Pharmacological and computational evaluation of Sapodilla and its constituents for therapeutic potential in hyperactive gastrointestinal disorders

Muhammad Bilal Riaz 1, Arif-ullah Khan 1*, Neelam Gul Qazi 1

1 Riphah Institute of Pharmaceutical Sciences, Riphah International University, Islamabad

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O B S T R A C T
Objective(s): This study was designed to investigate various gastrointestinal effects of Manilkara zapota (Sapodilla), exploring its anti-diarrheal, anti-secretary, anti-spasmodic, anti-ulcer and antimotility potential.

Materials and Methods: Antidiarrheal and anti-secretary activities were investigated using castor oil induced diarrhea and castor oil induced fluid accumulation. Isolated rabbit jejunal tissues (antispasmodic) were employed for in vitro experiments. Antulcer, antimotility and molecular docking were performed using ethanol-HCl induced ulcer assay, charcoal meal transit time and Auto Doc Vina.

Results: Mz.Cr exhibited protection against castor oil-induced diarrhea (P<0.05 vs. saline group) and dose-dependently inhibited intestinal fluid secretions (P<0.001 vs. castor oil group). Mz.Cr caused relaxation of spontaneous and K⁺ (80 Mm)-induced contractions with EC₅₀ values of 0.11 mg/ml (0.08-0.1, n=4) and 0.16 mg/ml (0.09-0.2, n=4) respectively (P<0.05 vs. saline group). It showed protective effect against gastric ulcers induced by ethanol-HCl (P<0.001 vs. saline group). Plant constituents: caffeoylquinic acid and methyl 4-O-galloylchlorogenate showed high binding affinities (E-value≥-6.5 Kcal/mol) against histaminergic H₂ receptors, H₁/K⁺ ATPase pump and voltage gated L-type calcium channels, while possesses moderate affinities (E-value8 Kcal/mol) vs. calmodulin, muscarinic M₁, M₃, adrenergic α₁, phosphodiesterase enzyme and dopaminergic D₂ receptors. Lupeol-3-acetate and β-amyrin-3-(3'-dimethyl) butyrate observed weak affinities.

Conclusion: In present study, M. zapota is reported to exhibits anti-diarrheal, anti-secretory, anti-spasmodic, anti-motility, anti-ulcer effects and computational binding affinities against gastrointestinal targets.

Introduction

Gastrointestinal ailments are very common among the people of Asia and medical practitioners believe that it is a root cause for the occurrence of several other co-morbidities. Modern day medicine has so far does not produced any efficacious remedial drug against gastrointestinal disorders. It only gives temporary relief but with side effects. However, traditional herbal medicines have got excellent economical and long lasting potential to treat digestive system disorders (1). These natural products have been a significant source and major contributor to the present day commercial medicines and several drug lead molecules. About 61% of drugs introduced worldwide are derived from natural products (2). Screening of crude plant extracts ease the way for discovery of novel bioactive compounds and their structure elucidation can open the window for new synthetic preparations. For particular therapeutic purposes, pure bioactive compounds can be made in suitable dosage form and their accurate doses can be find out (4). Edible fruits being potential sources of functional foods and its phytoconstituents often serves the purpose in treating and curing several chronic diseases. Use of edible fruit extracts have been reported by several researchers for their gastrointestinal activities (5).

Manilkara zapota L. commonly known as “Sapodilla” and locally “Chiku” belongs to the family of Sapotaceae and is an evergreen, depliated tree up to 15 m in height. Asia is a major cultivator of this species, though it is native to Mexico and Central America (6). M. zapota has been used traditionally in fever, hemorrhage, wound healing, ulcer, arthritis, pulmonary diseases, rheumatism, and as antifungal agent (7). Its use as laxative and for treating constipation and diarrhea, further enhance its ethnomedicinal importance. Fruits are used in traditional medicines as anti-oxidant, due to their polyphenolic content (8).

M. zapota is reported with presence of polyphenolic compounds like tannins and flavonoids (9). Also, triterpenes were previously isolated from these fruits. Its methanolic extracts contain dihydromyricetin, quercitrin, myricitrin, catechins and gallic acid (7). Recently some novel triterpenes have been identified as 4-caffeoylquinic acid (cryptochlorogenic acid), lupeol-3-acetate, methyl 4-O-galloylchlorogenate and β-amyrin-
3-(3’-dimethyl) butyrate (10).

In the present study, we report anti-diarrheal, anti-secretary, anti-spasmodic, anti-motility and anti-ulcer effects. Extensive folkloric uses and previous studies were used as a baseline data to validate aforementioned ethnomedicinal uses of the plant. Molecular docking of its constituents with known structure is done to find out the potential lead molecule responsible for pharmacological effects. The 2D and 3D structures of the plant constituents: 4-caffeoylquinic acid (cryptochlorogenic acid), lupeol-3-acetate, methyl 4-O-galloylchlorogenate and β-amyrin-3-(3’-dimethyl) butyrate are presented in Figure 1.

Materials and Methods

Experimental procedures

Superior quality of M. zapota fruit weighing 4 kg was purchased from local market in Feb 2017. Plant was authenticated by a taxonomist Dr. Mushtaq Ahmad, at Department of Plant Sciences, Quaid-a-Azam University, Islamabad. Voucher specimen no. (ISL-B-23) was collected after submitting sample of specimen of these species to the herbarium at same department. The fruit (4 kg) was air-dried, crushed into powdered form and extracted at room temperature with aqueous-methanol (70:30) three times to obtain M. zapota crude extract (Mz.Cr).

