection of stool samples from the two infected groups for confirming the incidence of infection as shown in Fig. 2a-b. Except negative normal control (uninfected) group, were infected orally with the prepared inoculum.

Experimental design: Before the experiment, all of the rats were given 2 weeks to acclimate. They were then kept in plastic cages with a controlled room temperature of 25°C, a 12 hrs day and night cycle, free access to tap water and a standard rat diet.

The experimental 21 male albino rats were randomly assigned to three groups of seven rats each, comprising three groups total (7 rats/cage). The groups were then divided into the following categories:

I: Non-infected (negative control group): Animals received 1 mL/100 g of normal physiological saline daily for successive 20 days (whole period of the experiment)

II: Infected and non-treated (positive control group): After washing oocysts 3 times in PBS and then counting by using the hemocytometer (Marienfeld, Germany), then diluted in distilled H_2O[27], to obtain 10^6 oocysts/mL and used for incidence of the infection for 2 successive days, then a collection of feces for confirming infection by the determined parasite at day 5

III: Infected and treated with CR: After induction of infection, then treatment of the infected animals with CR at the previously used dose (100 mg/kg)[26]. The infected rats were treated orally by CR daily starting on day 6 after confirming infection for successive 15 days. The tested compound was administered to the male rats using special oral gavage syringes that were used for the oocyst inoculation[28,29]. As the experimental outline was shown in (Fig. 3)

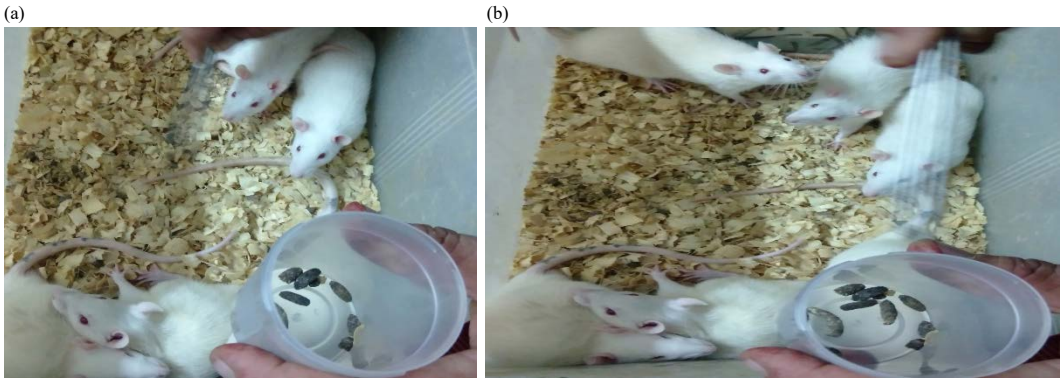

Fig. 2(a-b): Regular collection of stool samples from 1st day after inoculation for confirming the incidence of infection

