Research Article
In Vivo, Proteomic, and In Silico Investigation of Sapodilla for Therapeutic Potential in Gastrointestinal Disorders

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This study aims to delineate the effects of Manilkara zapota Linn. (Sapodilla) fruit chloroform (Mz.CHCl3) and aqueous (Mz.Aq) extracts tested through different techniques. Antidiarrheal activity and intestinal fluid accumulation were examined by using castor oil-induced diarrhea and castor oil fluid accumulation models. Isolated rabbit jejunum tissues were employed for in vitro experiments. Antimotility and antiulcer were performed through charcoal meal transient time and ethanol-induced ulcer assay, molecular studies were conducted through proteomic analysis, and virtual screening was performed by using a discovery studio visualizer (DSV). Mz.CHCl3 and Mz.Aq extracts attributed dose-dependent (50–300mg/kg) protection (20–100%) against castor oil-induced diarrhea and dose-dependently (50–300mg/kg) inhibited intestinal fluid secretions in mice. Mz.CHCl3 and Mz.Aq extracts produce relaxation of spontaneous and K+ (80Mm) induced contractions in isolated tissue preparations and decreased the distance moved by charcoal in the gastrointestinal transit model in rats. It showed gastroprotective effect in ulcerative stomach of rats and decreased levels of IL-18 quantified by proteomic analysis. Histopathological results showed ethanol-induced significant gastric injury, leading to cloudy swelling, hydropic degeneration, apoptosis, and focal necrosis in all gastric zones using hematoxylin and eosin (H&E) staining. Moreover, ethanol increased the activation and the expression of tumor necrotic factor (TNF-α), cyclooxygenase (COX-2), and nuclear factor kappa-light-chain-enhancer of activated B cells (p-NFκB). In silico results were comparative to in vitro results evaluated through virtual screening. Moreover, ethanol increased the activation and expression of tumor necrotic factor, cyclooxygenase, and nuclear factor kappa-light-chain-enhancer of activated B cells. This study exhibits the gastroprotective effect of Manilkara zapota extracts in the peritoneal cavity using a proteomic and in silico approach which reveals different energy values against target proteins, which mediate the gastrointestinal functions.

1. Introduction
Gastrointestinal disorders are highly prevalent among the people of Asia. Health physicians claimed that it is an origin for the occurrence of several other comorbidities. Till now, there is no significant drug that has been discovered for gastrointestinal disorders, which completely eradicates the disease [1]. Moreover, herbal therapies have shown excellent economical and long-lasting potential to treat gastric system disorders [2].

In silico and in vivo evaluation of fruit extracts helps to screen out novel bioactive molecules, and their further processing leads to the development of innovative therapies. Most significant and desirable therapeutic effects can be achieved with the purified form of isolated bioactive constituents that can be formulated into suitable dosage form along with the dosage regimen. Many chronic disorders have been treated by herbal remedies, particularly by consuming fruits as functional foods as well as their active constituents. Researchers have investigated the role of crude fruit extracts, which have the potential to combat gastrointestinal disorders. Manilkara zapota Linn. commonly known as Sapodilla or Naseberry is a terrestrial photosynthetic epiphytic plant
with a Mediterranean distribution and about 8–15 m in height, and it belongs to the family Sapotaceae; tillage is found easily throughout the regions of Asia, though *Manilkara zapota* Linn. originates in Mexico and Central America [3]. Fruits are edible and sweet with rich fine flavor and seeds are aperients, diuretic tonic, and febrifuge. Barks and leaves are used as tonic to treat cough, cold, diarrhea, dysentery, and paludism. Various constituents were isolated from *Manilkara zapota* L. which are methyl chlorogenate, caffeic acid, dihydromyricetin, quercitrin, 4-O-galloylchlorogenic acid, myricetin-3-O-α-L-rhamnoside, (+)-catechin, apigenin-7-O-α-L-rhamnoside, (-)-epicatechin, (+)-gallocatechin, methyl 4-O-galloylchlorogenate, and gallic acid [4, 5] along with some novel (-)-α-side, (+)-catechin, apigenin-7-O-L-rhamno-

This research aimed to delineate *Manilkara zapota* fruit extracts for antidiarrheal effects, antisecretory effects, isolated tissue preparation, gastrointestinal transient time, and antiulcer effects. On the basis of ethnomedicinal uses, we assumed that extracts modulate the expression of proteins in the stomach and thus potentially promote the molecular and organ/tissue damage associated with diseases [5]. Ulcer is an inflammatory disorder, so we hypothesize here whether *Manilkara zapota* extract could cure gastric ulcer by inhibiting NFkB-dependent proinflammatory cytokines. The present study aims to evaluate whether *Manilkara zapota* effects on inflammation and oxidative stress could eventually account for gastric cell protection. Moreover, in silico approach helps us to understand the biochemical mechanisms and unknok the complex signaling network, which controls cellular function including apoptosis and inflammation [8]. The chemical structure of *Manilkara zapota* constituent is shown in Figure 1.

### 2. Materials and Methods

#### 2.1. Materials. *Manilkara zapota* fruits (4 kg) were purchased from the local market in March 2018 and verified by a taxonomist Dr. Mushtaq Ahmad, at the Department of Plant Sciences, Quaid-a-Azam University, Islamabad. Voucher specimen no. ISL-B-23 was collected after submitting the specimen sample of species to the herbarium. Plants were dried, crushed, and extracted with chloroform (4 × 500 mL). The whole extracts were filtered through a standard procedure using Whatman No. 1 filter paper and then evaporated through a rotary shaker (Tokyo Rikakikai Type A 1000S) under reduced pressure at temperature not exceeding 55°C to yield 30 g dry extract. The leftover residue was suspended in 4 L of methanol-water (20:80) and successively extracted with the aforementioned technique. The solvent was evaporated under reduced pressure to yield 110 g of extract [5].