Chemicals

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

Experimental animals and housing conditions

Sprague-Dawley rats (180-220 g), BALB/c mice (25-30 g) and rabbits (1.0-1.2 kg), of either sex were obtained from animal house of the Riphah Institute of Pharmaceutical Sciences (RIPS) Islamabad. The animals were kept in plastic cages at standard temperature (23-25 °C). They were fed with standard animal feed and tap water ad libitum. Animals were fasted before each experiment for 24 hr. All the animal experimental protocols were approved by Research and Ethics Committee of RIPS (Ref. No. REC/RIPS/2017/008) which were performed in accordance with the guidelines of “Principles of Laboratory Animal care” (11).

Phytochemical analysis

Detection of major secondary metabolites presence such as glycosides, anthraquinones, steroids, flavonoids and tannins was carried out in Mz.Cr according to standard procedure (12) with slight modifications.

Castor oil induced diarrhea

Previously reported method was used for this study (13). All the test animals were fasted for 24 hr prior to commencement of experimentation. The floor of cage was lined with blotting paper in which animals were placed. First group was assigned as negative control group and received normal saline (10 ml/kg) orally, while second group was given with a dose of loperamide hydrochloride (10 mg/kg, p.o.) and assigned as positive control. Third, fourth and fifth groups received 50, 100 and 300 mg/kg body weight of the extract orally respectively. One hr after administration of the respective doses and treatments, all animals received (10 ml/kg, p.o.) of castor oil. Post treatment evaluation was carried out after waiting 4 hr in order to analyze the diarrheal droppings presence, absence of diarrheal droppings was documented as a positive result. Results were analyzed by applying Chi square test.

Assessment of intestinal fluid accumulation

Intestinal fluid accumulation was determined using the method as described previously (14). To study the intestinal fluid accumulation, entero-pooling assay was used. Overnight fasted mice were taken and put into five assigned cages with five mice in each. Group I and II were administered normal saline (10 ml/kg) and castor oil (10 ml/kg, p.o.) respectively. Extract doses of 50, 100 and 300 mg/kg intraperitoneally were given to Group III, IV and V respectively. Standard drug atropine at dose 10 mg/kg was given to last group, 1 hr prior induction with castor oil (10 ml/kg, p.o.). Mice were sacrificed after 30 min, then intestine was removed and weighed. The results were articulated as: (Pi/Pm) x 1000 where, Pi is the weight (g) of the intestine and Pm is the weight (g) of the animal.
Isolated tissue preparation

Rabbits fasted for 24 hr before experiment but they had a free access to water. Jejunal portion was isolated after cervical dislocation of rabbit and washed with Tyrode’s solution. Jejunal segment of 2 cm length was suspended in tissue bath containing Tyrode’s solution. Temperature of bath was kept at (37 °C) and proper aeration of 95% O2 and 5% CO2 (carbogen) is ensured. An initial load of 1 g was applied to each tissue and was allowed to equilibrate for 30 min before the addition of any drug. Following equilibration period, each preparation was then stabilized with sub-maximal concentration of ACh (0.3 μM) at 3 min interval until constant responses were recorded via a force displacement transducer (model FT-03) coupled with bridge amplifier and power Lab 4/25 data acquisition system connected to computer running Lab-Chart 6 software (AD Instrument, Sydney Australia). The effects of MzCr at doses (0.01-3mg/mL) was recorded as the % change in the voluntary contractions of jejunum (15).

Ethanol-HCl induced ulcer assay

Rats weighing 250-280 g of either sex were distributed in 5 groups (n=5). Group 1 served as a negative control received normal saline 10 ml/kg body weight, group 2 received 20 mg/kg, (p.o.) omeprazole as standard drug; group 3, 4 and 5 received 50, 100 and 300 mg/kg, (p.o.) of MzCr respectively. All the animals were treated with 1 ml/100 g of ethanol-HCl mixture (p.o.) i.e. (0.3 M Hydrochloric acid and ethanol 60%) after 1 hr to induce gastric ulcer. Animals were sacrificed via cervical dislocation 1 hr after administration of ethanol-HCl mixture. The stomachs were removed 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 as described previously (16). For each stomach lesion, ulcer index was calculated as mean ulcer score (US) as follows, US<0 5 mm2, 2: 0.5<US≤2.5 mm2, 3: 2.5 mm2<US≤5 mm2, 4: 5 mm2<US≤10 mm2, 5: 10 mm2<US≤15 mm2, 6: 15 mm2<US≤20 mm2, 7: 20 mm2<US≤25 mm2, 8: 25 mm2<US≤30 mm2, 9: 30 mm2<US≤35 mm2 and 10: US>35 mm2. For each stomach injury sum of the lengths (mm) of all sores was utilized as the ulcer index (UI). The gastro protective assessment was displayed as an inhibition percentage (%) calculated by the following formula:

\[ I(\%) = \frac{USc-USt}{USc} \times 100 \]

Where USc= ulcer surface area of control and USt= ulcer surface area of test drug group.

