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Potential Use of *Chromolaena odorata* Linn. Flavonoids against *Escherichia coli* Induced Diarrhoea in Mice

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J-JC and RG designed the experiment. C-QS, H-YL and J-QZ did the experiment. H-CRW and D-JK performed the statistical analysis. C-QS, FY and KAL wrote the manuscript.

Key words
***Chromolaena odorata* Linn., Bacteria, Immunoglobulin, TGF-β1, Mouse**

ABSTRACT

Flavonoids are important chemotaxonomic markers for the genus *Chromolaena*. *Chromolaena odorata* Linn. (CO) is used in folklore medicines to treat gastrointestinal diseases such as diarrhoea. This study examined the feasibility of total flavonoids from *C. odorata* (TFCO) as an antibacterial agent *in vitro*, and the mechanism of action of TFCO in combating *Escherichia coli*-induced diarrhoea in mice. MBC and MIC values of 0.25 and 0.125 g TFCO/ml showed it was effective as an antibacterial agent against *E. coli* (CMCC 44752) *in vitro*. Ninety Kunming mice were randomly divided into five groups of 18 mice per group (1 male: 1 female). The groups were control (saline), negative control (*E. coli* + 0 mg/kg TFCO), low dose (*E. coli* + 10 mg/kg TFCO), medium dose (*E. coli* + 40 mg/kg TFCO), and high dose (*E. coli* + 160 mg/kg). TFCO was administered by gavage 3 h post intraperitoneal (*i.p*) *E. coli* injection. Serum IgM, IgA, IgG, TGF-β1 mRNA expression in duodenal villi, and histopathology of duodenum were also studied. TFCO significantly increased the IgA and IgG concentration ($P<0.05$), reversed the intestinal mucosal damage caused by *E. coli*, and increased the relative expression of TGF-β1 mRNA. Therefore, TFCO has potential to improve immune function against *E. coli* infection, restored the intestinal structure and cured the *E. coli*-induced diarrhoea in mice.

INTRODUCTION

Herbal plants in the family Asteraceae have characteristic flavonoids and phenolics which account for their pharmacological significance (Pisutthanan *et al*., 2006). *Chromolaena odorata* Linn. (formally called *Eupatorium odoratum*) is a common invasive herbal plant in the Asteraceae family (Tonzibo *et al*., 2007). It is widely distributed in the humid tropics and sub-tropics of Africa and Asia where it causes agro-ecological destructions (Tonzibo *et al*., 2007). *C. odorata* has been explored in Indian Ayuveeda, traditional Chinese medicines and African folkore medicines to prevent and control several gastrointestinal disorders including diarrhoea (Panda *et al*., 2010; Aba *et al*., 2015). Toxicological evaluations and analytical studies have further revealed that, pyrrolizidine alkaloids levels in the leaves and stems of the herbal plant are insignificant and they can be further reduced by organic solvent extractions (Ogbonnia *et al*., 2010). Flavonoids obtained from ethanol and methanol extractions of herbal medicines are less toxic based on hepatotoxic, mutagenic, and cytotoxic evaluations (Ogbonnia *et al*., 2010; Asomugha *et al*., 2015). They have also been found to have broad-spectrum anti-bacteria effects against gram positive and gram negative bacteria (Omokhua *et al*., 2017). Infectious bacteria such as *Escherichia coli* cause diarrhoea which remains a major health problem in food animal's production especially swine and poultry (Gomes *et al*., 2016; Lin *et al*., 2017). *E. coli* -induced diarrhoea lowers production efficiency and their control also increases production cost and economic loss (Lin *et al*., 2017). Enterohemorrhagic *Escherichia coli* (EHEC) are the pathogenic subgroup of Shiga toxin (Stx)-producing *E. coli.* EHEC can cause non-bloody and bloody diarrhoea (Bryan *et al*., 2015). They multiply quickly in

