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Molecular and Immunological Studies of Phloretin and Escherichia Coli on Experimentally Induced Ulcerative Colitis in Rats

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Abstract

Background: In therapy of bowel disease, immunosuppressants or anti-inflammatory medications were used. Present article aimed to assess intestinal anti-inflammatory features activity of Escherichia coli (Migula) Castellani and Chalmers, ATCC 25922 and another Escherichia coli Egyptian isolate individually and in combination along with phloretin versus DSS-induced ulcerative colitis in rats.

Methods: Bacteria isolation and identification was done using 16S rRNA, VITEK and conventional methods, Also, E. coli ATCC 25922 108 CFU, nonpathogenic, and phloretin (100 mg/kg.b.w.) was administered orally individually and in combination for 15 days to rats with DSS-induced colitis.

Results: 16S rRNA sequence of isolate showed monophyletic clade with Escherichia genera according to Phylogenetic analysis. It was 97.21% 16S rRNA gene sequence similar to Escherichia coli. However, administration E. coli ATCC 25922 and phloretin individually or in combination significantly reduces plasma total cholesterol (TC), interleukin-1 β (IL-1β), tumor necrosis factor alpha (TNF-α) and Leucine rich alpha2-glycoprotein (LRG) as well as colon malondialdehyde (MDA) in DSS-treated rats.

Also, plasma triglycerides (TG) and HDL-cholesterol, reduced glutathione (GSH) and superoxide dismutase (SOD) showed significantly elevated in DSS-treated rats when administrated E. coli ATCC 25922, isolated E. coli, and phloretin individually or in combination. Moreover, E. coli ATCC 25922 and isolated one significantly down regulates of nuclear factor- Kβ (NF-Kβ), high-mobility group box protein 1 (HMGB1) as well as up regulates of trefoil factor 3 (TFF3) gene expression in DSS-treated rats.

Conclusion: Results clearly suggest that E. coli ATCC 25922 and isolated one when combined with phloretin displayed intestinal anti-inflammatory properties and improve gut microbiota in DSS model of colitis in rats.

Key words: E. coli ATCC 25922, phloretin, DSS, ulcerative colitis, inflammatory cytokines, oxidative stress biomarkers and TFF3.

1. Background

Ulcerative Colitis (UC) is the most common inflammation of gut, has a wide range of effects on one's health and quality of life, it's often catastrophic [1,2]. It primarily affects young people which causes lifetimes of relapsing and quitting activity for many of them [3,4]. Incidence rates vary considerably depending on the region. In 2017, UC incidence rates ranged from 0.97 to 57.9 per 100,000 in Europe, 8.8 to 23.14 per 100,000 in North America, and 0.15 to 6.5 per 100,000 in Asia and the ME [5]. Its incidence noted in Egypt is going up in the last decades [6].

Escherichia coli (E. coli) which known as *Enterobacteriaceae*, the most ubiquitous colonizing gut microbiota in intestines of human and numerous animals [7]. *E. coli* is a diverse group of bacteria, the majority of which are

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innocuous, they are critical to preserving the health of human intestinal tract by producing vitamin K2 and preventing growth of harmful bacteria [8]. Nonpathogenic *E.* coli strain is *E. coli* ATCC 25922 that has been certified as biosafety level 1 (BSL-1) and can be used in a variety of laboratory experiments [9]. In addition to being well-known as Gram-negative control bacterium, it is also universally perceived as a prokaryotic model organism in the disciplines of microbiology and biotechnology that served as host organism [10]. Phloretin Figure (1) is a flavonoid that belongs to the chalcone class of flavonoids and is occured naturally phytochemical [11]. Molecular formula C15H14O5, molecular weight 274.27 Da, Solid, Pearl white powder; Sweet aroma, Melting Point 263.5 °C, slightly soluble in water and sparingly soluble in ethanol [12]. Since phloretin is widespread in apples and strawberries, it has become a staple of the average person's diet. Phloretin has been found to have several biological properties, including anti-inflammatory, anti-oxidative, and anti-cancer effects. [13].

Phloretin, for example reduced (IL-1β)-induced proinflammatory cytokines in human lung epithelial cells via modulating the activation of the NF-B pathway [14].

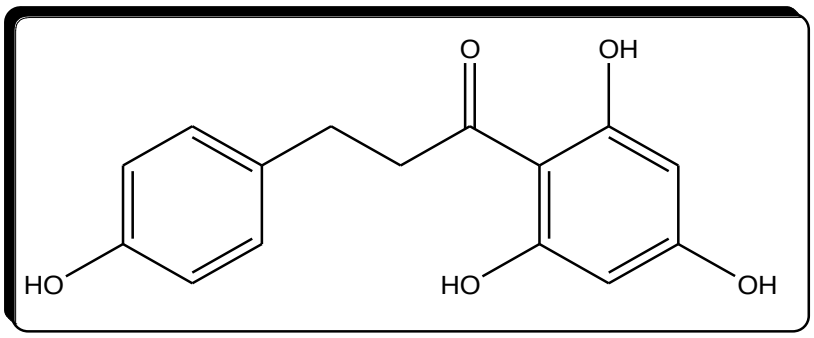


Figure. 1: Chemical structure of phloretin

Moreover, in rat model of sepsis caused by cecal ligation and puncture, [15] revealed that phloretin showed potent antioxidant and anti-inflammatory effects. According to recent study, phloretin is a powerful natural TLR2/1 inhibitor that can be used to treat TLR2-mediated inflammatory immunological disorders. [13]. our study aimed to estimate molecular and immunological effects of phloretin and *E. coli* on experimentally induced ulcerative colitis in rats.

In this article, we present a simple method for evaluating the combined therapeutic potential of Escherichia coli and phloretin in rat model of DSS-induced ulcerative colitis, that could open the gate to potential therapeutic use.

2. Materials and Methods

2.1. Materials

MP Biomedicals, based in UK, supplied the phloretin (97.5%) and Dextran Sulphate Sodium Salt, colitis grade (MW 36-50 kDa) (DSS, 98%) by purchased. All other chemicals used in this study were of the analytical grade, were stored according to industry standards, and came from typical commercial suppliers.

2.2. Samples collection

Clinical isolate of *E. coli* was collected from abdominal abscess from Al-Salam International Hospital.

Escherichia coli ATCC 25922 (Migula) Castellani and Chalmers was purchased from Microbiological Resources Center (Cairo MIRCEN).

2.3. Isolation and identification of bacterial isolate

For enrichment, nutrient broth-containing sample was incubated at 37°C overnight. After that, the culture was streaked onto MacConkey agar then cultured at 37° Celsius for 24 hours. Large Target colonies were inoculated on Eosin Methylene Blue agar media after that cultured up to 24 hours at 37° Celsius. Gram staining and biochemical tests including Citrate utilization, Catalase, Oxidase, Voges-Proskauer, Urease, Methyl Red, and indole production tests were conducted to verify E. coli [16].

Bacteria were conserved with 15% glycerol/nutrient broth at -80ºC until use [17].

2.4. Identification of bacteria by VITEK 2 system

VITEK 2 is a growth-based microbiology system that is automated, used to identify bacteria [18].

2.5. Molecular confirmation

Using PCR with 16S rRNA target, the isolate was molecularly confirmed. Bacterial genomic DNA was extracted from cells by DNA extraction kit by use protocol of Quick-DNA™ Fungal/Bacterial Miniprep Kit Catalog No. D6005 (ZYMO RESEARCH, Irvine, CA), then PCR was made by using Willowfort COSMO PCR RED Master Mix W10203001kit (Willowfort, Birmingham, UK) as described by the manufacturer.

