Original Article

Anti-diarrheal Activity of Caffeine: A Modulatory Effect with Loperamide and through 6FH5 (PIK3CG) Protein Interaction Pathway

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ABSTRACT

Background: Caffeine (CAF) is known for its central nervous system stimulatory effect. Although, CAF use in diarrhea, especially in Runner's diarrhea is still controversial, but it has been reported that dark tea containing CAF has anti-diarrheal effect on Sennae-mediated diarrhea in mice. **Aim:** To evaluate the anti-diarrheal effect of CAF and its modulatory effects on the standard anti-diarrheal drug loperamide (LOP), an opioid receptor agonist. **Materials and Methods:** CAF (15 mg/kg, i.p.) with or without LOP (3 mg/kg, p.o.) was administered to the *Swiss* mice (*Mus musculus*) previously treated with castor oil. Additionally, an *in silico* study was also performed to see the possible anti-diarrheal mechanism of CAF and LOP. **Results:** CAF increased the latent period, while decreasing the diarrheal defecation during the observation period (4 hr) in the test animals. Interestingly, CAF co-treated with the LOP exhibited a prominent anti-diarrheal effect in comparison to the negative control, CAF and LOP groups. Further, *in silico* study suggests that CAF have the most binding affinity with the 6FH5 (PIK3CG) protein (-8.22 KJ/mol) of adenosine receptor, while LOP with the μ-opioid receptor. **Conclusion:** CAF showed significant anti-diarrheal effect as well as strengthen the anti-diarrheal effect of LOP in castor oil-induced diarrheal mice. Protein 6FH5 might be the best conformor for the CAF in the treatment of diarrhea.

Key words: *Mus musculus,* Caffeine, Castor oil, Diarrhea, Adenosine receptor.

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INTRODUCTION

Diarrhea results three to more unformed stools in a day along with other stomachic symptoms.[1] Most often diarrhea lasts for a few days and results in dehydration. The process of dehydration causes loss of essential fluid and electrolytes from our body. Therefore, diarrhea is one of the life-threatening diseases in the world. Diarrhea occurs mostly in the developing countries and causes an economic burden on the poor patients.[2] Diarrhea is also evident to cause nausea, vomiting and abdominal cramps, thereby, forces the patients into bed rest.[3]

Generally, diarrhea is caused by an infection in the intestines by one or more pathogen (e.g., virus, bacterium, or parasite) or to gastroenteritis. These infectious agents are mostly acquired from the contaminated foods or water. Diarrhea alters the transportation process of water and essential electrolytes in our body, results hyper-secretory responses and giant contraction in the intestine. An ideal anti-diarrheal agent can correct the altered situations in our intestine.[4] Commonly used anti-diarrheal agents (e.g., loperamide, nifedipine, prazosin, propranolol) can cause a number of mild to severe side effects, such as nausea, vomiting, dizziness, drowsiness, tiredness, abdominal pain, constipation, abnormalities in heartbeat and so on.

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Caffeine ($C_8H_{10}N_4O_2$: 1,3,7-trimethylxanthine or 3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione), an adenosine receptor antagonist that acts centrally as a stimulant in our body.[5] Caffeine (CAF) has many important biological effects, including long-term memory enhancing,[6] health protecting effects,[7] and so on. It has been demonstrated that CAF intake may associate with some reversible and short-term physiological effects in our body. However, a moderate CAF intake is assumed to be safe in some diseases, including cardiovascular disease.[8]

One study reports that abuse of CAF may cause diarrhea in human.[9] However, there is a lack of scientific evidence behind this fact. On the other hand, to avoid CAF during Runner's diarrhea (acute exercise-induced diarrhea) is still controversial.[10] In contrary, in a recent study, the effect of dark tea in Folium Sennae mediated diarrhea in mice was investigated, suggesting the tea extract significantly progressed the diarrheic phenomena (e.g., loose stools, diarrheal index, intestine peristalsis) in moderate to high- dose groups. It may be due to the high amount of CAF and gallocatechin present in it.[11]

Upon understanding the needs, this study aims to evaluate anti-diarrheal effect of CAF in mice pre-treated with castor oil. The interaction capacity of CAF with the commonly used anti-diarrheal drug loperamide has been also investigated. Additionally, *in silico* study has been also performed to see the possible anti-diarrheal mechanism of CAF.