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the gastrointestinal tract and cause damage to tissues and organs such gastrointestinal tract, kidneys, spleen, liver, and even death in severe cases (Börjesson *et al*., 2016). Synthetic antibiotics are currently the most commonly used drugs for prevention and treatment of diarrhoea in food animals. However, this has led to the development of bacterial resistance, and antibiotic residues in meats thereby posing threats to biosafety. Consumer demands for "green label" feed additives as alternatives to synthetic antibiotics is on the increase (Liu *et al*., 2011). They are posited to be biologically safer, environmentally-friendly and economically cheaper compared to synthetic antibiotics (Liu *et al*., 2011). Phytochemicals have been used in folklore medicines and ethnoveterinary to prevent and treat several ailments. Increasing evidence supports the assertion that, flavonoids are beneficial for gastrointestinal health (Unnikrishnan *et al*., 2014).

Earlier studies from our laboratory using 16s RNA sequencing and Miseq amplification showed that, total flavonoids of *C. odorata* (TFCO) exerts competitive exclusions to prevent inoculation of disease-causing bacteria such as *E. coli* in the cecum of broiler chickens and enhances serum humoral immunity as a result (Zhang *et al*., 2018). In this study, total flavonoids were extracted from *C. odorata* aerial parts. The potential of the total flavonoids from *C. odorata* (TFCO) as an antibacterial agent was examined *in vitro*. Kunming mice were further used as a model to examine the antibacterial activity of the TFCO in combating *E. coli*-induced diarrhoea. Duodenal mucosal villi structure, IgM, IgA, and IgG concentrations, and a key factor in intestinal mucosal repair, TGF-β1 expressions were also monitored to get an overall understanding of the mechanism of action of TFCO. Detection and analysis of the flavonoid compositions is however beyond the scope of this study.

MATERIALS AND METHODS

The aerial parts of young and non-flowering *C. odorata* were collected the campus of Guangdong Ocean University, Zhanjiang- China (Guangdong Province, China) in summer (June). Herbs were identified and confirmed by the Botany Department of the College of Agricultural Sciences, Guangdong Ocean University. These aerial parts were cleaned, shade-dried, pulverized, and passed through a 150-mesh sieve. The powder was preserved in a refrigerator at 4°C until extraction. Total flavonoids were extracted by solvent- solvent extraction as described by Owoyele *et al*. (2008) with slight modification. Briefly, ten grams of the dried powder was dissolved in 500 ml 70% ethanol (a solid: liquid ratio of 1:60) for 24 h, extracted for 5 h at 70°C using an ultrasonic washing device (KQ-500DE, Kunshan Ultrasonic Instruments Co., China) and filtered. Filtrate was purified with petroleum ether (10:1) using ethyl acetate extraction. The primary purified extract was placed in an electro-thermal incubator, evaporated at 70°C and stored in a refrigerator at 4°C. 160 mg of the resulting powder material was then dissolved in 1 ml dimethyl sulfoxide solvent (DMSO: Water, 2:4 v/v) to prepare the stock solution of 160 mg/ml, then this stock was 4-fold diluted with saline to obtain 40 mg/ml, and 16-fold diluted with saline to obtain 10 mg/ml.

Bacterial studies

Strains of *Staphylococcus aureus* (ATCC 25923), *Salmonella pullorum* (ATCC 14028), *Escherichia coli* (CMCC 44752) and *Bacillus subtilis* (CMCC (B) 63501) were used. They were obtained from the Veterinary Medicine Department of Guangdong Ocean University. The antibacterial activity of the flavonoids of TFCO was screened using the agar disc diffusion assay. Bacterial suspensions were prepared by inoculating bacteria onto nutrient agar plate and culturing at 37°C for 16 h. A single colony was inoculated into liquid broth and allowed to grow for 16 h at 37°C, and the culture was diluted with liquid broth to a final concentration of 10^7 CFU/ml. 100 µl of inoculum was spread on the nutrient agar plates. Sterile filter paper disc (5 mm) loaded with 10 µl of TFCO was placed on the agar plates containing bacterial inoculum. The inoculated plates were left for 30 min at room temperature for diffusion of the TFCO into agar and then incubated at 37°C for 48 h. Microbial inhibition was determined by measuring the diameter of the clear zone surrounding the discs; the result was recorded in millimeter (mm). All the tests were performed in triplicate (Alshaikh and Perveen, 2017).

