Original Article

In vitro anti-inflammatory and antioxidant activity of *Nephrolepis cordifolia* and molecular docking of its active chemical constituents

Rohit Pal[#], Ghanshyam Teli[#], Bharti Sharma, Bhupinder Kumar, Pooja A Chawla*

Department of Pharmaceutical Chemistry, ISF College of Pharmacy, Moga, Punjab, India

[#]First two authors contributed equally to the research

Correspondence:
Pooja A Chawla, Department of Pharmaceutical Chemistry, ISF College of Pharmacy, Moga, Punjab, India. E-mail: pvchawla@gmail.com

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ABSTRACT

Nephrolepis cordifolia is a fern native to northern Australia and Asia. It has many common names including fishbone fern, tuberous sword fern, tuber ladder fern, erect sword fern, narrow sword fern and ladder fern, and herringbone fern. N. cordifolia is a wood fern that typically grows in woodland areas. Both fertile and sterile fronds are pinnate, up to 3 feet in length and 3 inches wide. There are many leaflets, or pinnae, ranging from 40 to 100 mm (1.5 to 4 inches) on each side of the rachis. Each pinna is oblong to lanceolate with an auricle that overlaps rachis. Rhizomes are orange/brown to pale brown with linear scales having hair like tips. Stolon is straw colored and produces small underground tubers. The presence of tubers distinguishes sword fern from the native N. cordifolia fern. The aim of the present study was to evaluate the antioxidant and anti-inflammatory activity of ethanolic extract of leaves of *N. cordifolia*. The active chemical constituents were docked to establish the possible mechanism of action.

Keywords: Anti-inflammatory, antioxidant, ethanolic extract, fern, *Nephrolepis cordifolia*

INTRODUCTION

Inflammation can be defined as a generalized, nonspecific but beneficial response of tissues to injury. It comprises a complex array of adaptive responses to tissue injury which are both local and systemic. The local responses result in recruitment of phagocytic cells and removal of endogenous or foreign material. The systemic responses may alter the "milieu interior" to allow these processes to occur more efficiently.[1,2] The cellular processes of inflammation fall into four major groups: Changes in blood flow caused by changes in smooth muscle cell function causing vasodilatation, alterations in vascular permeability engendered by cytoskeletal contraction in endothelial cells, migration of phagocytic leukocytes to the site of inflammation, and phagocytosis.[3] Natural products have long been recognized as an important source of therapeutically effective medicines. Of the 520 new drugs approved between 1983% and 1994, 39% were natural products or derived from natural products and 60–80% of antibacterial and anticancer drugs were also derived from natural product. Plants offer a vast source of compounds that present different effects in human.[4]

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Nephrolepis cordifolia is a terrestrial fern which grows vigorously by forming colony with short rhizome and small scaly tubers at their roots. They are able to grow in different situation: as epiphytic and epilithic plant with fronds which are normally 16–32 inches long and 4 inches wide and appear as bright green color. It can spread with the help of spores, stolons, tuber, and rhizomes and are commonly known as erect sword fern, lemon butter fern, ladder fern, and fish bone fern.[5] The different parts of *N. cordifolia* such as leaf, rhizome, and rachis are covered very densely by epidermal glands. These epidermal glands contain various phytochemical substances such as phenolic acids, flavonoids, glycosides, and alkaloids.[4,6] Due to presence of this substance, they show several pharmacological activities and they are found to be less soluble in water and highly soluble in organic solvents such as ethanol, acetone, and methanol. According to the phytochemical screening of *N. cordifolia,* extract components such as

reducing sugar, tannins, and cardiac glycosides are highly present.[4] In one of the experiment six different types of compounds were extracted with the help of *N. cordifolia* ethanol extract. All of the six isolated and identified compound are β-sitosterol, fern-9(11)-ene, myristic acid, oleanolic acid, triacontanol, and hentriacontanoic acid and they are isolated for the 1st time from this plant.[7] In one of the studies on chemical composition of essential oil of *N. cordifolia* it was found that nonanal (10.6%), β-ionone (8.0%), eugenol (7.2%), and anethol (4.6%) were present in very high concentration.[8]The current work was undertaken to study the anti-inflammatory and antioxidant activities of the leaves of N. cordifolia using human red blood cell (HRBC) method and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, respectively. Docking studies were performed to establish the probable MOA using AUTODOC VINA software.

