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Effect of Egyptian Propolis on Lipid Profile and Oxidative Status in Comparison with Nitazoxanide in Immunosuppressed Rats Infected with *Cryptosporidium spp.*

[1]Tamer H. Abd El-Aziz, [2]Nadia M. El-Beih, [1]Hamdy Soufy,
[1]Soad M. Nasr, [1]Fathia A.M. Khalil and [3]Mohamed Sharaf

[1]Department of Parasitology and Animal Diseases, National Research Center,
El-Behouse Street, Dokki, P.O. Box 12622, Giza, Egypt
[2]Department of Zoology, Faculty of Science, Ain Shams University, Cairo, Egypt
[3]Department of Phytochemistry and Plant Systematic, National Research Center,
El-Behouse Street, Dokki, P.O. Box 12622, Giza, Egypt

Abstract: The present study was conducted to evaluate the effect of Egyptian propolis on lipid profile and oxidative stress in immunosuppressed rats infected with *Cryptosporidium spp*. in comparison with nitazoxanide (NTZ) -as a standard drug- for treatment of cryptospordiosis. Evaluation of antioxidant activity of propolis was determined *in vitro* (DPPH free radical scavenging assay) and *in vivo* (dexamethasone-immunosuppressed rat model of cryptosporidiosis). One hundred and eighty adult male Sprague-Dawley rats of 190-220g BW were randomly divided into nine equal groups. Groups I, II and III were kept as normal control, immunosuppressed and infected control, respectively. Ethanolic extract of propolis (EEP), water extract of propolis (WEP) and NTZ were prepared at a dose 50, 50 and 100 mg/kg BW, respectively and orally administered to the groups of infected rats with different regimes. At the 15th day post infection (dpi), blood was collected from all groups for assessment of lipid profile. Antioxidant enzymes' activities and the levels of reduced glutathione (GSH) and GST-Pi1 mRNA were measured in ileum. Results revealed that both EEP and WEP showed DPPH anion scavenging activities at different concentrations but WEP possessed high antioxidant efficiency than EEP. The levels of total cholesterol, triglycerides (TGs) and low - density lipoprotein cholesterol significantly decreased in serum of infected rats treated with propolis, while, the level of TGs significantly decreased after NTZ administration compared to infected control rats. On the other hand, a marked increase in the levels of high-density lipoprotein cholesterol was recorded in serum of all treated-rats with WEP. In the infected rats treated with NTZ, EEP and WEP, there was a marked increase in the GSH level compared to infected rats. GST-Pi1 mRNA levels in the ileum of infected rats therapeutically treated with WEP was markedly elevated in comparison with infected control. In conclusion, propolis (EEP&WEP) administration exhibits anti-radical and antioxidant effects, which alleviates the oxidative stress caused by cryptosporidiosis.

Key words: Propolis · Rats · Cryptosporidiosis · Lipid Profile · Oxidative Status · GST-Pi1 mRNA Expression · Nitazoxanide

INTRODUCTION

Propolis (Bee glue) is a resinous sticky hive product that honeybees produce by mixing their own waxes and bee enzymes with resins collected from plants [1]. It is used as a folk medicine from ancient times.

The chemical composition of raw Egyptian propolis sample (Collected from Dakahlia Governorate) as investigated by GC/MS, 65 compounds were identified, such as aromatic acids: benzoic, cinnamic, trans-p-coumaric, 3,4- dimethoxycinnamic, ferulic and caffeic acids. Of the 19 esters identified, Egyptian propolis contained 11 caffeate esters including two new

Corresponding Author: Hamdy Soufy, Department of Parasitology and Animal Diseases, National Research Center, El-Behouse Street, Dokki, P.O. Box 12622, Giza, Egypt.

caffeate esters; tetradecenylcaffeate (Isomer) and tetradecanylcaffeate. Egyptian propolis contained some new triterpenoids including lupeol and alpha-amyrin. It also contained flavonoids, sugar and aliphatic acids. The investigators stated that Dakahlia propolis sample was a typical popular propolis [2]. The composition of the propolis depends upon the time, vegetation and the area of collection [3].

Propolis has several biological and pharmacological properties, as antimicrobial [4], anti-inflammatory [5], antioxidant [6, 7], antiparasitic [8], immune modularity and immune stimulant effects [9, 10], anti-atherosclerosis; reduced the hyper-cholesterolaemia, hypertriglycerdaemia and non-HDL-C, however, EEP had no significant effect on serum HDL-C [11].