MATERIALS AND METHODS

Reagents and chemicals

Castor oil was purchased from a local market of Bangladesh. Loperamide (LOP, IMOTIL® 2 mg Cap.) was purchased from the Square Pharmaceuticals Ltd., while CAF was collected from the ACME Laboratories Ltd., Bangladesh.

Experimental animals

Adult male albino mice (18-24 g), purchased from the Jahangir Nagar University (JU), Dhaka, were used in this study. The animals were kept in room having temperature: 25 ± 2°C, humidity: 50 ± 5% and 12 hr dark/light cycle. Standard diet and water were provided to the animals. They were subjected to this study after seven days (acclimatization period). The animals were randomly grouped into the test and control groups and the food was withdrawn 12 hr before the experimental hours.

Groups and treatments (castor oil-induced diarrhea in mice)

This study was done according to the method established by Awouters *et al.*[12] with slight modifications. Briefly, each animal was treated with 0.5 mL castor oil 30 min after the sample (Gr-II) and controls (Gr-I and Gr-III) treatment. Similarly, CAF (Gr-II) was co-treated (before 15 min) with Gr-III (Table 1). The animals (n = 5) were then observed for latency and total defecation up to 4 hr.

Computational study

In this case the binding mechanism of the CAF and LOP was determined against the therapeutic target receptors adenosine receptor (AR) and μ-opioid receptor (μ-OR), respectively.

Source of ligand and macromolecule

Ligand, CAF, was selected from the PubChem online database and crystallographic structure were downloaded in 'sdf' format. The protein, targeted receptor, were retrieved from the protein data bank, online database (www.rcsb.com). The target of the drug was retrieved drug bank in which specific receptor is selected. Crystallographic structure was collected in 'pdb' format.

Software and materials

Schordinger Suite version, Maestro 10.1, was subjected to perform ligand and protein preparation, grid generation and molecular docking study.[13] Drug discovery studio (version discovery studio 4.5), primary tools in drug discovery, has been utilized in various ligand- and structure-based methods for ameliorating pharmacophore modeling, virtual screening and non-bonding interaction.[14]

Ligand preparation

We targeted to select an adenosine receptor antagonist. CAF is considered as an adenosine receptor antagonist.[15] CAF (Pubchem CID: 2519), was incorporated in

Table 1: Animals fasting overnight are treated with the following substances at 10 mL/kg.

Treatment groups	Dose
Gr-I: VEH (i.p.)	10 mL/kg
Gr-II: CAF (i.p.)	15 mg/kg
Gr-III: LOP (p.o.)	3 mg/kg
Gr-IV: CAF (i.p.) + LOP (p.o.)	15 mg/kg + 3 mg/kg

VEH: 0.05% Tween 80 in 0.9% NaCl solution; CAF: Caffeine; LOP: Loperamide; i.p.: Intra-peritoneal; p.o.: Per oral

the *Schordinger software* for the preparation of ligand. Compounds' three dimensional structures were developed by using Ligprep 2.5 in Maestro v10.1 with force field OPLS_2005. Human μ-opioid receptor (μ-OR) was obtained *via* structure-preparation module implemented in MOE[16] followed by the protonation, minimization and Amber 99 partial charge application.

