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Antibacterial activity of *Commiphora molmol* against *E. coli* O157 infection

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ABSTRACT

Commiphora molmol is one of the plants that have been treasured since ancient times due to their effective applications in traditional medicine. The aim of this investigation was to evaluate the effect of *C. molmol* on mice infected with *E. coli* O157 with special reference to antibody production and histopathological changes. *C. molmol* water extract showed high antibacterial activity *in vitro* when compared with the hindrance activity of the reference antibiotic ciprofloxacin disk (CIP5) 5µg/ml. *In vivo* experimental study revealed that *E. coli* O157 count significantly decreased while Antibody titer detected by ELISA showed a trend to decrease in infected mice following the administration of *C. molmol* water extract. Histopathological findings showed the presence of less degenerative changes following the administration of *C. molmol* water extract. These findings revealed that *C. molmol* plant induced suppression to *E. coli* O157 infection and indicated the possibility of using *C. molmol* water extract as natural and safe alternative to synthetic antibiotics to control *E. coli* O157 infection.

KEY WORDS: *Commiphora molmol,* Antimicrobial activity, Antibody production, Histopathological changes, *E. coli* O157.

1. INTRODUCTION

The therapeutic effect of natural products has been documented since ancient times (Buenz, 2006; Norton, 2006). In spite the current progress in combinatorial chemistry, the contribution of drugs derived from natural products in drug discovery is still enormous. Most antibacterial agents used in clinical practice are either produced from natural products or similar to natural products (Bush, 2004; Appelbaum and Jacobs, 2005; Butler, 2005). *Commiphora molmol* (myrrh) is one of the plants that have been treasured since ancient times due to their effective applications in traditional medicine. *C. molmol* is a shrub like thorny tree of family Burseracae, which grows in North East Africa and Arabia. Myrrh is an oleo-gum-resin, obtained from *C. molmol* and is known in Arabic as "murr" which means "bitter" due to its bitter taste. Myrrh was used by ancient Egyptians for mummification and as an ingredient of the holy anointing oil of the Old Testament. It is collected through incisions in the bark of the plant (Hillson, 1988; Michie and Cooper, 1991; Hanus, 2005). *C. molmol* was used in the treatment of a variety of medical conditions (Hillson, 1988; Sheir, 2001; Tipton, 2003). Myrrh oil has been reported to have anti-inflammatory activity by reducing the production of proinflammatory cytokines (Tipton, 2003), antishistosomiasis chemotherapeutic activity (Sheir, 2001), anti-gastric ulcer activity (Al-Harbi, 1997), antithrombotic activity (Olajide, 1999) and antitumor activity (Qureshi, 1993).

Infectious diseases, caused by several infectious agents are responsible for great percentage of annual worldwide deaths (World Health Report 2009). *Escherichia coli* O157:H7 in particular, has been responsible for multiple food and waterborne outbreaks of diarrhea and/or hemorrhagic colitis (HC) worldwide (Mohawk and O'Brien, 2011). Antimicrobial agents are essential for controlling infectious diseases. Unfortunately, the effectiveness of antimicrobial agents is shrinking as a result of the development and spread of drug resistant pathogens (Lowy, 1998). Some pathogens became resistant to all kinds of antibiotics (Mandal, 2009). Antibiotic resistant strains represent serious threats to public health in humans as well as various animal groups such as pigs and cattle (Fischbach and Walsh, 2009). Natural products either separately or in combination with antibiotics has been used effectively to overcome the problem of antibiotic resistance in infectious diseases (Hemaiswarya, 2008; Genilloud, 2012).

Detailed understanding of the events that are triggered in a host after microbial infection is pivotal in the process of developing new therapies and vaccines for infectious diseases. The importance of experimental trials in this context emphasizes the importance of animal models in the field of infectious diseases. Mice are an ideal organism in which to understand both human and animal infectious diseases. The aim of this investigation was to evaluate the effect of *C. molmol* on mice infected with *E. coli* O157 with special reference to antibody production and histopathological changes.

