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In Vivo and *In Silico* Studies of Flavonoids Isolated from *Pistacia integerrima* as Potential Antidiarrheal Agents

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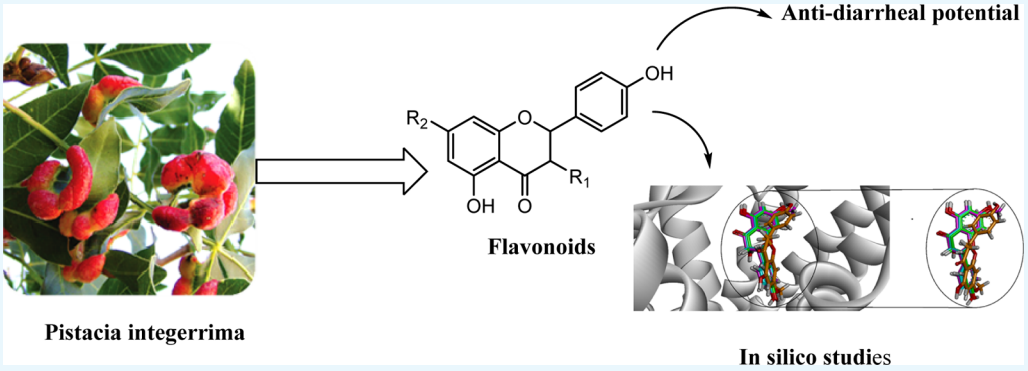

ABSTRACT: Pistacia integerrima leaf galls are used in several traditional medicines to cure many diseases such as diarrhea, asthma, fever, cough, vomiting, and hepatitis. The main goal of the present investigation was to assess the antidiarrheal effect of the *Pistacia integerrima* extracts/fractions and four isolated flavonoid compounds (**1**−**4**) on mice. An *in vivo* assay involving castor-oil-induced diarrhea was used to evaluate the antidiarrheal potential of extracts/fractions at 100, 200, and 400 mg/kg p.o., as well as isolated compounds at 5, 10, and 20 mg/kg p.o. Pretreatment of mice with extracts/fractions significantly attenuated castor-oil-induced diarrhea in a dose-dependent manner. Among all crude extracts and fractions, the ethyl acetate extract was the most effective with 100% protection against diarrhea followed by chloroform (75% protection) at 400 mg/kg p.o. Although all the isolated compounds exhibited strong antidiarrheal activity, isolated compounds **1** and **4** demonstrated 100% protection against diarrhea. Moreover, docking models were performed using the Molecular Operating Environment (MOE) and AutoDock software and suggested that the extracts and isolated compounds exert antidiarrheal activity by inhibiting mu-opioid and delta-opioid receptors. Therefore, our finding affords a strong pharmacological basis for the traditional use of *P. integerrima* galls in the treatment of diarrhea.

1. INTRODUCTION

Pistacia integerrima (J.L. Stewart ex Brandis), also known as Shaani, zebrawood, and Kakarsinghi, belongs to the family Anacardiaceae. *P. integerrima* is widely grown in various regions of Pakistan and in India, particularly in the Himalayan, at elevations of 7999 to 12,000 feet.[1−3] Horn-shaped leaf galls frequently develop on this tree and are harvested to prepare *kakad shringi*, an herbal product used to cure diarrhea in India.[4] *P. integerrima* has great medicinal values for treatment of various ailments, including blood cleans, analgesic, and anti-inflammatory effects. Various parts of the plant are used for the treatment of complex ailments such as diarrhea, fever, nausea, vomiting, and gastrointestinal disorders.[5,6] It is also used to treat hepatitis, oxidative stress, chronic bronchitis, asthma, psoriasis, disorders of the respiratory tract, hyperuricemia, and poor appetite.[7,8]

Phytochemically, various classes of compounds such as sterols, flavonoids, triterpenoids, and phenolic compounds have been purified from *P. integerrima* galls.[9−11] In this context, compounds isolated from various parts of *P. integerrima* have been documented analgesic, anti-inflammatory,[12] muscle relaxant,[13] antipyretic,[6] and in reducing and inhibition of gastrointestinal motility.[6] They also exhibit *in vitro* antioxidant,[14] antimicrobial,[15] multidrug-resistance reversal,[8] α-glucosidase inhibition,[16] β-secretase,[17] and phosphodiesterase-1[18] properties.