Protein preparation

Three dimensional crystal structure of macromolecule 1TB5 (PDE4B) *Homo sapiens*, 2YDO(ADORA2A) *Homo sapiens*, 3JWQ(PDE5A) *Homo sapiens*, 3PWH(ADORA2A) *Homo sapiens*, 3UZC(ADORA2A) *Homo sapiens*, 4DDF(pduT) *Salmonella typhimurium*, 4DKL(Oprm1) *Mus musculus*, Enterobacteria phage T4, 4DO9(POLB) *Homo sapiens*, 4UKK(RPS0A) *Homo sapiens*, 4UUU(CBS) *Homo sapiens*, 4UVC(KDM1A) *Homo sapiens*, 5HES(MAP3K20) *Homo sapiens*, 5UKL(GRK2) *Homo sapiens*, 6DDE(GNAI1) *Homo sapiens*, 2WEY(PDE1B) *Homo sapiens*, 2JBZ(acpS) *Streptomyces coelicolor*, 3K4S(PDE4D) *Homo sapiens*, 3REY(ADORA2A) *Homo sapiens*, 4NPV(PDE1B) *Homo sapiens*, 5C1M(Oprm1) *Mus musculus*, 5G55(Oprm1) *Mus musculus*, 5UPO(PDE1B) *Homo sapiens*, 6FH5(PIK3CG) *Homo sapiens*, 3UZA(ADORA2A) *Homo sapiens*, 3RFM(ADORA2A) *Homo sapiens* were selected from the protein data bank and converted into 'pdb' format. Then the 'pdb' file of protein is applied in Maestro version 10.1 for the preparation of each protein.[17] Bond orders and charges were accredited and hydrogen atoms were combined to the heavier atoms. The minimization was done by using the force field OPLS_2005 through setting most heavy atom RMSD (root-mean-square-deviation) to 0.30 Å.

Validating of active side and grid generation

Active side was validated properly due to having a great importance in moleculer docking study. Several binding sites were available in protein and then we selected one of the binding sites for the grid generation. The grids were developed by maintaining the default parameters of van der Waals scaling factor 1.00 and charge cutoff 0.25 subjected to OPLS 2005 force field. A three-dimensional bounding box (15 Å × 15 Å × 15 Å) was generated for the receptor.

Glide docking

Glide Standard Precision (SP) ligand docking was performed in Schrödinger-Maestro v10.1 to claim the binding affinity and bind energy. The higher the negative value of the glide score the more the binding affinity of the drug with the selected protein.

Frequency and optimization of ligand

The frequency and optimization of CAF have been carried out with GAUSSIAN 09 software.[18] B3LYP method with 6-31G(d,p) basis set was chosen to predict optimized structure, vibrational frequency analysis, HOMO-LUMO gap, chemical potentials.[19,20] The vibrational bands are calculated by applying Guess view molecular visualization program.[21]

IR frequency

The infra-red frequencies of CAF were calculated without scaling factor by using the density functional theory method (Figure 1).

The energies of HOMO, LUMO (Structure: Figure 2), hardness, softness and chemical potential[22-24] were calculated by using B3LYP/6-31G (d,p) (Table 2).

Statistical analysis

For the *in vivo* study, we performed one-way analysis of variance (ANOVA) and the results are expressed as mean ± standard deviation (SD). Newman-Keuls *post hoc* test was performed by using the software GraphPadPrism® - GraphPad Software, Inc. (Version: 6.0), considering $p < 0.05$ at 95% confidence intervals.

RESULTS

Castor oil-induced diarrhea

CAF and the standard drug LOP significantly ($p < 0.05$) increased latent periods in diarrheal mice as compared to the VEH group. CAF at 15 mg/kg (i.p.) showed latent period 33.62 ± 4.43 min, which was 24.93% more than the LOP group. CAF when co-treated with the LOP 3 mg/kg (p.o.) increased latency periods 35.75 and 51.77% more than the CAF and LOP groups, respectively (Table 3).

Table 4 indicates that CAF at 15 mg/kg significantly ($p < 0.05$) reduces diarrheal secretions in comparison to the VEH group. The highest reduction of diarrheal secretions by CAF was observed on the 4th hr (4.48 ± 1.08). More reduction of diarrheal secretions was observed in LOP group. The results also revealed that CAF co-treated with the standard drug LOP effectively reduced diarrheal secretions in comparison to the CAF and LOP groups in all respective observation hours. CAF + LOP was seen to stop diarrheal secretion at the 4th hr in test animals.