2. MATERIALS AND METHODS

Animals: A total of 20 adult male Swiss albino mice with an average weight of 22-25g were obtained from the Laboratory Animal House of the National Research Center, Dokki, Egypt. They were acclimatized to the laboratory conditions with a lighting schedule of 12 hours light for a period of 15 days before being used. Animals were fed ad libitum with standard laboratory diet. The environmental conditions were properly standardized with respect to temperature, humidity and light. Mice in all groups were given 5 mg/ml streptomycin in their drinking water one day before oral infection with the bacteria. The experiments were performed based on animal ethics guidelines of NRC Animals Ethics Committee.

Oil Extraction: The essential oil was extracted from commercial oleoresin of *C. molmol* by steam distillation method according to (Rohloff, 2002). The essential oil yield was 4%.

Preparation of Oil Solution: A total weight of 8 gm of oil and 8 gm of Tween 80 (Sigma, USA) were mixed together and the resulting mixture was diluted to 1000 cm^3 distilled water and mixed very well. The solution was kept at 4°C. The final concentration of oil used in oral administration to each mouse daily was equivalent to the effective dose of the whole resin used (Al-Harbi, 1994).

***In vitro* Evaluation of the Antimicrobial Activity of *C. molmol*:** Antibacterial activity of Commiphora aqueous extract was evaluated *in vitro* against *E. coli* O157 using agar well diffusion method (Sgouras, 2004; Bauer, 1966). Muller Hinton agar plates were inoculated with selected bacterial reference strains prepared in concentration equivalent with 0.5 McFarland (1.5×10^8) and streaked onto the agar plates using sterile swabs. A total of 50 µl of the oil solution were placed into the wells under sterile conditions. All plates were incubated at 37°C/24 hrs for bacterial growth. Zones of inhibition were measured in mm using a ruler. The experiment was carried out in duplicate and the mean of the zones of inhibition was calculated.

***In vivo* Evaluation of the Antimicrobial Activity of *C. molmol*:** A total of 20 male mice were used to study the effect of *C. molmol* oil on mice infected with *E. coli* O157. After 1 day of streptomycin treatment, mice were then starved for food for 12 h before inoculation. They were divided into 4 groups (Table.1).

Table.1. Experimental design

Group No.	Group Name	Treatment			No. of animals
		Tween 80	Commiphora Oil	*E. coli*	
1	Tween 80 Control	+	-	-	5
2	Commiphora Control	-	+	-	5
3	*E. coli* O157 Control	+	-	+	5
4	Commiphora + *E. coli* O157	-	+	+	5

The first group was served as Tween 80 control and received a daily dose of 0.5 cm^3/mouse of 0.005% Tween 80 through oral administration. The second group was served as Commiphora Control group and received a daily dose of 0.5 cm^3/mouse of the oil solution. The third group was served as *E. coli* O157 Control and was infected with standard strain of *E. coli* O157 through oral administration of a single dose equivalent with 0.5 McFarland (1.5×10^8) (0.5ml/mouse at day 7). The fourth group was served as Commiphora + *E. coli* O157 and was given a dose of (0.5ml/day form day 2- day 7) of Commiphora extract through oral administration followed by a single dose of 0.5ml *E. coli* O157 at the seventh day of experiment equivalent with 0.5 McFarland (1.5 ×108) through oral administration. The fourth group was further given a daily dose of 0.5 cm^3/mouse of the oil solution while the third group was further given a daily dose of 0.5 cm^3/mouse of 0.005% Tween 80 through oral administration. The administration of Tween 80 in the first and third groups (0.5 cm^3/mouse of 0.005% Tween 80) as well as the administration of oil solution in the second and fourth groups (0.5 cm^3/mouse) started 7 days before *E. coli* infection and were continued until the end of the experiment.