Cryptosporidiosis is now recognized as a worldwide health risk to humans, especially to those individuals with compromised immune system which is responsible for the gastrointestinal illness cryptosporidiosis in humans [12].

Tissue damage due to excessive and unregulated generation of reactive oxygen species (ROS) has been shown after infection [13]. Fortunately, there are defensive mechanisms which have been evoked by the host against these reactive intermediates include the activity of various enzymes such as glutathione transferases (GSTs), glutathione peroxidase (GPx), catalase (CAT), as well as reduced glutathione (GSH) that scavenge ROS. Reduced glutathione (GSH) is the main non-protein thiol found in cells which, in conjugation with glutathione peroxidase (GPx) and glutathione-S-transferase (GST), plays a significant role in protecting cells against ROS. Moreover, hydrogen peroxide is converted by catalase into water (H_2O) and oxygen (O_2).

One of the most important drug-metabolizing or detoxication enzymes in liver and intestine are glutathione-S-transferases (GSTs) [14] which exhibit unique patterns of tissue-specific gene expression by structurally diverse drugs, carcinogens and other xenobiotics. Although GST activity in the small intestine of the rat was similar to those recorded in the liver and kidney [15] and GST-Pi1 was abundant in human intestine and colon compared to liver [16], little is known about the GSTs activity and its gene regulation (GST- Pi1) in the rat's ileum.

In the present study, we report the first attempt to evaluate the antioxidant activity of propolis to alleviate the oxidative stress induced by cryptosporidiosis in immunosuppressed rats. Also, determination of lipid profile in the serum in addition to GSH level, antioxidant enzymes activities and gene expression of glutathione-S-transference Pi class (GST-Pi1 mRNA) in ileum were performed. The obtained results were compared with that of nitazoxanide (NTZ) which was used as a commercial standard drug for treatment of cryptosporidiosis.

MATERIALS AND METHODS

This study was carried out according to guidelines for animal experimentation and approved by the Institutional Animal Care and Use Committee, National Research Centre Animal Care Unit, Dokki, Giza, Egypt.

Chemicals: Ellman's reagent (DTNB) [5, 5`-dithio-bis (2-nitrobenzoic acid)] was purchased from Sigma-Aldrich Chemical Co. (Poole, Dorset, UK) for estimation of GSH level. Reduced Glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Fluka Chemikalien, (Buchs, Switzerland) for determination of GST activity. All chemicals are of analytical grade.

Propolis: Propolis sample was collected from beehives located in Dakahlia Governorate, Egypt. The sample was kept in the dark and stored at -20°C up to its processing.

Ethanolic Extract of Propolis (EEP) and Water Extract of Propolis (WEP): Ethanolic and water extracts of propolis were prepared with some modification as described by Murad *et al*. [17] and Nagai *et al*. [18], respectively. Briefly, EEP was prepared by cutting 50 g of crude propolis into small pieces, grounded, extracted with 500 ml of 80% ethanol (1:10 w/v) and stirred continuously by shaking incubator (150 rpm) in the absence of light at room temperature for a week. After a week, the extract was filtered. The supernatant was evaporated to dryness in a rotary evaporator (Heidolph 2000, Germany) under reduced pressure at 40°C and stored at 4°C. To prepare WEP, 45 g of crude propolis was cut into small pieces, finely grounded and extracted with 5 volumes of distilled water, shaking at 30°C for 3 days. The extract was filtered. The obtained supernatant of WEP was concentrated by freeze-dryer (Labconco Lyophilizer, USA). The obtained dried ethanolic and water extract of propolis were suspended in phosphate buffered saline (PBS) (pH 7.2). The dose of propolis used in this experiment was 50 mg/kg BW.

Antioxidant Activities of EEP and WEP *in vitro*: The antioxidant activity of EEP and WEP were studied *in vitro* by radical scavenging assay which was

described by the method of Hatano *et al.* [19] using the stable free radical 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich). DPPH radical scavenging assay is an antioxidant assay based on electron-transfer that produces a violet solution in methanol. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. Changes in colour (From deep violet to light yellow) were read at 517 nm using spectrophotometer after incubation for 30 min.

Dexamethasone phosphate (DEX_P): It was used as immunosuppressive agent for rats before the experimental infection with *Cryptosporidium spp.* After determination of the daily water consumption rate for rats, the dexamethasone phosphate; DEX_P (Forticortin, Merck, Germany) was administered in the drinking water at concentration equivalent to 0.25 mg/kg/day [20]. Rats were maintained on dexamethasone treatment throughout the periods of treatment except the group of normal control.

