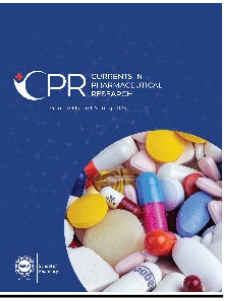
Currents in Pharmaceutical Research (CPR)
Volume 4 Issue 1, Spring 2026
ISSN$_{(P)}$: 3007-3235, ISSN$_{(E)}$: 3007-3243
Homepage: https://journals.umt.edu.pk/index.php/cpr



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DOI:	https://doi.org/10.32350/cpr.41.03
History:	Received: January 29, 2026, Revised: February 23, 2026, Accepted: April 02, 2026, Published: May 10, 2026
Citation:	Nisar S, Aslam N, Saeed MA, et al. In vitro, in vivo, and in silico investigation of methanolic extract of Withania coagulans (Stocks) against Diarrhea and Pyretic Albino Rats. *Curr Pharm Res*. 2026;4(1):50–71. https://doi.org/10.32350/cpr.41.03
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Conflict of Interest:	Author(s) declared no conflict of interest

A publication of
The School of Pharmacy
University of Management and Technology, Lahore, Pakistan

In vitro, In vivo, and In silico Investigation of Methanolic Extract of *Withania Coagulans* (Stocks) against Diarrhea and Pyretic Albino Rats

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ABSTRACT

The current research aimed to explore the potential anti-diarrheal and antipyretic properties of the *Withania coagulans* plant. To assess the quality and purity of the plant material, sensory analysis and physicochemical tests, including measurements of ash values, moisture content, and extractive values, were carried out. The methanolic extract of the plant was investigated for its effectiveness in reductreating diarrhea, influencing gastrointestinal motility, and countering entero-pooling induced by castor oil, and reducing fever. These investigations were conducted using doses of 250 and 500 mg/kg for anti-diarrheal and gastrointestinal motility tests, and 200 and 400 mg/kg for the antipyretic tests. The experiments were conducted on Wistar albino rats, with standard substances, such as atropine (3 mg/kg) and paracetamol (150 mg/kg) being employed as benchmarks for anti-diarrheal and anti-pyretic comparisons, respectively. Gas Chromatography-Mass Spectrometry (GC-MS) analysis uncovered the presence of diverse phytochemical compounds within the plant. Through in silico analysis of these isolated compounds using GCMS, it was determined that they exhibit binding affinity with COX and M3 receptors. The results indicated that the plant demonstrated significant anti-fever effects at 200 and 400 mg/kg doses. Moreover, its impact on treating diarrhea was more pronounced at doses of 250 and 500 mg/kg.

Keywords: antidiarrheal, anti-enteropooling test, antipyretic,

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gastrointestinal motility, physicochemical, phytochemical

1. INTRODUCTION

Withania coagulans is an ornamental plant. It is a member of family Solanaceae and genus *Withania*. This plant is familiar because it has milk-coagulating properties in its fruit. *W. coagulans* (Stocks) is a kind of coagulant [1]. The berries of *W. coagulans* (Stocks) are enormously utilized for milk coagulation, which is known as "cottage cheese" [2]. It is mainly consumed to treat dyspepsia, diabetes mellitus, liver problems, kidney issues, blood purifications, anti-inflammatory conditions, and blood pressure. The oil of *W. coagulans* plant has an elevated amount of two elements, that is, linoleic acid and b-sitosterol, that are assumed to be beyond hypocholesterolemia effect when taken simultaneously [3]. Furthermore, the oil of this plant also consists of wound-healing properties. *W. coagulans* is used in the medication of ulcers, rheumatism, dropsy, consumption, and sensible debility due to its capability to coagulate milk, antimicrobial, anticancer, anti-hyperglycemic, immune-suppressive, antioxidant, and peripheral nervous system depressant properties [4].

W. coagulans has a massive ratio of major and basic metabolites effective for various therapeutic activities. According to a study, withanolides extracted from the plant's fruits exhibited antidiabetic and cholesterol-lowering efficacy [5]. Withaferin-A (Wi-A) revealed noticeable cancer suppression properties when investigated *in vitro* as opposed to cells derived from human nasopharyngeal carcinoma. Wi-A shows strong anti-arthritic and anti-inflammatory effects without any harm Moreover, it also inhibits angiogenesis. Some withanolides play an advantageous role in the medication of neurodegenerative syndromes [6]. The seed of the plant is also used as a blood purifier in several parts of Asia. Additionally, the twigs of the plant are quite helpful in cleaning the teeth, and toothaches are relieved by inhaling plant's smoke. Nervous tiredness, incapacity, lack of sleep, malnutrition, and erectile-dysfunction are all treated with *W. coagulans* (Stocks) Dunal [7].

The seeds of *W. coagulans* plant contain 17.8% free sugars with a 1:1 ratio of D-galactose and D-arabinose, and maltose. Proteolytic investigation reveals that the polysaccharide has a deficiency of B-galactosidase [5]. It contains a large amount of withanolides, which are known as steroidal lactones. Withanolides are polyhydroxy C28 steroidal lactones that are

present naturally in the plant. A six- or five-membered lactone or lacto ring is associated with an untouched or reordered ergostane skeleton, and it is the basic structure of all withanolides [8].