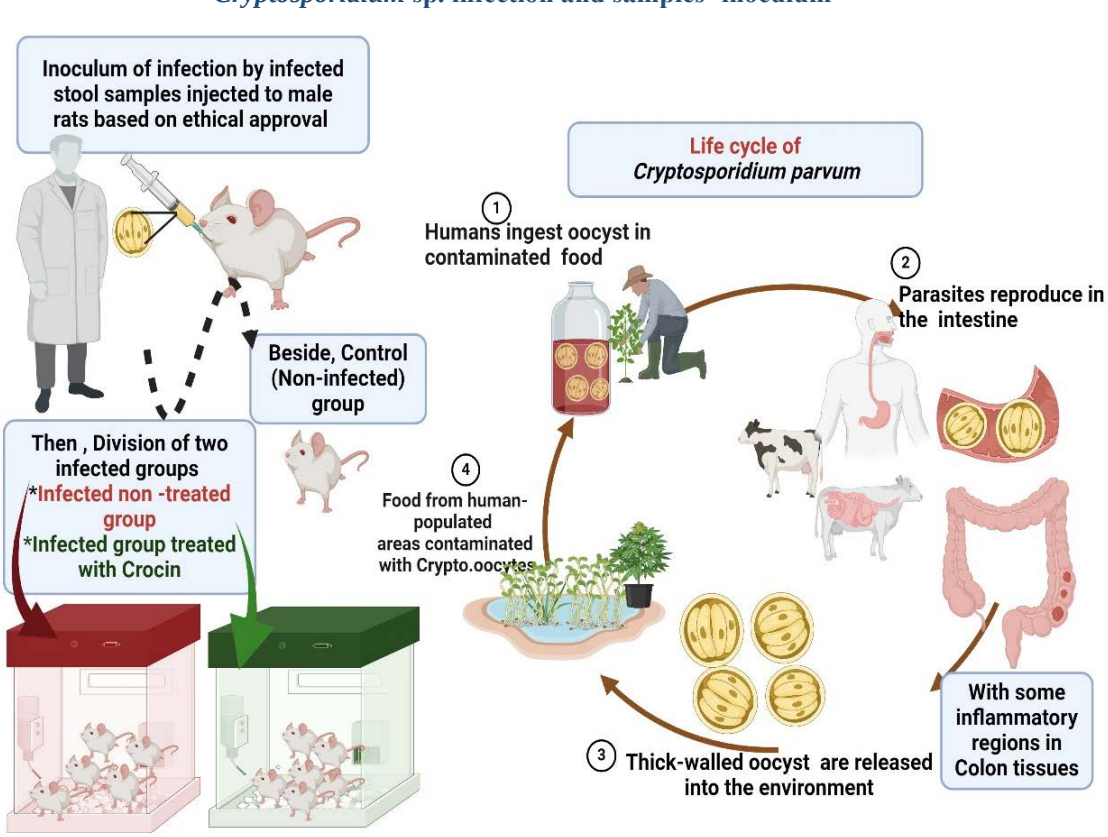

Fig. 3: Experimental outline and summary of *Cryptosporidium parvum* life cycle

Evaluation of the efficiency of the treatment

Parasitological and fecal examination: Eighteen fecal samples were collected and evaluated in the stained smears at the end of the treatment and the number of oocysts was detected/mg of feces using a hemocytometer (Marienfeld, Germany).

Evaluation of the hepatorenal functions: Twenty one blood samples were collected suddenly from the male rats after light anesthesia by (Ketamine/Xylazine) of different treated groups either infected or non-infected treated groups, samples were collected from the eye plexus as this is the highest and most purified blood and had more bleeding areas.

Samples of serum were used for the determination of both liver enzymes (AST and ALT) based on commercial kits (Biodiagnostic Co.).

Estimation of the antioxidant enzymes in liver tissues: Cold buffer saline was used to prepare liver tissue homogenates, which were subsequently used to estimate antioxidant enzymes and lipid marker peroxidation. The levels of malondialdehyde (MDA) were estimated using Ohkawa *et al.*[29] technique. The activity of superoxide dismutase (SOD) was estimated using the methods of Marklund and Marklund[30], Aebi[31] for measuring catalase (CAT) and Flohé and Günzler[32], for determination of glutathione peroxidase level (GPX), respectively.

Evaluation of lipid profile: The triglycerides (TG) and total cholesterol (TC) in the serum were measured in accordance with Lütjohann *et al.*[33]. The determination of HDL-C (High-Density Lipoprotein-Cholesterol) was based on the work of Warnick *et al.*[34]. Based on Friedewald *et al.*[35], the serum Low-Density Lipoprotein-Cholesterol (LDL-C) level was calculated. The triglyceride/5 is the value of VLDL-C and the risk ratios were calculated as follows: Risk ratios are; (I) Total cholesterol/HDL-C, (II) LDL-C/HDL-C and (III) triglycerides/HDL-C.

Transmission Electron Microscope (TEM) examination: Specimens from the colon tissues of the sacrificed male rats of the negative control group and infected groups either 2nd non-treated group or 3rd infected group treated with CR. Colon specimens were preserved in glutaraldehyde, then the tissues were suddenly fixed by serial procedures, then sections were imaged by JEOL TEM unit according to Hayat[36].

Statistical analysis: The statistical analysis Software Package for the Social Sciences (SPSS) version 26 (IBM Corp., USA) was used to analyze the data. Two-way Analysis of Variance (ANOVA) was used to compare the groups, along with multiple *post hoc* tests such as the Duncan's test. Statistical significance was defined as ($p<0.05$)[37].

RESULTS

Effect of treatment on fecal oocysts levels: Oocysts appeared in the stool samples from the 5th day of infection with *C. parvum* in all infected animal groups (Group II and III) until the 7th day without any apparent change or difference. On the 10th day, i.e., after three days of starting the treatment schedule, the number of shed oocysts significantly declined per mg of feces using a hemocytometer in the infected group treated with CR as shown in (Table 1).

Biochemical evaluation of hepatorenal functions and lipid profile: The results showed that infection with *Cryptosporidium parvum* afforded an increment in the liver enzymes (ALT and AST). Meanwhile, the treatment of the infected group with CR demonstrated a significant decline in the liver (Table 2).

