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MOLECULAR DOCKING SUPPORTED INVESTIGATION OF ANTIOXIDANT, ANALGESIC AND DIURETIC EFFECTS OF *COSTUS SPECIOSUS* RHIZOME

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ABSTRACT. The aim of the current study was to analyze the polyphenols and determines the antioxidant, analgesic and diuretic properties of the methanolic extract of C.speciosus rhizome. DPPH and ferric reducing antioxidant power (FRAP) assays were used to determine the antioxidant activity. Acetic acid-induced writhing and formalin-induced licking experiments were used to assess the analgesic effect. The total phenolic, flavonoid and flavonol contents were found 51.73± 0.25 mg GAE/g dry weight, 3.41± 0.07mg QE/g dry weights and 44.19± 2.24 mg QE/g dry weight, respectively. The plant extract exhibited weak antioxidant activity in the DPPH and FRAP assays, with an IC_{50} value of 1699±62 μg/mL and an EC_{50} value of 125±2 μg/mL, respectively. The extract significantly reduced the number of writhes at both doses (200 and 400 mg/kg body weight) as compared to the control. The extract (400 mg/kg) also significantly reduced the percent inhibition of licking by 31.96 and 62.69% compared to the control in the early and late phase, respectively. Compared to the standard drug furosemide, the plant extract also showed a weak diuretic effect. The docking study supported the analgesic activity of rhizome extract. The potent analgesic activity of the plant extract justifies the traditional and medicinal aspects.

KEY WORDS: *Costus speciosus*, Analgesic activity, Diuretic effect, Molecular docking

INTRODUCTION

The plants' therapeutic value lies in bioactive chemical compounds like tannins, glycosides, saponins, flavonoids, alkaloids and phenolic compounds that reduce the possibility of various pernicious disorders including cardiovascular, cancer and chronic diseases [1]. The endoplasmic reticulum, mitochondria, peroxisome, and phagocytic cell in the body can produce reactive oxygen spectrum (ROS), while environmental pollutants, heavy metals, industrial solvent, insecticides and radiation are the source of ROS [2]. Imbalance of ROS generation and antioxidant levels within the body collapse the cells' natural defence mechanism and oxidative stress occurs [3]. Oxidative stress is one of the leading key factor of DNA, nucleic acids, proteins and lipid damage, contributing to numerous physiological complications, including cardiovascular diseases, respiratory diseases, different types of cancer, diabetes mellitus [4, 5]. Plant-derived bioactive compounds like phenolics act as the natural heavy metal chelating agent that can scavenge pernicious free radicals during oxidative stress and put a promising role in human health by minimizing the risk of chronic health hazards [1]. Pain is a complex, uncomfortable sensory and emotional sensation associated with possible tissue injury and is often known as one of the disease's major signs and symptoms [6]. Although analgesic drugs effectively alleviate pain without significant alteration of consciousness; however the unwanted adverse effects of these drugs have limited their uses. For this reason, medicinal plants may become an alternative option

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of treatment to minimize these unwanted adverse effects [7]. Traditional painkillers or NSAIDs' main target is cyclooxygenase enzyme (COX) inhibition, resulting in the minimization of prostaglandin biosynthesis from arachidonic acid. Two COXs isoforms are available, named COX1 and COX2 [8]. Studies have shown that the constitutive COX1 enzyme in the stomach and kidney generates a cytoprotective mechanism, while the COX2 enzyme is associated with inflammatory response and other pathological conditions [9,10]. Non-selective NSAIDs inhibit both COX1 and COX2 enzyme, where the COX1 enzyme inhibition is responsible for the main side effects of NSAIDs, including peptic ulcer bleeding [11,12]. Coxibs (rofecoxib and valdecoxib) are well-known highly selective inhibitors of COX2. But, owing to potential side effects, such as cardiovascular effects, rofecoxib and valdecoxib have been withdrawn from the market. So designing natural compounds with high selectivity over COX2 is the scholars' main goal to minimize the adverse effects.

Imbalance of electrolyte composition and fluid volume within the body bring about numerous physiological complications. Diuretic drugs increase urine production in the kidney and maintain homeostasis by removing excess extracellular water from edematous tissue. They are utilized to treat several conditions, such as cardiac failure, high blood pressure, renal failure, liver disease and cirrhosis [14]. At present numerous commercial diuretic drugs are available such as thiazide, loop diuretics, K^+ sparing and these diuretics are associated with several adverse effects including an imbalance of electrolytes, metabolic alteration, sexual dysfunction. In the invention of novel diuretic drugs with a lower risk for adverse effects, medicinal plants may be a promising source [15].