Its fruits are utilized to cure liver issues, asthma, and biliary problems [9]. The fruit extract of *W. coagulans* has a diuretic potential. Withanolides, from *W. coagulans* plant, have been found extensively in the Antarctic as compared with other species of *Withania*. The extracts of *W. coagulans* plant exhibited hypotensive, respiratory stimulants, as well as muscle relaxation properties [10]. This specific plant was selected for the current study based on its chemical constituents. In review, from the detailed investigation of traditional use and pharmacological activities, it can be evaluated that there is no report regarding the effect of *W. coagulans* extract on diarrhea and pyrexia. Therefore, this plant may have a vital role in the treatment of diarrhea and pyrexia. Known phytochemical constituents established pharmacological activities with critical analysis of previous studies, explicit research gap, and clear study objectives. Recent studies have highlighted the anti-inflammatory and anti-diarrheal potential of *Withania* species. However, none has systematically evaluated the dual anti-diarrheal and antipyretic effects of *W. coagulans* methanolic extract using an integrated pharmacognostic, in vivo, and in silico approach.

2. MATERIALS AND METHODS

2.1. Extraction of Plant

Under standard conditions, the plant was grinded, dried, and soaked for 7 days in methanol. Methanolic extract was prepared by using a rotary evaporator. The extract was free of solvent and was dried in an oven and refrigerated at 2-4 °C in screw-scratched sterile container for further use.

2.2. Pharmacognostic Features

Pharmacognostic characteristics were determined in accordance with World Health Organization (WHO) to study plant characteristics [11].

2.3. Macroscopic and Microscopic Studies

The powder of dried whole plant of *W. coagulans* was subjected to macroscopic study, which covered the organoleptic characteristics, such as odor, color, taste, appearance, and shape [12].

2.4. Proximate Analysis

Loss on drying was measured and the percentage yield of the powder sample was calculated. This included percentage of total ash, acid insoluble ash, water-soluble ash, sulphated ash foaming index, swelling index determination, and alcohol soluble extract. The percentage of water-soluble extractives was measured by Fluorescence screening [11]. This method was used to evaluate natural drugs quantitatively and was used with little modification. Moreover, it was calculated using the previously used method. Color change was noted by using various solutions [13].

2.5. Phytochemical Screening

In series to investigate the presence of different constituents and profile of methanolic extract of *W. coagulans,* qualitative chemical tests of different kinds were conducted.

2.5.1. Fourier Transform Infrared Spectroscopy (FTIR) Analysis. FTIR analysis of methanolic extract of *W. coagulans* was performed to confirm the presence of different phytochemicals, ranging from 4000 to 400 cm^{-1}.

2.5.2. GC-MS Analysis. To identify phyto-constituents in *W. coagulans* extract, a Gas Chromatograph-Mass Spectrometer (GC-MS) (Shimadzu, Japan) was employed. GC was equipped with 0.25 mm column (diameter) with 30 m length. GC conditions were set as follows: column oven temperature, 80°C; injector temperature, 250°C; carrier gas, helium; flow rate, 1 mL/min, and pressure was kept at 82.7 Kpa. The starting and final temperatures were recorded to be 80°C and 220°C, and ion source temperature was 280°C. Scan started at 4.10 min and ended at 30 min with a mass scan range of 40–800 *m/z*. The spectrum obtained was compared with the fragmentation pattern available with NIST Library [14].

2.6. Biological Studies

2.6.1. Experimental Animals. Wister albino rats of both sexes, weighting 150-200 g, were acquired from animal house of The University of Lahore (UOL) for in vivo investigation of *W. coagulans* plant. All the above-mentioned animals were housed under standard research settings-maintained temperatures at 25°C. Animals were kept in polyvinyl cages, and each cage consisted of five animals. They were provided with food and water as well as were retained at this temperature for twelve hours

(dark/light). Institutional Research Ethics Committee (IREC-2021-32) of Faculty of Pharmacy, UOL, approved the experiment protocol.

Doses of 250 and 500 mg/kg for anti-diarrheal tests, and 200 and 400 mg/kg for antipyretic tests were selected based on: (1) acute toxicity results showing no mortality up to 5500 mg/kg, (2) preliminary dose-range finding studies (100–600 mg/kg) conducted prior to the main experiments, and (3) published literature on related *Withania* species, where 200–500 mg/kg demonstrated therapeutic effects without toxicity [7].

2.6.2. Acute Toxicity Test. The overall ratio of rats was distributed into three sets ensuring that each set contained four rats accordingly. The rats were provided with an extract that was mainly oral at dose level of 200 mg/kg. Afterwards. they were examined individually for about 48 hours for any neurological and behavioral fluctuations. The instabilities examined included convulsions, tremors, diarrhea, salivation, sleeping patterns, feeding behaviors, as well as lacrimation as signs of acute toxicity. After 48 hours, if two or three animals expired, the dose was then deliberated as virulent constituent. If a single animal expired, the dose was again imitated for verification of deadliness. If animals survived with that amount of dose, then same process was imitated for higher dose up to 5500 mg/kg [15]. The acute toxicity study was conducted following OECD Guideline 423 (Acute Oral Toxicity – Fixed Dose Procedure).

2.6.3. Castor Oil-induced Diarrhea. Rats were divided into four different groups and made to fast overnight, and each group contained six rats. Castor oil was given orally (1 ml per kg body weight) to induce diarrhea in these rats. Control group 1 was given 2 ml/kg normal saline orally, group 2 received 3 mg/kg atropine intraperitoneal, and group 4 and 5 were given 250, 500 mg/kg methanol extract, respectively. All these groups were treated one hour before castor oil administration orally. The number of diarrheal stools in every 1 hour up to 6 hours was calculated by placing a plain piece of paper in each group of rats. The mean number of diarrheal stools in treated group was compared with positive control group. Diarrheal inhibition percentage was determined in accordance to the following formula [16].

$$\% \text{ inhibition} = \frac{\text{Average number of WFC} - \text{Average number of WFT}}{\text{Average number of WFC}} \times 100$$

WFC=average number of wet faeces in control group, WFT = average

number of wet faeces in test group.

2.6.4. Charcoal Meal (Gastrointestinal Motility) Test. Rats of both sexes were separated into groups after making them fast for 18 hours. Percentage of inhibition and peristalsis index was expressed using the following formula [16].