#### 2.2. Chemicals. Atropine sulphate, acetylcholine, activated charcoal, ethanol, loperamide, methanol, omeprazole, papaverine, potassium chloride, and verapamil hydrochloride (Sigma Chemicals Co, St Louis, MO, USA) were used. Castor oil was obtained from KCL Pharma, Karachi, Pakistan.

#### 2.3. Experimental Animals and Housing Conditions. Experiments were performed in compilation with the rules of Research and Ethics Committee of RIPS (Ref. no. REC/RIPS/2018/021) along with the guidelines of “Principles of Laboratory Animal care.” Balb/C mice (20–25 g), rabbits (1.0–2.0 kg), and Sprague-Dawley rats (190–240 g) of either gender were used in the experimentation procedure and were kept in animal house of the Riphah Institute of Pharmaceutical Sciences (RIPS), Islamabad, provided with controlled environment (20–25°C).

#### 2.4. Phytochemical Analysis. Preliminary phytochemical analysis was carried out for the detection of major secondary metabolites such as saponins, glycosides, antheraquiones, alkaloids, steroids, proteins, flavonoids, and tannins according to the standard procedure with slight modifications [9].

#### 2.5. Castor Oil-Induced Diarrhea. The protocol was previously described by Sisay et al. [10]. Balb/C mice were randomly allocated to five groups for Mz.CHCl₃ and Mz.Aq, respectively. Animals were fasted for 24 hours (08:00–08:00) before experimentation. Each floor of cage was lined with blotting sheets in which animals were placed. Negative control group received normal saline (10 mL/kg) while positive control group received loperamide hydrochloride (2 mg/kg), and the remaining groups were given 50, 100, and 300 mg/kg of the extracts orally, respectively. After 1 hour of treatment, all mice received castor oil (10 mL/kg p.o.). Animals were individually observed for a period of 4 hours to analyze the onset of diarrhea and presence and absence of diarrheal dropping. The percentage of diarrheal protection was evaluated according to Rehman et al. [11] by using the chi-square (X²) test.

![Figure 1: 2D chemical structure of *Manilkara zapota* constituent (methyl chlorogenate) drawn through GaussView 5.0 Software and saved in PDB format.](image-url)
2.6. Assessment of Intestinal Fluid Accumulation. Intestinal fluid accumulation was determined using the method described by Shang et al. [10]. To study the intestinal fluid accumulation, enteropooling assay was used. Mice fasted overnight for 24 hours (08:00–08:00) were taken and placed in five assigned cages \( (n = 5) \). Groups I and II were given normal saline (10 mL/kg) and castor oil (10 mL/kg, p.o.), respectively. Mz.CHCl₃ doses of 50, 100, and 300 mg/kg intraperitoneally were administered to Groups III, IV, and V, respectively. Standard drug atropine at dose 10 mg/kg was given to II group, 1 hour prior induction with castor oil (10 mL/kg, p.o.). The same procedure was repeated for the Mz.Aq extract. Mice were sacrificed after 30 min, and the intestine was removed and weighed. The results were articulated as \( P_i \) and \( P_m \), where \( P_i \) is the weight (g) of the intestine and \( P_m \) is the weight of the animal, by using GraphPad Prism and analysis of variance one-way ANOVA followed by Tukey’s post hoc test.

\[
P_i/P_m 	imes 1000
\]

where \( P_i \) is the weight of the animal, by using GraphPad Prism and analysis of variance one-way ANOVA followed by Tukey’s post hoc test.

2.7. Isolated Tissue Preparation. Before experimentation, the rabbit was fasted for 24 hours (08:00–08:00) but had a free access to water. After cervical dislocation, jejunal portion of 2 cm was isolated and washed with Tyrode’s solution and suspended in tissue bath containing Tyrode’s solution to equilibrate for 30 minutes with environment along with proper supply of oxygen (95% \( \text{O}_2 \)) and 5% \( \text{CO}_2 \) (carbogen) under control temperature conditions before drug administration. Each preparation was stabilized with concentration of ACh (0.3 \( \mu \)M), until constant responses were recorded via a force displacement transducer (model FT-03) coupled with a bridge amplifier and a power Lab 4/25 data acquisition system connected to computer running Lab-Chart 6 software (AD Instrument, Sydney, Australia). Graded doses of Mz.CHCl₃ and Mz.Aq extracts were tested according to body weight, where they as positive control group received atropine sulphate (0.1 mg/kg, i.p.), while the negative control group received normal saline (10 mL/kg, p.o.). After one hour, animals receive charcoal meal (1 mL/kg). 30 minutes after all treatments, all the animals were sacrificed. The small intestine was excised, and the distance travelled by charcoal meal (5% activated charcoal suspension in distilled water) through the organ was expressed as a percentage of the length of the small intestine according to the following expression. The data were analyzed by using one-way ANOVA followed by Tukey’s post hoc test:

\[
\text{peristaltic index (PI)} \% = \frac{\text{distance moved by charcoal meal}}{\text{total length of the intestine}} \times 100.
\]

(1)

For further evaluation of % inhibition, peristaltic index is used:

\[
\% \text{inhibition} = \frac{\text{PIC} - \text{PIT}}{\text{PIC}} \times 100,
\]

(2)

where PIC is the peristaltic index of control and PIT is the peristaltic index of the test group.