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Diarrhea is a pathological condition that is commonly defined as the channel of three or additional indistinct stools per day (often along with other enteric symptoms) or the channel of more than 250 g of indistinct stool per day.[19] Diarrhea is considered as the second leading cause of death in children, with approximately 1.8 billion cases per year in children under 5 years of age. It is caused by viral, bacterial, and parasitic infections. Viral infections may be due to noroviruses and rotaviruses among others. Species such as *Salmonella typhi*, *Helicobacter pylori*, *Clostridium difficile*, and *Escherichia coli* are the main bacterial causes of diarrhea, whereas *Entamoeba histolytica* and *Giardia intestinalis* are involved in parasitic infections. In this regard, natural products, especially plants, are considered as alternative therapeutics for the treatment of diarrhea.[20−22]

On the basis of the abovementioned discussion, it is evident that the *P. integerrima* is rich in a number of phytochemicals that possess diverse pharmacological profiles. The aims of the present study are (i) to identify the drug target for identified phytochemicals that are responsible to exert pharmacological action and (ii) to search for therapeutic alternatives from natural sources. In the drug design paradigm, the identification of a molecular target for a particular disease is a key step to understand the mechanism of action. We have structurally elucidated the active constituents and the antidiarrheal activity of crude extracts/fractions of the plant and isolated flavonoids in castor-oil-induced diarrheal mice was determined. Finally, we further tried to determine the exact antidiarrheal targets that these isolated phytochemicals bind to exert pharmacological action using computational tools.

2. RESULTS

2.1. Effect of Crude Extracts/Fractions in Castor-Oil-Induced Diarrhea. Table 1 shows the results of the study on the effect of crude extracts against castor-oil-induced diarrhea in mice. Our findings reveal that the crude extract dose-dependently attenuated the induced diarrhea. The crude extract exhibited the maximum antidiarrheal effect (75%) at 400 mg/kg p.o. The ethyl acetate fraction was the most effective with 100% effect at 400 mg/kg p.o, whereas the *n*-hexane fraction was not effective at all test doses. On the other hand, the chloroform and aqueous fractions showed a maximum of 75% protection at 400 mg/kg p.o.

Table 1. Effect of the Crude Extracts/Fractions of Galls of *P. integerrima* against Castor-Oil-Induced Diarrhea in Mice[a]

treatment	dose	number of mice with diarrhea	% protection
saline	10 mL/kg p.o.	8	0
crude	100 mg/kg p.o.	5/8	37.5
	200 mg/kg p.o.	4/8	50
	400 mg/kg p.o.	2*/8	75
n-hexane	100 mg/kg p.o.	8/8	0
	200 mg/kg p.o.	7/8	12.5
	400 mg/kg p.o.	7/8	12.5
chloroform	100 mg/kg p.o.	4*/8	50
	200 mg/kg p.o.	3*/8	62.5
	400 mg/kg p.o.	2*/8	75
ethyl acetate	100 mg/kg p.o.	4*/8	50
	200 mg/kg p.o.	2*/8	75
	400 mg/kg p.o.	0*/8	100
aqueous	100 mg/kg p.o.	5/8	37.5
	200 mg/kg p.o.	4/8	50
	400 mg/kg p.o.	2*/8	75
loperamide	10 mg/kg p.o.	0**/8	100

[a]*$p < 0.05$ and **$p < 0.01$ analyses were performed using one-way ANOVA followed by the Tuckey post hoc test.

2.2. Effect of Flavonoids (1−4) on Castor-Oil-Induced Diarrhea. Results of the antidiarrheal effect of isolated compounds **1**−**4** against castor-oil-induced diarrhea are presented in Table 2. Our findings show that pretreatment of animals with compound **1** caused a marked dose-dependent antidiarrheal effect with 100% protection against diarrhea at 20 mg/kg p.o. When compound **2** was tested, it evoked a dose-dependent effect with a maximum of 87.5% antidiarrheal activity at 20 mg/kg p.o. On the other hand, compound **3** showed a maximum antidiarrheal action at 10 mg/kg p.o., which decreased to 62.5% at 20 mg/kg p.o, whereas compound **4** caused a significant dose-dependent effect with 100% protection against diarrhea at 20 mg/kg p.o. In traditional treatment, *P. integerrima* has been used as an antidiarrheal along with other uses. The current study revealed that the extracts/fractions and isolated flavonoids (**1**−**4**) from *P. integerrima* galls exert strong protection against diarrhea in castor-oil-induced diarrheal mice. Thus, the present study rationalizes the traditional use of *P. integerrima* galls as an antidiarrheal agent, as many people use this plant as a remedy for diarrhea.