2.6.5. Anti-enteropooling Test. Small intestine length was measured and gastric fluid was collected into graduated cylinder to determine the volume of intestinal contents. Intestine was reweighed and deviation between empty and filled intestine was calculated. The percentage of inhibition was determined by following formula [16].

2.6.6. Antipyretic Activity. Rectal temperature of all rats was assessed 1, 2, 3, 4, and 5 hours after extract administration and compared with control and standard [17].

2.7. Molecular Docking Studies

Compounds putative binding mode analysis for antipyretic activity was carried out using Auto Dock4 Tools [18]. Compounds structures were sketched and cleaned in 3D using Marvin Sketch. X-ray crystal structure of Prostaglandin-endoperoxide synthase 2 (PTGS2), also known as cyclooxygenase 2 (COX-2) and M3 muscarinic acetylcholine receptor, was obtained from RCSB protein databank with PDB ID 5F1A and 4DAJ [19,20]. X-ray protein structure contained co-crystallized ligand (salicylic acid) bounded inside substrate-binding pocket of human COX-2 enzyme. While, in case of M3 muscarinic acetylcholine receptor, it contained (1R,2R,4S,5S,7S)-7-{[hydroxy(dithiophen-2-yl) acetyl] oxy}-9,9-dimethyl-3-oxa-9 azoniatricyclo [3.3.1.0~2,4~] nonane. Binding site of co-crystallized ligands was used to carry out docking of the inhibitors. Before actual docking of test inhibitors, docking protocol was validated by redocking of co-crystallized ligand salicylate inside active pocket of enzyme by reproducing the co-crystallized ligand conformation. Compounds and standard control drugs docking was carried out using Lamarckian Genetic Algorithm followed by cluster analysis using AutoDock4 Tools [18]. Discovery Studio visualizer was then used for visual inspection and selection of conformations.

3. RESULTS AND DISCUSSION

3.1. Macroscopic and Microscopic Studies

W. coagulans is a grayish-green-colored plant, and the color of its fruit is yellow to brown. This plant has an indistinct odor, bitter taste, and a rough texture. Furthermore, the shape of its leaves is lanceolate with a hard fracture. The microscopic characteristics of powder of *W. coagulans* revealed that the plant contained calcium oxalate crystals, thick-walled cells of sclerenchyma cells, fibro-vascular tissue, cork cell, and thickened vessels (Figure 1). The percentage shown by methanolic extract was 8.246%. Whereas, the macroscopic study of the whole plant showed its physical features, such as its grayish green color with a 60-120 cm long shrub.

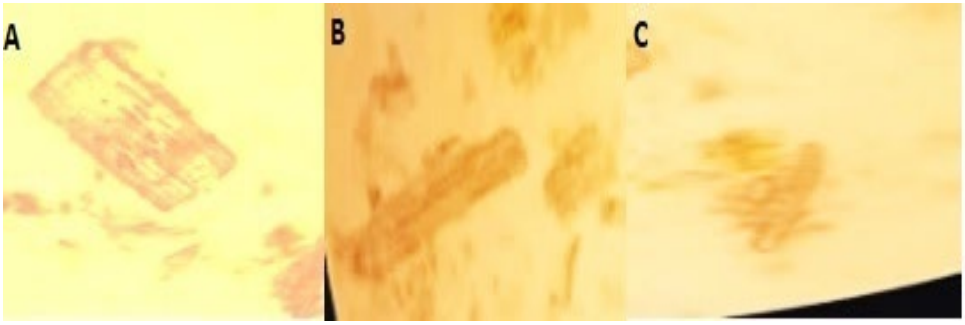

Figure 1. (A)Microscopic Evaluation of Calcium Oxalate Crystals, (B) Thick-walled Cell of Sclerenchyma Cells, (C) Fibro-vascular Tissue.

3.2. Physicochemical Evaluations

The physicochemical evaluation of crude drug is necessary to determine the quality and purity of powder. Extractive values (Table 1) determined chemical components present in raw drug when extracted with a specific solvent. Moisture content of powder was 1.5%, which was in limit (8-14%) and acceptable [21]. Total ash, acid insoluble ash, and water-soluble ash were found to be 12%, 3.3% and 4.4%, respectively. Sulfated ash method was used to determine the inorganic matter which was 9.6% and foaming index was found to be 0.7%, which was not within limit due to the absence of saponins in the plant. Swelling index was determined to confirm the presence of mucilage, which was up to 11%, among given official limit up to 5 ml. Water-soluble and alcohol-soluble extractive values were found to be 2.27 and 5%, respectively.

Table 1. Physicochemical Evaluation of *W. coagulans* Powder

Parameters	Obtained Values	Official Limits
Moisture Content (%)	1.5±0.17	8-14 %
Total Ash (%)	12±0.72	20 %
Acid Insoluble Ash (%)	3.3±0.15	< 10 %
Water-soluble Ash (%)	4.4±0.14	0.5-6 %
Foaming Index (ml)	0.7±0.08	100-1000 ml
Swelling Index (ml)	11±0.57	Up to 5 ml
Sulphated ash (%)	9.6±0.12	11 %
Alcohol-soluble Extractive (%)	2.27 ±0.015	Not Applicable
Water-soluble Extractive (%)	5±0.034	Not Applicable

Note. ±S.E.M (*n*=3)

3.2.1. Percentage Yield of *W. coagulans.* The extraction process was carried out through cold maceration, in which 500g of weighed powder of *W. coagulans* was extracted with analytical grade methanol. A percentage yield of 8.246% was attained from 41.23 g methanolic extract.