2.9. Ethanol-Induced Ulcer Assay. Rats were randomly assigned into groups weighing 190–240 g of either sex and kept for fasting period of 24 hours (09:00–09:00). Group 1 served as a negative control received normal saline 10 mL/kg body weight, and group 2 received 20 mg/kg (p.o.) omeprazole as standard drug. Groups 3, 4, and 5 received 50, 100, and 300 mg/kg (p.o.) of Mz.CHCl₃ and Mz.Aq extracts, respectively. All the animals were treated with 1 mL/100 g of ethanol (p.o.) after 1 hour of treatment. Animals were sacrificed through cervical dislocation and stomachs were removed and washed with normal saline, and lesion index was estimated by measuring each lesion in mm along its greater curvature. Surface area of each lesion was measured and scoring was done. For each stomach lesion, ulcer index was taken as mean ulcer score (US) (such as 0: no ulcer, 1: US \( \leq 0.5 \) mm², 2: 0.5 < US \( \leq 2.5 \) mm², 3: 2.5 < US \( \leq 5 \) mm², 4: 5 mm² < US \( \leq 10 \) mm², 5: 10 mm² < US \( \leq 15 \) mm², 6: 15 mm² < US \( \leq 20 \) mm², 7: 20 mm² < US \( \leq 25 \) mm², 8: 25 mm² < US \( \leq 30 \) mm², 9: 30 mm² < US \( \leq 35 \) mm²; and 10: US > 35 mm²) [14]. For each stomach injury, the sum of the lengths (mm) of all scores was utilized as the ulcer index (UI). The gastroprotective assessment was displayed as an inhibition percentage (%) calculated by the following formula:

\[
\% \text{inhibition} = \frac{(U_{Sc} - U_{Si})}{U_{Sc}} \times 100,
\]

(3)

where \( U_{Sc} \) is the ulcer surface area of control and \( U_{Si} \) is the ulcer surface area of the test drug group.

The data were analyzed by using one-way ANOVA followed by Tukey’s post hoc test. The gastric tissues were stored in a biofreezer (–80°C) for further proteomic analysis.

2.10. Enzyme-Linked Immunosorbent Assay (ELISA). IL-18 expression was measured using Rat IL-18 ELISA kit (E-EL-R0567) according to the manufacturer’s instructions (Elabscience). The tissues were homogenized at 15000 using Silent Crusher M (Heidolph), and the supernatant was collected after centrifugation (at 1350 \( \times g \) for 1 h). The concentration of IL-18 was determined by using an ELISA microplate reader [15]. Values are expressed as picograms of cytokines per milliliter (pg/mL) and evaluated by using two-way ANOVA followed by Tukey’s post hoc test.
2.11. Hematoxylin and Eosin (H&E) Staining and Immunohistochemical Analysis. Tissue sections on coated slides were deparaffinized with absolute xylene (100%) and rehydrated with ethyl alcohol (from 100% to 70%). The slides were rinsed with distilled water and immersed in hematoxylin for 10 min. The slides were then kept under running water in glass jar for 10 minutes and treated with 1% HCl and 1% ammonia water as previously reported by Gim et al. [16]. The slides were added to eosin solution for 5–10 min. After due time, the slides were rinsed in water and air-dried for some time. The dried slides were dehydrated in graded ethyl alcohol (70%, 95%, and 100%). The slides were cleared with xylene and were mounted with glass cover slip. The slides were pictured with light microscope (Olympus, Japan) and analyzed by ImageJ, a computer-based program. The number of images per slide was five per group, while focusing specifically on gastric cell size and shape, inflammation infiltrated cells, and vacuolation described by Shah et al. [8]. The TIF images were optimized to the same threshold intensity for all groups and examined in GraphPad Prism.

Immunohistochemical staining was performed as described previously with little modifications done by Gim et al. [16]. After deparaffinization, slides were processed for antigen retrieval step (enzymatic method) and then washed with PBS. The endogenous peroxidase was quenched by applying 3% hydrogen peroxide (H₂O₂) in methanol for 10 min. The slides were incubated with 5% normal goat serum containing 0.1% Triton X-100. After blocking, the slides were incubated overnight with mouse anti-TNF-α, p-NFkB, and mouse anti-COX-2 antibodies (dilution 1:100, Santa Cruz Biotechnology). The following morning, after washing with 0.1 M PBS, slides were incubated in biotinylated secondary antibody (dilution 1:50) according to the origin of the primary antibody and serum used. Following secondary antibody treatment, slides were incubated with ABC Elite kit (Santa Cruz Biotechnology) for 1 hour in a humidified chamber. The slides were washed with 0.1 M PBS, stained in DAB solution, washed with distilled water, dehydrated in a graded ethanol series, fixed in xylene, and cover-slipped in mounting medium. Immunohistochemical TIF images (five images per slide) were captured with a light microscope. ImageJ software was used to quantitatively determine hyperactivated p-NFkB, COX-2, and TNF-α, by optimizing the background of images according to the threshold intensity and analyzing p-NFkB, COX-2, and TNF-α positive cells at the same threshold intensity for all groups. The intensity is expressed as the relative integrated density of the samples relative to the saline by using one-way ANOVA followed by Tukey’s post hoc test.

2.12. Acute Toxicity. This study delineates lethal versus nonlethal dose of extracts in the animal model by using acute toxicity model. Mice were divided into three groups of five mice each. The test was performed using increasing doses (3 and 5 g) of the Mz extract given in 10 mL/kg volume. Saline (10 mL/Kg, p.o., negative control) was administered to one group. Animals were observed for change in behavior for about 4 hours, and mortality rate was studied for 24 hours after drug administration described by Ogwal-Okeng et al. [17].