Quantification, Isolation and Identification of *E. coli*: Fecal samples were collected from each animal one day before bacterial inoculation (Day 6), the day of inoculation (Day 7), 1st day post inoculation (Day 8), 5th day post inoculation (Day 12), and 7th day post inoculation (Day 14) to determine shedding and colonization of bacteria in infected mice. Serial dilutions of fecal suspensions were carried out according to modified NMKL: 125 (1996) and ISO/FDIS 7218 (2007) using Maximum Recovery Diluents (MRD) (Oxoid) and then spread using surface technique, onto violet red bile lactose MUG (Oxoid), Luria–Bertani agar, and sorbitol–MacConkey plates and incubated overnight at 37 °C to determine the number of viable bacteria per gram of feces. Isolates were identified biochemically using; Triple sugar iron agar (Oxoid), Lysine iron agar (Oxoid) and Urea agar base (Oxoid) in addition to Biochemical kits microbact 24E (Oxoid) and serotyping using Pathogenic *E. coli* O antisera (Mast assure). The level of colonization by strain O157 was calculated from the total number of sorbitol-negative colonies recovered from mice feces.

Preparation of *E. coli* Antigen: Gram-negative standard strain of *E. coli* O157 isolate was inoculated in Muller Hinton broth with 50 µl equivalent 0.5 McFrland (1.5×10^8) for 24 hours at 37°C. The bacterial growth was harvested

and centrifuged at 1500 xg for 20 minutes. The *E. coli* pellets were taken and washed 3 times with sterile normal saline by centrifugation at 1500 xg for 20 minutes under sterile conditions. *E. coli* cells were subjected to freezing and thawing for 20 times then homogenized. To be sure the bacteria were homogenized well, a sample was taken and inoculated on and streaked onto the Muller Hinton agar plates using sterile swabs and incubated at 37˚C/24hrs for observation of any bacterial growth. Homogenate was centrifuged at 10000 xg at 4^0C for 30 min. The supernatant was collected and its protein concentration was determined by the method of Lowry, (1951). The paste was re-suspended in 25 mM Tris-HCl buffer, pH 7.5. The Ethodin (6, 9-diamino-2-ethoxyacridine lactate) precipitation agent was dissolved in water to the desired final concentration (w/v).

***E. coli* Protein Assay:** Ethodin interferes with most commonly used protein measurement assays, e.g., Bradford, BCA, and spectrophotometric absorption measured at 280 nm. Thus, *E. coli* impurities remaining in the supernatant were measured in a sandwich ELISA utilizing polyclonal antibodies raised against an *E. coli* whole-cell lysate.

ELISA Assays: Mice sera samples were used for testing antibody levels by enzyme-linked immunosorbent assays (ELISA) as described by Vazquez (1996). Indirect ELISA method for anti- *E. coli* O157 IgG detection was employed. Polystyrene plates (Serowell, USA) were coated with loop1 of *E. coli* O157 homogenate control purified antigen diluted in 50 mM sodium carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. The plates were then washed three times with 0.1 ml of phosphate-buffered saline containing Tween 20, 0.05% v/v (PBS-T). Serum samples were diluted in PBS-T containing bovine serum albumin 1% w/v (PBS-TB) and 0.1 ml of dilutions of the respective samples were added to each well in duplicate and incubated at 37°C for 1 h. The plates were then washed four times with PBS-T, and 0.1 ml of goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma) diluted in PBS-TB was added and incubated at 37°C for 1 h. The plates were then washed four times with PBS-T, and 0.1 ml of substrate buffer (50 mM phosphate, 20 mM citric acid, pH 5. 5) containing H_20_2 0.5% v/v (6% purity) and OPD 0.1% w/v were added. After incubation at room temperature for 10 min the reaction was stopped with 2 N H_2S0_4. The optical density (OD) at a wavelength of 492 nm was determined in an AUTO-EIA System (Labsystems, Finland). OD shown by the background controls was subtracted from the OD of each test serum sample. *E. coli* O157 antigen was detected and quantified with a competitive indirect ELISA. Five two-fold dilutions of purified *E. coli* O157 control antigen were made in PBS-TB, and a 100-pl portion of each dilution was mixed with 100 pl of diluted *E. coli* O157 specific antiserum. The antigen antibody mixtures were then added to the wells of antigen-coated plates. Bound anti- *E. coli* O157 IgG was determined as described above. Thus, a decrease in OD by competition was used to construct a standard inhibition curve. Concentration of *E.coli* O157 in test samples was calculated by determining the amount of antigen that caused inhibition to the same extent as in the standard inhibition curve.