Costus is a genus belonging to family Costaceae and a class of perennial herbaceous plants. It is often individualized and distinguished from relatives such as *Zingiber* by its spiralling stems. Thus the genus is often called as spiral ginger [16]. It is abundant in Asia, America and Africa's various tropical and subtropical regions. Almost 175 species belong to the genus of *Costus* [17]. The *C. speciosus* is a rhizomatous, monocotyledonous herbaceous plant with rootstocks, tuberous stems, long, lanceolate leaves and white flowers in terminal clusters, growing up to 2-2.7 m in height. The rhizome juice of *C. speciosus* is historically used to treat inflammatory disorders, cancer, headache, diabetes, and anti-vermin, internally given with sugar to treat leprosy, formally used for smallpox in Malaysia. A large number of bioactive compounds are present in different parts of *C. speciosus*, including diosgenin, dioscin, curcumin, β-sitosterol, stigmasterol, campestral, prosapogenin A and B, β-carotene, tocopherols, 5-alpha-stigmast-9(11)en-3β-ol, methyl hexadecanoate, abscisic acid, fatty acids and a wide range of amino acids. [18].

In this study, the analgesic and diuretic effects of *C. speciosus* rhizome methanolic extract were evaluated in Swiss albino mice model along with antioxidant profile and docking study.

RESULTS AND DISCUSSION

Total phenolic, flavonoid, and flavonol contents

It was revealed from the results that the methanolic rhizome extract of *C. speciosus* contained a considerable amount of phenolic compounds (51.72 ± 0.25 mg GAE/g dry weight of extract). However, the quantity of total flavonol content (44.19 ± 2.24 mg QE/g dry weight) was more than the total flavonoid content in the rhizome (3.41 ± 0.07 mg QE/g dry weight). The existence of higher flavonoid and phenolic content is an indicator of excellent antioxidant activity [19]. The presence of lower flavonol, phenolic and flavonoid content might be responsible for the low antioxidant activity of *C. speciosus* rhizome extract.

Antioxidant activity assay

Table 1 represents the DPPH radical scavenging assay of *C. speciosus* rhizome extract. At all concentrations, the free radical scavenging activity of the *C. speciosus* methanolic rhizome extract

demonstrated low antioxidant activity relative to standard ascorbic acid. The methanolic rhizome extract exhibited an IC_{50} value of 1699 ± 62 µg/mL; on the contrary, the IC_{50} value for the ascorbic acid was 77.21 ± 2.01 µg/mL. The higher IC_{50} value is an indication of having lower DPPH radical scavenging activity. Therefore, it is reflected that *C. speciosus* rhizome showed poor antioxidant capacity in DPPH radical scavenging activity. In this assay, ascorbic acid (EC_{50} = 32.61 ± 0.80 µg/mL) showed the highest reducing power than rhizome extract (EC_{50} = 125 ± 2 µg/mL) of *C. Speciosus* (Table 1). The FRAP activity of rhizome extracts and standard ascorbic acid is increased with increasing concentration.

Table 1. Antioxidant activity of methanol *C. speciosus* extract and ascorbic acid (positive control) in DPPH and FRAP assay.

Sample	DPPH (IC_{50} µg/ml)	FRAP (EC_{50} µg/ml)
Ascorbic acid	77.21 ± 2.01	32.61 ± 0.80
MECS	1699 ± 62	125 ± 2

Values are expressed as Mean±SEM (n = 3). Note: DPPH, 1,1-diphenyl-2-picrylhydrazyl; FRAP, ferric reducing antioxidant power; IC_{50}, the half maximal inhibitory concentration; EC_{50}, the half maximal effective concentration; MECS, methanol extract of *Costus speciosus*.

Analgesic activity

Acetic acid-induced writhing is one of the well-known methods for assessing the effectiveness of analgesic drugs. *C. speciosus* induces dose-dependent analgesic activity in the acetic acid-induced writhing test after the oral administration of methanolic rhizome extract, and the values are shown in Table 2. The control group of mice cause 47.83 ± 2.90 writhes after injection of acetic acid. However, treatment with *C. Speciosus* rhizome methanolic extract at both doses of 200 mg/kg and 400 mg/kg decreased the number of writhes of 32.17 ± 3.95 (32.74% inhibition) and 23.50 ± 3.55 (50.87% inhibition), respectively. The results demonstrated that the plant extracts exhibited dose-dependent analgesic activity. On the other hand, 58.88% inhibition was observed for the standard.

Table 2. Effect of methanol extract of *C. speciosus* on the number of writhing responses in acetic acid induced writhing test.

Treatment	Number of writhing	% of inhibition
Control (DW) (10 mg/kg)	47.83 ± 2.892	-
Diclofenac (100 mg/kg)	19.67 ± 2.974***	58.88
MECS (200 mg/kg)	32.17 ± 3.945*	32.74
MECS (400 mg/kg)	23.50 ± 3.547***	50.87

Values are expressed as Mean±SEM (n = 6). $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.05$ (*) as compared to control (one-way ANOVA followed by Bonferroni's test). Note: MECS, methanol extract of *Costus speciosus*; DW, distilled water.