Nitazoxanide (NTZ): NTZ is a synthetic nitrothiazolyl-salicylamide derivative and an anti- protozoan agent which was used as a commercial standard drug for treatment of cryptosporidiosis.

Parasite: Oocysts of *Cryptosporidium spp.* were obtained from neonatal Holstein-Friesian calves, aged from 3 to 15 days, at governmental farm at Abu-Rawash-Giza governorate, Egypt. The oocysts were used to infect 2-day old female goat kids (10^6/animal). Following the onset of oocyst shedding, faeces were collected daily, mixed with an equal volume of 2.5% potassium dichromate (for preservation of Cryptosporidium oocysts) and stored at 4°C. Faeces were sieved sequentially through stainless steel screens. Oocysts were purified by discontinuous sucrose gradients [21], counted using a hemacytometer under bright field microscopy and stored in 2.5% potassium dichromate at 4°C.

Animals Used: One hundred and eighty adult male Sprague-Dawley rats of 190-220 g body weight, obtained from Animal Breeding Colony, the Animal House, National Research Center, Giza, Egypt were used in the present study. All the rats were examined to insure that they were free from known murine pathogens including *Cryptosporidium spp*. Rats were randomly divided into 9 equal groups, each of 20 rats and housed in plastic cages with wire mesh tops and wood shavings for bedding in well ventilated animal room under standardized conditions (20±3°C; relative humidity 50±5% and 12 hours light/dark cycle). All nutrients including water were supplied *ad libitum* to meet the requirements of the NRC [22]. Rats were acclimatized for 15 days before the start of the experiment.

Experimental Design: This experiment was carried out at the Experimental rat Unit of Lab Animal House, National Research Center, Dokki, Giza, Egypt. Ten days after the dexamethasone administration in drinking water, one hundred and eighty adult male Sprague-Dawley rats were randomly divided into nine equal groups, each of 20 rats. Groups I, II and III served as normal control, immunosuppressed and infected control with 4×10^6 *Cryptosporidium spp.* purified oocysts, respectively. Rats in groups IV, V and VI were infected with 4×10^6 *Cryptosporidium spp.* purified oocysts and treated orally on 5th day-post infection (dpi) with 100 mg/kg BW of NTZ and 50 mg/kg BW of EEP and WEP for 7 consecutive days, respectively. Groups VII, VIII and IX, infected rats were treated orally with 100 mg/kg BW of NTZ and 50 mg/kg BW of EEP and WEP for three days before infection and then administered again the same dose on the 5th dpi for 7 consecutive days, respectively. Scheme for the experimental design and plan of treatment were illustrated in Table (1) and Figure (1), respectively. All groups were observed daily for recording the mortality rate allover the experimental period. From each group, blood samples were taken for determination of serum lipid profile. Specimens from ileum tissue were taken for oxidative stress investigations and GST-Pi1 mRNA expression.

Blood Samples: Blood samples were collected by puncture of retro orbital plexus from each rat in all groups at the 15th dpi in the early morning before diet was offered. The blood sample was collected in a plain tube, left to clot at 37°C for 1 h and then centrifuged at 3000 rpm for 15 min for serum separation. Serum samples were stored at -20°C until further biochemical analyses.

Serum Lipid Profile Assessments: Determination of total cholesterol [23], triglycerides [24], high- density lipoprotein cholesterol (HDL-C) [25] was determined. Low- density lipoprotein cholesterol (LDL-C) level was calculated according to the equation of Friedewald *et al.* [26]. Test kits supplied by bioMérieux-France except LDL-C kit supplied by Bio-diagnostic, Dokki, Giza, Egypt.

Table 1: Scheme for the experimental design.

			Ethanol extract		Water extract		Nitazoxanide	
Groups	DEX	Infection	Before Infection	After Infection	Before Infection	After Infection	Before Infection	After Infection
I	-	-	-	-	-	-	-	-
II	+	-	-	-	-	-	-	-
III	+	+	-	-	-	-	-	-
IV	+	+	-	-	-	-	-	+
V	+	+	-	+	-	-	-	-
VI	+	+	-	-	-	+	-	-
VII	+	+	-	-	-	-	+	+
VIII	+	+	+	+	-	-	-	-
IX	+	+	-	-	+	+	-	-

GI: normal control. GII: immunosuppressed, GIII: immunosuppressed and infected with *Cryptosporidium spp.*, GIV: immunosuppressed, infected and treated with nitazoxanide (NTZ), GV: immunosuppressed, infected and treated with ethanolic extract of propolis (EEP), GVI: immunosuppressed, infected and treated with water extract of propolis (WEP), GVII: immunosuppressed, infected and treated with NTZ before and after infection. GVIII: immunosuppressed, infected and treated with EEP before and after infection, GIX: immunosuppressed, infected and treated with WEP before and after infection.