Table 2. Effect of the Flavonoids (1−4) Isolated From the Galls of *P. integerrima* on Castor-Oil-Induced Diarrhea in Mice[a]

treatment	dose	number of mice with diarrhea	% protection
saline	10 mL/kg p.o.	8*	0
1	5 mg/kg p.o.	4*	50
	10 mg/kg p.o.	3	62.5
	20 mg/kg p.o.	0*	100
2	5 mg/kg p.o.	4*	50
	10 mg/kg p.o.	3*	62.5
	20 mg/kg p.o.	1*	87.5
3	5 mg/kg p.o.	4*	50
	10 mg/kg p.o.	2*	75
	20 mg/kg p.o.	3*	62.5
4	5 mg/kg p.o.	4*	50
	10 mg/kg p.o.	3*	62.5
	20 mg/kg p.o.	0*	100
loperamide	10 mg/kg p.o.	0**	100

[a]*$p < 0.05$ and **$p < 0.01$ analyses were performed using one-way ANOVA followed by the Tuckey post hoc test.

2.3. Docking Studies. *2.3.1. Results of Docking Studies Using MOE Software.* We performed docking simulations to study the mechanism of the isolated antidiarrheal compounds (**1**−**4**) in the Molecular Operating Environment (MOE) (2016.0802) software package.[23] The isolated compounds were docked into the binding site of 5C1M. Before docking of target compounds, comprehensive redocking protocols were carried out to validate the docking algorithm. The native cocrystallized ligand was extracted and prepared in a comparable manner as for others. At first, docking was carried out using triangle matcher algorithm (placement stage) and scored by London dG scoring function. Subsequently, best

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scored poses were submitted to rigid receptor protocol (refinement stage). The final score was calculated with GBVI/WSA*d*G scoring function. Next, we changed the placement parameter from triangle matcher to alpha triangle and none. The use of one scoring function may produce bias, and rescoring was carried out using other two scoring functions (ASE and affinity dG). The best performance in terms of computed RMSD value, conformation, position, and pose (orientation) was obtained with triangle matcher and ASE scoring function. The overlaid position and orientations of the redocked and experimental native ligands into the binding site of 5C1M are shown in Figure 1a–c. The computed RMSD and binding energy of the native ligand into the binding site of 5C1M are presented in Table 3.

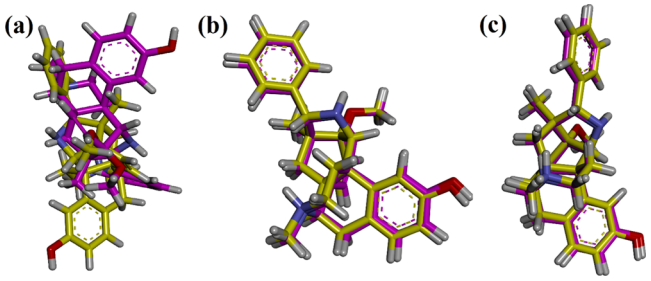

Figure 1. Overlaid orientations of redocked and experimental native ligand (BU72) using MOE with changed placement and scoring function parameters. (a) Placement: triangle matcher, scoring function London dG; (b) placement: triangle matcher, scoring function ASE; and (c) placement: alpha triangle, scoring function ASE.

Table 3. Results of the Redocking Experiment of the Native Ligand (BU72) into the Binding Site of 5C1M

parameters		RMSD (Å)	RMSD (refine, Å)	binding energy (kcal/mol)
placement (algorithm)	scoring function			
triangle matcher	London dG	3.18	0.92	−7.7408
triangle matcher	ASE	0.22	1.16	−9.1969
alpha triangle	ASE	0.22	0.96	−9.2004
none	ASE	0.21	0.21	−9.1989

Next, we docked our compounds into the binding site of 5C1M using the aforementioned parameters. The overlaid diagram of all four docked compounds into the binding site of μOR (5C1M) using MOE triangle matcher as a placement parameter and ASE scoring function is shown in Figure 2.