The results showed elevation of both TC, TG, LDL-C, VLDL-C and all risk ratios with a significant decline in HDL-C in the infected non-treated group, which is contrary to all lipid profiles in the infected group and treated with CR which confirmed the great hypolipidemic effect of CR with amelioration of physiological hepatic functions and declining risk ratio of incidence either atherosclerosis or any coronary or heart diseases (Table 3).

Table 1: *Cryptosporidium* oocysts's intensity shedding in the stool samples of the various treated groups

	Days post infection			
Groups	2	5	8	15
I: Negative control group	0	0	0	0
II: Infected non-treated group	0	8.22±1.25*	6.23±1.35	2.52±0.45*
III: Infected group and treated with CR	0	4.36±0.85***	2.96±0.95**	0.41±0.15**

Data are represented as Mean±SE, N = 7 rats, *$p\leq0.05$, **$p\leq0.001$ and ***$p\leq0.0001$

Table 2: Effect of CR on infected group on liver functions expressed (Mean±SE)

Parameter	Control group	Infected non-treated group	Infected group+CR
ALT (U/L)	12.15 ± 1.05^{c}	52.45 ± 3.25^{a}	24.22 ± 2.25^{b}
AST (U/L)	17.42 ± 2.47^{c}	57.41 ± 2.85^{a}	20.39 ± 2.78^{bc}

Means of each category with distinct letters are significant at the $p\leq0.05$ level, the mean value was arranged alphabetically, ALT: Alanine Aminotransferase and AST: Aspartate Aminotransferase

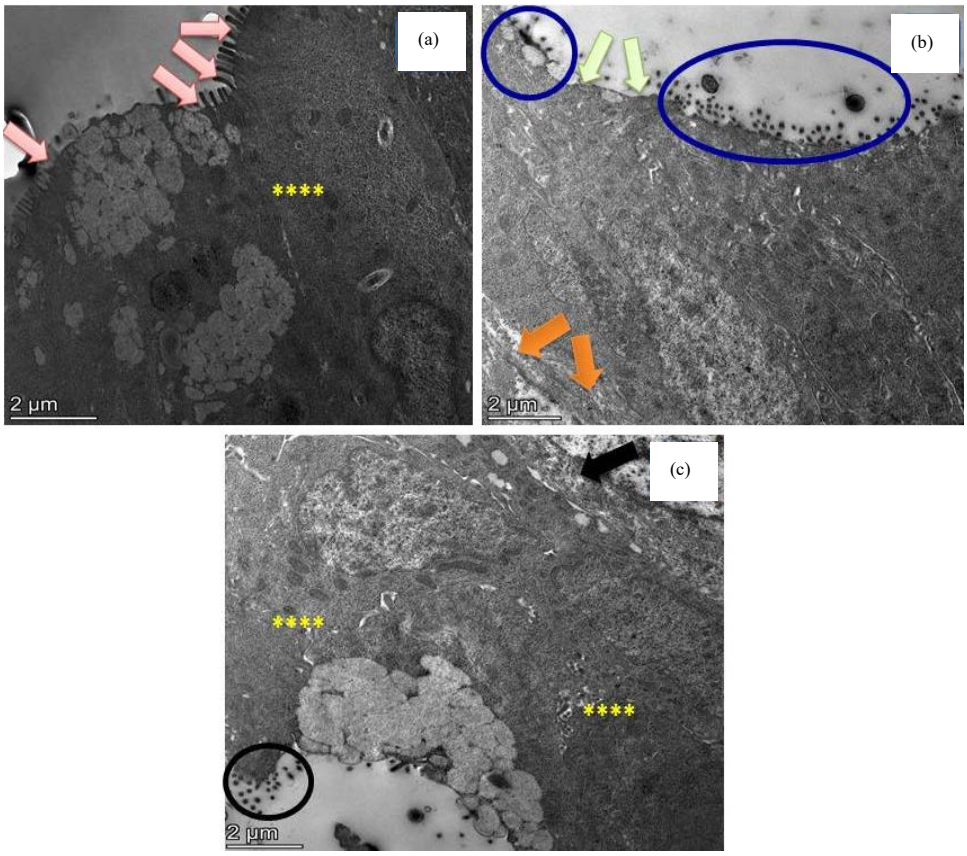

Fig. 4(a-c): TEM sections of colon tissues, (a) Control group, (b) Infected non-treated group and (c) Infected group treated with CR