Histopathological Examination: After sacrificing of the animals they were subjected to full necropsy. Tissue specimens were collected for histological examination. Each group was subject to in-situ testing in tissue of different organs. Tissue specimens including the brain, liver, kidney, intestine and brain were collected for histological examination. Specimens were fixed in 10% buffered neutral Formalin and processed by standard procedures. Sections of paraffin-embedded tissue were stained with hematoxylin and eosin then examined by light microscopy. Infected animals were assessed post-mortum for the presence of enteric or renal pathology associated with the disease. The presence of enteritis was determined by histopathologic analysis of the cecum. Enteritis was associated with hyperplasia combined with lymphoplasmacytic infiltration, inflammation, and erosion of the cecal mucosa. Renal pathology was associated with renal tubular dilatation and edema combined with the presence of renal thrombi or fibrin in the kidneys.

Statistical analysis: The experiment followed complete randomized design. The obtained data were subjected to analysis of variance (ANOVA) according to (Snedecor and Cochran, 1980). Duncan's multiple range was used to compare between means of treatment according to (Waller and Duncan, 1969).

3. RESULTS

In vitro antibacterial activity of the water extract of *C. molmol* against *E. coli* O157 was evaluated. The results showed high antibacterial activity of the tested extract when compared with the hindrance activity of the reference antibiotic ciprofloxacin disk (CIP5) 5µg/ml with inhibition zones of 19 mm and 11 mm, respectively using agar gel diffusion inhibition test.

In vivo antibacterial activity of water extract of *C. molmol* against *E. coli* O157 was evaluated using different parameters including the quantification of *E. coli* O157 in feces of infected mice (Table 2). Fecal samples were collected on days 6, 7, 8, 12 and 14 for bacterial count to assess the levels of both *E.coli* and *E. coli* O157 colonization. Inoculation of *E. coli* O157 was done on day 7 and the bacteria was recovered from feces of infected mice groups on days 8, 12 and 14 representing 1^{st}, 5^{th} and 7^{th} days post inoculation respectively. All of the inoculated mice became colonized by *E. coli* O157 strain. Enumeration of *E. coli* and *E. coli* O157 showed high *E. coli* count on day 6 with bacterial count from 10×10^2 to 17×10^2 CFU. All mice groups were negative for *E. coli* count at the day of challenge (day 7) due to oral administration of streptomycin. The Group 3 shed recoverable *E. coli* O157 into the feces on days 8, 12, and 14 with bacterial counts of 13×10^2, 14×10^2 and 36×10^2, respectively while in group 4 *E. coli* O157 count

was 0.4×10^2 CFU/ml at day 14, which was a very low count when compared with the group 3**.** Tween 80 control negative group (Group 1) showed very low non-pathogenic *E. coli* count on the day 14 reach 4×10^2 CFU/ml. Also, group 2 (Commiphora control) showed low non-pathogenic *E.coli* count at the day 14 (11×10^2 CFU/ml).

Table.2. Enumeration of *E. coli* and *E. coli* O157 in fecal samples

Group No.	Group Name	Bacterial Strain	Day6	Day7	Day8	Day12	Day14
1	Tween 80 Control	*E. coli* O157 count	-ve	-ve	-ve	-ve	-ve
		E. coli count	15×10^2	-ve	-ve	-ve	4×10^2
2	Commiphora Control	*E. coli* O157 count	-ve	-ve	-ve	-ve	-ve
		E. coli count	17×10^2	-ve	-ve	-ve	11×10^2
3	*E. coli* O157 Control	*E. coli* O157 count	-ve	-ve	13×10^2	14×10^2	36×10^2
		E. coli count	10×10^2	-ve	-ve	-ve	-ve
4	Commiphora + *E. coli* O157	*E. coli* O157 count	-ve	-ve	-ve	-ve	0.4×10^2
		E. coli count	10×10^2	-ve	-ve	-ve	-ve