Determination of MIC and MBC

Broth microdilution was performed as described by the Clinical and Laboratory Standards Institute (CLSI, 2017). To determine minimum inhibitory concentration (MIC), 2-fold serial dilutions of each antibiotic were prepared in test tubes with concentrations ranging from 0.002 g/ml to 2 g/ml. Inoculated tubes were incubated at 37°C for 24 h. All the tests were performed in triplicate. The lowest concentration of each antibacterial agent that inhibited the bacterial growth was then considered as the MIC. Minimum bactericidal concentration (MBC) was determined by culturing on agar plate. Plates were incubated at 37°C for 48 h, and viable colonies were counted. The lowest concentration that killed 100% of the initial bacterial population (with no colonies on the agar) was recorded as the MBC.

Animal studies

Ninety Kunming mice, six-eight weeks-old with similar initial weights (20±3 g), were fed *ad libitum* with a balanced isoenergetic diet that m*et al*l the nutritional requirements. All mice received free access to water. Mice were randomly assigned into five groups of 18 mice per group i.e. control (saline), negative control (*E. coli* + 0 mg/kg TFCO), low dose (*E. coli* + 10 mg/kg TFCO), medium dose (*E. coli* + 40 mg/kg TFCO), and high dose (*E. coli* + 160 mg/kg). 3 h post intraperitoneal (*i.p*) *E. coli* (3×10^8 *Escherichia coli* (CMCC 44752) injection, mouse-model of diarrhoea was made and TFCO administered by gavage. Six mice (3 males, 3 female) from each group were euthanized on 2, 5 and 8 d post injection.

Histopathology of duodenum

On 8 d, mice were euthanized and then autopsied. A portion of the duodenum cut from all animals was fixed in 10% formaldehyde overnight, dehydrated in a graded series of alcohol, and cleared in xylene prior to being embedded in paraffin. Serial 4-µm-thick paraffin sections were transferred to glass slides, deparaffinized and stained with hematoxylin and eosin for histopathological studies using a microscope (ECLIPSE E200, Nikon, Japan).

IgG, IgM and IgA

Blood was collected from all mice on days 2, 5, and 8 following the dose. Individual samples were centrifuged for 3 min at 3000 r/min, serum were separated and stored in a freezer at −20°C. The IgG, IgM and IgA were measured in 96-well plates, using an ELISA kit (Fanke Co., Shanghai, China) according to the manufacturer's instructions.

RNA extraction, primer design and real-time PCR

A small piece of the duodenum from each mouse was removed soon after euthanasia, washed with saline to remove intestinal contents, labelled, frozen immediately in liquid nitrogen, and stored in a freezer at −80°C. A separate 30 mg of duodenal tissue was collected and mixed with 1.5 ml Trizol (Takara Co., Otsu, Japan) to determine TGF-β1 expression. Reverse transcription-polymerase chain reaction (PCR) assay for mRNA expression of β-actin and TGF-β1 was carried out as described by Li *et al*. (2010). RNA was extracted with Trizol reagent (Takara Co. Otsu, Japan) according to the manufacturer's recommended procedure. RNA (2 µl) was reverse-transcribed using an M-MuLV First Strand cDNA Synthesis Kit (Takara Co. Otsu, Japan). All primer sequences were from the Ying Wei Jie Ji Co., Shanghai, China. PCR was performed using a real time PCR Kit (Takara Co. Otsu, Japan). 10 µl reaction mixture of SYBR Premix ExTaqTM II (Takara Co. Otsu, Japan) was added to 2 µl cDNA template, 0.8 µl forward primer (10 µmol/l), 0.8 µl reverse primer (10 µmol/l), 6.0 µl sterile distilled water and 0.4 µl ROX Reference Dye (50×). Cycling conditions were: 95°C for 35 sec; 40 cycles of 95°C for 20 sec, 64°C for 35 sec., 72°C for 20 sec; 60°C for 1 min and cooled to 4°C. PCR was performed in tandem with β-actin primers as an internal control. DNA sequences are shown as below.