2.13. Computational Studies. Through DSV, 3-dimensional structures of reference drugs were prepared (2016) by adding polar hydrogen atoms (H-atoms) and converted into PDB file and downloaded from PubChem database. Standard drugs for docking included phenylephrine, pirenzipine, atropine, domperidone, calmidazolium, verapamil, omeprazole, ranitidine, loperamide, and papaverine for receptors. Targets involved in gut physiology were selected and obtained from the website of RCSB protein data bank. Selected targets included adrenergic α1 receptor (PDB ID: P35348), calmodulin (PDB ID: 1CTR), dopaminergic D2 (PDB ID: 6CM4), histaminergic H2 (PDB ID: P25201), H+/K+ ATPase (PDB ID: 4UX2), muscarinic M1 (PDB ID: 5CXV), muscarinic M3 (PDB ID: 2CSA), mu-opioid (PDB ID: 6DDE), phosphodiesterase enzyme (PDB ID: 6DDE), and voltage gated L-type calcium channel (PDB ID: 1T3S). Structures were purified by removing water molecules and ligands in DSV and were saved in PDB format till further procedure. PyRx is used to perform molecular docking, and the results were analyzed through the best pose by using DSV and energy values (kcal/mol) achieved through PyRx in sdf and csv formats. Only one pose having the lowest value of energy (kcal/mol) was selected for postdock analysis through DSV after the evaluation of top ten conformations. 2D image was assessed to check the ligands and amino-acid residue interactions with the receptor including alanine (ALA), asparagine (ASN), aspartic acid (ASP), arginine (ARG), lysine (LYS), threonine (THR), glycine (GLY), glutamine (GLN), cysteine (CYS), methionine (MET), glutamic acid (GLU), histidine (HIS), phenylalanine (PHE), isoleucine (ILE), proline (PRO), tyrosine (TYR), serine (SER), threonine (THR), tryptophan (TRP), and valine (VAL). Docking signifies the binding of the receptor with the ligand in the physiological mechanism and hence provides a valuable marker for drug binding inside the body.

2.14. Statistical Analysis. Data were expressed as mean±SEM (n = 5) and median effective concentrations (EC50) having 95% confidence intervals. Statistical analysis of the results was analyzed using one-way ANOVA followed by Tukey’s post hoc test. Chi-square test was used in the case of the antidiarrheal data, where p < 0.05 was regarded as significant. Nonlinear regression using GraphPad program (GraphPAD, San Diego, CA, USA) was used to analyze the concentration-response curves.

3. Results

3.1. Phytochemical Profile. Qualitative phytochemical analysis of Mz.CHCl₃ and Mz.Aq showed the presence of flavonoids, alkaloids, phenols, saponins, triterpenes, lignin, unsaturated sterols, and carbohydrates validated through chemical assays.
3.2. Effect of Mz.CHCl₃ and Mz.Aq on Castor Oil-Induced Diarrhea. Mz.CHCl₃ and Mz.Aq exhibited dose-dependent (50–300 mg/kg) protective effect against castor oil-induced diarrhea in mice, and the negative control group (saline treated) did not show any protection against castor oil-induced diarrhea. *Manilkara zapota* extract showed marked response of 60%, 80%, and 100 in Mz.CHCl₃ and 20%, 40%, and 60% in Mz.Aq at doses of 50, 100, and 300 mg/kg, respectively (*p < 0.05 versus saline group). Loperamide hydrochloride (2 mg/kg), a well-known antidiarrheal medicine, showed 100% protection from diarrhea (**p < 0.01 versus saline group) in the positive control group (Table 1).

3.3. Effect of Mz.CHCl₃ and Mz.Aq on Enteropooling Assay. When tested against castor oil-induced enteropooling assay in mice, Mz.CHCl₃ exhibited a dose-dependent (50–300 mg/kg) antisecretory effect. Intestinal fluid accumulation in the saline treated group was 86.5 ± 0.93 (mean ± SEM, n = 5), whereas in the castor oil-treated group it was 122.96 ± 0.93 (**p < 0.001 versus saline group). Mz.CHCl₃ at the doses of 50, 100, and 300 mg/kg reduced the castor oil-induced fluid accumulation to 109.29 ± 2.62 (**p < 0.001 versus castor oil group), 94.78 ± 3.09 (**p < 0.001 versus castor oil group), and 77.68 ± 3.33 (**p < 0.001 versus castor oil group), respectively, while the effect of Mz.Aq at the doses of 50, 100, and 300 mg/kg reduced the castor oil-induced fluid accumulation to 104.28 ± 3.08 (**p < 0.001 versus castor oil group), 95.58 ± 3.11 (**p < 0.001 versus castor oil group), and 89.77 ± 1.62 (**p < 0.001 versus castor oil group), respectively. Atropine at the dose of 10 mg/kg decreased the intestinal fluid accumulation to 75.11 ± 0.42 (**p < 0.001 versus castor oil group), as shown in Figure 2.

3.4. Effect of Mz.CHCl₃ and Mz.Aq on Spontaneous and K⁺-Induced Contractions. Figure 3 shows the inhibitory effect of the plant extract, papaverine, and verapamil against spontaneous and K⁺ (80 mM) induced contractions. Mz.CHCl₃ was found to be equally effective against spontaneous and K⁺ (80 mM) induced contractions with EC₅₀ values of 2.170 mg/mL (1.676–2.810, n = 4) and 5.644 mg/mL (1.697–18.77, n = 4), respectively, as shown in Figure 3(a). Similarly, Mz.Aq showed spontaneous and K⁺ (80 mM) induced contractions with EC₅₀ values of 2.62 mg/mL (0.03–0.06, n = 4), as compared to spontaneous contractions (0.12 μM (0.10–0.20, n = 3)), as shown in Figure 3(d).

3.5. Effect of Mz.CHCl₃ and Mz.Aq on Charcoal Meal Transit Time. Mz.CHCl₃ delays the charcoal meal to travel through the small intestine in a dose-dependent manner. The distance travelled by the saline group was 92.6%. Mz.CHCl₃ at 50, 100, and 300 mg/kg dose showed % inhibition in charcoal meal transit by 9.74, 19.92, and 27.03%, respectively, whereas Mz.Aq showed % inhibition of 12.48%, 20.89%, and 25.91% at doses of 50, 100, and 300 mg/kg.
respectively (*p < 0.05, **p < 0.01, and ***p < 0.001 versus saline group). Atropine (0.1 mg/kg, i.p.) shows inhibitory effect of 81.40% (Table 2).

**Table 2:** Effect of *Manilkara zapota* extracts: chloroform (Mz.CHCl₃), aqueous (Mz.Aq), and atropine on charcoal meal transit time in rats.