MATERIALS AND METHODS

Collection of plant

The fresh leaves of the plant *N. cordifolia* were collected in the month of February to March from F.R.I (Forest Research Institute), Dehradun. The species was authenticated by in the Systemic Botany Discipline, Forest Research Institute, Dehradun. The fresh leaves of *N. cordifolia* were washed by running water and dried under the shade. The dried leaves were put in grinder and the powdered was obtained. The dried powdered was packed in the paper bags and stored in an airtight container until use.

Drugs and chemicals

Methanol AR (Thomas Baker Chemical Pvt. Ltd.), nitric acid, sodium hydroxide, HCl, conc. HNO_3, NH_4OH, $FeCl_3$, potassium dichromate, all other solvents were used during the experimental protocol was of analytical grade and procured from Merck-India.

Morphological study

The morphological study includes color, odor, taste, and shape were carried out on fresh leaves [Figure 1]. The result of the morphological features was determined and given in the Table 1.

Extraction process

The plant material (dried leaves), 10 g was taken with different solvents (Ethanol, Hydroalcoholic, and Aqueous) [Figure 2] and cold extraction method was used for extraction. After 24 h, the extract was properly dried by the use of the water bath. The dried extract was stored in the air tight container. The yield of different solvent extract is given in Table 2.

Phytochemical investigations

Test for carbohydrates

- Fehling's test: 1 ml of Fehling's solutions A and B was mixed and boil for 1 min. Equal volume of extract was added then it was heated in boiling water bath for 5–10 min, first a yellow, brick red ppt was observed
- Benedict's test: 2 ml of Benedict's reagent and test solution was mixed in test tube. It was heated in boiling water bath for 5 min. The solution was appeared to be green in color which indicates the presence of reducing sugar in test solution.

Test for monosaccharide

- Barford's test: Took equal volume of test solution and Barford's reagent. Kept for 1–2 min in boiling water bath and allowed to cool. Red ppt was obtained.

Test for hexose sugars

- Tollen's phloroglucinol test: Took 1–2 ml of test solution and add 3 ml of conc. HCl and 4 ml of 0.5% phloroglucinol, heated the mixture. Yellow to red color appeared.

Test for reducing polysaccharides (STARCH)

- Iodine test: To the 3 ml of test solution added few drops of dil. Iodine solution, appeared which was disappeared on boiling and reappears on cooling
- Tannic acid test: With test solution add 20% of tannic acid solution, gives yellow ppt.

Tests for alkaloids

Each extract was dissolved in HCl and filtrates were obtained. They were used for testing.

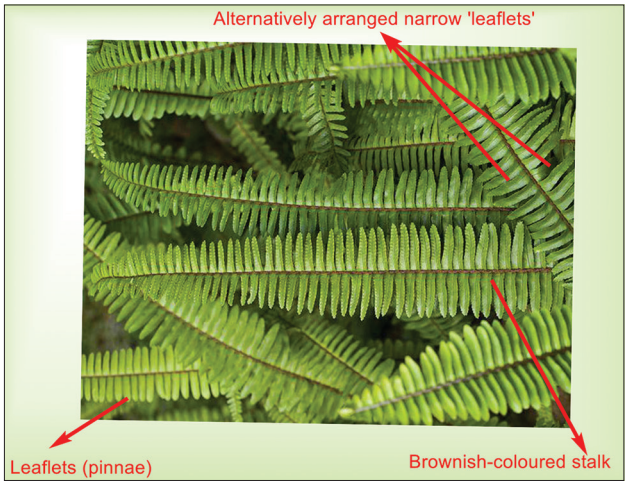

Figure 1: Morphological features of the leaves of *Nephrolepis cordifolia*

Table 1: Morphological features of the leaves of *Nephrolepis cordifolia*

Characteristic features	Observation
Color	Dull green to yellow green
Odor	Characteristic
Taste	Bitter to taste less
Shape	10–35 mm long and 4–11 mm wide