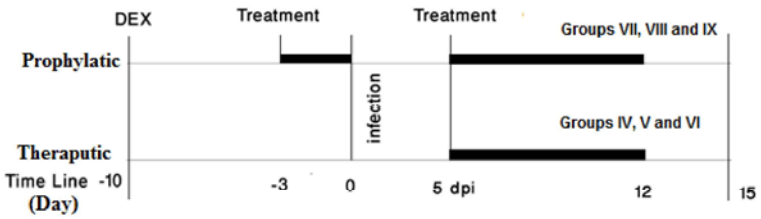

Fig. 1: Plan of prophylactic and therapeutic treatment with ethanolic and water extracts of propolis and nitazoxanide in immunosuppressed rats infected with *Cryptosporidium spp.* during the period of treatment (25 days).

Ileum Tissue Sample: All rats were euthanized by over dose of chloroform at the 15th dpi. From each rat, ileum was rapidly removed, washed with 0.9% ice-cold NaCl and stored at -80ºC for preparation of homogenate and RNA isolation.

Assessment of Antioxidant/oxidant Status: Preparation of ileum homogenate: Ileum tissue was homogenized in ice-cold 1.15% solution of potassium chloride in 50 mM L^{-1} potassium phosphate buffer solution (pH 7.4) to yield intestine homogenate 10% (W/V). Then the homogenate was centrifuged at 4,000×g for 5min at 4°C. The supernatant was collected and used for determination of GSH level and the activities of GST, GPx and CAT.

Ileum Oxidative Status Assessment and Determination of Total Proteins: GSH (mM g^{-1} tissue) content in ileum tissue homogenate was determined using Ellman's reagent according to the method described by Ellman [27]. The activity of GST (nM min^{-1} mg^{-1} protein) in ileum homogenate was determined according to Habig *et al*. [28]. Ileum GPx activity (µM min^{-1} mg^{-1} protein) was measured by the method of Paglia and Valentine [29]. The ileum CAT activity (U g^{-1} tissue) was measured according to Aebi [30]. Total protein concentration in ileum tissue homogenate was measured according to the method of Lowry *et al.* [31], using bovine serum albumin as a standard and the total proteins expressed as mg/ml homogenate. Total protein concentration was used to express the enzymatic activity as /mg protein. Test kits of GPx and CAT were supplied by Bio-diagnostic, Dokki, Egypt.

Glutathione-S-Transferase (GST) mRNA Expression in Ileum Tissue:

RNA preparation and cDNA synthesis: Total RNA was extracted from the frozen ileum according to the acid guanidinium thiocyanate - phenol - chlorophorm single step method of Chomczynski and Sacchi [32]. Prior to cDNA synthesis, 3 µl of total RNA was digested with Deoxyribonuclease I (RNase-free DNase I, Fermentase, Thermoscientific - USA) to avoid genomic contamination.

Table 2: Primers' sequence of glutathione-S-transferase (GST) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), predicted amplification product size and annealing temperature.

Primers	Primer sequence	Product size, bp	Annealing Temperature
GST			
Forward	5`-TGTCTGTATGGGCAGCTCC-3`	223 bp	58°C
Reverse	5`-CCTTCACRTAGTCATCCTTACC-3`		
GAPDH			
Forward	5´-CAAGGTCATCCATGACAACTTTG-3´	496 bp	58°C
Reverse	5´-GTCCACCACCCTGTTGCTGTAG-3´		

Subsequently, 1 µl DNase I-treated RNA was reverse transcribed in 20 µl reaction's mixture primed with Oligo dT_{18} using RevertAid First Strand cDNA Synthesis Kit (Fermentase, Thermoscientific, USA) according to the manufacturer's instructions.

Quantitative Real-Time RT-PCR (qRT-PCR): All qRT-PCR experiments were performed on the (Eppendorf, Realplex instrument, Germany) for the signal detection and analysis. Highly purified salt-free primer for glutathione-S-transferase Pi class (GST-Pi1) was synthesized according to Schülke *et al*. [33]. The housekeeping gene transcripts like glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal standard to control for error between samples. The sequences for the primers and the predicted sizes were illustrated in Table (2). For qRT-PCR, Maxima SYBR Green qPCR Master Mix (Fermentase, Thermoscientific-USA) was used in accordance with the manufacturer's instructions. After initial incubation at 95°C (10 min) 40 cycles followed, each comprising denaturation (95°C, 15 s), annealing/extension (60°C,60 s). In negative controls, the template cDNA was replaced with sterile distilled water. A negative control was performed for each reaction series. The threshold cycle (Ct), a number at which the increase in the signal associated with exponential growth of PCR products was recorded. The ratio between the Ct values of target gene (GST) and that for GAPDH (internal control) was calculated to normalize the initial variation in samples [34].