Table 3 represents the formalin-induced licking test results in mice of the rhizome extract. Animals administered methanol extract of rhizome of *C. speciosus* demonstrated a decrease in paw-licking time compared to the control group at both phases. The early phase results showed that methanolic rhizome extract induced only a mild inhibitory effect (20.51% and 31.96%, respectively) determined from the licking response at a dose of 200 mg/kg and 400 mg/kg. However, in the late phase (25-35 min), methanolic rhizome extract at a 200 mg/kg and 400 mg/kg dose showed remarkable analgesic activity (59.07% and 62.69%, respectively). Methanolic extract from aerial parts of *C. speciosus* has been reported to produce anti-inflammatory, analgesic and antipyretic activity in experimental animal models at doses of 400 and 800 mg/kg body

weight. The analgesic activity of *C. speciosus* rhizome may be due to the presence of various bioactive compounds. Diosgenin isolated from *C. speciosus* rhizome inhibited the upregulation of various pro-inflammatory cytokines, including interleukin (IL)-1β, IL-2, and tumour necrosis factor-a (TNF-a). Curcumin is responsible for inhibiting the pain associated mediators of prostaglandins, cyclooxygenase-2, leukotrienes, lipoxygenase, phospholipase, and nitric oxide). Previous studies have shown that microinjection of abscisic acid (5, 10, 15 µg/rat i.c.v) caused analgésic effect by activating PPAR β/α(peroxisome-proliferating-activated receptor β/τ) which stimulates IL-1 β, TNFα, chemo attractant protein-1 monocyte (MCP-1) generation. The presence of beta-sitosterol in rhizome extract might be responsible for the analgesic activity [19].

Table 3. Effect of methanol extract of *C. speciosus* on percent inhibition in hind paw licking test.

Treatment	Percent inhibition of licking (PIL)	
	Early phase (0-5 min)	Late phase (25-35 min)
Diclofenac (100 mg/kg)	45.61	86.94
MECS (200 mg/kg)	20.51	59.07
MECS (400 mg/kg)	31.96	62.69

Values are expressed as Mean±SEM (n = 6); MECS represents methanol extract of *Costus speciosus*.

Diuretic activity

The oral administration of rhizome extract increased the urine flow when compared with control (Table 4). The diuretic index values of rhizome extract at a dose of 200 mg/kg and 400 mg/kg were 3.25, 1.25, respectively, indicating a weak diuretic activity of the extract compared with furosemide standard. Lijuan *et al.* reported that 10 mg/100 mL rhizome extract of *C. speciosus* stimulates urination properties in rats [20].

Table 4. Diuretic activity of methanolic extract of rhizome of *C. speciosus.*

Treatment	Mean amount of urine volume (mL)	Diuretic index	Diuretic activity
Control (DW) (10 mL/kg)	0.1 ± 0.07	-	-
Furosemide (10 mg/kg)	2.05 ± 0.08***	20.5	-
MECS (200 mg/kg)	0.325 ± 0.14	3.25	0.159
MECS (400 mg/kg)	0.125 ± 0.08	1.25	0.061

Values are expressed as Mean±SEM (n = 6). $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.05$ (*) as compared to control (One-way ANOVA followed by Bonferroni's test). Note: MECS, methanol extract of *Costus speciosus*; DW, distilled water.

Molecular docking

A molecular docking study of 16 chemical compounds isolated from *C. speciosus* rhizome was performed by Autodock tools 1.5.6 and MGL 1.5.6 tools package and BIOVIA Discovery Studio (version 4.5) to obtain their binding affinities and to predict non-covalent interaction against the COX1 and COX2.To validate the entire docking procedure, ibuprofen was redocked into the COX1 active sites andSC-558 into COX2 active sites (Score-7.6 and-9.1, respectively). Binding with ARG513 amino acid residue indicates high selectivity of COX2 inhibitors. On the other hand, the formation of the salt bridge between ARG120 is responsible for the non-selectivity of COX1. Binding interactions might explain the difference in activities of selected ligands on COX1 and COX2 [21].The docking scores of the selected ligands on COX2 ranged from -5.4 to 12 (Table 5). Curcumin, beta-sitosterol, tocopherols, Dioscin, gracillin, and prosapogeninA formed hydrophobic interaction with ARG513, whereas curcumin formed electrostatic interaction with

ARG513 in the COX2 active site (responsible for high selectivity) (Table 6). Nevertheless, dioscin, gracillin and prosapogenin A showed a very low binding affinity with COX2. However, curcumin, tocopherols and beta-sitosterol showed minimum energy score, which was -8.4, -8 and -7.8, respectively. Cycloartenol, stigmasterol, and beta-sitosterol formed a hydrogen bond with GLN350 and hydrophobic interaction with HIS90. A hydrogen bond with GLN350 was formed by Sapogenin but showed no hydrophobic interaction with HIS90. Triacontanoic acid and triacontanol formed hydrogen bonds with HIS90 and GLN192 and showed hydrophobic interaction with VAL349. Diosgenin, lupeol and β amyrin showed hydrophobic interaction with HIS351, whereas octacosanoic acid formed hydrophobic interaction with VAL349. None of the compounds showed interaction with ARG120 (responsible for non-selectivity) (Figure 1).