Table 2: Morphological features of the leaves of *Nephrolepis cordifolia*

S.NO.	Solvents	Weight of plant material (g)	Colors of extract	Weight of extract	Extractive value (%w/w)
1.	Ethanol	10	Dark green	1.08	10.8
2.	Hydroalcoholic	10	Light green	0.46	4.6
3.	Aqueous	10	Yellow green	0.85	8.5

- Mayer's test: Mayer's reagent (Potassium Mercuric Iodide) was treated with filtrates. The yellow color ppt is appeared which give indication ok presence of alkaloids
- Wagner's test: Filtrates were Taken and treated with Wagerer's reagent (solution of Potassium Bismuth Iodide). Brown/reddish ppt was formed that indicates the presence of alkaloids
- Dragendroff's test: Filtrates were Taken and treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Reddish ppt was obtained which indicates the detection on alkaloids
- Hager's test: Filtrates were Took and treated with Hager's reagent (saturated picric acid solution). Detection of alkaloids was confirmed by the formation of ppt of yellow colour.

Test for flavonoids

- Alkaline reagent test: The extracts was taken and treated with few drops of sodium hydroxide solution. Indication for flavonoids was detected by formation of yellow color and on addition of dilute acid it turns colorless
- Lead acetate test: Extracts were treated with few drops of lead acetate solution. Indicates the presences of flavonoids by the formations of yellow color precipitate
- Shinoda test: To the extract 95% of ethanol was added with few drops of conc. HCl and 0.5 g of magnesium turnings were also added. The presence of flavonoids was confirmed by observation of pink color.

Test for cardiac glycoside

- Legal's test (cardenoloids): Took the extract, added pyridine, and 1ml of nitroprusside. The confirmation of cardiac glycoside was indicated by appearance of pink to red color.

Test for tannins and phenols

- Ferric chloride test: To the extract solution few drops of 5% of $FeCl_3$ solution were added the presence of phenolic compounds was determined by dark green coloration
- Lead acetate test: To the extract solutions add few drops of 10% of lead acetate solution formation of white ppt indicate the presence of phenolic compounds.[6]
- Gelatine test: To the extract solution few drops of 10% gelatine were added which results the formation of white ppt, indication for the presences of phenolic compounds
- Bromine water test: To 2–3 ml of the extract solution added few drops of bromine water. The decoloration of water indicates the presence of phenolic compounds.
- Acetic acid test: To 2–3 ml of the extract solutions add few drops of acetic acid, solution becomes red in color which indicated the presence of phenolic compounds.

Test for proteins

- Biuret test (general's test): To 3 ml of the extract solution added few drops of 4% NaOH and few drops of 1% $CuSO_4$ solution. The appeared of pink or violet color indicated the presence of proteins
- Xanthoprotein test (for protein containing tyrosine or tryptophan): To 2–3 ml of the extract solution add 1ml of conc. H_2SO_4, white ppt appeared which turns yellow on boiling. Now added NH_4OH ppt turns orange[9]
- Test for proteins containing sulfur: To 2–3 ml of the extract solution added few drops of 40% NaOH and few drops of lead acetate solution (10%). Black or brownish color appeared due to PbS formation which confirmed presences protein.[10]

In vitro anti-inflammatory (hypotonicity-induced HRBC) membrane stabilization method)

The anti-inflammatory activity of leaf extract of Linn was determined by HRBC membrane stabilization method. Blood was collected from healthy volunteers. The collected blood was mixed with equal volume of (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride in water). The blood was centrifuged at 300 rpm and packed cells were washed with isosaline (0.85%, pH 7.2) and 10% v/v suspension was made with isosaline. The assay mixture contained the drug (concentration as mentioned in Table 1) 1 mL phosphate buffer (0.15 M, pH 7.4), and 2 mL of hyposaline (0.36%) % 0.5 mL of HRBC suspension. Diclofenac was used as the reference drug. Instead of hyposaline, 2 mL of distilled water was used as control. All the assay mixtures were collected at 37 for 30 min and centrifuged. The hemoglobin content in the supernatant solution was estimated using colorimeter at 560 nm. The percentage hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water as 100%.[9] The percentage of HRBC membrane stabilization or protection was calculated using the following formula.