In silico study

In the IR spectrum CAF showed the highest frequency at 1750 cm^{-1} which is comparable to a result obtained from a Fourier transform infra-red spectroscopy (FTIR)

analysis, where the absorbance band was found at 1655 cm^{-1} at 5 ppm sensitivity technique. The thermodynamic properties of CAF were calculated: Free energy: -680.240968 Hartree, Enthalpy: -680.188514 Hartree and Dipole moment: 3.5390 Debye. CAF has also a low HOMO-LUMO gap (0.19). In molecular docking study, for the visualization of the ligand and protein, Discovery studio software (e.g., Discovery studio visualizer v16.1.0.15350 data) plays a significant role. The molecular docking is done by the schordinger v 10.1 and glide score are shown in the Table 5. Our findings suggest that CAF has highest binding affinity with 6FH5 (PIK3CG) receptor (binding affinity: -8.22 kj/mol). CAF has reasonable binding energy with this receptor which accounts for -32.839. The docking scores of 3PWH, 2JBZ and 5G55 receptors with CAF were - 8.088, 8.15 and 7.88 kj/mol, respectively.

In biological science, hydrogen bond plays a crucial role in DNA structure. Our study suggests that the CAF-6FH5 (PIK3CG) complex have well organized hydrogen bond. The strong hydrogen bond was recognized in the VAL882 (1.92514), ILE881 (2.80903) and GLU880 (2.44) amino acid residues. Several hydrophobic bonds were also present in MET953 (3.59417), TYR867 (5.34636), ILE963 (5.44852) amino acid residues. Binding affinities and non-bonding interactions are shown in Table 5 and 6 and Figure 3.

From our study, it is clear that, CAF has the most binding affinity with the 6FH5 (PIK3CG) protein. This CAF-6FH5 complex have a significant amount of hydrogen bond, expressing the strong binding interaction between the CAF and receptor. The receptor μ-OR represents an important opioid target for the occurrence of pain, diarrhea, chronic pulmonary edema, cough and shivering. Shown in Figure 4 are molecular docking results of μ-OR with LOP. For LOP, docking results show multiple hydrophobic interactions with the μ-OR. In addition, the methyl group of VAL236, ILE296, VAL300 and ILE322 display hydrophobic interactions with the aromatic rings of LOP while TRP293 and TYR326 show π-π stacking.

Table 2: Energy of HOMO, LUMO, Gap, Softness, Hardness by B3LYP/6-31G(d,p).

Compound Name	HOMO	LUMO	GAP	Hardness (η)	Softness (S)	Chemical Potential (μ)
Caffeine	-0.22	-0.03	0.19	0.08	12.78	-0.24

Table 3: Latent period of the test and control groups in mice pre-treated with castor oil.

Treatment groups	Latency (min)
VEH	10.22 ± 2.78
CAF	33.62 ± 4.43[ac]
LOP	25.24 ± 1.58[a]
CAF + LOP	52.33 ± 3.23[abc]

Values are mean ± SD ($n = 5$); one way ANOVA followed by Newman-Keuls *post hoc* test; $p < 0.05$ when compared to the [a]Gr-I, [a]Gr-II, [c]Gr-III; VEH: 0.05% tween-80 in 0.9% NaCl solution; CAF: Caffeine; LOP: Loperamide

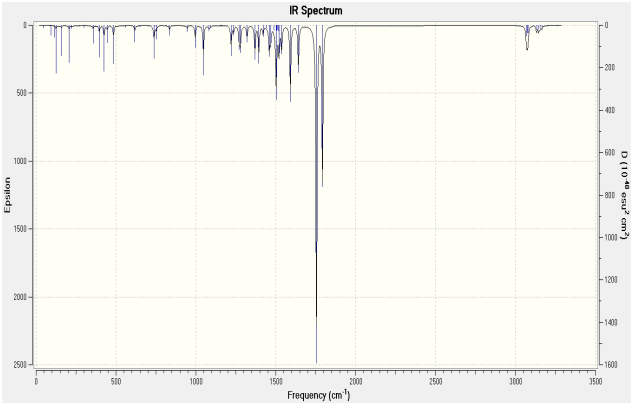

Figure 1: Infra-red frequency of caffeine.