The results of antibody titer against *E.coli* O157 detected by ELISA was illustrated in Chart.1. The OD of blank was (0.25) while the mean OD for Tween 80 control group was 0.29. In both Commiphora control group and *E. coli* O157 control group, the antibody titer was comparable at first day post infection (Day 8) with OD of 0.43. The results of antibody titer showed non-significant differences between Commiphora control group, *E. coli* control group and Commiphora + *E. coli* O157 group in all days as indicated by the OD values although *C. molmol* water extract administration in Commiphora + *E. coli* group showed a trend to decrease antibody titer when compared with antibody titer in *E. coli* control group with OD values of 0.55 in the and 0.61 7th day post inoculation (Day 14) respectively. This result showed the possible ability of *C. molmol* water extract, if the experiment time was extended to induced suppression to the *E. coli* O157 infection as indicated by antibody titer.

Histopathological finding showed diffused focal area of necrosis and infiltration of mononuclear cells in hepatic tissues of mice infected with *E. coli* O157. The hepatic cells showing necrobiotic changes are shown in Fig.1. Mononuclear cell infiltration, activation of kuffer cells and large multinuclear phagocytic cells (Fig.2). The hepatic tissues of Commiphora + *E. coli* O157 group (Fig.3), was normal with no characteristic foci of necrosis and no mononuclear cell infiltration if compared with Fig.1. On the meantime the mice treated with *Commiphora molmol* only (Fig.4), also showed normal hepatic tissues with no characteristic changes as compared with Fig.1. The renal tissue of infected mice with *E. coli* O157 showed focal area of necrosis of renal tissues, hyaline degeneration and necrobiotic changes of epithelial cells of renal tubules (Fig.5). Renal tissues mice treated mice with *Commiphora molmol*, after *E. coli* O157 infection showed congestion and dilatation of renal blood vessels as well as degenerative changes of epithelial cells of renal tubules (Fig. 6). Cross section of ileum of infected mice, showing degenerative changes of epithelial cells of intestinal vile and infiltration of mononuclear cells in sub epithelial layer. (Fig.7). While infected mice with *E. coli* and treated with *Commiphora molmol* (Fig.8), showing infiltration of mononuclear cells and edema in sub epithelial layer. Tissue section of stomach of infected mice showing invasion of the infected pathogen to the stomach mucosa cells (Fig.9). The infected mice with *E. coli* and treated with *Commiphora molmol* (Fig.10), showing congestion of the upper mucosa with less degenerative changes as compared with Fig.7.

DISCUSSION

Medicinal plant extracts are promising source of new antibacterial agents. The antibacterial activity of water extract of oleo-gum resins of *C. molmol* against *E. coli* was investigated in the current study. Although methanol is the preferred solvent for more consistent extraction of antimicrobial substances from medicinal plants compared to other solvents including hexane, ethanol and water (Ahmed, 1998; Karaman, 2003) water extract showed obvious antibacterial activity in the current study. Water was used for extraction because it is safer than other solvents and was historically used in plant extraction in traditional medicine. Our results were in agreement with El-Ashry (2003), who reported the effectiveness of different Commiphora species as antibacterial agents against some Gram-positive and Gram-negative bacteria. Also, in a different study, *C. molmol* exhibited antibacterial activity against some strains of *S. aureus, Salmonella enterica* and *Klebsiella pneumoniae* (Rahman, 2008). Simillar findings were found during the study of antimicrobial properties of *C. molmol* resin oil against clinical isolates of *S. aureus* (Adam and Selim, 2013). They concluded that ethanol and methanol extracts can be considered as effective anti-staphylococcal natural products. The antibacterial activity of oleo-gum resins extracted from other plant species including two Boswellia species against *P. aeruginosa, B. rivae and E. coli* was also doccumented (Camarda, 2007).