Figure 2: Different extract of *Nephrolepis cordifolia* (a) hydroalcoholic, (b) aqueous, (c) ethanolic extract

% Protection = 1- (Optical density of test sample/Optical density of control) × 100

Antioxidant DPPH assay

The antioxidant activity of the extracts was determined using the DPPH free-radical scavenging assay. Briefly, the universal bottle was contained 50 µL of *N. cordifolia* extracts in concentrations from 1 to 5 mg/mL and 5 mL 0.004% (w/v) solution of DPPH was added. The obtained mixture was vortexed, incubated for 30 min at room temperature in a relatively dark place and then was read using spectrophotometer at 517 nm. The blank was 80% (v/v) methanol. Ascorbic acid (Vitamin C) was used for comparison.[11] Measurements were taken in triplicate. DPPH scavenging effect was calculated using the following equation:

DPPH scavenging effect (%) = $\{A_0\text{-}A/A_0\} \times 100$

where A_0 is the absorbance of negative control (0.004% DPPH solution) and A is the absorbance in presence of extract.

Computational studies of the major chemical constituents of the *N. cordifolia*

Scientists have reported the chemical composition of *N. cordifolia* using gas chromatography mass spectrometry analysis. Among all the chemical composition compounds β-sitosterol (**1**), fern-9(11)-ene (**2**), β-ionone (**3**), and benzyl butyl phthalate (**4**)[4,12] [Figure 3] were present in the major amount. Therefore, the constituents were subjected to computational studies such as ADMET and docking.

ADMET prediction

ADME-Toxicity (ADMET) was calculated using the ADMET "Descriptor" module of pre ADMET versus 2.0 software. The following six mathematical models such as aqueous solubility, blood-brain barrier penetration, cytochrome P450 2D6 (CYP2D6) inhibition, hepatotoxicity, human intestinal absorption, and plasma protein binding were used to predict ADMET properties. The study of ADMET properties is important because they determine oral bioavailability, cell permeation, metabolism, and elimination (Pharmacokinetic characteristics) of drug molecules.

Docking

Energy minimized structure of human COX (PDB:6Y3C) was used as a receptor for docking studies. PDB files of compounds **1**, **2**, **3,** and **4** were generated from Pymol. AutoDock vina 1.1.2 was used for conducting docking experiments of human COX with **1** and **2**. After ensuring that protein and ligands are in correct orientations for docking, the receptor-grid files were generated using grid parameter file program. Grid box was set to whole protein with dimensions of 50, 40, and 56 Å with a grid center of 0.375Å. The interactions of the ligands with protein were visualized with discovery studio and figures were formed using discovery studio.

RESULTS

Morphological features of the leaves of *N. cordifolia*

The lower part (i.e. stipes) of the leaves was n glossy brown elongated (i.e. linear-lanceolate) scales whereas, its upright "leaves" (i.e. fronds) have a brownish-colored stalk (i.e. stipe) up to 15 cm long. The stalk was divided into numerous alternatively arranged narrow "leaflets" (i.e. pinnae). These "leaflets" (usually 10-35 mm long and 4–11 mm wide, but rarely to 6 cm long) have irregularly and often finely scalloped (i.e. crenate or crenulate) margins and are usually hairless (i.e. glabrous). The different morphological features are listed in Table 1.

Extractive value of leaves of *N. cordifolia* (decoction method)

The extraction was performed through decoction (cold extraction) methods using three different solvents (ethanol, hydroalcoholic and