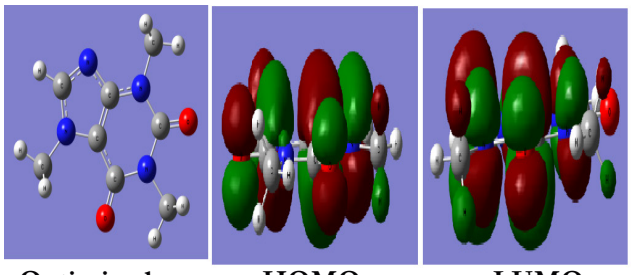

Figure 2: Optimized, HOMO and LUMO structures of CAF.

DISCUSSION

The castor oil test has been used for years for the screening and evaluation of anti-diarrheal drugs. Castor oil increases the overall secretion of water and essential electrolytes along with the stimulation of peristalsis and hastening the intestinal transit.[25] The pancreatic lipase hydrolyzed castor oil glycerol and ricinoleic acid after ingestion. Ricinoleic acid is responsible for the diarrheal effect in animals.[26] It is due to it can release prostaglandins and platelet-activating factor,[25] thereby, causes smooth muscle contractionin the small intestine.[27] It also promotes the release of nitric oxide (NO) and activation

Table 4: Diarrheal secretions of mice in test and control groups at 1st, 2nd, 3rd and 4th hr.

Treatment groups	1st h	2nd h	3rd h	4th h
VEH	16.23±2.44	12.38±2.68	10.78±1.57	8.48±2.74
CAF	6.78±1.59[a]	6.23±1.08[a]	5.62±1.08[a]	4.48±1.08[a]
LOP	4.19±2.38[ab]	3.78±2.68[ab]	1.84±1.36[ab]	1.44±1.78[ab]
CAF+LOP	1.23±0.19[abc]	1.21±0.18[abc]	0.20±0.22[abc]	0.00±0.00[abc]

Values are mean ± SEM ($n = 5$); one way ANOVA followed by Newman-Keuls *post hoc* test; $p < 0.05$ when compared to the [a]Gr-I, [b]Gr-II, [c]Gr-III in respective hour; VEH: 0.05% tween-80 in 0.9% NaCl solution; CAF: Caffeine; LOP: Loperamide

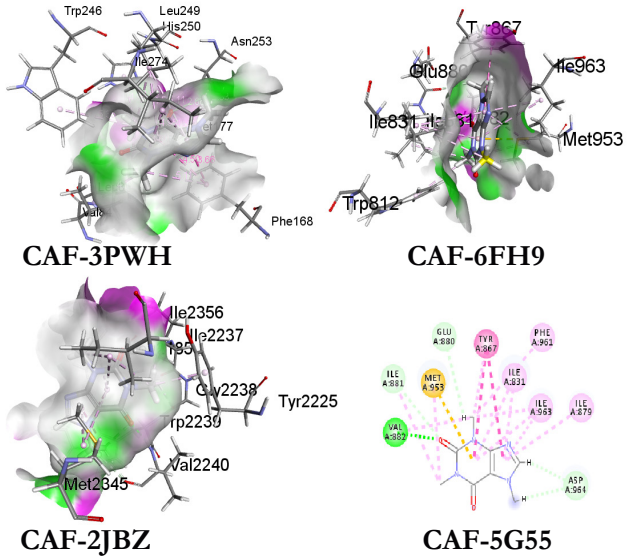

Figure 3: Non-bonding interactions of caffeine (CAF) with 3PWH, 6FH9, 5G55 and 2JBZ proteins (discovery studio visualizer v16.1.0.15350).