(a) Colon sections from the control group showing normal tissue architecture of colon with no inflammatory infiltration (yellow asterisks) and normal crypts villi (red arrows) (scale bar = 2 µm), (b) Colon sections with loss of crypts (green arrows) and ulceration associated with colitis (orange arrow), as well as severe inflammatory cell infiltration (blue circles) (scale bar = 2 µm) and (c) Colon sections of the infected group treated with CR showed a reduction of the inflammatory infiltration markedly (black circle), restoring the normal architecture (yellow asterisk) while restoring normal mucosa architecture (black arrow) (scale bar = 2 µm)

Table 3: Effect of CR on infected group on liver functions expressed (Mean±SE)

Parameter	Control group	Infected non-treated group	Infected group+CR
TC (mg/dL)	92.68±3.24^{c}	166.85±5.47^{a}	97.58±5.24^{b}
TG (mg/dL)	55.98±3.25^{c}	81.63±4.25^{a}	70.58±3.69^{b}
HDL-C (mg/dL)	42.48±2.87^{a}	33.58±3.58^{c}	40.58±2.98^{b}
LDL-C (mg/dL)	30.58±3.78^{c}	37.98±3.28^{a}	32.88±3.78^{b}
VLDL-C (mg/dL)	11.87±1.89^{c}	18.69±2.69^{a}	13.58±2.78^{b}
Risk ratio (I)	2.18	4.96	2.40
Risk ratio (II)	0.71	1.13	0.81
Risk ratio (III)	1.31	2.43	2.23

Means of each category with distinct letters are significant at the $p \leq 0.05$ level, the mean value was arranged alphabetically, TC: Total cholesterol, TG: Triglycerides, HDL-C: High Density Lipoprotein, LDL-C: Low Density Lipoprotein and VLDL-C: Very Low-Density Lipoprotein

Table 4: Changes in antioxidant enzymes in hepatic tissues of different treated infected treated group with crocin (CR)

Parameter	Control group	Infected non-treated group	Infected group+CR
SOD (U/g)	23.48±3.42^{a}	8.13±1.65^{c}	20.82±2.87^{b}
CAT (U/g)	17.15±2.54^{ab}	7.45±1.75^{c}	16.45±2.17^{b}
GPx (U/g)	20.45±3.23^{ab}	16.45±3.45^{c}	19.16±3.98^{b}
MDA (U/g)	29.99±3.69^{bc}	58.99±4.35^{a}	29.05±3.37^{c}

Non: Infected group (Mean±SE), Means of each category with distinct letters are significant at the $p \leq 0.05$ level, the mean value was arranged alphabetically, SOD: Superoxide dismutase, CAT: Catalase, GPx: Glutathione peroxidase and MDA: Malondialdehyde

Changes in oxidative stress in infected non-treated and infected treated group with CR: The infected non-treated group induced marked elevation in MDA levels with a significant decline in SOD, CAT and GPx. Treatment of the infected group with CR afforded a significant decline in lipid peroxidation (MDA) which means a reduction in the oxidative stress levels and induced marked elevation of the antioxidant enzymes (SOD, CAT and GPx) enzymes. Results obtained in (Table 4) showed a high elevation of all antioxidant enzymes in the group treated with CR while lowering the marker of oxidative stress.

Transmission Electron Microscope (TEM) examination: The TEM sections of colon tissues, Control group showed colon tissues with normal tissue architecture appearance with no inflammatory infiltration (Fig. 4a). Infected non treated group showed loss of crypts and ulceration associated with colitis, as well as severe inflammatory cell infiltration (Fig. 4b). Infected group treated with CR showing colon sections with reduction of the inflammatory infiltration markedly with restoring the normal architecture (Fig. 4c).

DISCUSSION

The current study revealed the potent antioxidant activities of CR in the infected treated group with CR via amelioration of liver enzymes (ALT and AST), with elevation of the antioxidant enzymes (SOD, GPx and CAT) and decline of the lipid peroxidation marker (MDA), CR improved the lipid profile via increasing HDL-C levels and decline of LDL-C and VLDL-C levels as compared to infected non-treated group, with reduction of the coronary disease risk ratios, concurrent with amelioration of the colon ultrastructure. The TEM sections of colon tissues showed loss of crypts and ulceration associated with colitis in the infected non-treated group. Meanwhile, colon sections with the infected group treated with CR showed a reduction of the inflammatory infiltration markedly while restoring normal mucosa architecture.