Table 5. Binding affinity of compounds isolated from *C. speciosus* rhizome with crystal structure of COX1 (1EQG) and COX2 (1CX2).

Drug candidates	Binding affinity (kcal/mol) 1EQG	Drug candidates	Binding affinity (kcal/mol)1CX2
Curcumin	-8.4	SC-558	-9.1
Abscisic acid	-7.9	Curcumin	-8.4
Tocopherols	-7.5	Tocopherols	-8.0
Ibuprofen	-7.4	Beta Sitosterol	-7.8
Triacontanoic acid	-6.8	Ibuprofen	-7.6
Octacosanoic acid	-6.6	Stigmasterol	-7.5
Triacontanol	-6.3	Sapogenin	-7.5
Stigmasterol	-5.8	Diosgenin	-7.5
Beta Sitosterol	-5.5	β amyrin	-7.5
SC-558	-5.3	Cycloartenol	-7.1
Sapogenin	-4.0	Triacontanol	-6.9
Cycloartenol	-3.1	Lupeol	-6.9
Diosgenin	2.6	Triacontanoic acid	-6.6
Lupeol	8.4	Octacosanoic acid	-6.5
β amyrin	9.1	Abscisic acid	-5.4
Gracillin	23.5	Gracillin	7
Prosapogenin A	23.6	Prosapogenin A	11.2
Dioscin	30.9	Dioscin	12

Table 6. Types of interactions for the complexes formed from compounds isolated from *C. speciosus* rhizome at the active site of both COX1 and COX2.

Drug candidate	Types of interactions	
	COX-1	COX-2
Ibuprofen	One hydrophobic interaction with ARG120 (5.09 Å) One hydrophobic interaction with TYR355 (5.24 Å)	One hydrophobic interaction with TYR355 (5.00 Å)
SC-558	Two hydrophobic interactions with ARG120 (4.55 and 5.43 Å) One electrostatic interaction with ARG120 (4.27 Å)	Three hydrogen bonding with ARG120 (1.91, 2.23 and 2.41 Å) Three hydrogen bonding with ARG513 (2.34, 2.57 and 2.86 Å)
Cycloartenol	One hydrophobic interaction with TYR355 (4.29 Å) One hydrophobic interaction with VAL349 (4.24 Å)	One hydrogen bonding with GLN350 (2.69 Å) Two hydrophobic interactions with PRO514 (4.44 and 4.81 Å) One hydrogen bonding with HIS90 (4.89 Å)
Abscisic acid	One hydrogen bonding with ARG120 (2.87 Å)	One hydrogen bonding with TYR355 (2.46)

Drug candidate	Types of interactions	
	COX-1	COX-2
	One hydrophobic interaction with TYR355 (4.77 Å)	Two hydrophobic interactions with SER353 (2.83 and 2.88 Å)
Beta Sitosterol	Two hydrophobic interactions with TYR355 (4.62 and 4.56 Å)	One hydrophobic interaction with ARG513 (4.71 Å) Two hydrophobic interactions with HIS90 (4.45 and 5.31 Å)
Curcumin	Two hydrogen bonding with ARG120 (2.41 and 2.07 Å) One hydrogen bonding with TYR385 (2.42 Å)	One hydrogen bonding with SER353 (2.21) One electrostatic interaction with ARG513 (4.76 Å)
Diosgenin	One hydrogen bonding with TYR355 (2.96 Å) One hydrophobic interaction with ARG120 (4.12 Å) One hydrophobic interaction with TYR355 (4.52 Å)	Two hydrophobic interactions with PRO514 (4.63 and 5.33 Å)
Lupeol	One hydrophobic interaction with TYR355 (3.89 Å) One hydrophobic interaction with ARG120 (3.38 Å)	One hydrophobic interaction with HIS351 (4.44 Å) One hydrophobic interaction with HIS356 (5.03 Å)
Octacosanoic acid	Two hydrogen bonding with GLY526 (2.53 and 2.99 Å) One hydrogen bonding with SER530 (3.07 Å)	One hydrophobic interaction with VAL349 (3.85 Å) One hydrophobic interaction with LEU359 (4.83)
Sapogenin	Two hydrophobic interactions with TYR355 (4.57 and 5.32 Å) One hydrophobic interaction with ALA527 (3.20 Å) One hydrophobic interaction with TYR355 (3.28 Å)	One hydrogen bonding with GLN350 (2.64 Å) One hydrogen bonding with HIS351 (2.66 Å) Two hydrogen bonding with HIS351 (4.03 and 5.16 Å)
Stigmasterol	Two hydrophobic interactions with TYR355 (5.29 and 4.68 Å) One hydrophobic interaction with ARG120 (4.98 Å)	Two hydrophobic interactions with HIS90 (4.79 and 5.18 Å)
Tocopherols	One hydrophobic interaction with TYR355 (4.45 Å)	One hydrophobic interaction with ARG513 (4.96 Å) One hydrophobic interaction with HIS90 (4.49 Å)
Triacontanoic acid	One hydrogen bonding with HIS90 (2.09 Å) One hydrogen bonding with TYR355 (2.09 Å)	One hydrogen bonding with HIS90 (2.33 Å) One hydrophobic interaction with TYR355 (4.48 Å)
Triacontanol	One hydrophobic interaction with ILE89 (4.18 Å)	Two hydrogen bonding with GLN192 (2.64 and 2.18 Å)
β amyrin	One hydrophobic interaction with ARG120 (4.67 Å)	One hydrophobic interaction with HIS351 (4.62 Å)
Dioscin	One hydrogen bonding with ARG120 (2.85 Å) One hydrophobic interaction with ARG120 (4.61 Å) Two hydrophobic interactions with TYR355 (5.39 and 4.30 Å) One hydrophobic interaction with HIS90 (5.01 Å)	One hydrogen bonding with GLN192 (2.78 Å) One hydrogen bonding with TYR355 (2.72 Å) One hydrophobic interaction with ARG513 (5.18 Å) One hydrophobic interaction with HIS90 (3.21 Å)