Charcoal meal transit time

Gastrointestinal transit time was estimated utilizing the charcoal meal test (17). Rats were fasted for 24 hr, the test groups received the extracts at 50, 100 and 300 mg/kg body weight doses, where as positive control group received atropine sulfate (0.1 mg/kg, IP), while the negative control group received normal saline (10 ml/kg, p.o.). 30 mins after all treatments, all the animals were sacrificed. The small intestine was excised after cervical dislocation of rabbit and washed with Tyrode’s solution. Jejunal segment of 2 cm length was suspended in tissue bath containing Tyrode’s solution. Jejunal segment of 2 cm length was suspended in tissue bath containing Tyrode’s solution. Relative concentration of ACh (0.3 μM) at 3 min interval until constant responses were recorded via a force displacement transducer (model FT-03) coupled with bridge amplifier and power Lab 4/25 data acquisition system connected to computer running Lab-Chart 6 software (AD Instrument, Sydney Australia). The effects of MzCr at doses (0.01-3mg/mL) was recorded as the % change in the voluntary contractions of jejunum (15).

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Acute toxicity

Mice were divided in 3 groups of 5 mice each. The test was performed using increasing doses of the plant extract (3 and 5 g/kg) given in 10 ml/kg volume. Saline (10 ml/kg, p.o. negative control) was administered to one group. Twenty-four hr post study the mice were observed for mortality (18).

Computational studies

3-D structures of the test compounds (β-amyrin-3-(3’-dimethyl) butyrate, methyl 4-O-galloylchlorenogenate, 4-cafeoylquinic acid and lupeol-3-acetate) were constructed by using the software of Gauss View 5.0 (Figure 2). Three dimensional structures of reference drugs were prepared through Discovery Studio Visualizer (2016) as shown in Figure 3. Reference drugs included phenoxy benzamine, verapamil, calmidazolium, domperidone, ranitidine, pirenzapine, atropine, loperamide, omeprazole, papaverine and pyrilamine. 3-D structures of selected targets possibly involved in the gut physiology, were retrieved from the website of RCSB protein data bank as represented in Figure 4. Selected targets included adrenergic α, receptor (PDB ID:3534B), muscarinic M1 (PDB ID:5CV), muscarinic M2 (PDB ID:4IU4), dopaminergic D2 (PDB ID:6CM4), calmodulin (PDB ID:1CTR), mu-opioid (PDB ID:5C1M), voltage gated L-type calcium channel (PDB ID:1T3S), histaminergic H1 (PDB ID:3RZE), histaminergic H2 (PDB ID:2P501), H+/K+ ATPase (PDB ID:5YLU) and phosphodiesterase enzyme (PDB ID:3G4K). Autodock Vina which is a geometry based automatic docking tool is used through which molecular docking was performed. Evaluation of docking results was based on atomic energy in Kcal/mol (19). Assessment in 2-D design was made to check the most extreme restricting interactions of complex framed amongst amino acid residues and ligands including: valine (VAL), alanine (ALA), proline (PRO), arginine (ARG), lysine (LYS), glycine (GLY), glutamine (GLN), asparagine (ASN), cysteine (CYS), methionine (MET), glutamic acid (GLU), histidine (HIS), phenylalanine (PHE), isoleucine (ILE), tyrosine (TYR),...
serine (SER), threonine (THR), aspartic acid (ASP) and tryptophan (TRP).

**Statistical analysis**

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

**Results**

**Phytochemical profile**

Qualitative phytochemical analysis of Mz.Cr showed the presence of flavonoid, phenols, triterpenes, lignin, unsaturated sterols and carbohydrates.

**Effect of Mz.Cr on castor-oil induced diarrhea**

Mz.Cr exhibited a dose-dependent (50-300 mg/kg) protective effect against castor oil-induced diarrhea in mice. The negative control group (saline treated) did not show any protection against castor oil-induced diarrhea.

| Treatment (mg/kg) | No of mice (out of 5) with diarrhea | Protection (%) |
|------------------|------------------------------------|---------------|
| Saline (10 ml/kg)+castor oil | 5 | 0 |
| Mz.Cr (50 mg/kg)+castor oil | 4 | 20 |
| Mz.Cr (100 mg/kg)+castor oil | 2 | 40 |
| Mz.Cr (300 mg/kg)+castor oil | 1* | 80 |
| Loperamide (10 mg/kg)+castor oil | 0** | 100 |

*P<0.05, **P<0.01 compared to saline group, data analyzed by Chi-squared test

Pretreatment of animals with the Mz.Cr showed 20% protection from diarrhea at 50, 40% at 100 and 80% protection at 300 mg/kg (P<0.05 vs. saline group). Loperamide (10 mg/kg) showed 100% protection from diarrhea (P<0.01 vs. saline group) in the positive control group (Table 1).

**Effect of Mz.Cr on intestinal fluid accumulation**

When tested against castor oil-induced intestinal fluid accumulation in mice, Mz.Cr exhibited a dose-dependent (50-300 mg/kg) anti-secretory effect. Intestinal fluid
accumulation in the saline treated group was 81.9±0.84 (mean±SEM, n=5), whereas in the castor oil-treated group it was 122.5±0.55 (P<0.001 vs. saline group). Mz.Cr at the doses of 50, 100 and 300 mg/kg reduced the castor oil-induced fluid accumulation to 108.30±0.47 (P<0.001 vs. castor oil group) and 84.98±0.67 (P<0.001 vs. castor oil group) respectively. Atropine at the dose of 10 mg/kg decreased the intestinal fluid accumulation to 74.34±0.69 (P<0.001 vs. castor oil group) as shown in Figure 5.