2.6. Animals

Female albino rats weighing around 150±10 g was obtained from animal house of Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. They were housed in plastic cages with stainless steel covers at the National Cancer Institute Animal House. The animals were maintained at a temperature of 22±1°C and a humidity of 55–60% in a light-controlled room. The animals were kept for 1 week to acclimatize and provided with standard diet and water *ad libitum*.

2.7. Experimental setup

This study aimed to investigate the protecting impact of *Escherichia coli* and phloretin in combination against DSS-induced ulcerative colitis. This experiment was carried out in accordance with the guidelines of Applied Health Sciences Technology Animal Care and Use Committee (Approval No. 20220523). Nine groups of six adult female albino rats, each weighing approximately 150±10 g, were formed. Table 1 lists the experimental groups in brief.

Table 1. Treatment groups descriptions

Group	Name of groups	Treatment description
I	Control negative	3 mL of distilled water, orally for 15 days
II	Nutrient broth	Rats was administrated 15%glycerol/nutrient broth (200 µL), orally for 15 days
III	DSS	Rats was administrated 5% DSS (which was dissolved in DW to prepare 5% DSS solution) then allowed the rats to drink freely for 15 days
IV	Phloretin	Rats was administrated Phloretin (100 mg/kg), orally for 15 days (19,20).
V	DSS + Phloretin	Rats was administrated DSS (5% in DW + Phloretin (100 mg/kg), orally for 15 days
VI	DSS + Standard *E. coli* ATCC 25922	Rats was administrated DSS (5% in DW + Standard *E. coli*, ATCC 25922 10^8 CFU (200 µL) by gavage for 15 days
VII	DSS + Isolated *E. coli*	Rats was administrated DSS (5% in DW + isolated *E. coli* 10^8 CFU (200 µL) by gavage for 15 days
VIII	DSS +Standard *E. coli* ATCC 25922 + Phloretin	Rats was administrated DSS (5% in DW + Standard *E. coli*, ATCC 25922 10^8 CFU (200 µL) by gavage + Phloretin (100 mg/kg), orally for 15 days
VIIII	DSS +Isolated *E. coli* + Phloretin	Rats was administrated DSS (5% in DW + isolated *E. coli* 10^8 CFU (200 µL) by gavage + Phloretin (100 mg/kg), orally for 15 days

At the completion of the trial, on the15th day, all rats were euthanized, blood was drawn from each animal's retroorbital vein as well as placed in heparin-containing tubes and centrifuged at 1000 xg for 20 minutes cholesterol (TC) [21], triglycerides (TG) [22] as well as High-density lipoprotein cholesterol (HDL-C) [23], tumor necrosis factor alpha (TNF- α) [24], interleukin-1 β (IL-1β) [25], Leucine rich alpha2-glycoprotein (LRG) [26] levels were measured in separated plasma.

Colons were also collected, washed in phosphate buffered saline, then dried among two filters papers. Colons' distal sections were then partitioned, one for histological research and the other frozen at − 80 °

Celsius retained up until biochemical analysis of thiobarbituric acid reactive substances (TBARS) [27], reduced glutathione (GSH) [28], superoxide dismutase (SOD) [29] in addition to quantitative real-time (qRT-PCR) for nuclear factor- Kβ (NF-Kβ), high-mobility group box protein 1 (HMGB1) and trefoil factor 3 (TFF3) gene expression.

2.8. Real-time PCR

Colon tissues' total RNA was extracted using RNA-spinTM total RNA extraction kit of (QiaGen GmbH, Hilden, Germany). Reverse transcription kit used for preparing cDNA, total RNA (1 –5 g) (Applied Biosystems, Foster City, CA). By using SYBR Green PCR master mix, cDNA was used for qPCR (iNtRON Biotechnology, Korea) as described by the manufacturer. Table (2) elucidate the evaluated gene sequences (NF-Kβ, HMGB1 and TFF3) and β-actin as a housekeeping primer (Primer Design Ltd, USA) utilized in RT-PCR.

Table (2): Primers sequences utilized in RT-PCR

Gene	**Sequence**	
NF-Kβ [30]	forward	5'- CATGAAGAGAAGACACTGACCATGGAAA -'3
	reverse	5'- TGGATAGAGGCTAAGTGTAGACACG -'3
HMGB1 [31]	forward	5'- TGATTAATGAATGAGTTCGGGC–'3
	reverse	5' - TGCTCAGGAAACTTGACTGTTT -'3
TFF3 [32]	forward	5'-TAATGCTGTTGGTGGTCCTG-'3
	reverse	5'-CAGCCACGGTTGTTACACTG-'3
β-actin [33] (housekeeping)	forward	5'-TGACTGACTACCTCATGAAGATCC-'3
	reverse	5'-TCTCCTTAATGTCACGCACGATT-'3

2.9. Histological assessment:

Colon tissues were divided into slices and pieces, and then fixed in 10% formaldehyde solution. After that it was dehydrated, embedded in paraffin then sectioned, and stained with hematoxylin and eosin [34]

2.10. Statistical analysis

Findings were presented as mean ± SD for each of the nine distinct determinations. SPSS/18 was used to statistically evaluate all data SPSS Windows, Version 18.0.0(Inc., Chicago, IL, USA) [35]. One-way analysis of variance was performed to evaluate hypothesis, followed by least significant difference test ($p< 0.05$). Two-sided P value.

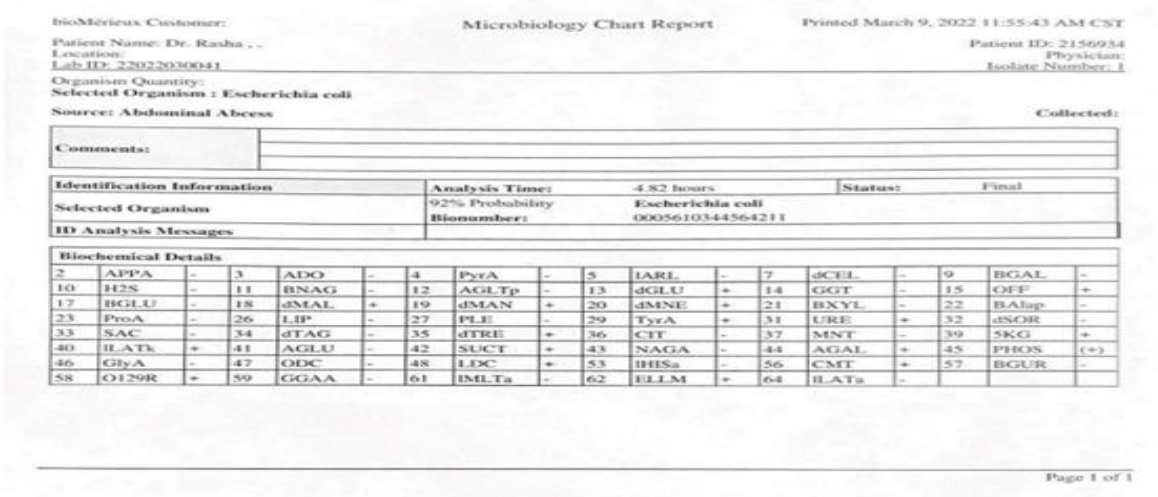
3. Results

3.1. Isolation and identification of bacterial isolates

The specimen collected from abdominal abscess of the patient was cultured onto MacConkey Eosin Methylene Blue (EMB) agar. Appearance of pink colonies on MacConkey agar prove that isolate bacteria capable to ferment lactose and gave green colonies with metallic sheen on Eosin Methylene Blue agar**.**