Drug candidate	Types of interactions	
	COX-1	COX-2
Gracillin	One hydrogen bonding with TYR355 (1.55 Å) One hydrogen bonding with SER353 (1.78 Å) Two hydrophobic interactions with HIS90 (4.79 and 5.05 Å) Two hydrophobic interactions with TYR355 (5.29 and 4.10 Å)	One hydrogen bonding with ARG513 (2.24 Å) One hydrophobic interaction with ARG513 (5.44 Å) One hydrogen bonding with TYR355 (2.55 Å) One hydrophobic interaction with TYR355 (5.24 Å) One hydrogen bonding with HIS90 (2.82 Å) One hydrophobic interaction with HIS90 (4.32 Å)
Prosapogenin A	Two hydrophobic interactions with TYR355 (5.38 and 4.88 Å)	One hydrogen bonding with GLN192 (2.09 Å) One hydrophobic interaction with ARG513 (4.31 Å) One hydrophobic interaction with HIS90 (4.25 Å) One hydrophobic interaction with TYR355 (5.04 Å)

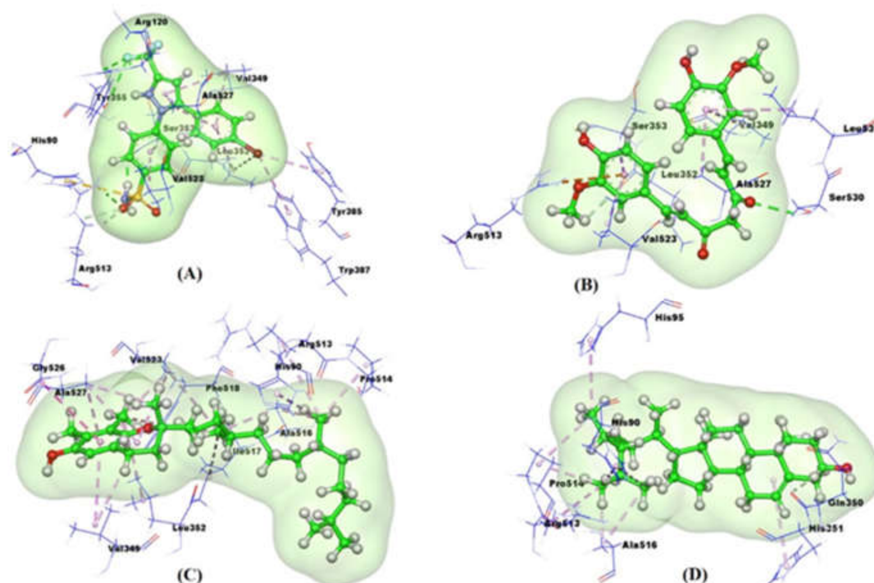

Figure 1. 3D structure of 1CX2 interaction with selected ligands. (A) SC-558, (B) curcumin, (C) tocopherols and (D) beta sitosterol.

The docking score COX1 with the selected ligands ranged from -3.1 to 30.9 (Table 6). The docking results of COX1 with the compounds revealed that abscisic acid, curcumin, cycloartenol, dioscin, diosgenin and lupeol formed hydrogen bond with ARG120 residue (binding with ARG120 is responsible for non-selectivity) but formed hydrophobic interaction with VAL116 (Table 6). Dioscin and diosgenin showed a very poor binding affinity with COX1 enzyme. Triacontanoic acid formed a hydrogen bond withTYR355 andHIS90 amino acid residue. Sapogenin, stigmasterol, tocopherols and beta-sitosterol was found to show hydrophobic

interaction with VAL116 and TYR355. Octacosanoic acid, beta amyrin and triacontanol formed hydrogen bond with SER530. Gracillin formed a hydrogen bond with TYR 355 and prosapogenin formed two hydrogen bonds with HIS90. It has been reported that diosgenin isolated from *C. speciosus* rhizome is recognized as an excellent anti-inflammatory agent [22]. Our study showed that diosgenin non-selectively binds with ARG120 in COX1 and could be an outstanding candidate for anti-inflammatory drug design (Fig. 2a and 2b).