3.3. Fluorescence Analysis

Fluorescence study is important for first line validation of crude extracts. In fluorescence screening, wavelength of fluorescent light is always larger than stimulating light. In fluorescence analysis, the whole plant powder was served with various reagents under ordinary, short UV light (254nm) and long UV light (365nm). The results showed that powder of the plant exhibited different colors with different reagents, for instance it showed green color at daylight, brown color at 254 nm, and red color at 365 nm. These secondary metabolites are responsible for pharmacological activities [11].

Table 2. Fluorescence Screening of Dried Powder of *W. coagulans*

Reagents	Visible Light	Short UV (254nm)	Long UV (366nm)
Methanol	Green	Yellowish	Greenish Blue
Chloroform	Light Brown	Green	Pinkish White
Ferric Chloride	Dark Brown	Reddish Brown	Orange
Iodine	Yellowish Green	Blue	Purple

Reagents	Visible Light	Short UV (254nm)	Long UV (366nm)
Potassium Hydroxide	Green	Yellowish Brown	Light Greenish Brown
Sodium Hydroxide	Dark Brown	Yellowish Green	Reddish Brown
Sulphuric Acid	Black	Purple	Blue
Ammonia	Brown	Blue	Dark Greenish Brown
Distilled Water	Green	Orange	Greenish Blue

3.4. Phytochemical Analysis

Phytochemical analysis of the *W. coagulans* extract determined the presence of alkaloids, tannins, flavonoids, carbohydrates, phenols, steroids, mucilage, fats, and oil. Whereas, glycosides, gum, saponins, proteins, and amino acids were not present.

3.4.1. FTIR Analysis. FTIR analysis of powder and ethanolic extract of *W. coagulans* (Stocks) determined the presence of different organic compounds and functional groups. Different phytocompounds were identified based on functional groups due to its peak value in infrared region. The powder of *W. coagulans* (Stocks) showed (Figure 2a) prominent peaks at 1022 cm^{-1}, 1316 cm^{-1}, 1623 cm^{-1}, 2923 cm^{-1}, and 3308 cm^{-1}. This corresponds to C-O structure vibration of hydroxyl ketones, carboxylic acid, primary amines, alkanes, and hydrogen bonded O-H in carboxylic acid, respectively [10]. Ethanolic extract of *W. coagulans* (stocks) exhibited same prominent peaks at 1022 cm^{-1} and 2923 cm^{-1}. The characteristic peaks at 1316 cm^{-1} and 1623 cm^{-1} slightly shifted to 1388 cm^{-1} and 1656 cm^{-1} in ethanolic extract. Wide peak at 3308 cm^{-1} shifted to 3369 cm^{-1}, becoming sharp and intensive. Previously, similar results regarding the fruits of *W. coagulans* were found by [22]. The study revealed that *W. coagulan's* (Stocks) powder and ethanolic extract possess various functional groups of phytochemicals, such as alkanes, aldehydes, carboxylic acids, amines, and aromatic compounds.

3.4.2. GC-MS Analysis. *The extract of W. coagulans* was subjected to GC-MS analysis, which led to the identification of 29 constituents. These included triterpenes, sesquiterpenes, ketones, alcohols, hydrocarbons, phenols, and fatty acids as well as their methyl esters (Figure 2b). Among

identified compounds, 7-tetradecenal, (Z)- (17.76%), *n*-hexadecanoic acid (13.80%), squalene (11.41%), *cis*-hexahydroindan (9.46%), 2-methoxy-4-vinylphenol (7.16%), octadecanoic acid (4.64%), 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (4.34%), 1-(6-oxabicyclo [3.1.0] hex-1-yl) ethanone (3.98%), and 2-isopropenyl-5-methylhex-4-enal (3.23%), were the major constituents (>3% concentration). Table 3 (Figure. 3) shows the retention time (min), area of identified peaks, and their respective concentration.

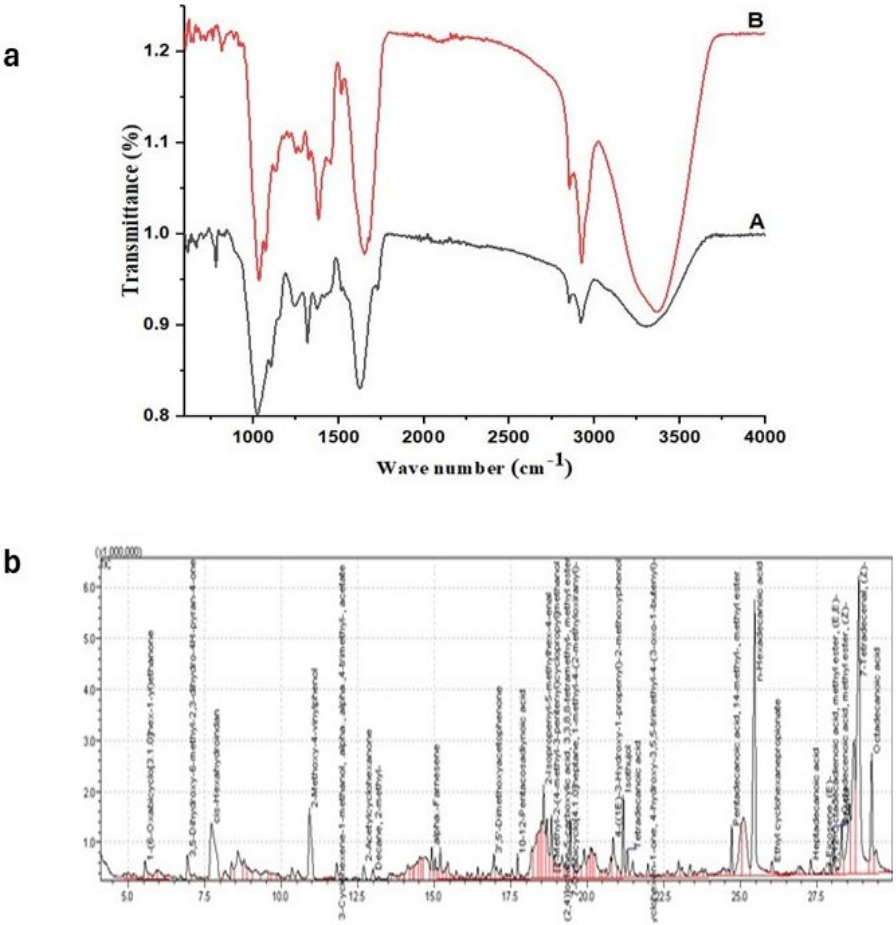

Figure 2. a) FTIR Spectra of *W. coagulans* Powder A and ethanolic extract B, b) The GC Chromatogram of *W. coagulans* Extract