<table>
<thead>
<tr>
<th>Doses (mg/kg, p.o.)</th>
<th>Mean length of intestine (cm)</th>
<th>Distance moved by charcoal (cm)</th>
<th>% intestinal transient</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (10 mL/kg)</td>
<td>92.6 ± 1.6</td>
<td>90 ± 1.3</td>
<td>97.1</td>
<td>—</td>
</tr>
<tr>
<td>Mz.CHCl₃ (50 mg/kg)</td>
<td>87.4 ± 1.3</td>
<td>76.6 ± 1.9**</td>
<td>87.64</td>
<td>9.74</td>
</tr>
<tr>
<td>Mz.CHCl₃ (100 mg/kg)</td>
<td>90.8 ± 0.8</td>
<td>70.6 ± 0.8*</td>
<td>77.75</td>
<td>19.92</td>
</tr>
<tr>
<td>Mz.CHCl₃ (300 mg/kg)</td>
<td>84.4 ± 0.5</td>
<td>59.8 ± 0.3***</td>
<td>70.85</td>
<td>27.03</td>
</tr>
<tr>
<td>Mz.Aq (50 mg/kg)</td>
<td>73.6 ± 0.9</td>
<td>86.6 ± 0.2</td>
<td>84.98</td>
<td>12.48</td>
</tr>
<tr>
<td>Mz.Aq (100 mg/kg)</td>
<td>74.2 ± 0.5</td>
<td>57 ± 0.7*</td>
<td>76.81</td>
<td>20.89</td>
</tr>
<tr>
<td>Mz.Aq (300 mg/kg)</td>
<td>77 ± 0.7</td>
<td>55.4 ± 0.9**</td>
<td>71.94</td>
<td>25.91</td>
</tr>
<tr>
<td>Atropine (0.1 mg/kg, i.p.)</td>
<td>90.8 ± 0.9</td>
<td>16.4 ± 0.6***</td>
<td>18.06</td>
<td>81.40</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01, and ***p < 0.001 compared to control saline group. One-way analysis of variance followed by Tukey’s post hoc test, n = 5.

3.6 Effect of Mz.CHCl₃ and Mz.Aq on Ethanol-HCl-Induced Ulcer. Mz.CHCl₃ in dose-dependent manner (50–300 mg/kg) exhibited an antiulcer effect. Mz.CHCl₃ at 50, 100, and
300 mg/kg caused 20, 58, and 76% (**p < 0.001 versus ethanol group) inhibition, respectively. Omeprazole (20 mg/kg) exhibited 86% inhibitory effect. Mz.Aq showed antulcer effect in healing wound at doses of 50, 100, and 300 mg/kg and % inhibition was 38, 56, and 76%, respectively (Table 3). Macroscopic observation showed the gastric mucosa of rats (Figure 4).

3.7. Effect of Mz.CHCl3 and Mz.Aq on IL-18 Expression in the Stomach of Ethanol-Induced Ulcer Models. Figure 5 illustrates the effect of Manilkara zapota chloroform (Mz.CHCl3) and aqueous extract (Mz.Aq) on IL-18 in gastric tissues. One-way analysis of variance (ANOVA) followed by Tukey’s post hoc test revealed that Manilkara zapota extracts significantly reduced the IL-18 expression in treated groups as compared to the negative control group (*p < 0.05 and **p < 0.01 versus ethanol group) (**p < 0.001 versus saline). The levels are significantly decreased in treated versus disease group. In Mz.CHCl3, at doses of 100 and 300 mg/kg, the level of inflammatory mediator release from the tissue was relatively less at higher doses, in contrast to the response shown by Mz.Aq at doses of 100 and 300 mg/kg. In comparison, Mz.Aq has more significant results with respect to omeprazole.

3.8. Histopathology and Immunohistochemistry (IHC) Analysis. Figure 6 shows IHC and H&E staining in order to distinguish necrotic cells from intact ones. Ethanol produces robust cellular changes in the highly prone areas of gastric cells, while treatment with Mz.Aq extracts attenuates this damage. A substantial difference was observed in the control group relative to the ethanol-administered group. The damaged area exhibits abnormal morphological features including alteration in mucosal cell size and shape, alteration in color staining (cytoplasmic eosinophilia/pyknosis and basophilic nature of nucleus), and vacuolation. No noticeable alterations were noticed in the histological preparations of control animals. Significantly more intact cells were there in the Mz.Aq extract treated groups compared to the ethanol-operated group (*p < 0.05, **p < 0.01, and ***p < 0.001). Severity scores of stomach injury in different groups (n = 5) are calculated via relative density of value (AU) and relative integrated density. Data are mean ± SEM. ***p < 0.001 versus ethanol group; *p < 0.05 versus ethanol group.

3.9. Acute Toxicity. The extracts did not cause any significant behavioral and pathological changes and did not show any mortality up to the dose of 5 g/kg.