One of the main causes of human diarrhea has been suggested to be a *Cryptosporidium* infection[38]. Diarrhea is a self-limiting illness that lasts 2 to 4 weeks, but in some clinical cases, it can be fatal. In light of the suggestions, trials were created to evaluate the effectiveness of specific active compounds or even some plant extracts for treating cryptosporidiosis in light of the WHO's plan to replace synthetic medical products with products from some medicinal plants[39]. Herbals have selective actions against parasites without reduction of host cell viability[40].

A protozoan parasite called *Cryptosporidium parvum* may be the source of waterborne illnesses. The parasite's ability to produce energy and maintain cellular metabolism depends on glycolysis. The primary regulators of glycolysis are the liver enzymes of *C. parvum*. The goal of anti-cryptosporidial medications is to specifically target and obstruct *C. parvum's* metabolic and biochemical pathways[41]. This is a crucial situation for the inhibition of numerous cellular receptors and the display of essential amino acids.

A few studies have examined the effectiveness of natural substances, in particular, essential oils against *C. parvum*[42]. Thus, the current study set out to evaluate the efficacy of CR, the saffron plant's active component, in treating male rats harboring a *C. parvum* infection. This is the first study that assesses CR anti-cryptosporidial activity *in vivo* in male rats. The results demonstrated that CR had positive structural effects on biochemical markers and antioxidant enzymes and it induced a promising effect against *C. parvum*.

By assessing the hepatic markers of oxidative stress, a lipid peroxidation biomarker, was elevated and SOD was lowered, suggesting that parasitic infection by *C. parvum* treatment induced an oxidant-antioxidant imbalance in the hepatic tissues. This result was consistent with the earlier research on *Giardia lamblia*[14]. It has been proposed that important inflammatory mediators are induced as a result of an increase in reactive oxygen species.

The results of earlier studies demonstrate that in renal tissue, CR reduced the rise in lipid peroxidation brought on by cisplatin which is consistent with the antioxidant effect of CR observed in hepatic tissues and its evident effect on the colon ultrastructure[43]. Rats' age-related renal oxidant-antioxidant imbalance has also been demonstrated to be improved by CR supplementation[14].

Lastly, CR avoided methotrexate-induced renal damage by maintaining the oxidant-antioxidant balance[44]. Reactive oxygen species and inflammatory molecules are released in connection with all inflammatory disorders[45].

The CR's antioxidant and free radical scavenging abilities underpin its anti-inflammatory effects. Additionally, it has been demonstrated that CR slows the development of diabetic nephropathy by adjusting the inflammatory cascade and the oxidative burden[46].

The current finding proved the great ameliorative effect of CR on the biochemical biomarkers and the vitality of the liver tissues with confirmation of the potent and promising antioxidant activities of CR against oxidative markers and its role was very clear in declining the oocysts number after the treatment regimen and declining the marker of lipid

peroxidation with enhancing the antioxidant enzyme levels including (SOD, CAT and GPx) enzymes with restoring the normal colon architecture to the normal status which proved it's potent capacities as a potent antioxidant agent and anti-parasitic agent in case of infection with *C. parvum*. Thus, the formula of CR could display good gastrointestinal stimulation, decline the effect of *C. parvum* and alleviate any other side and undesired effects. Finally, the parasitological, biochemical and ultrastructural investigation revealed the significant amelioration effect induced by CR against *C. parvum* infection.

CONCLUSION

The current study demonstrated that in male albino rats infected with cryptosporidiosis, CR exhibits a promising therapeutic effect. The CR holds a great promising effect as antioxidant, hepatoprotective and antiparasitic alternative agents for cryptosporidiosis with declined any other side effects. The CR exhibits a promising anti-parasitic effect against cryptosporidiosis and it is recommended to perform more experimentation regarding the efficacy of CR combined with other treatments and evaluate their efficacy prospectively.

SIGNIFICANCE STATEMENT

The current study was conducted to assess the parasitological effect of the protozoan *Cryptosporidium parvum*, which is known to parasitize humans, particularly children and individuals with compromised immune systems and is the cause of cryptosporidiosis and to assess the therapeutic efficacy of CR. Which has a high capacity to scavenge free radicals and a variety of pharmacological effects to be an alternative for a lot of anti-parasitic medications that may produce a number of undesirable side effects. The CR showed potential therapeutic effects as an antioxidant, hepatoprotective and antiparasitic agent. A lot of studies could be performed prospectively on some CR metal complexes and/or combined with other active compounds with a comparative evaluation of their novel effects as potent novel antiparasitic agents.

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