Table 3. Identified Components of *W. coagulans* Extract using GC-MS Analysis

Compound Name	Retention Time (min)	Area	Concentration (%)
1-(6-Oxabicyclo[3.1.0]hex-1-yl)ethanone	5.553	732424	3.98
3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	6.938	797372	4.34
Cis-Hexahydroindan	7.718	1738623	9.46
2-Methoxy-4-vinylphenol	10.925	1317010	7.16
3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-, acetate	11.816	185686	1.01
2-Acetylcyclohexanone	12.691	270621	1.47
Decane, 2-methyl-	13.008	80110	0.44
.alpha.-Farnesene	14.911	186453	1.01
3',5'-Dimethoxyacetophenone	16.950	187514	1.02
10-12-Pentacosadiynoic acid	17.718	78616	0.43
2-Isopropenyl-5-methylhex-4-enal	18.570	593666	3.23
[2-Methyl-2-(4-methyl-3-pentenyl)cyclopropyl]methanol	18.827	394460	2.15
Tricyclo[5.1.0.0(2,4)]octane-5-carboxylic acid, 3,3,8,8-tetramethyl-	19.179	158877	0.86
7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl)-	19.450	459639	2.50
4-((1E)-3-Hyroxy-1-propenyl)-2-methoxyphenol	20.844	378362	2.06
Isothujol	21.176	436071	2.37
Tetradecanoic acid	21.311	155924	0.85
2-Cyclohexen-1-one, 4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)	21.982	183979	1.00
Pentadecanoic acid, 14-methyl-, methyl ester	24.715	501637	2.73
n-Hexadecanoic acid	25.474	2537939	13.80
Ethyl cyclohexanepropionate	26.050	40549	0.22
Heptadecanoic acid	27.303	109839	0.60
3-Eicosene, (E)-	27.734	37697	0.21
9,12-Octadecadienoic acid, methyl ester, (E,E)-	27.940	59634	0.32
6-Octadecenoic acid, methyl ester, (Z)-	28.058	224740	1.22

Compound Name	Retention Time (min)	Area	Concentration (%)
Phytol	28.306	323678	1.76
Squalene	28.618	2097462	11.41
7-Tetradecenal, (Z)-	28.866	3265311	17.76
Octadecanoic acid	29.297	853323	4.64

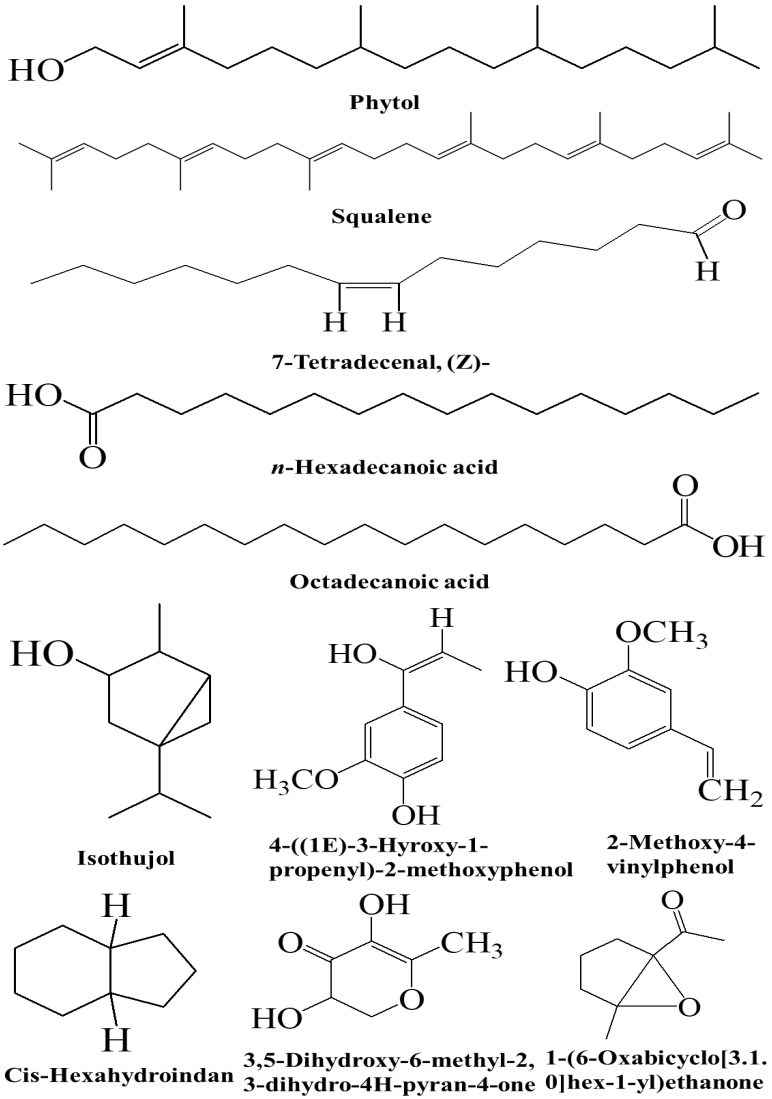

Figure 3. Structures of Few Majorly-identified Constituents through GC-MS Analysis in *W. coagulans* Extract

3.5. Acute Toxicity Study

Different dose, from 200 mg/kg up to 5500 mg/kg methanolic extract of *W. coagulans,* was administered orally. No major physical change was seen in Wister albino rats during the evaluation period of 24 hours. After the examination and observation of physical changes, it was declared that there was no sign of change in skin, eyes, mucous membrane, and behavioral changes. Tested dose did not show any mortality.