3.2. Microscopical inspection and biochemical characteristics

Gram staining and other microscopically noticeable traits were used to identify pure colonies. The bacterial isolate had peritrichous flagella yet was shaped like gram-negative rods that non-spore forming. Bacterial isolates were thought to be E. coli based on its microscopical features was exposed to the relevant biochemical tests. VITEK 2 system was used for identifying bacteria. Figure 2 showed that there was a 92% probability of *Escherichia coli.*



bioMérieux Customer: Microbiology Chart Report Printed March 9, 2022 11:55:43 AM CST

Patient Name: Dr. Rasha . . Patient ID: 2156934
Location: Physician:
Lab ID: 22022030041 Isolate Number: 1
Organism Quantity:
Selected Organism : Escherichia coli
Source: Abdominal Abcess **Collected:**

Comments:	

Identification Information	**Analysis Time:**	4.82 hours	**Status:**	Final
Selected Organism	92% Probability	**Escherichia coli**		
ID Analysis Messages	**Bionumber:**	0005610344564211		

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	+	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	+
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	(+)
46	GlyA	-	47	ODC	-	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	+	64	BLATa	-			

Page 1 of 1

Figure 2: Biochemical reactions by VITEK. Showed that there was a 92% probability of *Escherichia coli.*

3.3. Identification by 16s rRNA gene sequencing

Phylogenetic analysis was performed by SIGMA SCIENTIFIC SERVICE company figure (3). The BLAST result demonstrated that the 16S rRNA sequence of isolate has 97.21% similar sequence with *Escherichia coli* strain NBRC 102203 with accession number (NR 114042.1). The isolate was submitted in NCBI with accession number ON970911.

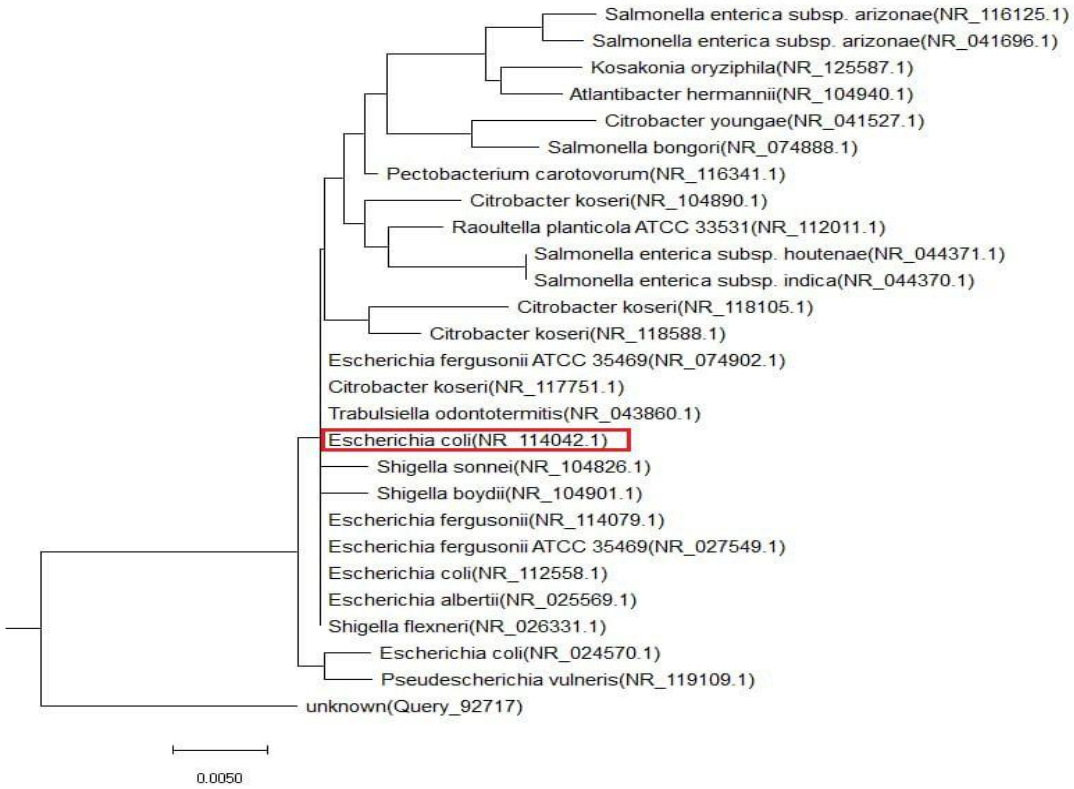


Figure 3: Phylogenetic tree of the isolated Escherichia coli has similar sequence with Escherichia coli strain NBRC 102203 with accession number NR 1140421.1

3.4. Result of biochemical assay

Table 3 showed plasma levels of high-density lipoprotein cholesterol **(**HDL-C), triglycerides (TG) as well as cholesterol (TC) in different groups of rats. In comparison to normal control group, oral administration of Dextran Sulphate Sodium (DSS 5%) resulted in a significantly increase ($p<0.05$) in plasma levels of TC and a significant depletion ($P< 0.05$) of plasma TG and HDL-C, respectively. Also, administration of normal rats with phloretin (100 mg/kg.b.w.) and nutrient broth was non-significantly changed HDL-C, TG and TC, in comparison to normal rats.

However, treatment with Phloretin (100 mg/kg.b.w.) resulted in a significant reduction in plasma TC level and increase in TG and HDL-C, when compared with DSS-treated control group of rats. Furthermore, administration of standard *E. coli* ATCC 25922 10^8 CFU significant decrease in plasma TC level, as well as increase in TG and HDL-C levels, in comparison to DSS-treated control group of rats ($P< 0.05$). In contrast, administration of isolated *E. coli* 10^8 CFU significantly decreased plasma TC level also increased in TG and HDL-C levels, when compared with DSS-treated control group of rats. In addition, administration of standard *E. coli* ATCC 25922 10^8 CFU + phloretin (100 mg/kg.b.w.) decreased plasma TC level, as well as increased TG and HDL-C levels, in comparison to DSS-treated control group of rats. However, administration of isolated *E. coli* 10^8 CFU + phloretin (100 mg/kg.b.w.) significantly decreased plasma TC level and increased plasma TG as well as HDL-C, when compared with DSS-treated control group of rats.