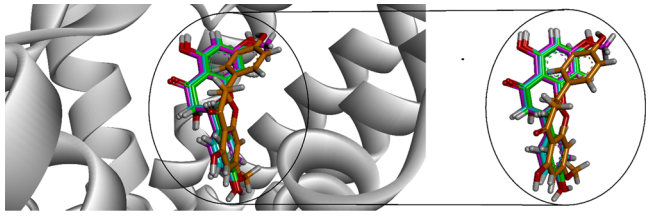

Figure 2. Overlaid orientation of all the four compounds docked into the binding site of rodent μOR (5C1M) using MOE triangle matcher as a placement parameter and ASE scoring function.

The 3-D interaction plots of the compounds are shown in Figure 3. Compound **1** forms hydrogen-bond interactions with

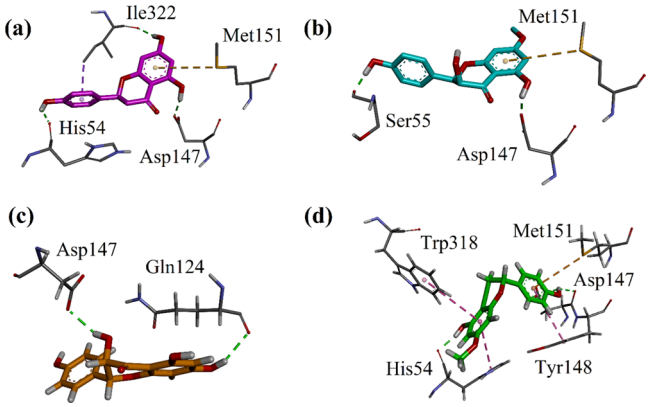

Figure 3. (a–d) Closeup 3-D interaction plot of compounds **1**–**4** at the binding site of rodent μOR (5C1M) using MOE triangle matcher as a placement parameter and ASE scoring function.

His54, Asp147, and Ile322. Met151 forms π–sulfur interaction with the phenyl ring (Figure 3a). Compound **2** displayed hydrogen-bond interactions with Ser55 and Asp147. On the other hand, Met151 forms π–sulfur interaction with the phenyl ring (Figure 3b). Compound **3** oriented itself toward Gln124 and Asp147 (Figure 3c). In a similar fashion, compound **4** forms hydrogen-bond interactions with His54 and Asp147. His54, Tyr148, and Trp318 establish π interactions with phenyl rings, whereas Met151 forms π–sulfur interaction with the phenyl ring (Figure 3d).

Next, we docked isolated compounds into the binding site of delta receptors. The 3-D structure of δOR from *Mus musculus* in complex with naltrindole was retrieved from the Protein Data Bank (PDB ID = 4EJ4). Validation of the docking protocol was performed using the redock method. Herein, the best performance in terms of computed RMSD value, conformation, position, and pose (orientation) was obtained with triangle matcher and London dG scoring function. The overlaid position and orientations of the redocked and experimental native ligands (naltrindole) into the binding site of 4EJ4 are shown in Figure 4.

Compounds **1** and **2** form hydrogen-bond interactions with Tyr129 and Ile304. Val217 and Val281 form π–alkyl interactions with the phenyl ring (Figure 5a,b). Compound **3**

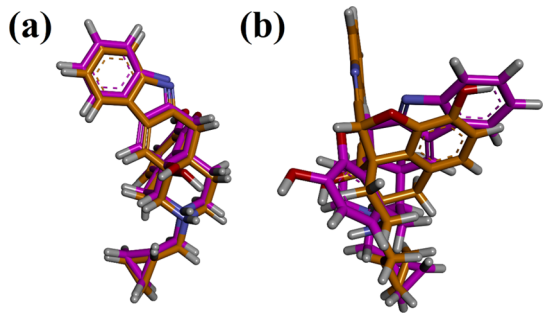

Figure 4. Overlaid orientations of redocked and experimental native ligand (naltrindole) using MOE with changed placement and scoring function parameters. (a) Placement: triangle matcher, scoring function London dG and (b) Placement: triangle matcher, scoring function ASE.