TGF-β1 F: 5'-GCCCTGGATACCAACTATTGC-3',
R: 5'-GCAGGAGCCACAATCATGTT-3.
β-actin: F: 5'-GTCCCGGCCAGCCAGGTCCAG-3',
R: 5'-CCTAAGGCCAACCGTGAAAAGATG-3'.

The relative change in TGF-β1 expression levels was determined from qPCR and calculated according to the $2^{-\triangle\triangle CT}$ method, $\triangle CT = CT_{TGF\text{-}\beta1} - CT_{\beta\text{-}actin}$.

Statistical analysis

All statistical analyses were performed using the statistical software SPSS version 17.0. Using ANOVA method, multiple comparisons were made using the LSD method, and the results are expressed as mean ± SD. $P<0.05$ was considered statistically significant. MIC and MBC for each antimicrobial agent are reported as minimum, maximum, median, and range (95% CI) values.

Ethical considerations

All mice were housed in an environmentally-controlled environment. All animal care and sacrifice were in accordance with the country standards. This research protocol was approved by the Animal Ethics Committee of Guangdong Ocean University (Approval No: NXY20160172). The guidelines of Animal Research: Reporting of *In Vivo* Experiment (ARRIVE) guidelines were adhered to.

RESULTS

TFCO concentration

The yield of TFCO obtained from the ethanol extraction was 18.8% (w/w).

MIC and MBC

The bacteriostatic circle diameter in the four types of bacteria (*Staphylococcus aureus*, *Salmonella pullorum, E. coli*, *Bacillus subtilis*) exposed to TFCO (0.5 g/ml) was > 11 mm. The antibacterial effect of TFCO was highest on *S. aureus* with an inhibition zone of 19.5 mm, and least on *E. coli* with 11 mm (see Table I).

The MIC and MBC for *S. aureus* were the lowest, with values of 0.0156 and 0.0313 g/ml respectively, indicating that TFCO was highly bacteriostatic against *S. aureus in vitro* (see Table II).

Table I. In-vitro bacteriostatic activity of *Chromolaena odorata* Linn. flavonoids tested at a concentration of 0.5 g/ml.

Bacteria	Bacteriostatic diameter/mm
Escherichia coli	11.00±0.05
Salmonella pullorum	13.33±0.89
Staphylococcus aureus	19.50±0.25
Bacillus subtilis	13.67±0.22

Table II. MIC and MBC of *Chromolaena odorata* Linn. flavonoids *in vitro*.

Bacteria	MIC[a] (g/ml)	MBC[b] (g/ml)
Escherichia coli	0.1250	0.2500
Salmonella pullorum	0.0313	0.1250
Staphyloccocus aureus	0.0156	0.0313
Bacillus subtilis	0.0625	0.1250

[a]minimum inhibitory concentration (g/ml); [b]minimum bactericidal concentration (g/ml).

Diarrhoea

Three hours post injection, *E. coli* injected mice (negative control and three treatment groups) exhibited clinical changes but the saline injected mice (blank control) did not. Observed clinical changes were slight reduction in physical activity and watery diarrhoea followed by bloody diarrhoea. On day 2, all mice developed diarrhoea except mice in the blank control. On days 3, 4, and 5, the diarrhoea incidence among *E. coli* injected (negative control and three treatment groups) mice was lowest in the high dose (160 mg/kg) TFCO group. Diarrhoea incidence for the high dose TFCO (160 mg/kg) was 58.3%, 25%, and 8.3% for days 3, 4 and 5 respectively. On day 6, none of the animals exhibited diarrhoea except the negative control (*E. coli* + 0 mg/kg TFCO).