Table 3. Effect of standard and isolated *Escherichia coli* both individually and in combination along with phloretin on plasma cholesterol, triglycerides, and HDL-cholesterol in normal and DSS-treated rats

No.	Groups	Cholesterol (mg/dl)	triglycerides (mg/dl)	HDL-C (mg/dl)
(I)	Control negative	87.85 ±6.25	175.90 ±8.09	37.98 ±5.48
(II)	15% Glycerol/Nutrient broth	88.13 ± 7.48	172.90 ± 6.81	34.28 ± 3.07
(III)	DSS (5% in DW)	168.48^{ab} ±8.13	109.67^{ab} ±6.97	18.46^{ab} ±2.80
(IV)	Phloretin (100 mg/kg.b.w.)	81.55^{c} ±4.45	171.76^{c} ±12.11	38.30^{ac} ±3.91
(V)	DSS + Phloretin (100 mg/kg.b.w.)	126.17^{abcd} ± 4.54	146.48^{abcd} ± 7.11	30.44^{ac} ± 3.80
(VI)	DSS + Standard *E. coli*, ATCC 25922 10^8 CFU	135.73^{abcde} ± 5.80	144.60^{abcd} ± 4.74	26.08^{abcd} ± 4.74
(VII)	DSS + Isolated *E. coli* 10^8 CFU	143.59^{abcdef} ±5.81	135.44^{abdef} ±5.54	25.63^{abcd} ±4.92
(VIII)	DSS +Standard *E. coli*, ATCC 25922 10^8 CFU + Phloretin	$94.13^{abcdefg}$ ±5.39	176.95^{cefg} ±6.22	34.18^{cfg} ±2.90
(VIIII)	DSS +Isolated *E. coli* 10^8 CFU + Phloretin	$114.93^{abcdefgh}$ ±8.38	$159.94^{abcdefgh}$ ±8.18	33.59^{acfg} ±3.44

DSS, 5% was given orally. It was given to all groups except the normal one. Isolated *Escherichia coli* 10^8 CFU, Standard *Escherichia coli* ATCC 25922 10^8 CFU and Phloretin were given orally for 2 weeks. Values represent the mean ± SD (n=6). Data shown are mean ± standard deviation of number of observations within each treatment.

a: Significant with Control negative; b: Significant with 15% Glycerol/Nutrient broth; c: Significant with DSS (5% in DW); d: Significant with Phloretin (100 mg/kg.b.w.); e: Significant with DSS + Phloretin (100 mg/kg.b.w.); f: Significant with DSS + Standard *E. coli*, ATCC 25922 10^8 CFU; g: Significant with DSS + Isolated *E. coli* 10^8 CFU; h: Significant with DSS +Standard *E. coli*, ATCC 25922 10^8 CFU + Phloretin

Table 4 showed the effect of standard and isolated *E. coli* both individually and in combination along with phloretin on plasma interleukin-1 β (IL-1β), tumor necrosis factor alpha (TNF-α) and leucine rich alpha2-glycoprotein (LRG) in normal and DSS-treated rats. In comparison to normal control group, oral administration of DSS (5%) resulted in a significant increase ($p<0.05$) in plasma IL-1β, TNF-α and LRG

levels, respectively. Also, administration of normal rats with phloretin (100 mg/kg.b.w.) and nutrient broth was non-significantly changed plasma IL-1β, TNF-α and LRG when compared with the normal rats.

However, in comparison to DSS-treated control group of rats, treatment by Phloretin (100 mg/kg.b.w.) significantly reduced plasma IL-1β, TNF-α and LRG levels.

Table 4. Effect of standard and isolated *Escherichia coli* both individually and in combination along with phloretin on plasma IL-1β, TNF-α and Leucine rich alpha2-glycoprotein (LRG) in normal and DSS-treated rats

No.	Groups	IL-1β (Pg/mL)	TNF-α (pg/ mL)	LRG (ng/mL)
(I)	Control negative	38.60 ±5.46	4.23 ±0.98	22.39 ±3.47
(II)	15% Glycerol/Nutrient broth	37.77 ± 5.28	3.88 ± 0.68	24.01 ± 3.47
(III)	DSS (5%)	105.08^{ab} ±9.54	12.24^{ab} ±1.26	121.17^{ab} ±8.91
(IV)	Phloretin (100 mg/kg.b.w.)	37.13^{c} ±4.33	4.02^{c} ±0.75	22.80^{c} ±2.33
(V)	DSS + Phloretin (100 mg/kg.b.w.)	59.56^{abcd} ± 3.60	6.01^{c} ± 0.37	40.20^{abcd} ± 5.00
(VI)	DSS + Standard *E. coli*, ATCC 25922 10^8 CFU	83.35^{abcde} ± 5.89	7.51^{c} ± 0.32	59.30^{abcde} ± 3.95
(VII)	DSS + Isolated *E. coli* 10^8 CFU	92.25^{abcdef} ±6.57	9.20^{abcdef} ±0.51	69.96^{abcdef} ±5.32
(VIII)	DSS +Standard *E. coli*, ATCC 25922 10^8 CFU + Phloretin	43.13^{abdefg} ±3.80	4.70^{cg} ±0.36	$27.11^{abcdefg}$ ±2.84
(VIIII)	DSS +Isolated *E. coli* 10^8 CFU + Phloretin	$55.67^{abcdfgh}$ ±5.36	5.67^{cg} ±0.51	$33.67^{acdefgh}$ ±2.81

DSS, 5% was given orally. It was given to all groups except the normal one. Isolated *Escherichia coli* 10^8 CFU, Standard *Escherichia coli* ATCC 25922 10^8 CFU and Phloretin were given orally for 2 weeks. Values represent the mean ± SD (n=6). Data shown are mean ± standard deviation of number of observations within each treatment.
a: Significant with Control negative; b: Significant with 15% Glycerol/Nutrient broth; c: Significant with DSS (5% in DW); d: Significant with Phloretin (100 mg/kg.b.w.); e: Significant with DSS + Phloretin (100 mg/kg.b.w.); f: Significant with DSS + Standard *E. coli*, ATCC 25922 10^8 CFU; g: Significant with DSS + Isolated *E. coli* 10^8 CFU; h: Significant with DSS +Standard *E. coli*, ATCC 25922 10^8 CFU + Phloretin

Furthermore, the groups which administered with (standard *E. coli* ATCC 25922 10^8 CFU or standard *E. coli* ATCC 25922 10^8 CFU + phloretin (100 mg/kg.b.w.) or isolated *E. coli* 10^8 CFU or isolated *E. coli* 10^8 CFU + phloretin (100 mg/kg.b.w.)) resulted in significant decrease in plasma IL-1β, TNF-α and LRG levels, respectively, when compared to DSS-treated control group of rats ($P< 0.05$).

Table 5 showed the Effect of standard and isolated *E. coli* both individually and in combination along with phloretin on colon reduced glutathione (GSH), superoxide dismutase (SOD) and Malondialdehyde (MDA) in normal and DSS-treated rats. In comparison to normal control group, oral administration of DSS (5%) resulted in a significant increase ($p<0.05$) in colon levels of MDA and a significant depletion ($P< 0.05$) of colon GSH and SOD levels, respectively. Also, administration of normal rats with Phloretin (100 mg/kg.b.w.) and nutrient broth was non-significantly changed colon GSH, SOD and MDA, when compared with the normal rats.

Table 5. Effect of standard and isolated *E. coli* both individually and in combination along with phloretin on colon GSH, SOD and MDA in normal and DSS-treated rats

No.	Groups	GSH (nmol/mg tissue)	SOD (U/mg tissue)	MDA (nmole/mg tissue)
(I)	Control negative	23.16 ±3.20	363.24 ±8.15	2.61 ±0.41
(II)	15% Glycerol/Nutrient broth	20.99 ± 2.40	369.38 ± 8.36	2.95 ± 0.32
(III)	DSS (5% in DW)	4.79^{ab} ±0.51	130.97^{ab} ±9.33	8.63^{ab} ±0.28
(IV)	Phloretin (100 mg/kg.b.w.)	23.08^{c} ±3.30	362.71^{c} ±9.97	2.79^{c} ±0.27
(V)	DSS + Phloretin (100 mg/kg.b.w.)	19.62^{abcd} ± 2.93	336.25^{abcd} ± 7.88	4.82^{abcd} ± 0.68
(VI)	DSS + Standard *E. coli* ATCC 25922 10^8 CFU	15.97^{abcd} ± 2.10	312.81^{abcde} ± 9.37	5.38^{abcd} ± 0.30
(VII)	DSS + Isolated *E. coli* 10^8 CFU	15.29^{abcd} ±2.46	309.32^{abcde} ±10.11	5.86^{abcd} ±0.28
(VIII)	DSS +Standard *E. coli* ATCC 25922 10^8 CFU + Phloretin	23.04^{cefg} ±3.42	$384.80^{abcdefg}$ ±12.1	3.88^{c} ±0.22
(VIIII)	DSS +Isolated *E. coli* 10^8 CFU + Phloretin	20.46^{cefg} ±2.27	$331.38^{abcdfgh}$ ±10.29	4.29^{abcd} ±0.33