Effect of Mz.Cr on spontaneous and K⁺ induced contractions

Figure 6 shows comparative inhibitory effect of the plant extract, papaverine and verapamil against spontaneous and K⁺ (80 mM)-induced contractions. Mz.Cr was found to be equally effective against spontaneous and K⁺ (80 mM)-induced contractions with EC₅₀ values of 0.11 mg/ml (0.09-0.2, n=4) and 0.16 mg/ml (0.09-0.2, n=4) respectively as shown in Figure 6A. Papaverine also showed similar pattern of non-specific inhibitory response (Figure 6B) with respective EC₅₀ values of 0.6 (0.3-1.3, n=4) and 0.4 μM (0.2-0.8, n=4), whereas, verapamil was found more potent against K⁺ (80 mM)-induced contractions with EC₅₀ value of 0.04 μM (0.03-0.06, n=4), as compared to spontaneous contractions (0.12 μM (0.10-0.20, n=3)) as shown in Figure 6C.

Effect of Mz.Cr on ethanol-HCl induced ulcer

Mz.Cr in dose dependent manner (50-300 mg/kg) exhibited an anti-ulcer effect. Mz.Cr at 50, 100 and 300 mg/kg caused 21.1, 42.2 and 73.26% (P<0.001 vs. saline group) inhibition respectively. Omeprazole (20 mg/kg) exhibited 88.8% inhibitory effect (Table 2). Macroscopic observation showed the gastric mucosa of rats (Figure 7).

Table 2. Protective effect of Manilkara zapota crude extract (Mz.Cr) and omeprazole against ethanol-HCl induced gastric ulcers in rats

| Treatment                  | Ulcer Index | Inhibition (%) |
|----------------------------|-------------|----------------|
| Saline 10 ml/kg+Ethanol-HCl| 9.0±0.07    | -              |
| Mz.Cr (50 mg/kg)+Ethanol-HCl| 7.1±0.20    | 21.1           |
| Mz.Cr (100 mg/kg)+Ethanol-HCl| 5.2±0.14    | 42.2           |
| Mz.Cr (300 mg/kg)+Ethanol-HCl| 2.4±0.14    | 73.26          |
| Omeprazole (20 mg/kg)+Ethanol-HCl| 1±0.11    | 88.8           |

***P<0.001 compared to control saline group, one-way analysis of variance, followed by Post hoc Tukey’s test, n=5
Effect of Mz.Cr on charcoal meal transit time

Mz.Cr hinders the charcoal meal to travel through the small intestine in a dose dependent manner. The distance travelled by the saline group was 82.29%. Mz.Cr at 50, 100 and 300 mg/kg dose shows inhibition of charcoal meal transit by 54.05, 51.57 and 47.25% respectively (P<0.001 vs. saline group). Atropine (0.1 mg/kg, IP) shows inhibitory effect of 44.23% (Table 3).

Acute toxicity

The extract did not show any mortality up to the dose of 5 g/kg.

Docking evaluation

Assessment of E-value is an important contributor which helps in docking evaluation. Apart from this, other contributing factors include hydrogen bonding, pi-pi bonding and other hydrophobic interactions between ligand-protein complexes. Results of post dock analysis are given in Tables 4-6 and Table 7, showing number and binding residues of hydrogen bonds, pi bonds

### Table 3. Effect of *Manilkara zapota* crude extract (Mz.Cr) and atropine on charcoal meal transit time in rats

| Treatment (mg/kg) | Mean length of Intestine (cm) | Distance Moved by Charcoal (cm) | Intestinal transit (%) |
|-------------------|-------------------------------|-------------------------------|------------------------|
| Saline (10 ml/kg) | 86.66±0.6                     | 71.32±0.6                     | 82.29                  |
| Mz.Cr (50 mg/kg)  | 86.32±0.3                     | 46.64±1.4                     | 54.05                  |
| Mz.Cr (100 mg/kg) | 85.32±0.3                     | 44.00±1.5                     | 51.57                  |
| Mz.Cr (300 mg/kg) | 84.99±0.4                     | 40.32±0.6                     | 47.25                  |
| Atropine (0.1 mg/kg, IP) | 86.60±0.4 | 39.64±1.4                     | 44.23                  |

***P<0.001 compared to control saline group, one-way analysis of variance followed by Post hoc Tukey’s test, n=5

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### Table 4. E-values (Kcal/mol) of best docked poses of methyl 4-O-galloylchlorogenate, β-amyrin-3-(3’-dimethyl) butyrate, lupeol-3-acetate, 4-caffeoylquinic acid and standard drugs against targets: adrenergic α1 receptor, muscranic M1, muscranic M3, dopaminergic D2, calmodulin, mu-opioid, voltage gated L-Type calcium channel, histaminergic H1, histaminergic H2, H+/K+ ATPase pump and phosphodiesterase enzyme