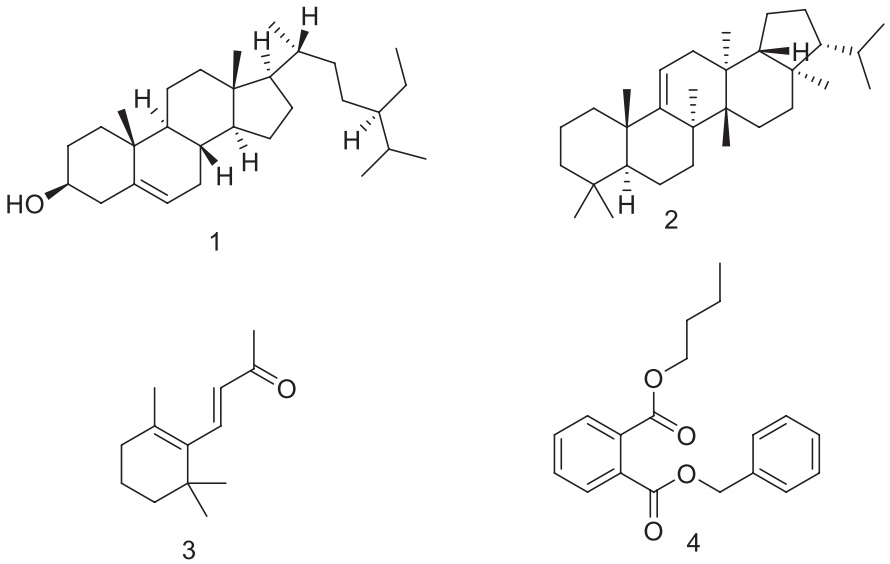

Figure 3: Active chemical constituents of *Nephrolepis cordifolia*

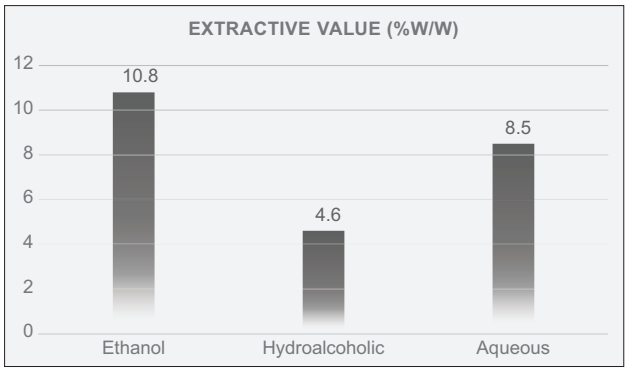

Figure 4: Graph showing the different extractive value of different solvent used for extraction

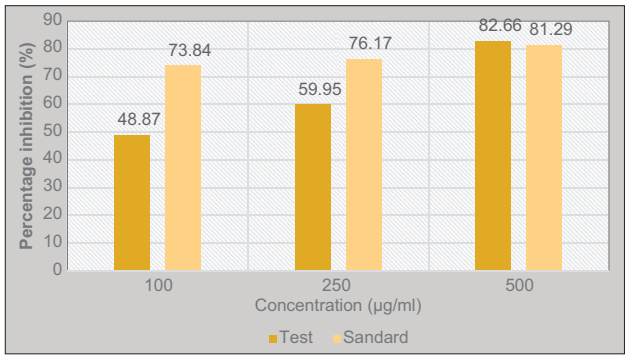

Figure 5: Graphical representation of anti-inflammatory activity human red blood cell method

Table 3: Phytochemical investigation of *Nephrolepis cordifolia*

Phytoconstituents	Method	Ethanolic extract	Aqueous extract	Hydro-alcoholic extract
Flavonoids	Shinoda test	+ve	+ve	+ve
	Lead acetate test	+ve	−ve	−ve
Alkaloids	Wagner test	+ve	+ve	+ve
	Hager's Test	+ve	+ve	+ve
	Dragendroff's test	−ve	+ve	+ve
	Mayer's test	+ve	+ve	−ve
Tannins and Phenols	Lead acetate test	+ve	+ve	+ve
	Bromine water test	+ve	+ve	+ve
	Acetic acid solution test	+ve	−ve	+ve
	Dil.potasium permanganate test	+ve	−ve	+ve
	Dil. Iodine solution test	+ve	+ve	−ve
	Dil. HNO_3 test	−ve	+ve	+ve
	Potassium dichromate test	+ve	+ve	−ve
Carbohydrates	Fehling's test	+ve	+ve	−ve
	Benedict's test	+ve	+ve	−ve
	Barfoed's test	+ve	+ve	−ve
	Tannic acid test	+ve	−ve	−ve
	Iodine test	−ve	+ve	+ve
	Tollen's test	−ve	+ve	+ve
Cardiac glycoside	Legal test	+ve	+ve	−ve
Proteins	Biuret test	+ve	+ve	+ve
	Xanthoprotein test	−ve	−ve	−ve
	Test for protein containing sulfur	+ve	−ve	−ve

aqueous). Among these solvents, ethanol yields greatest extractive value with 10.8 % w/w. The hydroalcoholic and aqueous extraction yields 4.6 and 8.5 % w/w extractive value. These are shown in Table 2 and Figure 4.