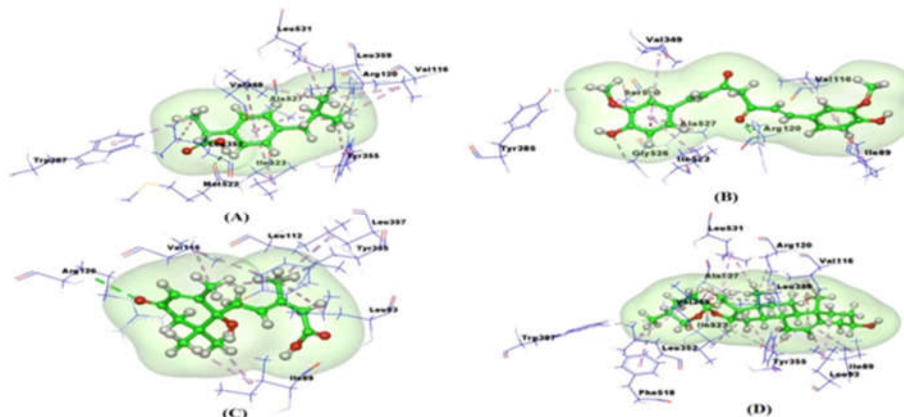

Figure 2a. 3D structure of 1EQG interaction with selected ligands (A) ibuprofen, (B) curcumin, (C) abscisic acid and (D) diosgenin.

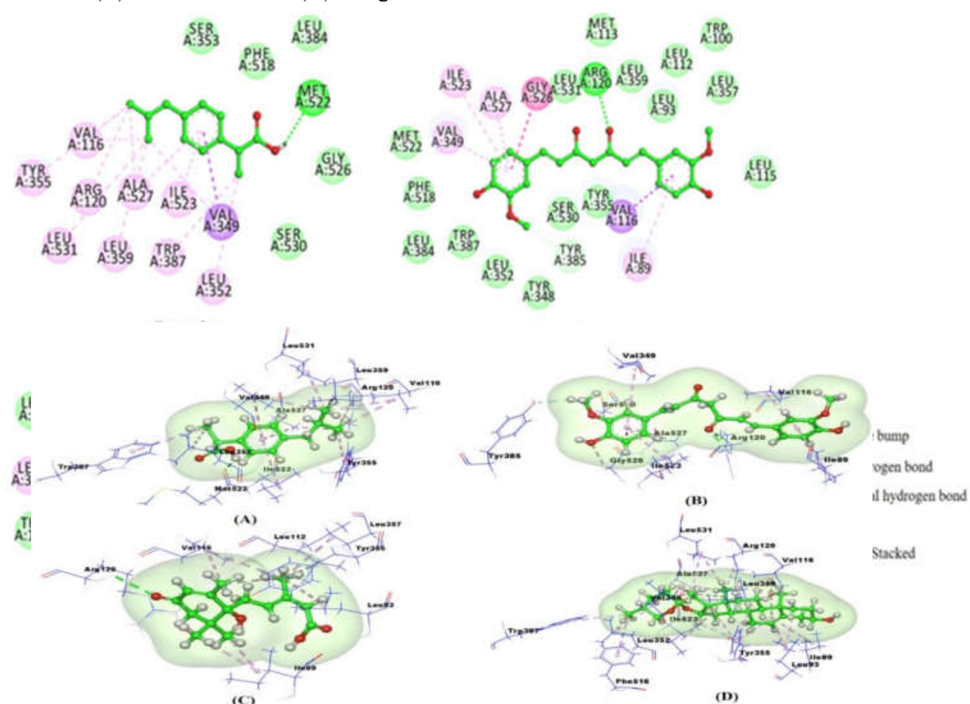

Figure 2b. Non-covalent interactions of selected compounds with 1EQG protein.

EXPERIMENTAL

Collection, identification and preparation of methanolic rhizome extract rhizomes of C. speciosus were gathered from Chandranath hill near Sitakunda in Chittagong, Bangladesh and subsequently Bangladesh national herbarium, Dhaka, Bangladesh identified the plant part (Accession number: DACB 43651). For extract preparation, briefly, undesired plant parts were separated from *C. speciosus* rhizomes and then washed thoroughly with fresh water. Rhizomes were then air-dried under shade for four weeks, pulverized and amassed in a clean flat bottom glass container. Total 400 g of grounded *C. speciosus* rhizome was macerated in methanol for fifteen days, and after that, using filter cloth and No. 1 Whatman filter paper, the solution was filtered. Then, with a rotary evaporator (RE-EV311-V, LabTech S.R.L, Italy), the filtered solution was condensed into dryness, followed by room drying and a water bath. The resulting brownish gluey concentrate was used as a crude methanol extract of *C. speciosus* rhizome throughout the experiment.