| Target Proteins | PDB ID | β-amyrin-3-(3’-dimethyl) butyrate | 4-Caffeoylquinic acid | Methyl 4-O-galloylchlorogenate | Luteol-3-acetate | Standard drugs |
|-----------------|--------|----------------------------------|----------------------|-------------------------------|----------------|---------------|
| Adrenergic α1   | 3538   | -10.3                            | -8.4                 | -9.9                          | -8.0           | -8.0          |
| Muscranic M1    | 5CXV   | -10.0                            | -7.6                 | -9.1                          | -10.9          | -9.0          |
| Muscranic M3    | 4U14   | -9.3                             | -7.0                 | -9.6                          | -9.5           | -9.6          |
| Dopaminergic D2 | 6CM4   | -9.7                             | -8.4                 | -9.4                          | -9.5           | -10.6         |
| Calmodulin      | 1CTR   | -8.9                             | -6.3                 | -7.1                          | -8.4           | -8.3          |
| Calcium channel | 1TSS   | -9.3                             | -7.4                 | -7.4                          | -8.9           | -7.6          |
| Histaminergic H1| 3RZE   | -8.5                             | -7.1                 | -6.9                          | -8.0           | -5.7          |
| H+/K+ ATPase    | 5YLI   | -9.7                             | -9.2                 | -10.9                         | -10.3          | -8.4          |
| Histaminergic H2| P2Z521 | -8.7                             | -8.6                 | -9.7                          | -8.8           | -6.1          |
| Mu-opioid       | 5C1M   | -10.5                            | -7.3                 | -8.4                          | -9.4           | -9.2          |
| Phosphodiesterase enzyme | 3G4H | -10.5                             | -9.1                 | -8.8                          | -9.7           | -8.3          |

Standard inhibitors or activator of pathways are: (A) phenoxy benzamine, (B) pirenzapine, (C) atropine, (D) domperidone, (E) calmozolium, (F) verapamil, (G) pyrilamine, (H) omeprazole, (I) ranitidine, (J) loperamide and (K) papaverine

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**Figure 7.** Panels [I] and [II] shows (A), (B), (C) and (D) interactions of methyl 4-O-galloylchlorogenate, β-amyrin-3-(3’-dimethyl) butyrate, lupeol-3-acetate and 4-caffeoylquinic acid against targets: calmodulin receptor and calcium channel respectively. (E) represents calmozolium and verapamil interactions. 
**Table 5.** Hydrogen bonds (H-bonds) formed by methyl 4-O-galloylchlorogenate, β-amyrin-3-(3'-dimethyl) butyrate, lupeol-3-acetate, 4-cafeoylquinic acid and standard drugs against targets: adrenergic α1 receptor, muscranic M3, muscranic M2, dopaminergic D3, calmodulin, mus-opioid, voltage gated L-Type calcium channel, histaminergic H1, histaminergic H2, H+/K+ ATPase pump and phosphodiesterase enzyme

| Target Protein | PDB ID | Amino Acids | n-H bonds | Amino Acids | n-H bonds | Amino Acids | n-H bonds | Amino Acids | n-H bonds | Amino Acids | n-H bonds | Amino Acids |
|---------------|--------|-------------|----------|-------------|----------|-------------|----------|-------------|----------|-------------|----------|-------------|
| 4-caffeoylquinic acid | 3RZE | ASN 123 | 4 | GLU 110 | 4 | LEU 105 | 2 | THR 127 | 1 | TYR 127 | 1 | 4E 477 |
| lupeol-3-acetate | 3RZE | ARG 544 | 6 | THR 181 | 4 | THR 173 | 3 | ARG 544 | 6 | THR 181 | 4 | 4E 477 |
| methyl 4-O-galloylchlorogenate | 3RZE | ARG 544 | 6 | THR 181 | 4 | THR 173 | 3 | ARG 544 | 6 | THR 181 | 4 | 4E 477 |

**Table 6.** Pi-Pi bonds (p-p bonds) formed by methyl 4-O-galloylchlorogenate, β-amyrin-3-(3'-dimethyl) butyrate, lupeol-3-acetate, 4-cafeoylquinic acid and standard drugs against targets: adrenergic α1 receptor, muscranic M3, muscranic M2, dopaminergic D3, calmodulin, mus-opioid, voltage gated L-Type calcium channel, histaminergic H1, histaminergic H2, H+/K+ ATPase pump and phosphodiesterase enzyme

| Proteins | PDB ID | 4-caffeoylquinic acid | methyl 4-O- galloylchlorogenate | lupeol-3-acetate | Standard drugs |
|----------|--------|-----------------------|---------------------------------|-----------------|----------------|
| n-H bonds | Amino Acids | n-H bonds | Amino Acids | n-H bonds | Amino Acids | n-H bonds | Amino Acids |
| Adrenergic α1 | 3RZE | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 |
| Muscranic M3 | 3RZE | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 |
| Dopaminergic D3 | 3RZE | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 |
| Calmodulin | 3RZE | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 |
| Calcium channel | 3RZE | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 |
| Histaminergic H1 | 3G4K | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 |
| Histaminergic H2 | 3G4K | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 |
| H+/K+ ATPase | 3G4K | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 |
| Phosphodiesterase enzyme | 3G4K | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 | ARG 174 | 0 |

Standard inhibitors or activators are: (A) phenoxy benzamine, (B) pirenzapine, (C) atropine, (D) domperidone, (E) calmidazolium, (F) aspartic acid, (G)omeprazole, (I) ranitidine, (J) loperamide and (K) papaverine. Amino acids are: ALA alanine; ARG arginine; ASN asparagine; ASP aspartic acid; CYS cysteine; GLN glutamine; GLU glutamic acid; GLY glycine; HIS histidine; ILE isoleucine; LYS lysine; MET methionine; PHE phenylalanine; PRO proline; SER serine; THR threonine; TRP tryptophan and TYR tyrosine.