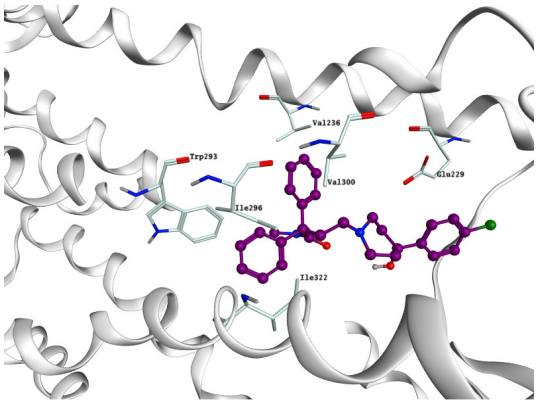

Figure 4: Molecular docking interaction of the μ-opioid receptor with loperamide.

Table 5: Binding energy and docking score of caffeine against adenosine receptor.

Proteins	Binding energy	Docking score
1TB5 (PDE4B) *Homo sapiens*	-31.382	-4.886
3JWQ(PDE5A) *Homo sapiens*	-33.818	-5.421
3PWH(ADORA2A) *Homo sapiens*	-30.086	-8.088
3UZC(ADORA2A) *Homo sapiens*	-28.26	-7.599
4DDF(pduT) *Salmonella typhimurium*	-25.383	-6.13
4DKL(Oprm1) *Mus musculus*, Enterobacteria phage T4	-22.698	-5.752
4DO9(POLB) *Homo sapiens*	-32.33	-5.915
4UKK(RPS0A) *Homo sapiens*	-28.271	-6.664
4UUU(CBS) *Homo sapiens*	-28.808	-6.313
4UVC(KDM1A) *Homo sapiens*	-32.14	-6.445
5HES(MAP3K20) *Homo sapiens*	-29.781	-6.395
5UKL(GRK2) *Homo sapiens*	-32.618	-6.587
6DDE(GNAI1) *Homo sapiens*	-25.879	-6.163
2WEY(PDE1B) *Homo sapiens*	-35.858	-5.805
2JBZ(acpS) *Streptomyces coelicolor*	-32.704	-8.158
3K4S(PDE4D) *Homo sapiens*	-32.328	-5.48
3REY(ADORA2A) *Homo sapiens*	-27.745	-7.423
4NPV(PDE1B) *Homo sapiens*	-29.745	-4.694
5C1M(Oprm1) *Mus musculus*	-24.481	-6.051
5G55(Oprm1) *Mus musculus*	-33.836	-7.886
5UPO(PDE1B) *Homo sapiens*	-35.359	-5.477
6FH5(PIK3CG) *Homo sapiens*	-32.839	-8.22
3UZA(ADORA2A) *Homo sapiens*	-29.719	-7.375
3RFM (ADORA2A) *Homo sapiens*	-29.054	-7.743

of adenylyl cyclase which causes an increase in cyclic adenosine monophosphate (cAMP) concentration. The increased cAMP level (a) stimulates intestinal peristaltic activity, (b) changes membrane permeability, (c) reduces Na^+K^+ATPase pump activity, leading to decrease in Na^+ and K^+ absorption. The overall factors cause an accumulation of water, Na^+ and K^+ in the lumen of the intestine.[28,29]

CAF is evident to decrease the production and release of NO in experimental animals.[30] Moreover, CAF is also found to decrease the levels of pro-inflammatory mediators, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin (IL)-3, -6 and -12 and nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-ϰB) *via* inhibitor of nuclear factor kappa B (IϰBα) phosphorylation in zebrafish.[31] A short-term CAF treatment is evident to reduce adenylyl cyclase activity in mouse model.[32] In a recent study, it has been seen that CAF intracts with the cAMP-induced chemotactic signal pathways in *Dictyostelium*, probably through multiple targets, including phosphoinositide 3-kinases (PI3K) and mammalian target of rapaycin complex 2 (mTORC2).[33] In this study, we have seen that CAF significantly ($p< 0.05$) increased the latent periods in diarrheal mice as compared to the VEH group. On the other hand, CAF when co-treated with the standards drug LOP resulted in the highest increased latency, while reducing in diarrheic secretions in castor oil induced diarrheal mice.