Duodenal villi structure

In the blank control group, the duodenal mucosa in mice was normal, with intact intestinal villi and small intestinal glands and no obvious pathological change (Fig. 1a). The negative control (*E. coli* + 0 mg/kg TFCO) group showed marked duodenal mucosal damages with breakages in the intestinal villi and sparsely arranged intestinal epithelial cells (Fig. 1b). In the low dose (*E. coli* + 10 mg/kg TFCO) group, duodenal villi rupture and atrophy were visible (Fig. 1c). In the medium dose (*E. coli* + 40 mg/kg) group, some villi exhibited swelling and sloughing off (Fig. 1d). In the high dose (*E. coli* + 160 mg/kg group), the duodenal villi had returned to normal, with only a few occasional damaged intestinal villi (Fig. 1e).

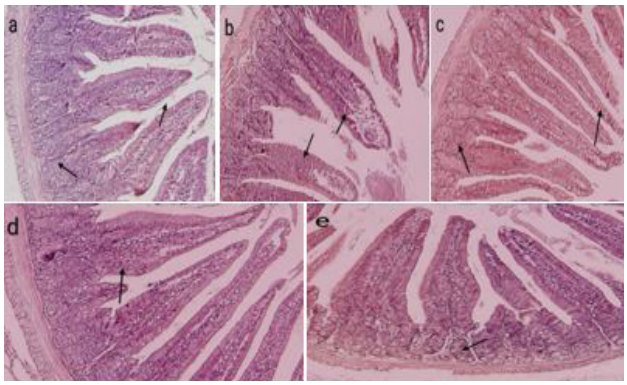

Fig. 1. Histopathology of duodenal villi (hematoxylin eosin stain, × 100) in controls and mice exposed *E. coli* and *chromolaena odorata* Linn. Flavonoids (COF). Control group: duodenal villi and small intestinal glands are complete and clear. (b) *E. coli* + 0 mg COF/kg group; Rupture of duodenal villi intestinal epithelial cells arranged sparsely. (c) *E. coli* + 10 mg COF/kg group, duodenal villi damaged with some atrophy. (d) *E. coli* + 40 mg COF/kg group, epithelial cells of duodenal villi abscission and partially visible swelling of the intestinal villi. (e) *E. coli* + 160 mg COF/kg group, showing normal duodenal villi. The arrows indicates duodenal villi.

Relative expression of TGF-β1 mRNA in duodenum

On day 2 after *E. coli* infection, the relative expression of TGF-β1 mRNA in the mice given TFCO was significantly higher than that of the blank control group ($P<0.01$) and this was most marked in the high-dose group ($P<0.05$). At 5 days after *E. coli* infection, the relative expression of TGF-β1 mRNA declined in all the treatment groups, but was still significantly higher than the control group ($P<0.01$) but there was no significant difference between the TFCO treatment groups ($P>0.05$). After 8 days of *E. coli* infection, the relative expression of TGF-β1 mRNA in only the high-TFCO-dose group was still significantly higher than that of the control group and the positive control (*E. coli* + 0 mg/kg TFCO) group ($P<0.05$) (Table III).

Relationship between duodenal villi histopathology and expression of TGF-β1 mRNA

There was a relationship between the villus length, crypt depth and TGF-β1 mRNA expression. The correlation coefficient between TGF-β1 mRNA and duodenal crypt depth and duodenal villi length were correlated at 0.9956 and 0.9831 respectively. The corresponding regression formulae were $y=0.0002x-0.0252$ ($R^2=0.9956$) and $y=0.0003x-0.1406$ ($R^2=0.9831$). The repair of the villi in the duodenum was also closely related to TGF-β1 mRNA expression.

Table III. Effect of *Chromolaena odorata* Linn. flavonoids on relative expression of TGF-β1mRNA in mouse duodenum.