DSS, 5% was given orally. It was given to all groups except the normal one. Isolated *Escherichia coli* 10^8 CFU, Standard *Escherichia coli* ATCC 25922 10^8 CFU and Phloretin were given orally for 2 weeks. Values represent the mean ± SD (n=6). Data shown are mean ± standard deviation of number of observations within each treatment.

a: Significant with Control negative; b: Significant with 15% Glycerol/Nutrient broth; c: Significant with DSS (5% in DW); d: Significant with Phloretin (100 mg/kg.b.w.); e: Significant with DSS + Phloretin (100 mg/kg.b.w.); f: Significant with DSS + Standard *E. coli*, ATCC 25922 10^8 CFU; g: Significant with DSS + Isolated *E. coli* 10^8 CFU; h: Significant with DSS +Standard *E. coli*, ATCC 25922 10^8 CFU + Phloretin

However, when compared to DSS-treated control group of rats, treatment with phloretin (100 mg/kg.b.w.) resulted in a significant reduction in colon MDA level and a significant increase in colon SOD and GSH levels. Furthermore, administration of standard *E. coli* ATCC 25922 10^8 CFU significantly decreased colon MDA level and increased colon SOD and GSH levels, respectively, in comparison to DSS-treated control group of rats ($P< 0.05$). In contrast, administration of isolated *E. coli* 10^8 CFU significantly decreased colon MDA level and significantly increased colon SOD as well as GSH level, in comparison to DSS-treated control group of rats. In addition, administration of standard *E. coli* 10^8 CFU + phloretin (100 mg/kg.b.w.) significantly decreased colon MDA level also, increased in colon SOD and GSH levels, respectively, in comparison to DSS-treated group in control of rats ($P< 0.05$). However, administration of isolated *E. coli* 10^8 CFU + phloretin (100 mg/kg.b.w.) significantly decreased colon MDA level, as well as significantly increased colon GSH and SOD levels, in comparison to DSS-treated group in control of rats.

Figure 4 showed the Effect of standard and isolated *E. coli* both individually and in combination along with phloretin on colon nuclear factor- Kβ (NF-Kβ), high-mobility group box protein 1 (HMGB1) and trefoil factor 3 (TFF3) in normal and DSS-treated rats. When compared to normal control group, oral administration of DSS (5%) significantly increased ($p<0.05$) colon levels of NF-Kβ and HMGB1, as well as significantly depleted colon TFF3. Also, administration of normal rats with Phloretin (100 mg/kg.b.w.) and nutrient broth was non-significantly changed colon NF-Kβ, HMGB1 and TFF3, when compared with the normal rats.

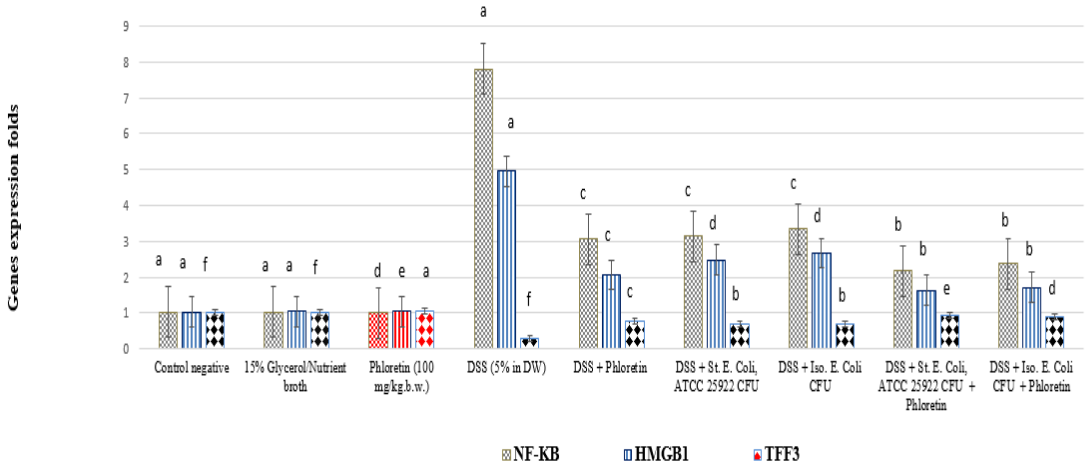


Figure 4: Effect of standard and isolated *Escherichia Coli* both individually and in combination along with phloretin on colon nuclear factor- Kβ (NF-Kβ), high-mobility group box protein 1 (HMGB1) and trefoil factor 3 (TFF3) in normal and DSS-treated rats. Oral administration of DSS (5%) significantly increased colon levels of NF-Kβ and HMGB1, and significantly decreased colon TFF3. Treatment with phloretin (100 mg/kg.b.w.) significantly reduced colon levels of NF-Kβ and HMGB1 and significantly increased colon TFF3 in compared with DSS group of rats. Groups of (standard *E. coli ATCC 25922* 10^8 CFU, standard *E. coli* ATCC 25922 10^8 CFU + phloretin (100 mg/kg.b.w.), isolated *E. coli* 10^8 CFU or isolated *E. coli* 10^8 CFU + phloretin (100 mg/kg.b.w.)) resulted in significant decrease in colon NF-Kβ and HMGB1 levels, respectively, and a significant increase in colon TFF3 levels, when compared to DSS-treated group in control of rats.

However, when compared to DSS-treated control group of rats, treatment with phloretin (100 mg/kg.b.w.) significantly reduced colon levels of NF-Kβ and HMGB1, as well as a significantly increased colon TFF3. Furthermore, the groups which administrated with (standard *E. coli* ATCC 25922 10^8 CFU or standard *E. coli* ATCC 25922 10^8 CFU + phloretin (100 mg/kg.b.w.) or isolated *E. coli* 10^8 CFU or isolated *E. coli* 10^8 CFU + phloretin (100 mg/kg.b.w.)) resulted in significant decrease in colon NF-Kβ and HMGB1 levels, respectively, and a significant increase in colon TFF3 levels, when compared to DSS-treated group in control of rats ($P< 0.05$). TFF3 and β-actin images of agarose gel electrophoresis by RT-PCR support our outcomes (Figure 5).