Statistical Analysis: All data were subjected to statistical analysis including the calculation of the mean and standard error. Differences between control and treated groups were tested for significance using a one-way analysis of variance followed by Duncan's multiple range test. Data of the antioxidant activities of EEP and WEP *in vitro* in the same concentration was evaluated by Student *t*-test at level $P<0.05$. Differences were considered significant at $P<0.05$ level [35] using SPSS version 15.0 computer program.

RESULTS

Mortality Rate: The mortality rate among all treated groups was lower than the controls (46%) except for that observed in NTZ-treated group (55%). In the rats treated with water extract, the mortality rate was the lowest among all treated groups (30%).

Antioxidant Activities of EEP and WEP *in vitro*: Both propolis extracts showed DPPH anion scavenging activities at different concentrations (0.125, 0.25, 0.5, 1 and 2 mg/ml). At different concentrations, the antioxidant activity percentages (AA%) of WEP were significantly higher than those of EEP (Table 3).

Serum lipid Profile: Results of lipid profile of rats were illustrated in Table (4). Compared to normal control rats (Group I), the present data revealed that DEX administration (Group II) caused significant elevation in serum level of TC, TGs and LDL-C. However, there was no significant change in serum HDL-C. The serum level of TC, TGs and LDL-C was significantly elevated in *Cryptosporidium*-infected rats (Group III) in comparison with immunosuppressed rats (Group II).

Compared to infected control (Group III), NTZ administration (Groups; IV and VII) caused a significant reduction in TGs level, while no significant changes were noticed in the other lipogram values.

In experimentally infected rats treated with EEP (Groups; V and VIII) or WEP (Groups; VI and IX), there was a significant decrease in the TC and TGs concentration in comparison with infected control (Group III).

With regard to HDL-C, there was a marked increase in infected rats treated with WEP (Groups; VI and IX), while no significant increase was noticed in infected rats treated EEP (Groups; V and VIII) compared to infected control (Group III). Conversely, the level of LDL-C was significantly decreased in groups; V, VI and IX.

Table 3: Antioxidant activity (AA%) of the ethanol and water propolis extracts at different concentrations. (Mean ± SE)

	Percentage of antioxidant activity (AA %)		
Concentrations (mg/ml)	Ethanol extract of propolis	Water extract of propolis	*t*-value
0.125	30.27±0.25[a]	78.12±1.01[a]	-46.039***
0.250	45.00±0.16[b]	79.26±1.24[a]	-27.480***
0.500	63.87±0.17[c]	79.63±0.08[a]	-81.984***
1.000	71.54±0.23[d]	83.41±0.30[b]	-31.943***
2.000	78.02±0.08[e]	86.63±0.90[c]	-9.509**
F-value	11264.788***	18.012***	

Means with different superscripts in the same column are significantly different at $P<0.05$.
** = $P<0.01$. *** = $P<0.001$.

Table 4: Lipogram in different experimental groups of dexamethasone-immunosuppressed rats treated with nitazoxanide (NTZ), ethanol extract of propolis (EEP) and water extract of propolis (WEP) before and after *Cryptosporidium* spp. infection. (Mean ± SE)

Parameters				
Groups	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
I	70.94 ± 3.44[a]	97.26 ± 4.70[a]	31.07 ± 2.46[a-c]	20.42 ± 0.72[a]
II	108.57 ± 2.04[b]	135.42 ± 7.84[b]	31.55 ± 1.92[a-c]	49.93 ± 3.75[b]
III	151.66 ± 5.97[c]	259.12 ± 5.79[e]	28.55 ± 1.70[ab]	71.29 ± 4.33[cd]
IV	143.68 ± 6.26[c]	194.59 ± 7.76[d]	27.76 ± 2.79[a]	77.01 ± 6.09[d]
V	124.66 ± 4.10[b]	168.21 ± 4.29[c]	35.00 ± 2.66[bc]	56.01 ± 1.80[b]
VI	120.88 ± 3.27[b]	143.23 ± 7.18[b]	38.07 ± 0.37[cd]	54.17 ± 2.13[b]
VII	144.28 ± 9.49[c]	171.66 ± 4.30[c]	34.36 ± 2.61[a-c]	75.59 ± 9.21[d]
VIII	124.13 ± 4.37[b]	141.49 ± 5.75[b]	35.55 ± 1.83[b-d]	60.28 ± 3.98[bc]
IX	121.07 ± 4.65[b]	141.24 ± 4.05[b]	41.99 ± 2.10[d]	50.84 ± 4.58[b]

Means with different superscripts in the same column are significantly different at $P<0.05$.
HDL-C= High density lipoprotein cholesterol. LDL-C= Low density lipoprotein cholesterol.