Chemicals and drugs

Folin-Ciocalteu reagent, methanol and formalin were imported from Merck KGaA, Darmstadt, Germany. Aluminum chloride ($AlCl_3$), sodium carbonate, sodium acetate and potassium acetate were imported from Merck (India) Limited. Quercetin, gallic acid, DPPH and acetic acid were purchased from Sigma Chemicals, St. Louis, USA. Diclofenac sodium, furosemide and physiological saline IV were provided by Square Pharmaceuticals Ltd., Dhaka, Bangladesh (0.9 percent NaCl). For the experimental purpose, all other reagents used were of analytical grade and pure.

Test animals

The central animal house of the Department of Pharmacy, Jahangir Nagar University, Dhaka, Bangladesh was provided young and healthy Swiss-albino mice (18-25 g) of either sex for the experiment. Experimental animals were housed in plastic cages (40 cm x 30 cm x 17 cm) at standard laboratory conditions and acclimatized at naturally illuminated ambient of half/half-light-dark cycle. During the experimental phase, water and meals were available ad libitum. The Institutional Ethics Committee of Noakhali Science and Technology University, Bangladesh, has approved all animal procedures and guidelines for conducting animal experiments.

Preliminary phytochemical screening

Qualitative phytochemical screening of the extract was done to identify alkaloids, tannins, saponins and cardiac glycosides [23]. Mayer's, Wagner's and Dragendroff's tests were accomplished to detect the presence of alkaloids. The frothing, ferric chloride and Balget test were conducted to confirm saponins, tannins and cardiac glycosides.

Determination of the amount of phenolic compounds

Total phenolic content

The modified Folin-Ciocalteu method was used to discern the total phenolic content in rhizome extract [24]. 1 mL of *C. speciosus* rhizome extract (1 mg/mL) was blended with 5 mL of the Folin-Ciocalteu reagent (10 times diluted by distilled water) and after that 4 mL of sodium carbonate (7.5% w/v) was added. The resultant was vortexed to mix well and then incubated in darkness for 30 minutes at room temperature. Using a UV-spectrophotometer, the absorbance of the mixture

was recorded at 765 nm. The result was evolved as mg gallic acid equivalent per gram of dry weight of the extract (mg GAE/g), calculated by extrapolating the standard calibration curve of gallic acid standards (0 to 300 mg/L).

Total flavonoid content

Total flavonoid content of CS rhizome was ascertained spectrophotometrically as the method stated by Lin and Tang [28]. In a test tube, 1.5 mL of 95% methanol, 0.1 mL of 10% aluminium chloride hexahydrate, 0.1 mL of 1 M potassium acetate and 2.8 mL distilled water were mixed, and then 1 mL of 1 mg/mL rhizome extract was added with this mixture. The absorbance was read at 415 nm after 30 min of the incubation period. The total flavonoid concentration was deduced from the standard curve and measured in mg of quercetin equivalents per gram of dry weight of the extract (mg QE/g).

Total flavonol content

Kumaran and Karunakaran's method was applied to determine the total flavonol content of the extract [29]. 1 mL of $AlCl_3$ (aluminium chloride) and 3 mL of sodium acetate solution were mixed. After that, 1 mL crude extract of 1 mg/mL was added and then incubated for 2.5 hours at room temperature. The absorption was measured at 440 nm, and the total flavonol content was estimated from the standard curve, and the result was evolved as mg of quercetin equivalent per gram of dry extract (mg QE/g).

DPPH radical scavenging assay

DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay was conducted as the modified method explained by Cavar [30]. In methanol, 0.1 mM DPPH solution was prepared, and then 0.1 mL rhizome extract of each concentration was mixed with 3 mL of 0.1 mM freshly prepared DPPH solution. The solution was left in the dark for 30 min at room temperature, and the absorption was estimated at 517 nm utilizing a spectrophotometer against the corresponding blank. All determination was done in triplicate, and the following formula estimated the scavenging activity or percentage inhibition of the DPPH radical.

$$\% \text{ Radical scavenging capacity} = \frac{(A_0 - A_t)}{A_0} \times 100$$

where A_0 = absorbance value of blank sample; A_t = absorbance value of the test samples. The concentration of extract responsible for 50% inhibition of DPPH radical is calculated as IC_{50} value, and the lower IC_{50} value indicates a potent antioxidant.

Ferric reducing antioxidant power (FRAP)

The ferric ion reducing ability of the CS rhizome extract was assessed as the method described by Shahid-Ud-Daula with some modifications [25]. 100 mL sodium acetate buffer (300 mM, pH 3.6), 10 mL of TPTZ (2,4,6-tripyridyl-s-triazine) solution (10 mM TPTZ in 40 mM hydrochloric acid),and 10 mL ferric chloride heptahydrate (20 mM) were mixed to prepare FRAP reagent. Before use, the freshly prepared FRAP reagent was heated at 37 °C. Then 3 mL of FRAP reagent was added to 0.3 mL of *C. speciosus* rhizome extract at different concentrations (5-500 µg/mL).Next, the mixture solution was incubated at room temperature for 5 min, and the reaction mixture absorbance was reported at 593 nm. Then, the EC_{50} value was discerned, and the result was compared with standard ascorbic acid at the same concentration.