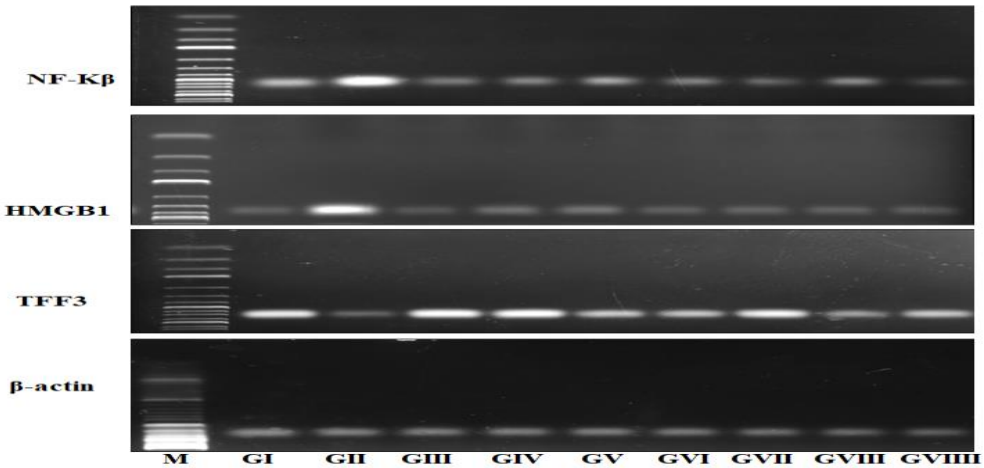


Figure 5: An agarose gel electrophoresis shows PCR products of colon nuclear factor- Kβ (NF-Kβ), high-mobility group box protein 1 (HMGB1), trefoil factor 3 (TFF3) and beta actin (β-actin) in different studied groups. M: DNA marker with 100bp. Group I (GI): negative control group (a) Was administrated DW, Group II (GII): Was administrated 15% Glycerol/Nutrient broth (b), Group III (GIII): Was administrated DSS (5% in DW), Group IV (GIV): Was administrated phloretin (100 mg/kg.b.w.), Group V (G V): Was administrated DSS + Phloretin (100 mg/kg.b.w.), Group VI (GVI): Was administrated DSS + Standard *E. coli* ATCC 25922 10^8 CFU (f), Group VII (GVII): Was administrated DSS + Isolated *E. coli* 10^8 CFU, Group

VIII (GVIII): Was administrated DSS +Standard *E. coli* ATCC 25922 10^8 CFU + Phloretin (100 mg/kg.b.w.), Group VIIII (GVIIII): Was administrated DSS + Isolated *E. coli* 10^8 CFU + Phloretin (100 mg/kg.b.w.).

Histopathological examination of rat's colon tissues of 3 different groups in figure 6. Group I: Normal rat as negative control (a), Group II: Was administrated 15%glycerol/nutrient broth (b), Group IV: Was administrated phloretin (100 mg/kg.b.w.) (c). The three groups showed normal colonic mucosa, with numerous mucosal glands, lined by simple columnar epithelium (black arrow), with normal goblet cells (blue arrow). No granulomatous reaction seen, no evidence of inflammatory cell infiltrate and no ulcerations (H&E; 200X).

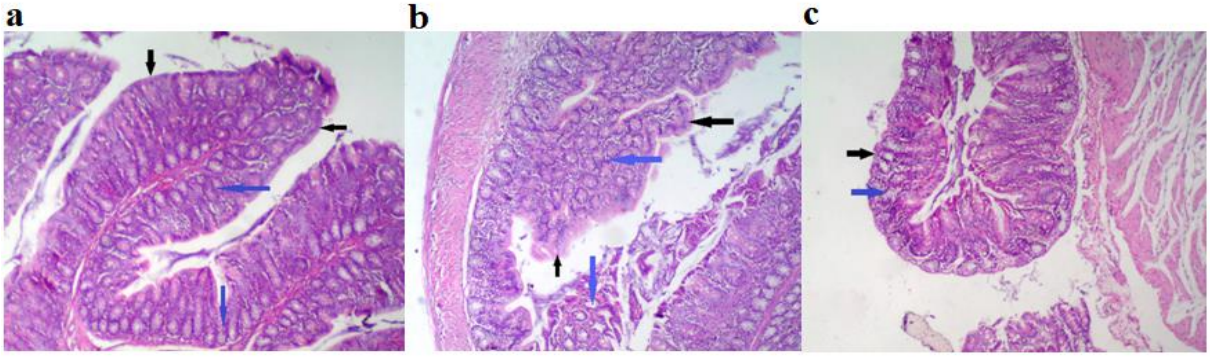


Figure 6: Histological examination of rat's colon tissues of 3 different groups. Group I: Normal rat as negative control (a), Group II: Was administrated 15%glycerol/nutrient broth (b), Group IV: Was administrated phloretin (100 mg/kg.b.w.) (c). The three groups showed normal colonic mucosa, with numerous mucosal glands, lined by simple columnar epithelium (black arrow), with normal goblet cells (blue arrow). No granulomatous reaction seen, no evidence of inflammatory cell infiltrate and no ulcerations (H&E; 200X).

Histopathological examination in figure 7 Photomicrograph of colon tissue of DSS 5% positive control (a) showed numerous superficial ulcerations (black arrow) and large aggregates of lymphocytes in submucosa forming a granuloma (blue circle) and scattered lymphocytes in mucosa and submucosa (blue arrow). Photomicrograph of colon tissue of DSS + Phloretin (100 mg/kg.b.w.) (b) histological examination of the tissue revealed small aggregates of lymphocytes (blue circle) with no ulceration of the mucosa (black arrow) with reappearance of goblet cells (blue arrow) no granuloma formation (H&E; 200X), Indicating significant improvement compared to group received 5% DSS.

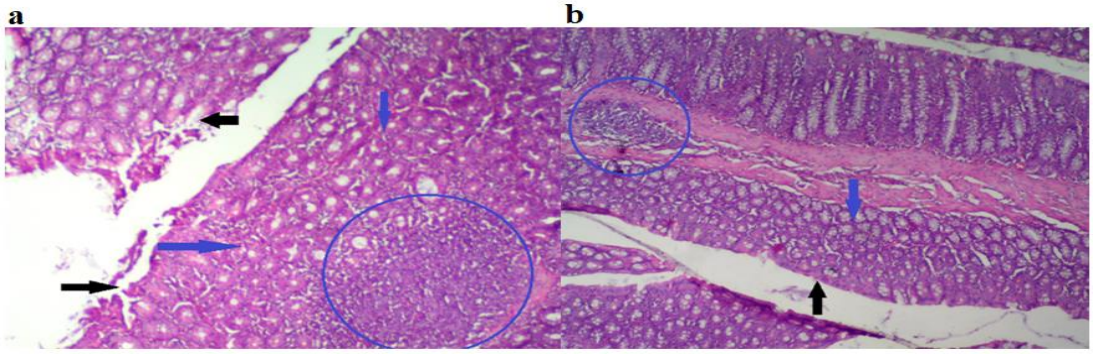


Figure 7: Sections stained with hematoxylin and eosin (H&E; 200X) histological examination of rat's colon tissues of different groups. Group III: Was administrated 5% DSS (a), showed numerous superficial ulcerations (black arrow) and large aggregates of lymphocytes in submucosa forming a granuloma (blue circle) and scattered lymphocytes in mucosa and submucosa (blue arrow), Group V: Was administrated DSS + Phloretin (100 mg/kg.b.w.) (b), histological examination of the tissue revealed

small aggregates of lymphocytes (blue circle) with no ulceration of the mucosa (black arrow) with reappearance of goblet cells (blue arrow) no granuloma formation (H&E; 200X).

Histopathological examination in figure 8 Photomicrograph of colon tissue of DSS + Standard *E. Coli* ATCC 25922 10^8 CFU (a) treated group with significant improvement compared to group (model of colitis) of the mucosal part (blue arrow), improved surface epithelium (red arrow) with focal residual ulcerated portions (black arrow) and mild lymphocytic infiltration (blue circle). The surface epithelium showed healing with no ulceration. The glands retained the adequate mucin production. Photomicrograph of colon tissue of combined DSS + Isolated *E. Coli* 10^8 CFU (b) treated group with improved colonic glands with partial healing of the surface epithelium (black arrow) The glands retained the adequate mucin production and without lymphocytic infiltration.