3.5.1. Castor Oil-induced Diarrhoea. Test doses of 250 and 500 mg/kg having anti-diarrheal potential of methanolic extract of *W. coagulans* and percentage inhibition are shown Figure 4A. The extract at doses of 250 and 500 mg/kg produced distinct action on diarrhea, and standard drug atropine at dose of 3 mg/kg showed comparable anti-diarrheal effects, such as that of methanolic extract. According to research, about 2-4 million cases of diarrhea are reported every year. Furthermore, there is also an increase in rate of death due to diarrhea, especially in infants [16].

3.5.2. Charcoal Meal (Gastrointestinal Motility) Test. Different test doses of 250 mg/kg and 500 mg/kg of methanolic extract of *W. coagulans* produced different effects (Figure 4b; Table 4). At 250 mg/kg dose, percentage inhibition was 53%, and at 500 mg/kg, the percentage inhibition was 77%. Intestinal mobility shown by rats at dose of 250 mg/kg extract was less significant (p<0.02), while at 500 mg/kg, it was more significant (p<0.01). Level of significance showed that 500 mg/kg dose produced same effects as shown by a standard drug. The plant extract inhibited the movement of charcoal marker in dose-dependent manner. The plant extract (500 mg/kg) had similar effect to that of atropine. Atropine effects ring-shaped and long muscles of intestinal wall. A decrease in colon muscles mobility increases the rest of matter in gut. It was assumed that depletion in movement of charcoal marker was due to the antispasmodic effect of the extract [1]. Methanolic extract also reduced intestinal fluid accumulation in dose-dependent manner. At 500 mg/kg dose, *W. coagulans* showed a considerable inhibition, that is, 52% (p<0.01) and at 250 mg/kg, the inhibition was 39% (p<0.02). The percentage inhibition of standard group (atropine) was 50% (p<0.01). The plant extract remarkably decreased the volume of fluid at higher dose, that is, 500 mg/kg, similar to that of standard drug. The 250 mg/kg extract had less effect when compared with standard drug.

Table 4. Effect of *W. coagulans* Extract on Gastrointestinal Motility in Rats

Treatment	Dose	Distance Travelled by Charcoal Meal (cm)	% Inhibition	Peristalsis Index
Normal Saline	2 ml/kg	33.17±1.38		
Normal Saline + Castor Oil	2 ml/kg +2ml	21.96±5.33	33%	65%
Atropine	3 mg/kg	8.17± 1.25[b]	72%	20%
MEWC	250 mg/kg	14±1.39[a]	53%	33%
MEWC	500 mg/kg	6.67±0.88[b]	77%	16%

Note. [a,b,c] $p < 0.02$, $p < 0.01$, and $p < 0.001$, respectively, ± S.E.M (n=6).

3.5.3. Castor Oil-induced Enteropooling. The methanolic extract reduced intestinal fluid accumulation in dose-dependent manner as shown in Figure 4C. The results revealed that the methanolic extract of *W. coagulans* contains constituents that reduce diarrhea by inhibiting intestinal fluid accumulation and intestinal motility. After 30 minutes of castor oil administration, diarrhea appeared for the next 4 hours in all rats of the control group. Number of stools was reduced by intraperitoneal injection of atropine at 3 mg/kg (66%) inhibition. While, similar decrease in number of stools was exhibited by the extract at 500 mg/kg (66 %) inhibition. Castor oil contains ricinolic acid, which causes irritability and swelling of intestinal mucosa, due to prostaglandins release.

3.5.4. Anti-pyretic Activity. Different test doses of 250 and 500 mg/kg having anti-pyretic potential of methanolic extract of *W. coagulans* are shown in Figure 4D. The effect of extract at 400 mg/kg was observed to be more as compared to the standard group. The outcomes represented that the methanolic extract of *W. coagulans* possesses remarkable antipyretic effect in dose-dependent way, and its effect was comparable to that of standard antipyretic drug paracetamol. There was significant decrease in body temperature (p<0.0001) after 1-hour administration of methanolic extract at dose of 200 and 400 mg/kg. The remarkable reduction in temperature of animals was due to alkaloids, tannins, and flavonoids present in the plant. Antipyretic activity of methanolic extract of *W. coagulans* was due to the suppression of prostaglandin production in hypothalamus [23].