The phytochemical investigation result is tabulated in Table 3.

The different extracts of N. cordifolia leaves were used for the entablature of phytochemicals. The chemical tests such as flavonoids, alkaloids, tannins, phenols, carbohydrate etc were tested. The results for the different chemical test are given in the Table 3.

In vitro anti-inflammatory activity

N. cordifolia ethanolic extracts of leaves at different concentrations (100, 250, and 500 µg/mL) showed significant stabilization toward HRBC membrane. The percentage protection of ethanolic extract at concentration 250 and 500 µg/mL was higher than that the standard [Figure 5]. However, the percentage protection was found to be increased at higher concentrations. The results are tabulated in Table 4.

Antioxidant activity by DPPH radical scavenging method

The leaves extract of *N. cordifolia* plant showed antioxidant potential when compare to standard ascorbic acid by DPPH scavenging assay method tabulate in Table 5. The absorbance at 517 nm by UV visible spectrophotometer was found to be as 0.209 and 0.030 for the ethanolic extract and standard ascorbic acid, respectively [Figure 6].

Table 4: Results of *in vitro* anti-inflammatory activity (human red blood cell method)

Treatment	Concentration (µg/ml)	Absorbance at 660nm	Percentage inhibition (%)
Control	--	1.154	--
Ethanolic extract	100	0.564	48.87
	250	0.274	59.95
	500	0.200	82.66
Diclofenac	100	0.302	73.84
	250	0.275	76.17
	500	0.217	81.29

Table 5: Results of antioxidant activity by 2,2-Diphenyl-1-picrylhydrazyl radical scavenging method

Treatment	Concentration (µg/ml)	Absorbance at 517 nm	Percentage inhibition (%)
Control	--	0.244	--
Ethanolic extract	5	0.242	0.99
	10	0.240	25.56
	15	0.237	39.55
	20	0.221	48.89
	25	0.218	59.53
	30	0.209	67.02
Standard (Ascorbic acid)	5	0.238	2.61
	10	0.171	29.66
	15	0.046	80.8
	20	0.041	83.01
	25	0.038	83.22
	30	0.030	84.02

Computational studies

ADMET prediction

The different parameters were calculated theoretically and tabulated in Table 6.

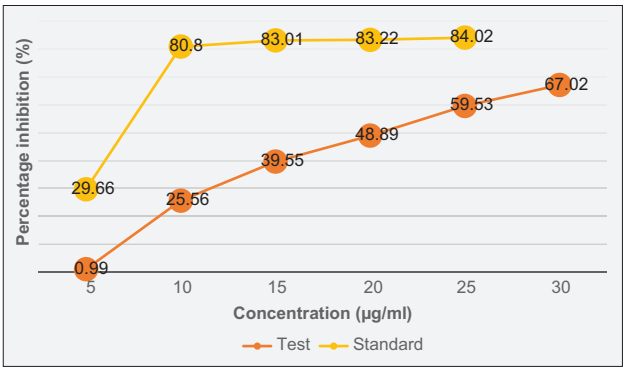

Figure 6: Graphical representation of results of antioxidant activity by 2,2-Diphenyl-1-picrylhydrazyl radical scavenging method

Docking

To establish the possible mechanism of *N. cordifolia*, molecular docking studies were carried out. The docking was performed on main targets of commonly used anti-inflammatory drug named as COX-1. For this purpose, compounds **1**, **2**, **3**, and **4** were selected because they are main chemical constituent and present in the abundant. The binding energy of the compounds **1**, **2**, **3**, and **4** and diclofenac sodium (standard) −8.2, −8.5, −5.6, −5.8, and −5.96 kcal/mol, respectively. Docking pose of compound **2** with COX-1 bound to the receptor in a pocket and formed various interactions such as Van der Waals (THR89, GLU524, ARG120, ARG83, GLY471, LEU123, GLN44, THR62, ASN122, ARG79, and THR76), alkyl (VAL119), and pi-alkyl (HIS43 and TYR64). Compound **1** fitted in the active site of COX-1 and showed interaction with various amino acid residues such as PHE99, LEU357, VAL119, ARG83, THR89, ARG120, PRO86, LEU112, LEU115, LEU93, LEU92, TRP100, VAL116, and PRO84. Diclofenac sodium was used as the standard and it showed hydrogen bonding interaction with ARG83, PRO84, and THR89 amino acid of COX-1. The 3D and 2D possess are presented in Figure 7 and 8, respectively.