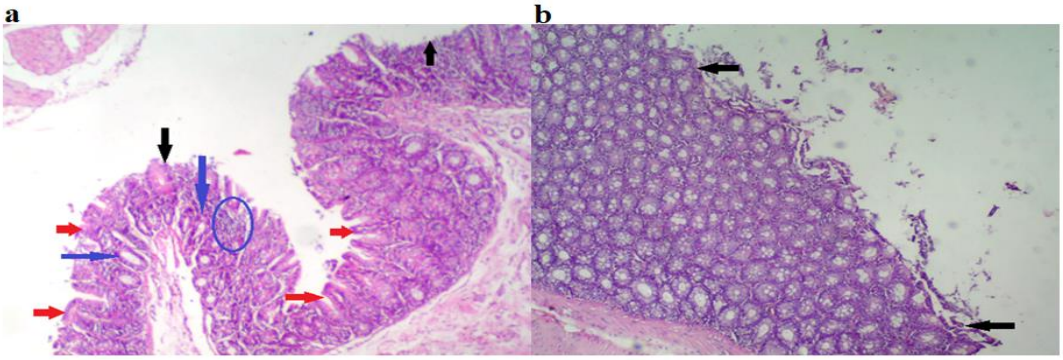


Figure 8: Sections stained with hematoxylin and eosin (H&E; 200 X) histological examination of rat's colon tissues of different groups. Group VI: Was administrated DSS + Standard *E. coli* ATCC 25922 10^8 CFU (a) Photomicrograph of colon tissue with significant improvement compared to group (model of colitis) of the mucosal part (blue arrow), improved surface epithelium (red arrow) with focal residual ulcerated portions (black arrow) and mild lymphocytic infiltration (blue circle), Group VII: Was administrated DSS + Isolated *E. coli* 10^8 CFU (b) Photomicrograph of colon tissue with improved colonic glands with partial healing of the surface epithelium (black arrow).

Histopathological examination in figure 9 Photomicrograph of colon tissue of combined DSS + Standard *E. coli* ATCC 25922 10^8 CFU + Phloretin (100 mg/kg.b.w.) (a) treated group with remarkable improvement compared to DSS treated group of the mucosal part with normal mucous content (blue arrow), approximately normal surface epithelium (black arrow). Photomicrograph of colon tissue of combined DSS + Isolated *E. coli* 10^8 CFU + Phloretin (100 mg/kg.b.w.) (b) treated group with approximately normal histological pattern of the mucosa with mild depletion of mucin in some mucosal glands (blue arrow) and normal surface epithelium (black arrow). The surface epithelium showed healing with no ulceration.

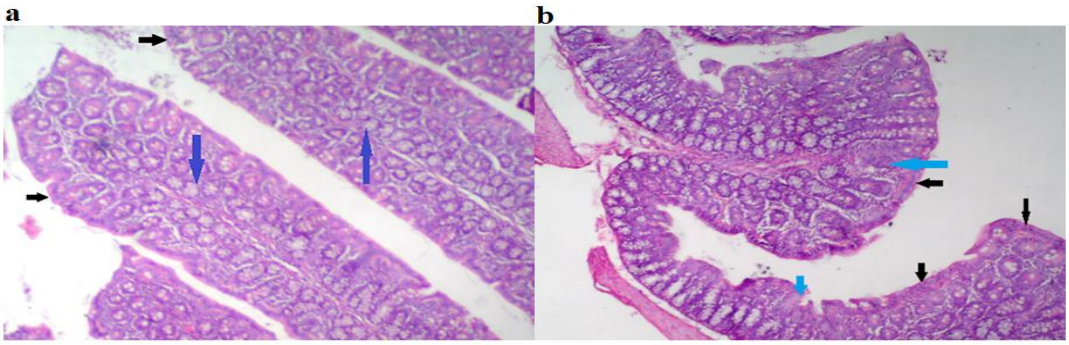


Figure 9: Sections stained with hematoxylin and eosin (H&E; 200 X) histological examination of rat's colon tissues of different groups. Group VIII: Was administrated DSS + Standard *E. coli* ATCC 25922 10^8 CFU + Phloretin (100 mg/kg.b.w.) (a) Photomicrograph of colon tissue with remarkable improvement compared to DSS treated group of the mucosal part with normal

mucous content (blue arrow), approximately normal surface epithelium (black arrow), Group VIIII: Was administrated DSS + Isolated *E. coli* 10^8 CFU + Phloretin (100 mg/kg.b.w.) (b) Photomicrograph of colon tissue with approximately normal histological pattern of the mucosa with mild depletion of mucin in some mucosal glands (blue arrow) and normal surface epithelium (black arrow).

4. Discussion

The isolated bacterial strain's genomic DNA was extracted. Using universal primer, 16S rDNA was amplified and sequenced. The 16S rDNA gene region was sequenced for a total of 1,300 bp and utilized to identify a bacterial strain. This test sequences were compared to GenBank's non-redundant sequence collection [36] database was performed with BLAST [37,38]. 16S rDNA sequence of isolate bacteria has 97.21% sequence similar to *Escherichia coli* NBRC with accession number NR 114042.1 in BLAST result then the isolated bacteria submitted in NCBI with accession number ON970911.

Subsequently, phylogenetic tree based on the 16S rDNA gene sequence was created to highlight the affinity between the isolate bacterium and selected relatives of the *Escherichia coli* genera. Phylogenetic study of 16S rDNA sequences confirms that the isolate bacteria represent a strain in genera *Escherichia coli*.

As previously noted, ulcerative colitis is assumed to be an inflammatory disease mediated by the T helper cell type 2 (TH2), whereas Crohn's is regarded to be a T helper cell type 1 (TH1)-mediated inflammatory disease, although this assertion is losing favor as discussed previously [39]. However, in experimental models, it remains important that the notion of models with a cytokine milieu is balanced in favor TH 2 (IL-4, IL-5, and IL-13) reflect UC and with cytokine milieu is balanced in favor TH 1 (INF-, IL-2, and TNF-) mimic Crohn's disease [40]. Despite the risks associated with these assumptions, murine models provide a valuable way to measure levels of miRNA and inflammatory cytokines in situations that may be attributed to human disease and inflammatory processes.

During this study, we observed that the DSS-treated rats demonstrated an increase in TC as well as decrease in plasma TG and HDL-C levels in comparison with control rats.

As suggested, the administration of *E. coli* ATCC 25922 and isolate *E. coli* both individually and in combination along with phloretin enhanced the total blood cholesterol profile in rats with DSS. Our study hypothesized that, administration of *E. coli* ATCC 25922 as well as isolate *E. coli* improves levels of TC, TG and HDL-C by producing fructose metabolizing enzymes and Pyrroloquinoline quinone (PQQ). In addition, production of fructose metabolizing enzymes by oral administration of *E. coli* ATCC 25922 as well as isolate *E. coli* converted fructose to mannitol which is further converted to short-chain fatty acids (SCFAs) in intestine through intestinal flora. This is corroborated by the fact that mannitol treatment boosted butyrate amounts in pigs' large intestines [41]. Existence of PQQ permits glucose dehydrogenase to create gluconic acid, which is then consumed by *Bifidobacteria* and *Lactobacilli* species, resulting in synthesis of SCFA [42].