Essential oils from different medicinal plants used in traditional medicine were also found to be effective against different bacterial species (Inouye, 2001). They reported that, vapor of most of the 14 essential oils used in the study showed effective antibacterial activity against *Haemophilus influenzae, Streptococcus pneumoniae, Streptococcus pyogenes* and *Staphylococcus aureus*. The vapors as well as extracts of the oleo-gum resins of

Commiphora molmol and *Boswellia papyrifera*, as two representatives of Arabian medicinal plants, were investigated for their antibacterial activity against different bacterial strains including methicillin resistant *Staphylococcus aureus* (MRSA). The methanol extracts showed the highest antibacterial activity whereas the ethyl acetate extracts showed some degree of activity and the petroleum ether and water extracts showed no or least activity (Abdallah, 2009; Abdallah & Khalid, 2012).

ELISA methods have been used to diagnose many infectious diseases and their application to infections in mice for the detection of *E. coli* and K88 antigens, respectively has been described previously (Mills, 1982; 1983; Mills & Tietze, 1984; Hernhdez, 1989). However, knowledge of the appropriate conditions is necessary for optimization of the assay and interpretation of results. Coating studies allowed the binding capacity of the O157 antigen on plate wells to be determined and the optimal concentration to be established. Clegg (1980), in an ELISA to detect IgG against the CFAA antigen of ETEC from man established an optimal coating concentration of purified CFNI antigen of 1 pg/ml. In the present study, purified O157 antigen adsorbed to polystyrene plates at low concentrations, and antigen concentrations higher than the optimal decreased the OD values (Chart.1). This effect has been reported previously, and probably results from the detachment of the antigen from the plate during the various washing steps (Holmgren and Svennerholm, 1973; Clegg, 1980).

The severe histopathological changes in different organs observed in the current study may be due to the Shiga toxin (Stx) produced by *E. coli* O157 which has the ability to reach susceptible organs and induce the damaging effect (Mohawka, 2010; Rasooly, 2010). The results showed the ability of *Commiphora molmol* water extract to reduce or inhibit the damages of organs. This protective effect may be due to the ability of *C. molmol* to down-regulate the inflammatory mediators and cytokines which might be sufficient to reduce cellular damage caused by Stx produced by *E. coli* O157 (Ahmad, 2015).