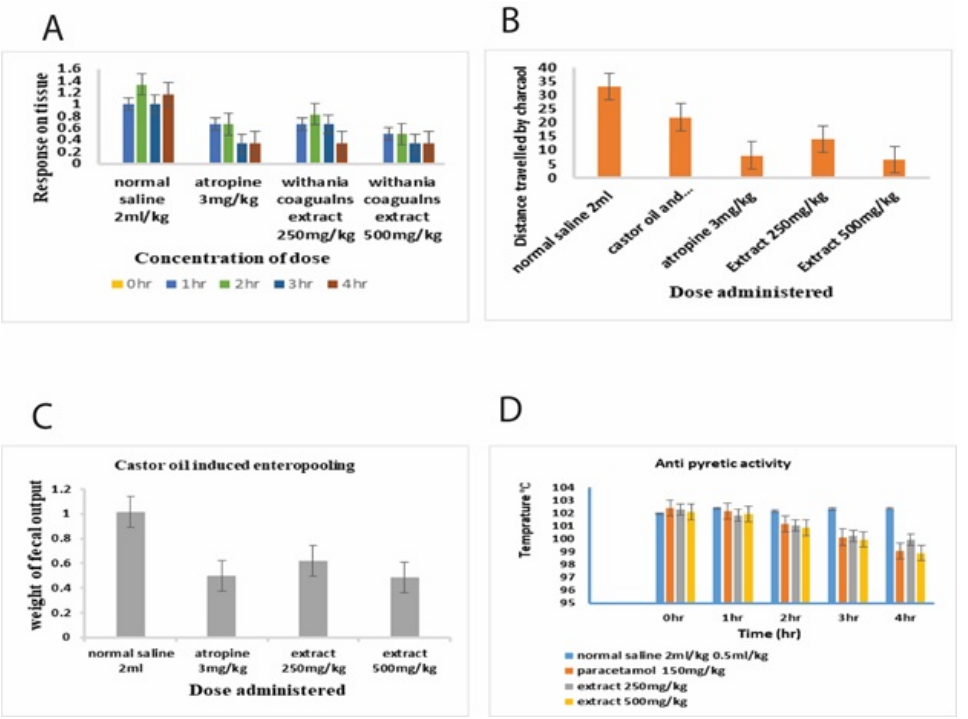

Figure 4 A) Anti-diarrheal Effect of *W. coagulans*. B) Effect of *W. coagulans* on Gastrointestinal Motility. C) Effect of *W. coagulans* on Castor Oil-induced Enteropooling. D) Antipyretic Activity of *W. coagulans* on Rats

3.6. Molecular Docking Studies

Molecular docking studies of isolated compounds were conducted to reveal their putative binding mode against antipyretic target Prostaglandin-endoperoxide synthase 2 (PTGS2), also known as cyclooxygenase 2 (COX-2) enzyme and antidiarrheal target - M3 muscarinic acetylcholine receptor. All compounds were screened and docking protocol was validated before the actual docking of isolated compounds. Docking of standard co-crystallized inhibitor reproduced co-crystallized ligand conformation within RMSD value of less than 2Å. All compounds were determined to bind with substrate-binding pocket with considerable binding affinity of a minimum -3.56 Kcalmol^{-1} and -0.11 Kcalmol^{-1} (as determined in case of compound M5) to a maximum binding affinity of -6.22 Kcalmol^{-1} and -5.51 Kcalmol^{-1} (as determined in case of compound M9) inside COX-2 enzyme and M3 muscarinic acetylcholine receptor, respectively. While standard

drugs, paracetamol in case of COX-2 and atropine in case of M3 muscarinic AChR, showed binding affinity of -5.95 $Kcalmol^{-1}$ and -8.93 $Kcalmol^{-1}$ respectively (Table 5).

Table 5. Binding Affinity Profile of Top-ranking Conformations

Compound	Binding Affinity for Top-ranking Cluster	
	COX-2	M3 Muscarinic AChR
M1	-5.27	-5.14
M2	-4.87	-4.25
M3	-5.39	-1.86
M4	-5.74	-4.93
M5	-3.56	-0.11
M6	-5.9	-4.08
M7	-5.59	-4.29
M8	-5.28	-3.68
M9	-6.22	-5.51
M10	-4.51	-3.10
M11	-5.27	-3.97
Paracetamol	-5.95	-
Atropine	-	-8.93

Upon visual inspection of top-ranking compound M9 inside COX-2 enzyme, it was found that substrate-binding pocket was surrounded by hydrophobic amino acid residues thus, favoring the formation of hydrophobic protein-ligand interactions. Therefore, compound M9 contained two hydrophobic rings perfectly aligned inside hydrophobic substrate-binding pocket. Additionally, it was found to form several hydrophobic interactions with amino acid residues Leu352, Tyr385, Trp387, Phe518, Met522, and Val523 similar to hydrophobic interactions formed by co-crystallized ligand salicylic acid that formed hydrophobic interactions with amino acid residues Leu352, Met522, and Gly526. The molecular docking of top-ranking compound M9 inside the M3 muscarinic acetylcholine receptor revealed that substrate-binding pocket was surrounded by hydrophobic amino acid residues similar to previous interaction with the COX-2 enzyme. Thus, it appears to be favoring the formation of hydrophobic protein-ligand interactions. Two hydrophobic rings formed hydrophobic interactions with amino acid residues Tyr148, Tyr529, Cys532, and Tyr533 similar to hydrophobic interactions formed by

co-crystallized ligand 7-{[hydroxy(dithiophen-2-yl) acetyl] oxy}-9,9-dimethyl-3-oxa-9-azoniatricyclo [3.3.1.0~2,4~] nonane. Graphical illustration of compound M9 interactions within M3 muscarinic acetylcholine receptor is shown in (Figure 5a), and its binding conformation is represented in (Figure 5b).