Table 5: Reduced glutathione concentrations and antioxidant enzymes activities in ileum homogenates in different experimental groups of dexamethasone-immunosuppressed rats treated with nitazoxanide (NTZ), ethanol extract of propolis (EEP) and water extract of propolis (WEP) before and after *Cryptosporidium* spp. infection. (Mean ± SE)

		Antioxidant Enzymes Activities		
Parameters Groups	Reduced Glutathione (GSH) (mM g^{-1} tissue)	Glutathione S-transferase (GST) (nM min^{-1} mg^{-1} protein)	Glutathione peroxidase (GPx) (μM min^{-1} mg^{-1} protein)	Catalase (CAT) (U g^{-1} tissue)
I	5.45 ± 0.28[a-c]	0.07 ± 0.01[a]	0.49 ± 0.03[bc]	0.08 ± 0.00[a]
II	4.74 ± 0.27[ab]	0.24 ± 0.02[e]	0.55 ± 0.04[b-d]	0.11 ± 0.00[bc]
III	4.62 ± 0.10[a]	0.12 ± 0.01[b]	0.67 ± 0.06[d]	0.13 ± 0.00[d]
IV	5.79 ± 0.57[b-d]	0.13 ± 0.01[b]	0.44 ± 0.03[ab]	0.10 ± 0.00[bc]
V	5.16 ± 0.42[c-e]	0.17 ± 0.01[cd]	0.66 ± 0.02[cd]	0.10 ± 0.01[bc]
VI	6.62 ± 0.48[de]	0.12 ± 0.00[b]	0.86 ± 0.08[e]	0.11 ± 0.01[cd]
VII	5.92 ± 0.27[cd]	0.13 ± 0.01[bc]	0.32 ± 0.03[a]	0.10 ± 0.01[ab]
VIII	7.07 ± 0.51[ef]	0.17 ± 0.01[d]	0.65 ± 0.05[cd]	0.10 ± 0.00[bc]
IX	7.76 ± 0.32[f]	0.15 ± 0.02[b-d]	0.94 ± 0.09[e]	0.13 ± 0.01[d]

Means with different superscripts in the same column are significantly different at $P<0.05$.

Reduced Glutathione Results of GSH Andantioxidant Enzymes Activities in Ileum Tissue: Results of *GSH and antioxidant enzymes activities in ileum tissue* were shown in Table (5).

Compared to normal control group (Group I), rats exposed to DEX (Group II) showed non-significant decrease in the level of GSH content. Also, there was non-significant decrease in GSH level in the infected group (Group III) compared to DEX group (Group II).

Compared to infected group (Group III), the level of GSH was significantly increased after NTZ treatment (Groups; IV and VII). Infected rats treated with EEP (Group V and VIII) or WEP (VI and IX) demonstrated a marked increase in the GSH level in comparison with infected rats (Group III).

In DEX-immunosuppressed rats (Group II), a significant increase in GST activity was observed in comparison with normal control (Group I). In contrast,

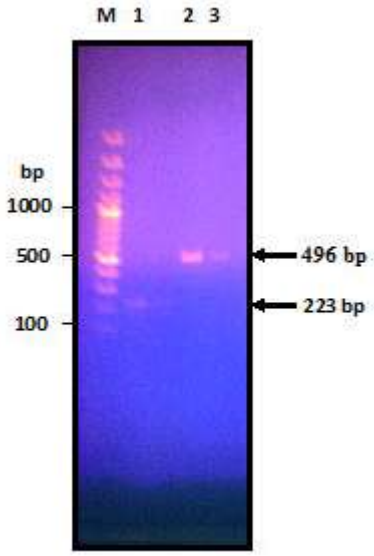

Fig. 2: PCR agarose gel electrophoresis using specific primers for glutathione s-transferase (lane 1) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control (Lane 2 and 3), M, 100 bp ladder.