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and hydrophobic interactions respectively. Formation of bonding and interaction by β-amyrin-3-(3'-dimethyl) butyrate, methyl 4-O-galloylchlorogenate, 4-cafeoylquinic acid, lupeol-3-acetate and standard drugs against selected targets are shown in Figures 5-10 respectively.

### Table 7. Hydrophobic interactions formed by methyl 4-O-galloylchlorogenate, β-amyrin-3-(3'-dimethyl) butyrate, lupeol-3-acetate, 4-caffeoylquinic acid and standard drugs against targets: adrenergic α1 receptor, muscarinic M3 receptor, dopaminergic D2 receptor, calmodulin, mu-opioid, voltage gated L-Type calcium channel, histaminergic H1, histaminergic H2, H+/K+ ATPase and phosphodiesterase enzyme

| Target proteins | PDB ID | β-amyrin-3-(3') dimethyl butyrate | 4-cafeoylquinic acid | methyl 4-O-galloylchlorogenate | Lupeol-3-acetate | Standard drugs |
|----------------|--------|----------------------------------|---------------------|--------------------------------|-----------------|----------------|
| Adrenergic α1   | 35348  | ILE 353(3), 307, 193             | TYR 193(2), 196     | ILE 193(2), 294(2), 194(2), 296(2) | ILE 193(2), 196(2) | ALA 194, ILE 98 |
| Muscarinic M3   | 35844  | ARG 123, LEU 143, 2A, 2B       | TYR 193             | TYR 208, ILE 103, 2A, 2O    | TYR 193, 2O     | ALA 109, ILE 109 |
| Muscarinic M3   | 35844  | ARG 123, LEU 143, 2A, 2B       | TYR 193             | TYR 208, ILE 103, 2A, 2O    | TYR 193, 2O     | ALA 109, ILE 109 |
| Depaminergic D2 | 6Q64   | TYR 177, TRP 143, PHE 124, ILE 222 | TYR 143             | TYR 221, TRP 121, PHE 221   | TYR 133, 129    | CYS 132, ILE 132 |
| Calmodulin      | 1CTR   | VAL 91, 108                       | ALA 119, 117, 116, 18 | ALA 119, 117, 116, 18       | ALA 119, 119    | VAL 119, ILE 119 |
| Calcium channel | 2ESC   | TYR 108                          | LEU 24, 58, 39, 175 | TYR 118, ILE 120             | TYR 133, 129    | CYS 132, ILE 132 |
| Histaminergic H1| 3KZ2   | VAL 71, ILE 148, TRP 164, PHE 166 | ASP 163             | VAL 71, ILE 148, TRP 164, PHE 166 | ILE 148, 149    | ALA 1674, 1673 |
| H+/K+ ATPase    | 5YU1   | VAL 71, ILE 148, TRP 164, PHE 166 | ASP 163             | VAL 71, ILE 148, TRP 164, PHE 166 | ILE 148, 149    | ALA 1674, 1673 |
| Histaminergic H2| 2F5021 | ILE 119, ARG 109, 45             | ALA 170             | ALA 170, ARG 109, 45         | ALA 170, ARG 109 | ALA 170, ILE 170 |
| M-oxipod        | 5C18   | VAL 106, ILE 296, 122, 388, 118 | MET 151             | ILE 296, 388, 118, 151       | ILE 296, 388, 118 | ILE 296, 388, 118 |
| Phosphodiesterase| 3O4K   | LEU 426, 487, 387, PHE 415, ARG 423, ASP 415 | LEU 426, 487, 387, PHE 415, ARG 423, ASP 415 | LEU 426, 487, 387, PHE 415, ARG 423, ASP 415 | GLN 535, ASP 464 | GLN 535, ASP 464 |

Standard inhibitors or activators are: (A) phenoxybenzamine, (B) pirenzepine, (C) atropine, (D) domperidone, (E) calmidazolium, (F) verapamil, (G) pyrilamine, (H) omeprazole, (I) ranitidine, (J) loperamide and (K) papaverine. Amino acids are: ALA, alanine; ARG, arginine; ASN, asparagine; ASP, aspartic acid; CYS, cysteine; GLN, glutamine; GLU, glutamic acid; GLY, glycine; HIS, histidine; ILE, isoleucine; LYS, lysine; MET, methionine; PHE, phenylalanine; PRO, proline; SER, serine; THR, threonine; TRP, tryptophan; TYR, tyrosine and VAL, valine.

Figure 8. Panels [I] and [II] shows (A), (B), (C) and (D) interactions of methyl 4-O-galloylchlorogenate, β-amyrin-3-(3'-dimethyl) butyrate, lupeol-3-acetate and 4-cafeoylquinic acid against targets: histaminergic H1 receptor and H+/K+ ATPase respectively. (E) represents pyrilamine and omeprazole interactions.
Discussion

Based on ethnopharmacological use of M. zapota in hyperactive gut diseases, such as colic and diarrhea, its extract was evaluated for the possible anti-diarrheal, anti-secretory, charcoal meal gastrointestinal motility and anti-ulcer effects in rodents. Isolated intestinal tissue was used for the elucidation of possible underlying mechanism(s) to rationalize aforementioned ethnomedicinal uses of the plant and it was further supported by virtual screening tools.