CAF has antioxidant[34] and anti-inflammatory[35] activities and through these effects it can protect our body from oxidative stress, pro-inflammatory and inflammatory mediators. Substances having antioxidant and anti-inflammatory effects may have anti-diarrheal activities.[36,37] Inflammatory bowel diseases (e.g., Crohn's disease, ulcerative colitis) also cause chronic diarrhea. CAF was found to down-regulate CHI3L1 mRNA expression, thereby, reduce in bacterial invasion in mice.[38]

The viral human immunodeficiency virus (HIV)-1 Trans activating factor (HIV-1 Tat) protein after activating glial cells results the release of neuroinflammatory responses, which is responsible for the diarrhea as

Table 6: Binding energy and non-bonding interactions of caffeine (CAF) with 3PWH, 6FH9, 2JBZ and 5G55 proteins (PyRx virtual screening).

Ligand-protein complex	Binding energy (kcal/mol)	H-bond (amino acid…ligand)	Bond distance (Å)	Hydrophobic bond	Bond distance (Å)
CAF-3PWH	-8.088	ASN253(Conventional Hydrogen Bond)	1.78911	PHE168 (Pi-Pi Stacked)	4.50436
		ASN253 (C-H…O)	2.83242	VAL84 (Alkyl… Alkyl)	4.12047
				LEU85 (Alkyl… Alkyl)	5.35311
				TRP246 (Pi-Alkyl)	5.34899
				HIS250(Pi-Alkyl)	4.94156
CAF-6FH9	-8.22	VAL882(Conventional Hydrogen Bond)	1.92514	MET953(Pi-Sulfur)	3.59417
		ILE881((C-H…O)	2.80903	TYR867(Pi-Pi T-shaped)	5.34636
		GLU880(C-H…O)	2.44219	ILE881 (Alkyl… Alkyl)	4.60565
				ILE963(Pi-Alkyl)	5.44852
CAF-2JBZ	-8.158	VAL2240(Conventional Hydrogen Bond)	2.00766	TRP2239 (Pi-Pi Stacked)	5.26535
		GLY2238(C-H…O)	2.9161	ILE2356 (Alkyl… Alkyl)	4.68417
				TYR2225(Pi-Alkyl)	5.17955
CAF-5G55	-7.88	VAL882 (Conventional Hydrogen Bond)	1.9619	TYR867 (Pi-Pi T-shaped)	5.65378
		ASP964(C-H…O)	2.28967	VAL882 (Alkyl… Alkyl)	5.22171
		GLU880(C-H…O)	2.686	TYR867(Pi-Alkyl)	3.22602

well as neurotoxic effects in animals.[39] CAF is evident to block HIV-1 Tat protein in SH-SY5Y cells.[40] CNS also controls gastrointestinal motility and secretion, therefore modulates gastrointestinal functions.[41] CAF is widely known CNS stimulant methylxanthine alkaloid class drug, therefore, it can act through this system.

The thermodynamic properties and optimized structure of CAF suggest that it is chemically stable.[42] Free energy can predict the binding properties. The more the negative values the more it is favorable to binding and interaction of the targeted protein. This study suggests CAF has higher free energy value, thus it has higher binding and interaction properties. On the other hand, if any drug has small HOMO-LUMO gap, then it will be more chemically reactive.[43] CAF has a small HOMO-LUMO gap, thus it will be more chemically reactive. Hydrogen bonding, a typical weak intermolecular interaction that stabilizes the energetically-favored ligands.[44] The *in silico* study suggests that the multi-targeted small molecule CAF binds with low affinity towards the targets, especially with the 3PWH, 6FH9, 2JBZ and 5G55 proteins.