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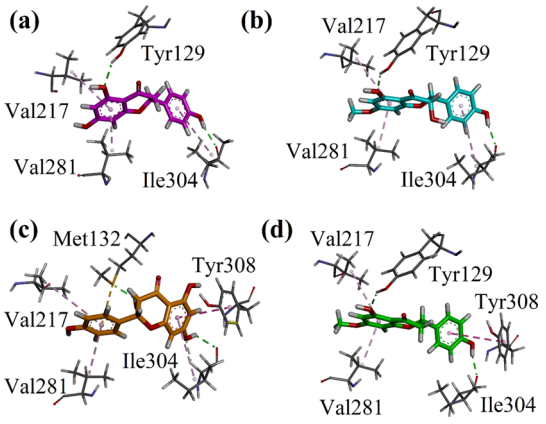

Figure 5. (a–d) Closeup 3-D interaction plot of compounds **1**–**4** at the binding site of rodent δOR (PDB ID = 4EJ4) using MOE triangle matcher as a placement parameter and London dG scoring function.

forms hydrogen-bond interactions with Met132 and Ile304. Met132 forms π–sulfur interaction with the phenyl ring. Val217 and Val281 form π–alkyl interaction with the phenyl ring to stabilize the ligand–enzyme complex (Figure 6c). Compound **4** forms hydrogen-bond interactions with Tyr129 and Ile304 (Figure 5d).

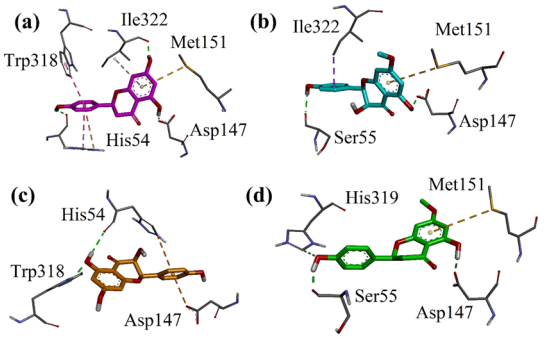

Figure 6. (a–d) Closeup 3-D interaction plot of compounds **1**–**4** at the binding site of rodent μOR (5C1M) using Autodock.

2.3.2. Results of Docking Studies Using Autodock Software. We have also performed docking studies using Autodock 1.5.6. Results of the 3-D interactions in the binding site of rodent μOR (PDB ID = 5C1M) and rodent δOR (PDB ID = 4EJ4) are shown in Figures 3 and 4, respectively. For μOR, except for compound **3**, all other compounds form π–sulfur interactions with Met151 and hydrogen-bond interactions with Asp147. The carboxylate anion of Asp147 forms π-anion type of interactions with the phenyl ring. His54 and Tyr318 form π–π interactions, whereas His54, Ser55, Ile312, and His319 also form hydrogen-bond interactions (Figure 6a–d). Computed binding energy data, ligand efficiency, and inhibition constant values are listed in Table 4. The computed ligand efficiencies for both compounds **1** and **4** are −0.34, while the predicted inhibition constants for **1** and **4** are 11.34 and 5.42 μM, respectively.

For delta-opioid receptors (δOR, PDB ID = 4EJ4), all studied compounds are oriented toward π–σ interactions with Val217 and Val281. Compounds **1** and **3** form hydrogen-bond interactions with Tyr129. Except for compound **1**, all other compounds form hydrogen-bond interactions with Ile304 (Figure 7a–d). Results of binding energies computed *via* MOE and Autodock for compounds **1**–**4** are presented in Tables 4 and 5, respectively.

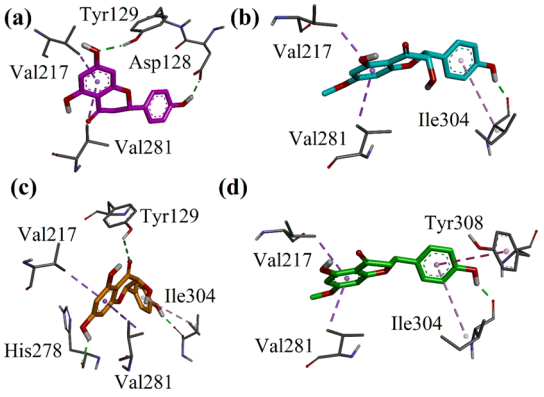

Figure 7. (a–d) Closeup 3-D interaction plot of compounds **1**–**4** at the binding site of rodent δOR (PDB ID = 4EJ4) using Autodock.