Mz.Cr showed protective effect against castor oil induced diarrhea, similar to effect produced by loperamide, a standard drug (11). Castor oil induces diarrhea through its active metabolite i.e. ricinoleic acid. It is responsible for causing diarrhea through a series of actions including activation of small intestinal peristaltic activity with reduction of Na⁺-K⁺ATPase activity. These changes eventually result in disturbance in the intestinal mucosa, electrolyte permeability, hypersecretion of intestinal contents, and a slogging of the transport time in the intestine (20). Thus, a potential agent may exhibit its anti-diarrheal activity by these mechanisms. Intracellular Ca²⁺ levels had a huge impact on secretory functions of the gastrointestinal organs which lead towards consequences such as discharge of gastric acids and intestinal fluid release. This effect might be affected by some drugs that hinder calcium influx (21). Mz.Cr shows protection against castor oil induced intestinal fluid secretions in mice. The anti-diarrheal and anti-secretory activities of Mz.Cr might be because of gastrointestinal relaxant component(s) present in the Mz.Cr.

Spontaneous contracting rabbit jejunum preparation is conventionally used to determine the spasmylytic impact, without the utilization of spasmogen (agonist). In jejunum, papaverine (Ca²⁺ influx and phosphodiesterase (PDE) inhibitor) and Mz.Cr both possess repressive effect on spontaneous as well as high K⁺-induced contractions with similar effect, whereas verapamil, a specific calcium antagonist have inhibitory effect against the K⁺-induced contractions. Against spontaneous and K⁺-induced contractions Mz.Cr produces inhibitory pattern like papaverine does, which depicts that plant may be involved in dual mechanism(s) with CCB, in producing relaxation effect, like PDE enzyme(s) inhibition. PDE enzyme inhibitors augment the intracellular level of cyclic AMP which results in relaxation of smooth muscles (22). Traditionally M. zapota is used in colic and diarrhea, which is observed through its anti-diarrheal, anti-secretory, anti-ulcer and anti-spasmodic effects. This is expected as both Ca²⁺ antagonists and PDE inhibitors possess an anti-diarrheal, anti-secretory and anti-spasmodic properties (15).

Various aggressive and protective factors play important role in acid release inside gastrointestinal tract. Any imbalance in these factors results in rupturing of mucosal protection and expose gastric lining to gastric acid leading to the sores called ulcers. To explore the
anti-ulcer effect of Mz.Cr, ethanol-HCl induced gastric model was used which through variety of mechanisms stimulates ulcers including mucus exhaustion, mucosal damage, release of superoxide anion, hydroperoxide free radicals, all these mechanisms prolonged the tissue oxidative stress and release of inflammatory mediators (16). Marked inhibition on certain ethanol-HCl induced gastric lesions formation as compared to control group showed gastro protective effect of Mz.Cr. The potential of Mz.Cr to produce anti-ulcer effect might be due to its CCB effect, as Ca²⁺ antagonist are well known to demonstrate such effects (23). In pathophysiology of gastric ulcers, oxidative stress plays a vital role. Antioxidant and nitric oxide free radical scavenging activity has been reported by M. zapota (6), which may be responsible for its effectiveness as anti-ulcer agent.

In the small intestinal transit test, Mz.Cr produces suppression of the propulsion of charcoal marker at all test doses just like atropine sulphate a standard drug, that has been reported to have anticholinergic effect on intestinal transit (24). A decrease in the motility of gut muscles increases the stay of substances in the intestine, thus allows better water absorption. This finding suggests that Mz.Cr has the ability to influence the peristaltic movement of intestine thereby indicating the presence of an anti-motility activity. It is therefore presumed that the reduction in the intestinal propulsive movement in the charcoal meal model may be due to antispasmodic properties of the Mz.Cr (25).

The observed therapeutic effects of M. zapota may be due to the presence of phytochemicals, tannins and flavonoids, as these phytoconstituents are well known for gastrointestinal effects. Anti-diarrheal, anti-secretory, anti-ulcer and anti-spasmodic activities may be due to flavonoids. Beneficial role of tannins in diarrhea cannot be ignored (26).

In acute toxicity testing, the Mz.Cr did not show any mortality up to the maximum dose (5 g/kg) tested, which shows the wide therapeutic range of M. zapota.

Molecular docking is an effective tool for evaluating the affinity of various protein targets that may possibly be associated with the pathophysiology of gastric disorders. The traditionally acclaimed use of M. zapota in the management of gastric related diseases has been supported with scientific evidence using virtual screening tool.

In this study, Auto Dock Vina program was used through PyRx (27). It uses gradient optimization method and it improves accuracy of binding mode predictions. Hydrogen bonding is reported to be significant in formation of ligand protein complex. In this study, we assessed affinity of ligands through E-value and number of hydrogen bonds against protein targets which imparts their influential effect in gastrointestinal diseases. Lower de-solvation energy is an indication of favorable ligand protein complex which is achieved with lower E-values (28). According to certain instances, no of pi-pi interactions formed by the ligand-target structural complex contributed to increase the stabilization of complex which is comparable to the stable interaction formed by H-bond. Other hydrophobic bonding likewise improves the partiality of ligand’s affinity for particular protein target (29). The affinity of ligands for respective targets was assessed on the basis of atomic energy value, hydrogen bonds, pi-pi interactions and hydrophobic bonding.