Table 6: Changes in cycle threshold (C_t) ratio of GST and GAPDH (internal control) in different experimental groups of rat infected with *Cryptosporidium* spp. and treated with nitazoxanide, ethanol and water propolis extracts (Mean ± SE).

Groups	$C_{t(GST)}/C_{t(GAPDH)}$ ratio
I	0.93 ± 0.01^{b}
II	0.87 ± 0.01^{ab}
III	0.86 ± 0.01^{ab}
IV	0.89 ± 0.05^{ab}
V	0.85 ± 0.04^{a}
VI	1.00 ± 0.02^{c}
VII	0.87 ± 0.03^{ab}
VIII	0.87 ± 0.04^{ab}
IX	0.91 ± 0.01^{ab}
F-value	10.503***

Means with different superscripts in the same column are significantly different at $P < 0.05$.

*** = $P < 0.001$.

GST activity was markedly decreased in infected rats (Group III) compared to DEX group (Group II). A significant increase in GST activity was observed in the infected rats treated with EEP (Groups; V and VIII), while WEP (Groups; VI and IX) and NTZ (Groups IV and VII) showed non-significant changes compared to infected group (Group III).

In rats exposed to DEX in drinking water (Group II), the data revealed non-significant increase in GPx activity in comparison with normal control (Group I). Also, non-significant increase in the GPx activity was observed in infected rats (Group III) compared to DEX group (Group II). However GPx activity was significantly increased in infected rats treated with WEP (Groups; VI and IX), there was a significant decrease in NTZ-treated rats (Groups IV and VII) compared to infected rats (Group III).

Compared to normal control (Group I), a significant increase was observed in CAT activity in rats administered DEX (Group II). Also, a marked increase was recorded in infected rats (Group III) in comparison with DEX group (Group II). In the experimentally infected rats treated with EEP (Groups; V and VIII) and NTZ (Groups IV and VII), there was a relevant decrease in CAT activity in comparison with infected group (Group III). On the other hand, non-significant changes were noticed in CAT activity in WEP-treated group (Groups; VI and IX).

GST mRNA expression in the ileum tissue: The efficiency of reverse transcription reaction was assessed by PCR using cDNA as a template and specific primers for GST and GAPDH (Figure, 2). Results of GST mRNA expression in the ileum are illustrated in Table (6).

Compared to normal control (Group I), rats exposed to DEX (Group II) caused a non-significant reduction in GST-Pi1 mRNA level in the rat's ileum. Also, the same record was found in rats infected with Cryptosporidium oocysts (Group III) in comparison with DEX-treated group (Group II).

Compared to infected group (Group III), the GST-Pi1 mRNA level in ileum was not significantly changed after NTZ treatment (Groups; IV and VII).

The GST-Pi1 mRNA level was markedly elevated in ileum of infected rats therapeutically treated with WEP (Group VI) in comparison with infected control (Group III), while infected rats treated with EEP (Groups; V and VIII) and WEP-treated before and after infection (Group IX) showed non-significant changes in GST-Pi1 mRNA level.

DISCUSSION

The present work has addressed several parameters to better understand the response of multiple antioxidant networks after cryptosporidiosis and the role played by propolis in the modulation of lipid profile, mucosal antioxidant defense capacity as well as GST-Pi1 mRNA gene expression in ileum was evaluated.

There is an increasing interest for use natural antioxidants rather than synthetic ones to prevent the deleterious effects of free radicals. WEP and its main

constituents (caffeoylquinic acids derivatives) have greater antioxidant effect (2-fold more potent) than EEP [36, 37] which was confirmed *in vitro* by DPPH free radical scavenging assay.

In the present study, DEX administration caused significant increase in serum level of TC, TGs and LDL-C, while non-significant difference in serum HDL-C level was reported. DEX has been successfully used to induce hyperlipidemia in rats [38]. The hyperlipidemic action of DEX could be due to the stimulation of liver and intestine to secrete VLDL-C and inhibition of VLDL-C and VLDL-TGs removal from plasma by lipoprotein lipase depression after glucocorticoid treatment [39]. Moreover, the activity of lecithin cholesterol acetyl transferase has also been inhibited in rats treated with DEX which in turn leads to increase in free cholesterol [40].

Studies of Farid *et al*. [41] have confirmed that infection by the gastrointestinal nematode *Nippostrongylus brasiliensis* significantly reduced serum paraoxonase-1 activity that is an HDL-associated enzyme with anti-atherogenic properties. This reduction was associated with an atherogenic lipid profile in rats.