Table 5. Docking Results of Compounds 1–4 in the Binding Site of 4EJ4 Using MOE and Autodock

	MOE dock (kcal/mol)		
compounds	TM/LdG	TM/ASE	Autodock
1	−6.8859	−6.8963	−7.91
2	−7.0782	−7.5327	−7.75
3	−6.9294	−6.9290	−7.40
4	−7.5813	−7.5971	−8.17
native ligand (naltrindole)	−8.4121	−7.0070	−9.13

3. DISCUSSION

In the present study, the crude extract and aqueous fractions of galls of *P. integerrima* showed remarkable protection against diarrhea. Of the fractions, the ethyl acetate was the most potent with absolute protection. Therefore, it was subjected to bioactivity-guided isolation, which led to four flavonoids (**1**–**4**) that caused profound activity. As shown in Table 2, compounds **1** and **4** exhibited antidiarrheal activity comparable to loperamide (standard drug), and a marked reduction in diarrheal droppings (electrolyte out-fluxes) was observed. It is worth mentioning that castor oil brings about diarrhea in

Table 4. Docking Results of Compounds 1–4 in the Binding Site of 5C1M Using MOE and Autodock

	MOE dock (kcal/mol)			Autodock		
compounds	TM/LdG	TM/ASE	αT/ASE	binding energy	ligand efficiency	inhibition constant (μM)
1	−5.9678	−6.6298	−5.9263	−6.75	−0.34	11.34
2	−6.3822	−6.5051	−6.4546	−6.63	−0.30	13.82
3	−5.8981	−6.0642	−6.0813	−6.40	−0.30	20.19
4	−6.6108	−6.8520	−6.6895	−7.18	−0.34	5.42

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experimental animals as a consequence of the development of ricinoleic acid from hydrolysis of castor oil. This causes a prominent change in the transport of electrolytes and water and brings about hypersecretion, resulting in strong contraction of the intestine smooth muscles.[24] Accordingly, the antidiarrheal drugs might act by interfering with the gut motility and/or diarrheal droppings (electrolyte outfluxes).[25,26]

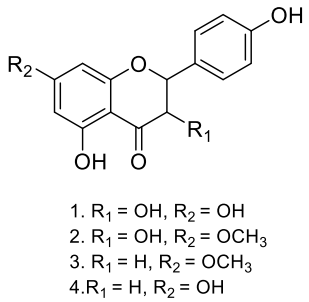
The computational approach we used is docking simulations. The standard drug used for the *in vivo* assay was loperamide. Loperamide is a synthetic piperidine moiety-based opioid that binds to intestinal mu-opiate receptors (μORs).[27] Targeting mu- and delta-opioid receptors leads to inhibition of diarrhea.[28,29] Hence, we performed docking simulations on μOR and δOR. We have performed docking simulations using MOE (2016.0802). Moreover, we used another docking algorithm to find if the observed trend is retained. In addition, we have performed docking studies using Autodock 1.5.6, which uses Genetic algorithm. The docking results from both software used have shown a similar trend in binding pose and interaction pattern. Within this context, ligand efficiency (LE) indices have been recently used to quantify the binding affinity of a drug to the number of nonhydrogen atoms,[30,31] whereas Hopkins et al. 2004 presented an exlicit formulation LE = $-(\Delta G)$/NHA.[32] Our in silico results strongly agree with the experimental data. In this respect, compounds **1** and **4** showed a remarkable dose-dependent effect with 100% protection against diarrhea at 20 mg/kg p.o. Binding energy data, LE, and inhibition constant confirmed these results (Table 4). Similarly, computed LEs for compounds **1** and **4** are −0.34, whereas the predicted inhibition constants for **1** and **4** are 11.34 and 5.42 μM, respectively. Based on the computed inhibition constant and ligand efficiency index, we can conclude here that the extracts and isolated compounds (**1**−**4**) may exert antidiarrheal effects by inhibiting mu- and delta-opioid receptors.

4. MATERIALS AND METHODS

4.1. Plant Material Collection. The galls of *P. integerrima* were obtained from the mountain area of Razagram in July and August (P.O Khall Distt: Dir Upper, KPK, Pakistan). The obtained sample of the plant was identified and authenticated by Dr. Barkatullah, a botanist from Department of Botany, Peshawar University, KPK, Pakistan. A voucher specimen (Bot20037) was placed at the herbarium located at the Department of Botany, University of Peshawar, Pakistan.