It has been found that 4-caffeoylquinic acid showed excellent score of binding against M₁ receptor with lowest E-value. This binding efficacy is greater than majority of the target proteins with better affinity as compared to the other test compounds and standard drugs. Thus, this result suggests that it showed maximum affinity for binding with M₁ receptor. Order of affinity of the test compounds for M₁ receptor was; 4-caffeoylquinic acid>methyl 4-O-galloylchlorogenerate>β-amyrin-3-(3'-dimethyl) butyrate>lupeol-3-acetate. Order of affinity of the test compounds for adrenergic α₁ receptor was found to be; phenoxy benzamine>4-caffeoylquinic acid>methyl 4-O-galloylchlorogenerate>β-amyrin-3-(3'-dimethyl) butyrate. Compounds with higher affinity all together formed stronger pi-pi bonds, high number of hydrophobic interactions and polar hydrogen bonding against M₁ and α₁ receptors, piranzapine showed only π–π interaction while phenoxy benzamine showed H-bonding along with π–π interactions as well. The order of affinity for ligands against M₂ receptor was found as; 4-caffeoylquinic acid>atropine>methyl 4-O-galloylchlorogenerate>β-amyrin-3-(3'-dimethyl) butyrate>lupeol-3-acetate. Order of affinity of the test compounds for dopaminergic D₁ receptor was found as; 4-caffeoylquinic acid>methyl 4-O-galloylchlorogenerate>lupeol-3-acetate>β-amyrin-3-(3'-dimethyl) butyrate>domperidone. Alongside hydrogen and hydrophobic interactions, different types of interactions, for example alkyl, pi-alkyl and vander waal interactions are appeared with high proclivity by test compounds. Amino acids; TYR 408, LEU 94, TRP 413 and ASP 114 are found to be important. Methyl 4-O-galloylchlorogenerate, 4-caffeoylquinic acid and domperidone exhibited bonding with ASP 114, a stable amino acid residue (30). The affinity order of ligands against calmodulin was found as; 4-caffeoylquinic acid>methyl 4-O-galloylchlorogenerate>calmomidazolium>lupeol-3-acetate>β-amyrin-3-(3'-dimethyl) butyrate. In addition, hydrogen bond is considered to be vital for complex of ligand with calmodulin. The affinity order for test compounds for voltage gated L-Type calcium channel was found as; methyl 4-O-galloylchlorogenerate>4-caffeoylquinic acid>verapamil>lupeol-3-acetate>β-amyrin-3-(3'-dimethyl) butyrate. Methyl 4-O-galloylchlorogenerate, 4-caffeoylquinic acid and lupeol-3-acetate showed interactions with ARG569 which helps in making non-covalent bonds (salt bridge) (31). Order of affinity of test compounds for histaminergic H₂ receptor was found to be: piraanzapine>methyl 4-O-galloylchlorogenerate>4-caffeoylquinic acid>verapamil>lupeol-3-acetate>β-amyrin-3-(3'-dimethyl) butyrate. Ligands are not engaged with making any solid interactions on stated restricting sites. Order of affinity of test compounds for H/K ATPase receptor was found as; omeprazole>4-caffeoylquinic acid>β-amyrin-3-(3'-dimethyl) butyrate>lupeol-3-acetate>methyl 4-O-galloylchlorogenerate. Hydrogen and hydrophobic associations are observed to be essential but no such interactions of test compounds with stated restricting site were seen. In this regard, SER 477 is
considered as important and vital amino acid. The affinity order of ligands against histaminergic H1 receptor was found as: ranitidine>4-caffeoylquinic acid>β-amyrrin-3-(3’-dimethyl) butyrate>lupeol-3-acetate>methyl 4-O-galloylchlorogenate. Order of affinity of the test compounds for mu-opioid receptor was found as: 4-caffeoylquinic acid>methyl 4-O-galloylchlorogenate>loperamide>lupeol-3-acetate>β-amyrrin-3-(3’-dimethyl) butyrate. Order of affinity of test compounds for phosphodiesterase enzyme was found as: papaverine >methyl 4-O-galloylchlorogenate>4-caffeoylquinic acid >lupeol-3-acetate>β-amyrrin-3-(3’-dimethyl) butyrate. Ligands having high restricting proclivity shaped interacts with TYR272 and VAL270.

It is revealed that 4-caffeoylquinic acid and methyl 4-O-galloylchlorogenate showed more affinity than lupeol-3-acetate and β-amyrrin-3-(3’-dimethyl) butyrate. Hydrophobic interactions were shown by ligands with high affinity. Essential amino acids of arginine family are important in the binding site which is involved in interactions with all these ligands (32).

Conclusion

M. zapota exhibited anti-diarrheal, anti-secretary, anti-spasmodic, anti-motility and anti-ulcer effects. The plant constituents: caffeoylquinic acid and methyl 4-O-galloylchlorogenate showed high binding affinities (E-value=-6.5 Kcal/mol) against histaminergic H1 receptors, H+/K+ ATPase pump and voltage gated L-type calcium channels, while showed moderate affinities (E-value=9 Kcal/mol) against histaminergic H2, muscarinic M1, muscarinic M3, mu-opioid, whereas revealed lower affinities (E-value=2.9 Kcal/mol) vs. calmodulin, adrenergic α1, phosphodiesterase enzyme and dopaminergic D2 receptors. Lupeol-3-acetate and β-amyrrin-3-(3’-dimethyl) butyrate exhibited weak affinities against aforementioned targets.

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Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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