3.10. Docking Evaluation. Energy values and H-bonds are the two main contributing factors for docking evaluation. However, formation of π-π bonds, π-alkyl bonds, π-sigma bonds, and Vander Waal forces plays a leading role between ligand-receptor complexes. In this study, methyl chlorogenate showed variable binding affinities against different protein receptors. Table 4 summarizes the atomic energy values (kcal/mol) and residues involved in H-bonding, π-π bonding, and other hydrophobic interactions of best dock poses of methyl chlorogenate and reference drugs in comparison with adrenergic α1 receptor, muscarinic M1, muscarinic M3, dopaminergic D2, calmodulin, mu-opioid, voltage gated L-type calcium channel, histaminergic H2, H+/K+ ATPase pump, and phosphodiesterase enzyme are plotted. Figures 7–16 illustrate the 2D view of interactions of methyl chlorogenate along with standard drugs. Against α1 adrenergic receptor, methyl chlorogenate showed E-value of −7.5 kcal/mol and formed 2 H-bonds and 4 hydrophobic interactions whereas phenylephrine showed E-value of −6.6 kcal/mol and formed 5 H-bonds, 1 π-π bond, and 4 other hydrophobic interactions. The 2D interactions are shown in Figure 7. Against M1 muscarinic receptor, methyl chlorogenate showed E-value of −7.5 kcal/mol and formed 6 H-bonds, 2 π-π bonds, and 1 hydrophobic interaction, whereas pirenzepine showed E-value of −8.8 kcal/mol and formed 2 H-bonds, 1 π-π bond, and 1 other hydrophobic interaction. Its 2D interactions are shown in Figure 8. Against M3 muscarinic receptor, methyl chlorogenate showed E-value of −6.1 kcal/mol and formed 2 H-bonds and 5 hydrophobic interactions, whereas pirenzepine showed E-value of −5.6 kcal/mol and formed 2 H-bonds, 1 π-π bond, and 19 other hydrophobic interactions as shown in 2D interactions in Figure 9. Against D2 dopaminergic receptor, methyl chlorogenate showed E-value of −7.8 kcal/mol and formed 9 H-bonds, 1 π-π bond, and 1 hydrophobic interaction, whereas domperidone showed E-value of −9.3 kcal/mol and formed 3 H-bonds, 5 π-π bonds, and 8 other hydrophobic interactions, as shown in Table 4, and 2D interactions as shown in Figure 10. Against calmodulin receptor, methyl chlorogenate showed E-value of −6.2 kcal/mol and formed 3 hydrophobic interactions, whereas calmidazolium showed E-value of −8.2 kcal/mol and formed 20 other hydrophobic interactions given in Table 4 and 2D interactions shown in Figure 11. Against calcium channel receptor, methyl chlorogenate showed E-value of −7.3 kcal/mol and formed 2 H-bonds, 1 π-π bond, and 6 hydrophobic interactions, whereas verapamil showed E-value of −6.7 kcal/mol and formed 1 H-bond, 2 π-π bonds, and 7 other hydrophobic interactions given in Table 4 and 2D interactions as shown in Figure 12. Against H+/K+ ATPase

<table>
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<tr>
<th>Treatment</th>
<th>Ulcer index</th>
<th>% inhibition</th>
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<tbody>
<tr>
<td>Saline 10 mL/kg + ethanol</td>
<td>10.0 ± 0.0</td>
<td>—</td>
</tr>
<tr>
<td>Mz.CHCl3 (50 mg/kg) + ethanol</td>
<td>8.0 ± 0.31*</td>
<td>20</td>
</tr>
<tr>
<td>Mz.CHCl3 (100 mg/kg) + ethanol</td>
<td>4.2 ± 0.2**</td>
<td>58</td>
</tr>
<tr>
<td>Mz.CHCl3 (300 mg/kg) + ethanol</td>
<td>2.4 ± 0.44***</td>
<td>76</td>
</tr>
<tr>
<td>Mz.Aq (50 mg/kg) + ethanol</td>
<td>6.2 ± 0.3***</td>
<td>38</td>
</tr>
<tr>
<td>Mz.Aq (100 mg/kg) + ethanol</td>
<td>4.4 ± 0.14***</td>
<td>56</td>
</tr>
<tr>
<td>Mz.Aq (300 mg/kg) + ethanol</td>
<td>2.4 ± 0.24***</td>
<td>76</td>
</tr>
<tr>
<td>Omeprazole (20 mg/kg) + ethanol</td>
<td>1.4 ± 0.24***</td>
<td>86</td>
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</table>

*p < 0.05, **p < 0.01, and ***p < 0.001 compared to control saline group. One-way analysis of variance, followed by Tukey’s post hoc test, n = 5.
receptor, methyl chlorogenate showed $E$-value of $-7.9$ kcal/mol and formed 6 H-bonds and 7 hydrophobic interactions, whereas omeprazole showed $E$-value of $-8.1$ kcal/mol and formed 2 $\pi-\pi$ bonds and 12 other hydrophobic interactions given in Table 4 and 2D interactions as shown in Figure 13.

Against $H_2$ histaminergic receptor, methyl chlorogenate showed $E$-value of $-7.4$ kcal/mol and formed 3 H-bonds and 4 hydrophobic interactions, whereas ranitidine showed $E$-value of $-5.7$ kcal/mol and formed 4 H-bonds, 1 $\pi-\pi$ bond, and 7 other hydrophobic interactions given in Table 4 and 2D interactions as shown in Figure 13.

Figure 4: Gross-appearance of gastric mucosa in rats: (a) pretreated with saline, 10 mL/kg, severe injuries are seen, as ethanol (1 mL/100 g) produced excessive hemorrhagic necrosis of gastric mucosa pretreated with *Manilkara zapota* extracts: (b–d) chloroform (Mz.CHCl₃), (e–g) aqueous (Mz.Aq) at doses of 50, 100, and 300 mg/kg, respectively, and (h) pretreated with omeprazole (20 mg/kg). The injuries were reduced with the increase of Mz.CHCl₃ and Mz.Aq doses and omeprazole compared with ulcer control. At 300 mg/kg, Mz.CHCl₃ and Mz.Aq showed most efficacious gastroprotective action.
Figure 5: Effect of Manilkara zapota extracts: chloroform (Mz.CHCl₃) and aqueous (Mz.Aq) in comparison with saline, ethanol, and omeprazole groups on IL-18 in ethanol-induced ulcer model. IL-18 levels were measured by ELISA assay. *p < 0.05, **p < 0.01, and #p < 0.001. Analyzed by one-way ANOVA followed by Tukey’s post hoc test.