CONCLUSION

In summary, findings from this investigation suggest that CAF at 15 mg/kg (i.p.) displayed remarkable anti-diarrheal effect in castor oil-induced diarrheal mice. It exhibited better anti-diarrheal effect when co-treated with the standard drug, LOP. We suppose, CAF-mediated potentiating anti-diarrheal effects on the Swiss mice may be due to its inhibitory capacity of NO, adenylyl cyclase and cAMP, thereby decreasing in cAMP concentration dependent stimulatory effects of intestinal peristaltic activity, alteration of the membrane permeability, thereby, reduce in Na^+K^+ATPase pump activity and an increase in water, Na^+ and K^+ levels in the lumen of the intestine in experimental animals. *In silico* study suggests that CAF exhibited strong binding affinity with the 6FH5 (PIK3CG) protein of the AR, while LOP with the μ-OR which might be responsible for their anti-diarrheal effects in animals. We suppose, the synergistic anti-diarrheal effect of CAF and LOP may resulted through the interactive capability of CAF with AR and LOP with μ-OR. More studies are highly appreciated to understand the exact molecular mechanism(s) behind the combined anti-diarrheal effect of CAF and LOP. This study accords an information regarding the safety of CAF or caffeinated products (e.g., tea, coffee etc.) during diarrhea. It also focuses on its interaction capability with the standard anti-diarrheal drug, such as LOP. Our study may provide supportive message to the future pre-clinical and clinical studies for the investigation of outcomes of CAF and CAF-related products in diarrhea.

Ethical Statement

All protocols were approved by the Ethical Committee at the Life Science Faculty, Bangabandhu Sheikh Mujibur Rahman Science and Technology University (BSMRSTU) (Approval No. 20150109015), Gopalganj, Bangladesh.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

μ-OR: μ-opioid receptor; **AR:** Adenosine receptor; **CAF:** Caffeine; **cAMP:** Cyclic adenosine monophosphate; **COX:** Cyclooxygenase; **IκBα:** Inhibitor of nuclear factor kappa B; **IL:** Interleukin; **iNOS:** Inducible nitric oxide synthase; **LOP:** Loperamide; **mTORC2:** Mammalian target of rapamycin complex 2; **NF-κB:** Nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells; **NO:** Nitric oxide; **PI3K:** Phosphoinositide 3-kinases.

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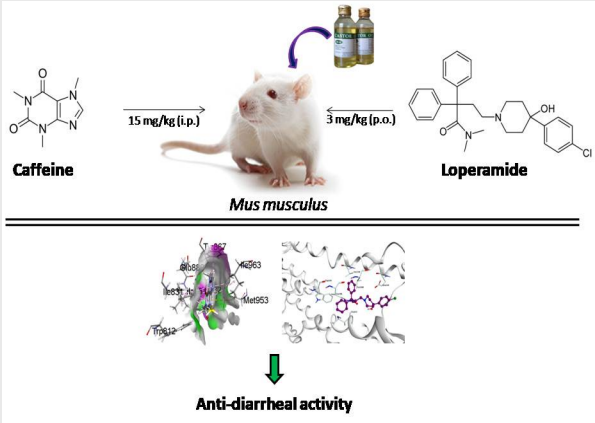
PICTORIAL ABSTRACT



SUMMARY

CAF at 15 mg/kg (i.p.) showed anti-diarrheal effect in castor oil-induced diarrheal mice. CAF co-treated with the standard drug, LOP exhibited better anti-diarrheal effect in experimental animals. In *in silico* study, CAF showed strong binding affinity with the 6FH5 (PIK3CG) protein of the AR, while LOP with the μ-OR which might be responsible for their synergistic anti-diarrheal effect in experimental animals. More investigations are highly appreciated to understand the exact molecular mechanism(s) behind the combined anti-diarrheal effect of CAF and LOP in animal model.

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