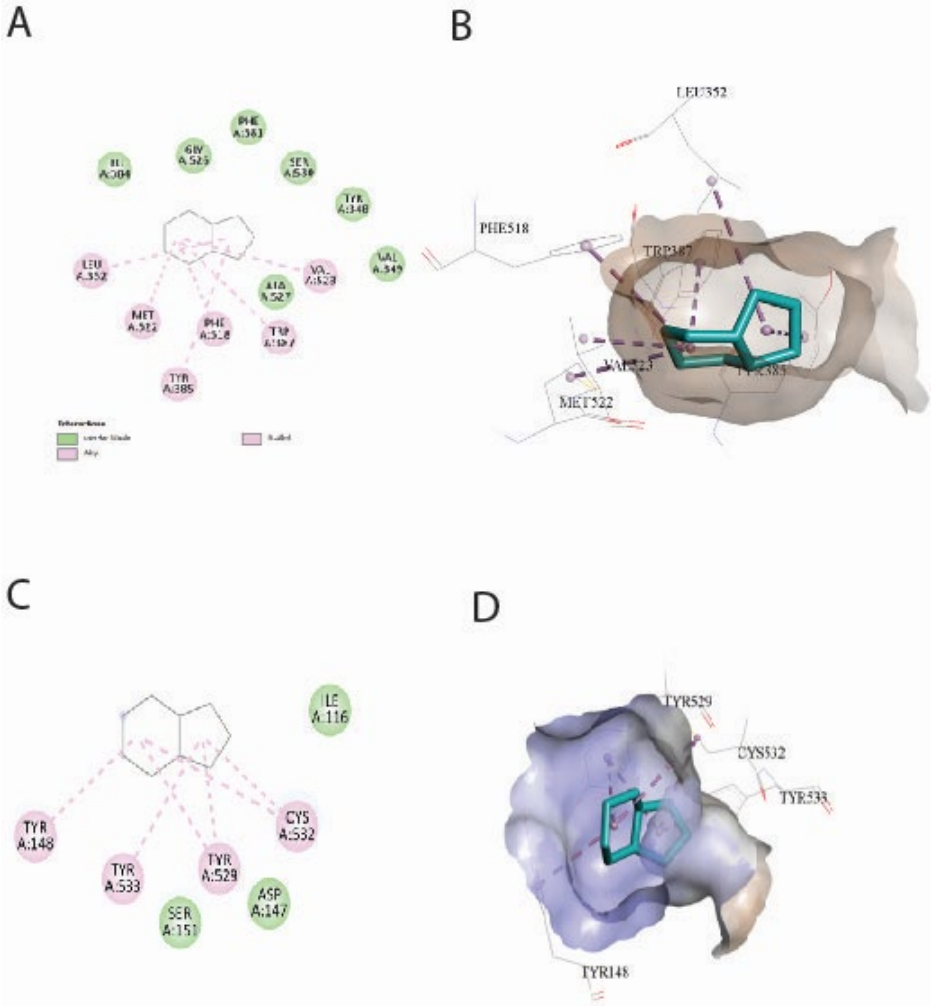

Figure 5. Graphical Illustration of the Compound M9 Interactions with the COX-2 Active Site. Alkyl and Pi-alkyl Interactions are shown in Pink-colored Dashed Lines, Respectively (A), Putative Binding Mode of Compound M9 Inside COX-2 Active Site (B), Graphical Illustration of the Compound M9 Interactions within M3 Muscarinic Acetylcholine Receptor

Ligand Binding Site (C), Alkyl and Pi-alkyl Interactions are Shown in Pink-Colored Dashed Lines, Respectively. (D) Putative Binding Mode of Compound M9 Inside M3 Muscarinic Acetylcholine Receptor ligand Binding Site

3.7. Conclusion

The methanolic extract of *W. coagulans* exhibited promising anti-diarrheal and antipyretic properties in Wistar albino rats. Primary contributors to anti-diarrheal effect of plant are its predominant chemical constituents known as 'tannins'. Observed activity is likely attributed to the presence of alkaloids, tannins, and flavonoids. Tannins play a role in diminishing mucosal secretions, and enhancing resilience of intestinal mucosa. Meanwhile, flavonoids and alkaloids work to suppress release of autocoids and prostaglandins, thereby counteracting secretion triggered by castor oil. Antipyretic impact of plants is ascribed to combined presence of flavonoids and tannins. It can be inferred that their mechanism involves interfering with synthesis of prostaglandins and inhibiting the release of cytokines, which contribute to fever response. In silico investigations have unveiled binding interactions between isolated compounds and COX and M3 receptors. This data corroborates findings from in vivo and in vitro studies, collectively providing substantial evidence for antipyretic and anti-diarrheal potential of methanolic extract.

3.8. Future Recommendations

Future studies should focus on:

- Bioactivity-guided fractionation to isolate and characterize individual compounds responsible for anti-diarrheal and antipyretic activity.
- Evaluation of synergistic effects among identified compounds, particularly n-hexadecanoic acid, squalene, and 7-tetradecenal.
- Development of standardized extract formulations with validated marker compounds for quality control.
- Mechanistic studies measuring direct PGE_2, TNF-α, and IL-1β levels in animal models.
- Chronic toxicity studies (90-day) to establish long-term safety profile.
- Clinical pilot studies in human subjects with mild to moderate diarrhea

or low-grade fever.

Given that, diarrheal diseases remain the second leading cause of death among children under five globally. Moreover, antipyretic agents are among the most commonly used medications worldwide, *W. coagulans* represents a promising candidate for developing affordable, accessible phytomedicines. The plant's established safety profile (LD_{50} > 5500 mg/kg) and traditional use history support its potential as a complementary therapy, particularly in resource-limited settings where conventional medications may be unavailable or unaffordable.

Author Contribution

Somal Nisar: writing- original draft. **Nazia Aslam:** supervision. **Muhammad Asad Saeed:** supervision. **Sherjeel Adnan:** formal analysis. **Zeeshan Masood:** data curation. **Hammad Ahmad:** methodology. **Rafia Andleeb:** investigation. **Kanwal Shahzadi:** validation. **Syed Jawad Ali Shah:** conceptualization. **Hafiz Majid Rasheed:** writing-review & editing. **Muhammad Farooq:** writing-review & editing.

Conflict of Interest

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

Data Availability Statement

All data analyzed during this study are included in this published article.

Funding Details

No funding has been received for this research.

Generative AI Disclosure Statement

The authors did not use any type of generative artificial intelligence software for this research.

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