Gross examination of stomach cells (chief cells, parietal cells, and submucosa)

Saline (10mL/kg) Ethanol Mz.Aq (300mg/kg) Omeprazole

TNF-α

p-NFκB

COX-2

Figure 6: Continued.
2D interactions as shown in Figure 14. Against mu-opioid receptor, methyl chlorogenerate showed $E$-value of $-9.2 \text{ kcal/mol}$ and formed 6 H-bonds and 3 hydrophobic interactions, whereas loperamide showed $E$-value of $-9.5 \text{ kcal/mol}$ and formed 1 H-bond, and 4 other hydrophobic interactions given in Table 4 and 2D interactions as shown in Figure 15. Against phosphodiesterase enzyme receptor, methyl chlorogenerate showed $E$-value of $-8.9 \text{ kcal/mol}$ and formed 4 H-bonds, 1 π-π bond, and 4 hydrophobic interactions, whereas papaverine showed $E$-value of $-8.1 \text{ kcal/mol}$ and 2 H-bonds, 2 π-π bonds, and 5 other hydrophobic interactions given in Table 4 and 2D interactions as shown in Figure 16.

### 4. Discussion

On the basis of ethnopharmacological utilization of *Manilkara zapota* in gastric disorders, such as gastritis, constipation, and diarrhea, *Manilkara zapota* extract was evaluated for its antidiarrheal, antisecretory, antispasmodic, charcoal meal gastrointestinal motility, and antiulcer effects. In vitro, in silico, and proteomic approach were used for the explication of possible underlying mechanisms to rationalize the aforementioned ethnomedicinal uses of the plant. *Manilkara zapota* fruit extracts exhibited protective effects against castor oil-induced diarrhea, similar to the effect produced by loperamide, a standard drug, while its possible underlying mechanism was estimated through isolated tissue preparations also associated with the reduction in gastric motility. Castor oil is responsible for increasing intestinal fluid as well as diarrheal effect through its active metabolite, i.e., ricinoleic acid [10]. It changes the electrolyte and water transport and generates enormous contractions in the transverse and distal colon. Gastrointestinal motility is maintained through the release of various mediators which are histamine, prostaglandins, acetylcholine, substance P, 5-hydroxytryptamine, nitric oxide, and cholecystokinin which provides stimulatory effects in gut and ultimately increases cytosolic Ca$^{2+}$ along with the release of mediators that block the above-described pathways accompanying with nonspecific inhibitory action that would relieve gut disorders [11]. *Manilkara zapota* extracts demonstrated protective effect against castor oil-induced intestinal fluid secretion in mice. *Manilkara zapota* extracts contain a gut relaxant constituent which mediates antidiarrheal and antisecretory effects.

Papaverine (a standard phosphodiesterase enzyme (PDE) inhibitor), chloroform, and aqeous extracts of plant caused a dose-dependent inhibition of spontaneous and high K$^+$-induced contractions in isolated rabbit preparations by producing a similar pattern of nonspecific inhibitory response whereas verapamil was found more potent against K$^+$ (80 mM) induced contractions. These data proposed that plant extracts produce their relaxant effect through the dual mechanisms with CCB and producing relaxation effect, like PDE enzyme inhibition [18]. Antidiarrheal effect of the Mz fruit extract, mediated possibly through the dual blockade of phosphodiesterase enzyme receptors and Ca$^{2+}$ channels, provides evidence for its effectiveness in diarrhea, though additional mechanisms can be ruled out. The phytochemical screening of *Manilkara zapota* fruit extract revealed the presence of different classes of chemicals, such as flavonoids, polyphenols, alkaloids, carbohydrates, saponins, and anthraquinones. Moreover, polyphenols target gut microbiota activity through its metabolites [19]; hence, the presence of these phytochemicals in *Manilkara zapota* may be accountable for its efficacy in diarrhea, though further research is required to explore the precise nature of chemical constituents mediating alleged biological activities. For charcoal meal transient time activity, *Manilkara zapota* extract in the small intestine produces suppression of the propulsion of charcoal marker in test doses just like standard drug atropine sulphate that has prominent anticholinergic effect on intestinal transit. A diminished GIT motility tone causes increase in the stay of substances in intestine, which permits better water absorption. These findings proposed that *Manilkara zapota* extract has an effect on the peristaltic movement of intestine which indicates its antimotility effects. In gastrointestinal tract, various aggressive and protective factors play an important role in the production and release of acids. Disturbance in these factors results in rupturing of mucosal protection which exposes gastric lining...
Table 4: Binding energy (kcal/mol) and post dock analysis of the best conformational pose of methyl chlorogenate with adrenergic $\alpha_1$ receptor, muscarinic M$_1$, muscarinic M$_3$, dopaminergic D$_2$, calmodulin, mu-opioid, voltage gated L-type calcium channel, H$^+$/K$^+$ ATPase pump, histaminergic H$_2$, and phosphodiesterase enzyme.

<table>
<thead>
<tr>
<th>Target proteins</th>
<th>$E$-value (kcal/mol)</th>
<th>Methyl chlorogenate</th>
<th>Amino acids forming H-bonds</th>
<th>Amino acids forming $\pi$-$\pi$ bonds</th>
<th>Hydrophobic interactions</th>
<th>Standard drugs</th>
<th>$E$-value (kcal/mol)</th>
<th>Amino acids forming H-bonds</th>
<th>Amino acids forming $\pi$-$\pi$ bonds</th>
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<td>TYR 82(2) TYR 106(2) TYR 381 ILE 180</td>
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BioMed Research International 11
<table>
<thead>
<tr>
<th>Target proteins</th>
<th>Methyl chlorogenate</th>
<th>Standard drugs</th>
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<td></td>
<td>E-value (kcal/mol)</td>
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<td>Mu-opioid</td>
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<td>Phosphodiesterase enzyme</td>
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ALA, alanine; ASN, asparagine; ASP, aspartic acid; ARG, arginine; CYS, cysteine; GLU, glutamic acid; GLY, glycine; GLN, glutamine; HIS, histidine; LYS, lysine; PHE, phenylalanine; SER, serine; THR, threonine; TRP, tryptophan; TYR, tyrosine; VAL, valine.
Figure 7: (a, b) The interactions of methyl chlorogenate and phenylephrine against the target adrenergic α₁ receptor, respectively, evaluated through Biovia Discovery Studio 2016.