Groups	Number of days following TFCO administration		
	2	5	8
Control	0.015±0.003^{B}	0.015±0.004^{B}	0.016±0.002^{b}
0 mg/kg	0.260±0.079^{Ab}	0.173±0.018^{A}	0.015±0.003^{b}
10 mg/kg	0.266±0.011^{Ab}	0.181±0.018^{A}	0.018±0.002^{ab}
40 mg/kg	0.347±0.135^{Ab}	0.199±0.045^{A}	0.020±0.004^{ab}
160 mg/kg	0.434±0.030^{Aa}	0.196±0.026^{A}	0.022±0.003^{a}

Compared with the control group, the data in the same column with different lowercase (^{a}, ^{b}) and uppercase letters (^{A}, ^{B}) letters indicate significant differences at $P<0.05$ and $P<0.01$ respectively.

IgM, IgA and IgG

Figure 2a shows that at day 5, the IgM concentrations of mice in the TFCO-treated (Low dose, medium dose, and high dose) groups were significantly higher than in the blank control group ($P<0.05$), but not between the TFCO treated groups.

At day 5, the IgA concentrations in the TFCO treatment groups were significantly different from the control group ($P<0.01$) but not between the treatment groups ($P>0.05$) (Fig. 2b). At day 8, the IgA concentration decreased in the TFCO-treated groups but was still significantly higher than that of the control (saline) group ($P<0.01$). The high dose (*E. coli* +160 mg/kg TFCO) group was significantly higher ($P<0.01$) than medium dose (*E. coli* + 40 mg/kg TFCO) and low dose (*E. coli* + 10 mg/kg TFCO) groups.

At 5 days following *E. coli* infection, the IgG concentrations were significantly ($P<0.05$) elevated in the treatment groups compared to the control (saline) group (Fig. 2c). Among the treatment groups, the high dose (*E. coli* + 160 mg/kg TFCO) group was significantly different ($P<0.05$) from the low TFCO dose (*E. coli* + 10 mg/kg).

At 8 days following *E. coli* infection, the IgG concentration further increased in all groups with the high dose (*E. coli* + 160 mg/kg TFCO) group significantly different ($P<0.01$) from the control (saline) and negative control (*E. coli* + 0 mg/kg group) groups. The medium (*E. coli* + 40 mg/kg) and low dose (*E. coli* + 10 mg/kg) groups were also significantly different ($P<0.05$) from the control (saline) group. Among the TFCO treatment groups, the IgG concentration in the high dose (*E. coli* + 160 mg/kg) group was significantly different ($P<0.05$) from the medium TFCO (*E. coli* + 40 mg/kg) and low TFCO (*E. coli* + 10 mg/kg) groups.