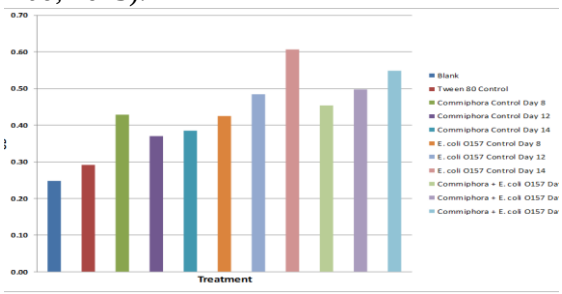

Chart.1. antibody titer against E.*coli* detected by Eliza

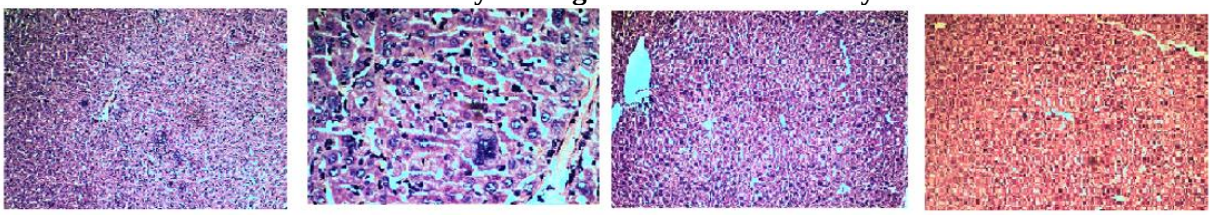

Figure.1, 2, 3, 4

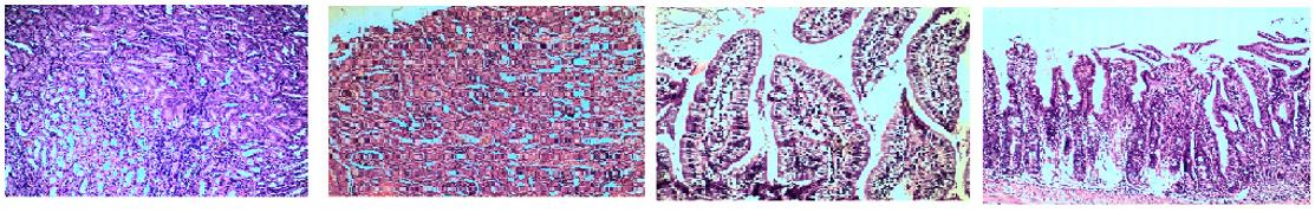

Figure.5, 6, 7, 8.

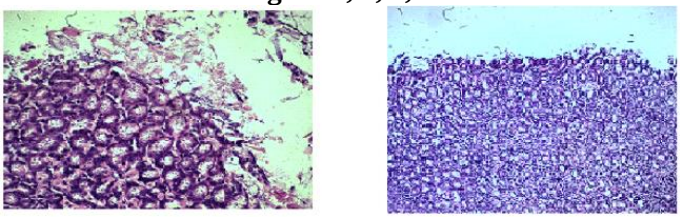

Figure.9, 10.

Histopathological findings in different tissues of mice infected with *E. coli* and treated with *Commiphora molmol*

Figures (1-10), Histopathological findings in different tissues of mice showing the effect of *Commiphora molmol* against *E. coli* O157 infection. Fig.1. Hepatic tissues of mice infected with *E. coli* O157 (*E. coli* O157 Control group) showing diffused focal area of necrosis and infiltration of mononuclear cells. The hepatic cells showing

necrobiotic changes (H&E.X 100); Fig.2. Hepatic tissues of *E. coli* O157 Control group showing mononuclear cell infiltration, activation of Van kuffer cells and large multinuclear phagocytic cells (H&E.X 400); Fig.3. Hepatic tissues of Commiphora + *E. coli* O157 group showing that the hepatic tissues are within normal changes, no characteristic foci of necrosis, no mononuclear cell infiltration (H&E. X 200); Fig.4. Hepatic tissues of Commiphora Control group showing that the hepatic tissues are within normal changes, and no characteristic changes as compared with fig.1(H&E. X100); Fig.5. Renal tissues of *E. coli* O157 Control group showing focal area of necrosis of renal tissues, hyaline degeneration and necrobiotic changes of epithelial cells of renal tubules (H&E.X100); Fig.6. Renal tissues of Commiphora + *E. coli* O157 showing congestion and dilatation of renal blood vessels as well as degenerative changes of epithelial cells of renal tubules (H&E.X100); Fig.7. Cross section of ileum of *E. coli* O157 Control group showing degenerative changes of epithelial cells of intestinal vile and infiltration of mononuclear cells in subepithelial layer (H&E. X100); Fig.8. Cross section of ileum of Commiphora + *E. coli* O157 group showing infiltration of mononuclear cells and edema in subepithelial layer (H&E.X100); Fig.9. Tissue section of stomach of *E. coli* O157 Control group showing invasion of the bacetria to the stomach mucosa cells (H&E. X 400); Fig.10. Tissue section of stomach of Commiphora + *E. coli* O157 group showing congestion of the upper mucosa with less degenerative changes as compared with fig.6.(H&E. X 100).

4. CONCLUSION

Commiphora molmol water extract induced suppression of the *E. coli* O157 infection in mice as indicated by the reduction in bacterial count as well as the trend of reducing the levels of antibody production in challenged mice. *C. moloml* also showed a protective effect against the damaging histopathological changes that occurred in different organs following bacterial infection. Although further studies are needed to confirm the results using more animals as well as to standardize the use of *C. moloml*, these results indicate the possibility of using *C. molmol* as safe alternative natural antibiotic to control *E. coli* O157 infection.

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