4.2. Extraction and Purification of Natural Constituents. Approximately 14 kg of the bark of *P. integerrima* was collected, washed, and air-dried in the shade. Plant material was crushed and soaked in methanol for 10−14 days with occasional shaking and stirring.[33] The extract obtained was filtered and then concentrated by means of a rotary evaporator. The repeated extraction with methanol led to the formation of 600 g of crude methanol extract. Dried extracts thus obtained were screened for their pharmacological properties. The solid mass was dissolved in distilled water, and a sequence of separations of pure constituents was carried out with solvents of different polarities such as hexane, chloroform, ethyl acetate, and butanol. The green chloroform crude extract (15.2 g) was subjected to silica gel 60 column chromatography. Elution was carried out with different ratios of methanol and chloroform (100:0 to 0:95). Solvents used in the extraction and separation were of commercial grade of high quality purchased from Musaji Adam and Sons.

The obtained fraction was subjected to thin-layer chromatography [silica 60 (F254; Merck)], and 10 different subfractions were obtained (PS-1 to PS-10). Subfraction PS-10 was obtained at a ratio of 95:05 (methanol: chloroform) fraction. PS-10 was subjected to repeated column chromatography using methanol and chloroform (9.8:0.2). This resulted in the isolation of a pure yellow amorphous solid that was characterized and identified as 3,5,7,4/-tetrahydroxy-flavanone (**1**).[24,25] Further elution of the column with methanol and chloroform (97:3) resulted in the isolation of yellow needle-like crystals of 3,5-dihydroxy-2-(4-hydroxyphenyl)-7-methoxychroman-4-one (**2**).[24] Characterization and structure elucidation of the pure phytochemicals was accomplished with a panel of spectroscopic methods such as UV, IR, NMR, and mass spectrometry. Spectral data obtained were compared with those reported in the literature.[13,18] Eluting the column with methanol and chloroform (95:05) resulted in the isolation of a dark yellow powder, which yielded two phytochemicals as shown by TLC. They were further purified by preparative chromatography using a mixture of methanol and chloroform (90:10), which led to the isolation of 5-hydroxy-2-(4-hydroxyphenyl)-7-methoxychroman-4-one (3) and naringenin (4) (Figure 8).[34,35]



1. R_1 = OH, R_2 = OH
2. R_1 = OH, R_2 = OCH_3
3. R_1 = H, R_2 = OCH_3
4. R_1 = H, R_2 = OH

Figure 8. Chemical structures of flavonoids (**1**−**4**) isolated from *P. integerrima* galls.

4.3. Experimental Animals. BALB/c mice having weight 22−28 g of both sex were used in the current screening. All animals were given free contact to standard diet and water ad libitum. Then, all animals were placed under normal situations at 25 °C ± 2 °C and the light cycle was maintained as 12 h dark and 12 h light as stated in the animals by laws of Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. Experiments were agreed by the ethical committee (SOU/Pharm-23), University of Swabi, Khyber, Pakistan. Animals, with free entree to water, were ravenous for 24 h prior to the experiments.

4.4. Castor-Oil-Induced Diarrhea. The animals were divided into various groups of eight mice each (n = 8). Before experimentation, all animals groups were starved and permitted free entree to drinking water 24 h earlier to experiments. One group was orally fed with 10 mL/kg saline and considered as a negative control. Another group was given loperamide 10 mg/kg p.o. and was considered as a standard. The rest of the groups received extracts/fractions of *P. integerrima* at 100, 200, and 400 mg/kg p.o. or pure compounds at 5, 10, and 20 mg/kg p.o. Every mouse had an equal quantity of castor oil (10 mL/kg p.o.). A feeding needle was used to introduce castor oil for 1 h after the treatments.[26,36] Afterward, all groups were observed

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for the onset of diarrhea excreta. The complete absence of diarrhea was observed when diarrheal drops were not appearing. The percent inhibition of defecation was calculated as follows:

$$\%\ \text{inhibition of defecation} = \frac{N_{\text{def, castor oil}} - N_{\text{def, drug}}}{N_{\text{def, castor oil}}} \times 100$$

where $N_{\text{def, castor oil}}$ and $N_{\text{def, drug}}$ are the numbers of defecations caused by castor oil and the drug, respectively.