On the other hand, Phloretin as a common polyphenol was shown to have considerable reducing effect on TC while enhancing TG and HDL cholesterol in rats treated with DSS. The obtained results in our study are in the line with previously reported animal studies [43-46]

Also, other studies were reported that the elevation of HDL levels is attributed with phenolic compounds supplementation in rats [47,48]. In the current research, the combination between *E. coli* and Phloretin produce a synergistic effect and is more declared than when we administrated them individually.

In the present study, the elevation of plasma IL-1β, TNF-α and leucine rich alpha2-glycoprotein (LRG) levels in DSS-treated rats was attributed with ulcerative colitis

IL-1β and TNF-α as common inflammatory cytokines play multifunctional part in the inflammatory response to a sterile, infectious threat and targeting LRG is presently regarded a highly intriguing innovative technique in diagnosis of infectious diseases [49]. Majority of gastrointestinal dysfunctions progress along inflammation. As a result, we reported plasma level of various inflammation indicators involving TNF-α, IL-1β, and LRG. All biomarkers raised in DSS-treated rats, attributing the inflammation related to this inflammatory disease. Also, Increased

proinflammatory cytokines in UC had been shown in several studies [50-52] and plasma LRG [53].

Current results validated the *in vivo* anti-inflammatory activity of *E. coli* ATCC 25922 and isolated *E. coli* compared to DSS-stimulated ulcerative colitis via lowering TNF-α, IL-1β, and LRG plasma level. Our resulted were supported by related reports demonstrated that secreting pyrroloquinoline quinone (PQQ) by *EcN* 1917 protects liver and colon against toxicants-induced tissue damage via their anti-oxidative properties [54,55]. Also, we suggested that *E. coli* ATCC 25922 and isolated *E. coli* administration ameliorated neurotransmitter biomarkers which alter in response to oxidative stress [56]. Additionally, *E. coli* ATCC 25922 and isolated *E. coli* making PQQ was found to be more declared than given PQQ orally in contrast to some drugs-stimulated oxidative conditions [57,58].

Important study revealed that pro-inflammatory gene expression was significantly blocked by phloretin administration and decreased IL-8 and IP-10 promoter signal pathways [59,60]. Our present study suggested that *E. coli* ATCC 25922 and isolated *E. coli* regulated the gut microbiota by producing PQQ in the presence of phloretin to inhibit production of LRG, IL-1β and TNF-α in DSS-treated rats.

In the present study, we noticed that elevation of colon MDA and depletion of colon GSH and SOD significantly in DSS-treated rats. UC is be associated with having an inflammatory reaction combined along with stomach mucosal damage through increasing in oxidative stress and depletion of antioxidant biomarkers [61]. Rucker et al [62] showed that lymphocyte, plasma cell, and macrophage infiltration and production of pro-inflammatory substances during inflammation of the stomach tissue such as TNF-α, IL-1 and IL-8.

However, administration of *E. coli* ATCC 25922 and isolated *E. coli* inhibit over production of MDA and increase levels of SOD and GSH in rats treated with DSS. Our obtained data was in agreement with the results of Rucker et al [62] and Misra et al [41] who suggested PQQ, which is a potent antioxidant compound released by *Escherichia coli Nissle* (*EcN)* 1917, is generated not by people or their microbiota, but by numerous gram-negative bacteria. It has the ability to stimulate nerve cell regeneration, improve mitochondrial activity and reproductive capacity, and sustain mitochondrial and neural function [63].

Also, phloretin, is widely reported as an antioxidant flavonoid, and possess anti-inflammatory, antioxidant, and neuroprotective effect inside cell-based models [64,65]. Furthermore, the literature reveals that phloretin represent a novel type of neuroprotectants that use their nucleophilic enolate forms to scavenge electrophilic metal ions and unsaturated aldehydes [66]. Our study suggested that the gut microbiota recovery by *E. coli* ATCC 25922 as well as isolated *E. coli* and the produced PQQ which accompanied with phloretin to produces synergistic antioxidant effect against DSS-induced UC.

Furthermore, our results exhibited that elevation of colon NF-kβ, HMGB1 as well as depletion of TFF3 gene expression in DSS-treated rats. Also, NF-kβ, HMGB1 and TFF3 is attributed with gastrointestinal mucosa inflammation [67]. It is co-produced and secreted with mucin (MUC) [68] [69]. Several studies have shown the protective function of all three TFFs in the gastrointestinal tract and their up-regulated expression at the site of mucosal damage [70,71].

In agreement with their alleviative inflammatory potential in the DSS-mediated colitis mentioned above, administration of the *E. coli* ATCC 25922 and isolated *E. coli* inhibited several pro-inflammatory mediators. To better understand the method of action while administering *E. coli* ATCC 25922 and isolated *E. coli* during colitis induction we examined the intestinal recovery closely, and enhance gut microbiota, when the TFF3 gene expression was up regulated in DSS-treated rats which indicates the existence of cellular immune responses to clean the cellular debris and initiate repair processes [72]. However, it may act through other pathways in addition to controlling goblet cell proliferation and TFF3 expression. (TFF-3) is a tissue repair factor involved in epithelial restoration as well as epithelial cell migration [73].

In addition, Phloretin alleviated inflammation by lowering IL-6, IL-8, (ICAM)-1 production, and mRNA expression in TNF-stimulated HaCaT human cells [58]. Furthermore, phloretin has been shown to have neuroprotective properties that reported in rats via activating Nrf2 pathway as well as suppressing oxidative stress [59].

Histological investigation of colonic sections revealed deformed mucosal colon architecture with inflammatory alterations in colonic architecture such as crypt formation, Surface epithelium loss, inflammatory cell infiltration, and total epithelial

architectural destruction The severity of the condition elevates with chronicity. These macroscopic and microscopic distortions detect in our investigation agreed with previous research on UC generated by DSS models in rats [59]. *E. coli* ATCC 25922 and isolate *E. coli* both individually and in combination along with phloretin resulted in significant decrease in inflammatory infiltrates in both lamina propria and submucosa, as well as a dose-dependently protected against changes in mass index and colon length. The use of *E. coli* ATCC 25922 or isolate *E. coli* with phloretin in UC is in accordance with its pathogenesis. The study shows therapeutic efficacy in UC induced by DSS. The study was conducted in vivo in rats, so we recommend doing other studies on human in the future.

5. Conclusions

In summary, our data suggest that, using isolate *E. coli* with accession number ON970911 or *E. coli* (ATCC 25922) could be promising to be combined with phloretin against ulcerative colitis due to its proinflammatory cytokine inhibition role and antioxidant effect as indicated by plasma IL-1β, TNF-α, leucine rich alpha2-glycoprotein (LRG) levels as well as MDA, GSH and SOD levels respectively. Moreover, when compared with DSS-treated rats induced ulcerative colitis, the molecular mechanisms effects were revealed by the downregulation of colon NF-Kβ and HMGB1 levels, which indicates decreasing the secretion of pro-inflammatory cytokines, and the upregulation of colon TFF3 levels which indicates the existence of cellular immune responses to clean the cellular debris and initiate repair processes. Finally, we recommend further studies for phloretin in combination with isolate *E. coli* with accession number ON970911 or *E. coli* (ATCC 25922) as a new novel technique for the treatment of ulcerative colitis.

6. Conflicts of interest

"There are no conflicts to declare."

7. Funding sources

This research did not receive any specific grants from funding agencies in the public, commercial, or non-profit sectors.

8. Acknowledgments

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