Figure 8: (a, b) The interactions of methyl chlorogenate and pirenzepine against the target muscarinic M₁ receptor, respectively, evaluated through Biovia Discovery Studio 2016.
Figure 9: (a, b) The interactions of methyl chlorogenate and atropine against the target muscarinic M₃ receptor, respectively, evaluated through Biovia Discovery Studio 2016.

Figure 10: (a, b) The interactions of methyl chlorogenate and domperidone against the target dopaminergic D₂ receptor, respectively, evaluated through Biovia Discovery Studio 2016.
different enzyme and acid productions leading to the sores called ulcers [20]. Ethanol-induced gastric model was used to explore the beneficial effect of Manilkara zapota extract through a variety of mechanisms that stimulate ulcers including free radicals OH$^-$, NO production, mucus exhaustion, mucosal damage, release of superoxide anion, which ultimately prolonged the tissue oxidative stress, and release of inflammatory mediators like TNF-\(\alpha\), p-NF\(\kappa\)B, and COX-2, which are measured through the proteomic analysis. The potential of Manilkara zapota extract to produce an antiulcer effect might be due to its CCB effect, as Ca$^{2+}$ antagonist is well known to demonstrate such effects [2]. In pathophysiology of gastric ulcers, oxidative stress plays a vital role. Antioxidant and nitric oxide free radical
scavenging activity has been reported by *Manilkara zapota*, which may be responsible for its effectiveness as an antiulcer agent [3]. Furthermore, ELISA was done to quantify the protein expression of IL-18, and Mz.Aq significantly attenuates necrosis in ulcerative tissue as compared to Mz.CHCl3 achieved through proteomic analysis [15]. Mz.Aq extract administration exerts more protective effect as compared to Mz.CHCl3 in ulcerative model, so, for IHC and H&E staining, Mz.Aq was selected for further analysis. Mz.Aq protects the gastric cells through the inhibition of inflammatory markers like TNF-α, p-NFκB, and COX-2. The results were quite significant as compared to ethanol. TNF-α, COX-2, and p-NFκB are widely considered as prototypic proinflammatory cytokines due to their principal interactions.
role in initiating the cascade of the activation of other cytokines and growth factors in the inflammatory response [20]. After injury or during inflammation, TNF-α and NFκB signaling pathway is activated which plays a vital role in inflammation. Ethanol induces inflammation in gastric mucosa in the control group, whereas Manilkara zapota extracts decreased the levels of TNF-α, p-NFκB, and COX-2 as compared to omeprazole when given alone to rats. Manilkara zapota extracts have broader therapeutic index as no mortality was observed at a dose of 5 g/kg.

Figure 15: (a, b) The interactions of methyl chlorogenate and loperamide against the target mu-opioid receptor, respectively, evaluated through Biovia Discovery Studio 2016.

Figure 16: (a, b) The interactions of methyl chlorogenate and papaverine against the target phosphodiesterase enzyme, respectively, evaluated through Biovia Discovery Studio 2016.
The ligand methyl chlorogenate (phytochemical constituent) was docked in the catalytic active pocket of α1-adrenergic receptor, muscarinic M1 and M3, domperidone D2, calmodulin, voltage gated L-type calcium channel, gastric hydrogen potassium ATPase, histamine H3, mu-opioid, and phosphodiesterase enzyme receptor targets that may be possibly associated with the pathophysiology of gastric disorders [21]. Docking is used as a preliminary tool used to evaluate the affinity of ligands to their particular protein targets. Molecular docking has a significant role in drug discovery and development, which includes structure-based evaluation and finding target specificity along with binding affinity. For virtual screening, DSV was used through PyRx [22]. It has gradient optimization method and it predicts the binding mode of ligand-receptor complex more precisely. Affinity of ligands with receptor could be assessed through E-value and hydrogen bonding that relates their influential effect in gastrointestinal diseases [23]. Based on E-value against different protein targets, the order of ligand affinity was found as mu-opioid > phosphodiesterase enzyme > H+/K+ATPase > D2 > α1 > M1 > H3 > voltage gated L-type calcium channel > Calmodulin receptor > M3.

5. Conclusions

Manilkara zapota Linn. holds a vital place in the traditional system of medicines. The current explored potential of the plant signifies its importance to the pharmaceutical industry due to exhibited anti-diarrheal, antisecretory, antispasmodic, antimotility, and anti-ulcer effects. Most histopathological changes in gastric tissues and associated derangement in related polyclonal antibodies were largely prevented by the Manilkara zapota extract, thus paving the way to a new therapeutic approach towards the management of inflammation-induced gastric mucositis. The plant constituent methyl chlorogenate showed moderate binding affinities (E-value ≥ -6.1 kcal/mol) against muscarinic M1, muscarinic M3, histaminergic H3 receptors, H+/K+ ATPase pump, calmodulin, adrenergic α1, and dopaminergic D2 receptors and voltage gated L-type calcium channels, while it showed high affinities (E-value ≥ 8 kcal/mol) against mu-opioid and phosphodiesterase enzyme.

Data Availability

The data used to support the findings of this study are available from the authors upon request.

Disclosure

This work was performed in partial fulfillment of the requirements for the degree of M.Phil. in Pharmacology by “Sameen Fatima Ansari” at the Faculty of Pharmaceutical Sciences, Riphah International University of Pharmaceutical Sciences, Islamabad, Pakistan.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

Arif-ullah Khan supervised the experimentation. Arif-ullah Khan and Fawad Ali Shah designed the experiment. Sameen Fatima Ansari performed the experimentation and wrote the paper. Komal Naeem and Neelum Gul Qazi helped in performance, review, and editing. All authors read and approved the manuscript.

References