Acetic acid-induced writhing test

The method explained by Ibrahim was used to perform acetic acid-induced writhing assay with minor modifications. Twenty-four healthy Swiss albino mice, with six mice in each group, were divided into four groups and fasted for 18 hours prior to the beginning of the experiment. The control group t group of mice was provided with distilled water (20 mL/kg) as the control group; the second group of mice has received 100 mg/kg diclofenac as a standard group, the next two groups were given 200 mg/kg and 400 mg/kg of methanolic extract, respectively. Thirty minutes after the treatment, acetic acid solution (0.6%, 0.1 mL/10 g) was administered intra peritoneally to all mice to induce the characteristic writhing. Again 5 min of interspace was given to confirm the proper absorption, and the number of writhes was enumerated for 15 min.

Formalin-induced licking test

The analgesic activity of the rhizome extract was also determined by a formalin-induced hind paw licking test according to the procedure explained by Dubuission and Dennis, with some modifications [26]. Twenty-four mice were separated into four groups of six mice per group and kept in an interval of 18hr fasting condition. The first group of mice was received distilled water 20 ml/kg as the control group; the second group of mice was given 100 mg/kg diclofenac as the standard group. 1 h prior to formalin injection, group three and four were given 200 mg/kg and 400 mg/kg of methanolic extract, respectively. 0.02 mL of 2.5% formalin was given subcutaneously to all mice plain rear of the left hind paw. The time used in leaking and biting was read as the early answer during the first five minutes. After that, during the last 25-35 min, the time used in laying and biting was read as the late response.

In vivo diuretic activity test

Diuretic activity of the *C. speciosus* rhizome extract was determined by a previously described method [33]. In this experiment, mice were divided randomly into four groups, each having four mice samples. Mice were given physiological saline (0.9% NaCl) at an oral dose of 1 mL/100 g body weight to ascribe uniform salt and water load after experiencing overnight fasting condition. The first group of mice was given distilled water of 0.1 mL/10 g as standard; the second group of mice received 10 mg/kg of furosemide as standard; the next two groups received 200 mg/kg and 400 mg/kg of methanolic extract, respectively. After immediate administration, the experimental mice were individually housed in a metabolic cage and placed in a beaker to collect urine. Urine was then collected for 6 hours after dosing and appropriately measured. No food or water was supplied to the animals during this time of the experiment.

Molecular docking study

Protein preparation

A molecular docking analysis has been performed at both the COX1 and COX2 active sites in order to explain all potential binding interactions of chemical compounds isolated from the *C. speciosus* rhizome. For this study, we chose the best known COX2 inhibitor SC-558 crystallized COX2 protein (PDBID: 1CX2) and previously used COX1 (PDBID: 1EQG) enzyme. Both PDB structures had been acquired from the online protein databank (www.rcsb.org). A swiss-PDB viewer software package (version 4.1.0) was used to check and optimize both the crystal structure of COX1 and COX2 based on their least energy. Repeated chains, heteroatoms, co-crystallized ligand, undesired surfactants, and water molecules were erased with the help of the PyMol molecular graphics system (version 2 Schrodinger, LLC) [27].

Ligand preparation

3D structure of 16 chemical compounds of *C. speciosus* rhizome was collected from Chemspider and Pubchem database. UCSF Chimera software package (version 1.14) was used to optimize the standard drugs and other ligands via AMBER 14 force field. The selective COX inhibitor SC-558 and non-selective COX inhibitor ibuprofen were used as reference drugs. SC-558 and ibuprofen were redocked to validate the docking method.

Molecular docking protocols

COX1 and COX2 proteins were docked with 16 chemical compounds isolated from *C. speciosus* rhizome by Autodock Vina protocol by Autodock tools 1.5.6 and MGL 1.5.6 tools package [28]. The possible binding affinity and non-covalent interaction of the selected COX1 and COX2 enzymes were predicted by molecular docking via the Autodock Vina protocols. The docking grid box covering the desired binding site residues was set around the COX1 and COX2 active sites. For the visualization and identification of the non-covalent interaction of the docked protein-ligand complex, PyMol (version 2) and BIOVIA Discovery Studio (version 4.5) were utilized.

CONCLUSION

This study confirmed the presence of secondary metabolites like tannins, alkaloids, cardiac glycosides and saponins. A considerable amount of phenolic and flavonol contents was determined in the rhizome extract. The docking study confirmed the analgesic activity of the rhizome extract of *C. speciosus.* Curcumin showed excellent binding interaction by binding with selective ARG513 and non-selective ARG120 amino acid residue on COX2 and COX1. From The findings of present study, it can be concluded that the biological activity of the *C. speciosus* rhizome extract could be a safe alternative to the synthetic NSAIDs.

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