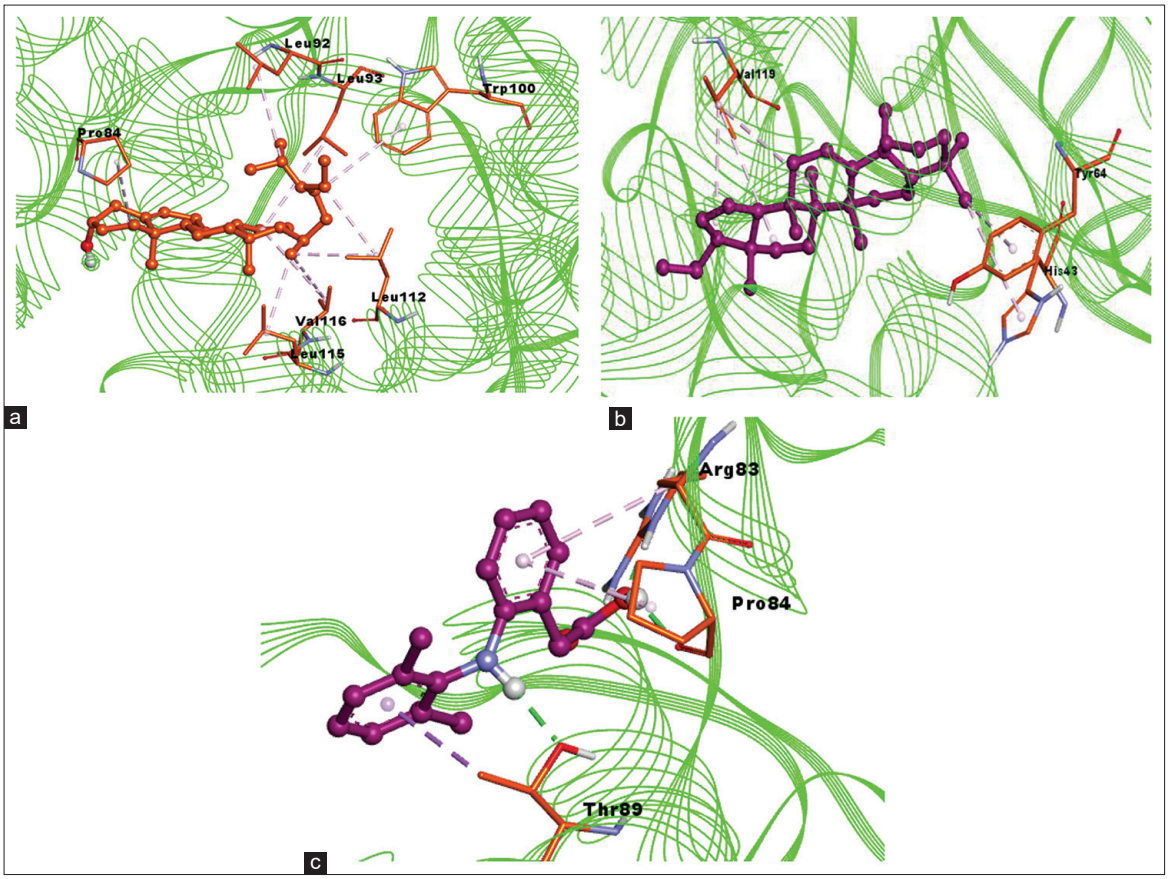

Figure 7: 3D poses of compounds with COX-1 (PDB ID: 6Y3C): (a) compound 1, (b) compound 2, and (c) Diclofenac sodium