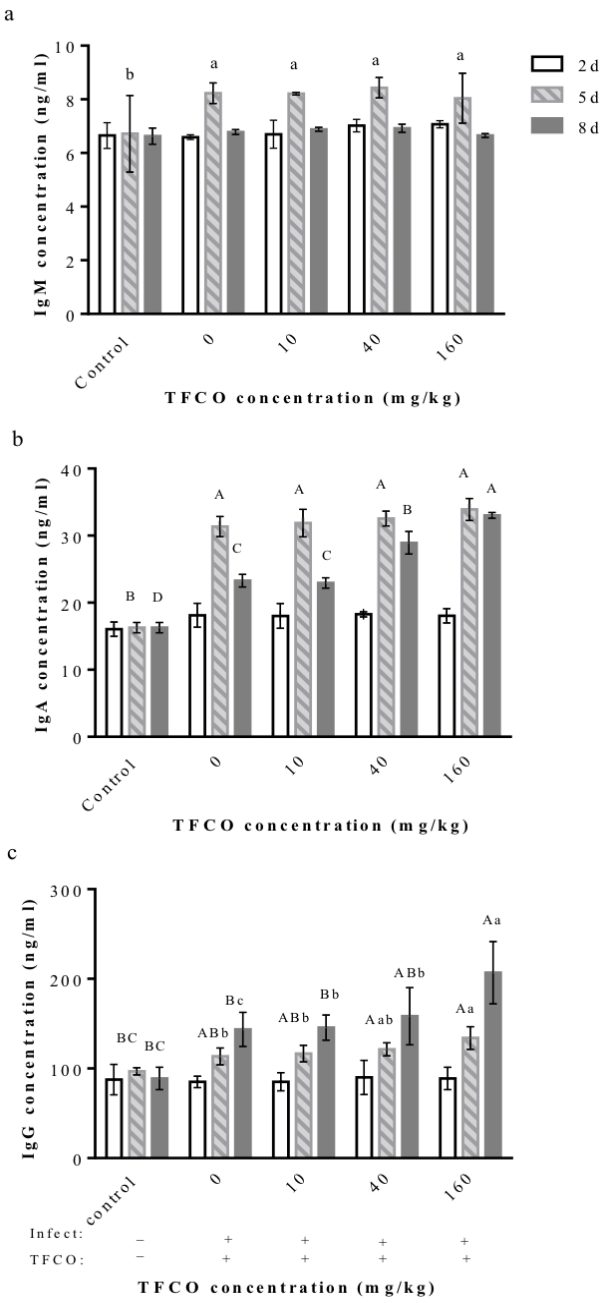

Fig. 2. IgM, IgA and IgG concentrations in control and *E. coli* and *Chromolaena odorata* Linn. flavonoids (TFCO) exposed mice.

The 0 mg/kg group is the positive control. The negative sign (−) refers to groups not infected with *E. coli* or treated with TFCO. The positive sign (+) refers to groups infected with *E. coli* or treated with TFCO. Figure 2a–c show serum IgM, IgA and IgG concentrations respectively. Compared with the control group, the different lowercase (^{a}, ^{b}, ^{c}) and uppercase (^{A}, ^{B}, ^{C}) letters indicate significant differences at $P<0.05$, and $P<0.01$ respectively.

DISCUSSION

Total flavonoids from *C. odorata* (TFCO) obtained by ethanol extraction have been identified as kaempferol-3-methoxy, rhamnetin, tamarixetin, quercetin, kaempferol, apigenin, luteolin and dihydrokaempferide (Yuan *et al*., 2007; Zhang *et al*., 2018). In this study, TFCO was moderately effective against *E. coli*, *Salmonella pullorum*, *Staphylococcus aureus* and *Bacillus subtilis in vitro* (Luo *et al*., 2015). In a related study, ethanol extract of *C. odorata* also exhibited inhibitory activity against *S. aureus* (ATCC 29213), *E. coli* (ATCC 25922) and other gastrointestinal disease-causing bacteria, with MIC values ranging from 0.39 to 3.12 (Omokhua *et al*., 2017). From this study, the MIC and MBC values of TFCO against *E. coli* were higher than that for *S. aureus* but still regarded as moderately sensitive. *E. coli* as a gram -negative bacteria and is less inhibited by extracts than gram-positive bacteria such as *S. aureus* (Palombo and Semple, 2001). The observed higher MIC and MBC values against gram-negative *E. coli* suggests that the TFCO inhibits bacterial growth rather than penetrating into the bacteria cell walls (Omokhua *et al*., 2017).

In healthy gut, pathogenic bacteria and commensal intestinal tract beneficial bacteria exist in a steady-state (Wang *et al*., 2017). Upon heavy bacterial such as *Escherichia coli* (*E. coli)* infection, the equilibrium is disrupted (Lin *et al*., 2017). *E. coli* administered as an intraperitoneally (*i.p.*) injection may cause intestinal dysbiosis with intestinal mucosal injury, including effects on intestinal villi and intestinal epithelial cell necrosis, which can result in intestinal absorption and secretion dysfunction and may also cause severe diarrhoea and even death (Gomes *et al*., 2016; Zhang *et al*., 2017).