4.5. Docking Studies. *4.5.1. Docking Studies Using MOE.* We have performed docking simulations using Molecular Operating Environment (MOE 2016.0802). The procedure for protein/ligand preparation and docking is as follows:

4.5.1.1. Protein Preparation. The 3-D structure of μOR from *M. musculus* (house mouse) bound to the agonist BU72 was obtained from the Protein Data Bank (PDB accession code = 5C1M). The 3-D structure of the delta-opioid receptor (δOR) from *M. musculus* in complex with naltrindole was retrieved from the Protein Data Bank (PDB id = 4EJ4). After downloading enzymes, we have prepared them for docking studies. Here, we performed 3D protonation (at pH 7, a temperature of 300 K, and a salt concentration of 0.1 M), energy minimization, and active site determination.[37−39] The Amber10:EHT force field was used for energy minimization. Active sites of the proteins were determined in two ways: Centroid of the native ligands of each complex was used to determine the active site. In another method, we used blind docking and computed energies in both methods were compared.

4.5.1.2. Ligand Preparation. Structures of the ligands were drawn in MOE window and were energy-minimized upto a gradient of 0.0001 using the Amber10:EHT force field.

4.5.1.3. Docking Validation. The docking procedure was validated by redocking of the native ligands. Comprehensive redocking protocols were carried out to validate the docking algorithm. Native cocrystallized ligands were extracted and prepared in a comparable manner as for others. Docking was carried out using triangle matcher algorithm (placement stage) and scored by London dG scoring function. Subsequently, best scored poses were submitted to a rigid receptor protocol (refinement stage). The final score was calculated with GBVI/WAS dGSFscoring function. Next, we changed the placement parameter from triangle matcher to alpha triangle and none, whereas rescoring was carried out using two other scoring functions (ASE and affinity dG). Results of the redocking experiments were evaluated using root-mean-square deviation (RMSD) for higher ranked pose, and further improvement was obtained by pose optimization. The reasonable performance (RMSD < 2.0 Å) protocol was adapted for the docking of all isolated compounds. The ligand enzyme complexes with the lowest binding energy were analyzed using the MOE ligand interaction module. Finally, Discovery Studio Visualizer was used for the 3-D interaction plot.[39]

4.5.2. Docking Studies Using Autodock. We have used the prepared structures of both ligands and target enzymes for docking simulations using AutiDock. AutoDock Tools (ADT, 1.5.6.) was used for calculation of Gasteiger partial charges. For docking, default parameters were used with grid dimensions 45 × 35 × 35 (5C1M) and 60 × 40 × 55 (4EJ4) and a grid spacing of 0.375 Å.

4.6. Statistical Analysis. The experimental results are presented as the mean ± standard error of the mean (SEM). The obtained results were measured in triplicate and the data were subjected to two-way analysis of variance (ANOVA). The statistical analysis was performed with the help of students's test for significance with the help of GraphPad Prism-6 (USA, http://www.graphpad.com); differences were measured significant at $p \leq 0.05$.

5. CONCLUSIONS

In the current study, various extracts and fractions of *P. integerrima* galls showed marked antidiarrheal effects against castor-oil-induced diarrhea. Moreover, bioassay-based extraction and isolation led to the identification of four organic compounds (**1**−**4**), which were characterized as flavonoids. The isolated compounds caused strong antidiarrheal effects and thus provided a chemical background for the activity of extracts/fractions. Computed binding energy data, inhibition constant, and LE confirmed that compounds have high affinity with selected targets. Hence, it is suggested that the extracts and isolated compounds may act as antidiarrheals by inhibiting mu- and delta-opioid receptors.

This is the preliminary study to identify the antidiarrheals in various extracts and fractions of *P. integerrima* galls. The results of this preliminary study rationalized the traditional uses of the plant as a remedy for diarrhea. The results of this investigation revealed that *P. integerrima* galls contain pharmacologically active compounds having antidiarrheal potential, while the binding energy data and LE indices computed *via* computational tools suggested that the extracts and isolated compounds may act as antidiarrheals by inhibiting mu- and delta-opioid receptors. However, additional *in vitro* and *in vivo* experiments are required to establish the exact mechanism of action of isolated compounds from *P. integerrima* species.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00298.

Extraction and isolation of flavonoids (**1**−**4**) isolated from *P. integerrima* galls (PDF)

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Notes

The authors declare no competing financial interest.

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