Results of serum lipids revealed that the EEP had a lowering effect on serum TC and TGs which may be attributed to the presence of flavonoids, steroids, phenolic acids and their esters among propolis constituents [42]. Fuliang *et al.* [43] reported that oral administration of propolis significantly lowered total cholesterol and triglycerides in serum of rats. Alves *et al.* [44] reported that the hypocholesterolemic effect of propolis could be a result of a direct effect on the liver or an indirect effect through thyroid hormones which affect reactions in almost all the pathways of lipid metabolism.

The inducible antioxidant capacity of the small intestine mucosa after cryptosporidial infection and propolis treatment has not been well documented.

GSH is an important non-protein thiol which, in conjugation with GPx and GST, plays a significant role in protecting cells against the cytotoxic effect of ROS. The decrease in GSH level in the immunosuppressed group may be attributed to the hyperlipidemic action of DEX [45]. DEX induced an increase in the levels of VLDL-C and TGs, thus accelerating their oxidation, the process which serves as the potential source of the toxic ROS [46]. In this study, a non-significant decrease in the GSH levels was recorded in rats infected with *Cryptosporidium*. A similar decrease in the GSH was observed in large intestine of rat infected with rat pinworm *Syphacia muris* [47]. The decrease in GSH levels may be due to decrease in the precursor of amino acids levels (Cysteine and cystine) available for GSH synthesis [48]. Flavonoids exhibit a wide range of biological effects including free radical scavenging and antioxidant activities. Therapeutic activities of propolis depend mainly on the presence of flavonoids. The increase in GSH concentration in rats infected with cryptosporidium and treated with EEP and WEP may be attributed to flavonoide which modulates expression of an important enzyme γ-glutamylcysteine synthetase in the synthesis of the GSH [49].

The glutathione-S-transferases (GSTs) are a family of proteins that catalyze the conjugation of glutathione on the sulfur atom of cysteine to various electrophiles [50]. Our results showed that the existence of Cryptosporidium in ileum produced a marked decrease in GST activity. It was confirmed by Verhulst *et al.* [51], who observed that patients with a chronic *Helicobacter pylori* infection had a decreased GST activity in their gastric mucosa. This might be due to a reduced detoxification capacity and therefore, an increased risk for developing enteritis. A significant increase in the total GST activity was observed in small and large intestine in *Trichinella spiralis* infected mice [52].

GPxs constitute a family of isoenzymes that catalyse the reduction of H_2O_2 or organic hydroperoxides to water or their corresponding alcohols using reduced glutathione (GSH) as an electron donor and/or other reducing equivalents [53]. In experimental cryptosporidiosis of rats, non- significant increase in GPx activity was observed. The increase in GPx activity in rats could be due to greater demand for this enzyme related to increases in hydrogen peroxide and/or lipid peroxides which are decomposed by this enzyme. A decrease in GPx activity due to glucocorticoids' treatment has also been reported in the hippocampi of rats [54]. DEX treatment decreases CAT activity in the hypothalamus [55]. A marked increase of GPx activity in rats administered DEX was achieved. Our result was in agreement with Virgili *et al.* [56] who mentioned that after treatment with DEX, GPx, CAT and GST activity markedly increased in duodenum and jejunum of zinc-deficient rats.

In the present study, GPx level appeared to be unaffected by propolis. But, a relevant increase was recorded in the infected rats treated with WEP which reflect the major role of GPx in cryptosporidiosis control in rat intestine. Some authors have underlined the occurrence of alterations in GPx and CAT activities upon the administration of propolis. Jasprica *et al.* [57] reported that propolis caused increase in GPx and CAT activities. Furthermore, Soboèanec *et al.* [58] showed that Croatian native propolis increased the activity of CAT and had no effect on the activity of GPx.

Interestingly, propolis extracts administration especially WEP significantly increased the level of GST-Pi1 mRNA which may be due to the phenolic compounds. This finding was also confirmed by Hayes *et al*. [59], Yen [60] and Yeh *et al*. [61] who studied the gene activating effects of phenolic compounds on SOD, GPx, CAT and GST mRNA levels in different tissues. Furthermore, Atta *et al*. [7] studied the enhancing effects of Egyptian propolis on SOD, Gpx and CAT mRNA levels in renal tissues of rats.

CONCLUSION

Propolis extracts modulate the activities of intestinal antioxidant enzymes and GSH levels in rats' cryptosporidiosis especially the water extract which demonstrated anti radical activity. Also, from the previous data it was concluded that GPx plays the main antioxidant enzyme present in the intestine which represents the first line of defense against the ingested toxins and xenobiotics.

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