Following injury to mucosal epithelial cells in the intestinal tract caused by pathogenic microorganisms (Coddens *et al*., 2017; Song *et al*., 2017), the intestinal epithelium undergoes a complex process of wound repair. In this study, *i.p* injection of TFCO at 160 mg/kg (high dose) post *E. coli* infection, ameliorated injury in the gut. A molecular study was further conducted to determine the intestinal repair process. TGF-β1 is a core factor that contributes to the recovery of injured intestinal epithelial cells (Ando *et al*., 2007). TGF-β1 is a pleiotropic cytokine secreted by immune and non-immune cells in the gut, which plays an important role in promoting the differentiation of regulatory T-cells (Fujio *et al*., 2016). TGF-β1 provides beneficial effects by enhancing the epithelial barrier and providing protection against pathogenic bacteria insults (Howe *et al*., 2005). It enhances the migration of intestinal epithelial cells, regulate entry and exit to the extracellular matrix, and plays a key role in intestinal tissue remodelling, and thereby accelerate the healing of intestinal injury (Chen *et al*., 2015). In this study, the relative expression of TGF-β1 mRNA in the high TFCO (160 mg/kg) dose group further supports the assertion that TFCO can promote mucosal repair. This was also consistent with the duodenal villi histopathology in the high dose TFCO (160 mg/kg) group. The relationships between the duodenal villi length, the depth of the crypts and the relative expression of TGF-β1 mRNA showed a linear correlation for 2, 5 and 8 days in all mice infected with *E. coli* indicating that TFCO can promote the expression of TGF-β1 mRNA, to repair duodenum insult. Degree of intestinal wound repair depends on the extent of damage, adjacent epithelial cell migration, proliferation and differentiation (Tsukahara *et al*., 2017), and also the influence of a variety of growth factors, such as fibroblast growth factor, epidermal growth factor, and keratinocyte growth factor (Elshaer and Begun, 2017). The ameliorative potentials of *C. odorata* extracts against tissue injuries have demonstrated *in –vitro* and cell culture studies (Phan *et al*., 2001; Isirima and Siminialayi, 2018), and the results from this study further supports these assertions. Venous stenosis and tissue disruption in the spleen of Wistar rats infected *Salmonella typhi* was reversed by *C. odorata* methanol extract at 200 and 400 mg/kg (Isirima and Siminialayi, 2018). A complex mixture of lipophilic flavonoid aglycones i.e. flavanones, flavonols, flavones and chalcones present in ethanol extract of the plant has potential to protect fibroblasts and keratinocytes (Phan *et al*., 2001). This is likely due to the inhibition of the bacteria biochemical activities such as release of cytotoxins (Isirima and Siminialayi, 2018). In addition, the flavonoids are antioxidative and can ensure cellular oxidative redox to mitigate cytotoxicity (Phan *et al*., 2001).

Advances in microbiomics have revealed that gut microbial dynamics changes affect the body's immune factors and functions (Zhang *et al*., 2018). When mice are infected with *E. coli*, the body's immune system becomes active and releases antibodies and other antigen immunomodulatory substances into the bloodstream. Our results showed that TFCO can improve the antibody titer and enhance serum immunity, as a result of it bacteriostatic effect against *E. coli*. This resulted in the elevated response of the serum IgA ($P<0.01$) and IgG ($P<0.05$) levels and such effects were most marked in the highest TFCO (*E. coli* + 160 mg/kg) group. This finding agrees with a recent report by Zhang *et al*. (2018) which reported that, TFCO reduces the population of fermicutes including *E. coli* and enhances serum humoral immunity in broiler chickens. Specific IgGs prevented enteroinvasive *E. coli* induced diarrhoea. Thus, IgG was able to provide effective protection to prevent bacteria-induced diarrhoea (Xu *et al*.,

2006). This partly accounts for effectiveness of *C. odorata* in the treatments of diarrhoea (Aba *et al*., 2015).

CONCLUSION

TFCO exhibited good bacteriostatic effect *in vitro* and *in vivo*. It was effective in enhancing antibody release, inducing expression of TGF-β1 mRNA, which can promote the repair of injury to duodenal villi and crypts. This study provides a theoretical basis for exploring the mechanism of the TFCO against *E. coli*-induced diarrhoea in mice. The study further supports the potential utilization of *C. odorata* as antibacterial agents in food animal productions for biosafety and sustainable livestock productions.

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Statement of conflicts of interest

The authors have declaredno conflict of interest.

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