Table 6: Theoretical ADMET parameters

Comp. code	Aqueous solubility	Blood-brain barrier penetration	CYP P450 2D6 inhibition	Hepatotoxicity	Skin Permeability	PP binding
1.	0.00145254	19.8883	Inhibitor	None	−0.593439	100.000000
2.	0.00014335	20.2017	Inhibitor	None	−1.72082	100.000000
3.	0.00014679	20.2067	Inhibitor	None	−1.67082	100.000000
4.	0.00014367	13.8765	Inhibitor	None	−1.45787	100.000000

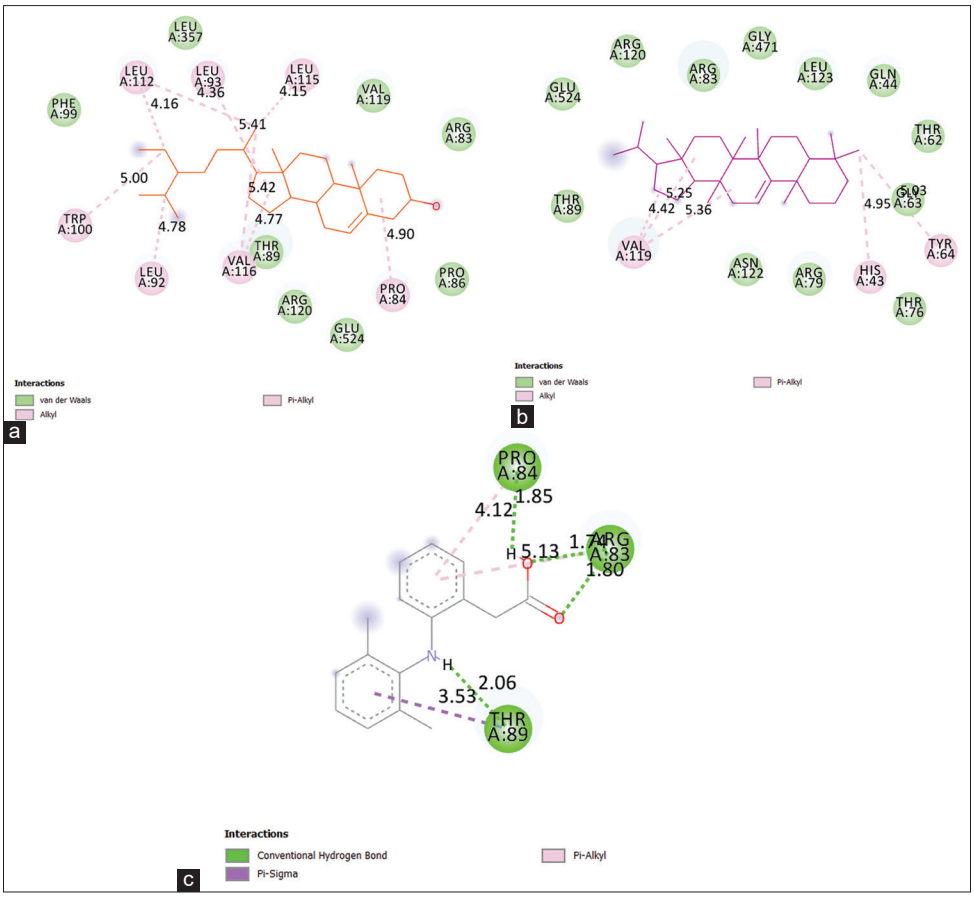

Figure 8: 2D poses of compounds with COX-1 (PDB ID: 6Y3C): (a) compound 1, (b) compound 2, and (c) diclofenac sodium

CONCLUSION

The replacement of synthetic with natural anti-inflammatory and antioxidant (because of implications for human health) agents may be advantageous. In the present study, the evaluation of anti-inflammatory and antioxidant activity was done using the HRBC and DPPH methods. The results showed that at the concentration of 250 and 500 μg/ml the ethanolic extract of the *N. cordifolia* leaves showed promising effect with 59.95 and 82.66% inhibition, respectively. In the antioxidant assay, the appreciable results were showed with 67.02% inhibition at the concentration of 30 μg/ml. **In** addition, the computational studies (ADMET and docking) were also performed on the active constituents of the *N. cordifolia* plants. As the results of docking studies, compound **2** was found to be more potent than diclofenac sodium with binding energy -8.5 and -5.96 kcal/mol, respectively.

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