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Research paper

An investigation on *in vitro* anti-inflammatory and antiproliferative potential of isolated Labdane diterpenoids from *Andrographis paniculata* (Burm. f.) wall. Ex nees: An important medicinal plant prescribed in Ayurveda

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ABSTRACT

Introduction: The inflammatory response is a highly regulated process, and its dysregulation can lead to the establishment of chronic inflammation and in some cases, to death. Diterpenoids have found to be an important class of phytochemicals possessing anti-inflammatory potential.

Methods: In present study, some Labdane diterpenoids isolated from *Andrographis paniculata* like, Andrographolide (compound **A**); 14-deoxy-11,12-didehydroandrographolide (compound **B**); 14-Deoxyandrographolide (compound **C**) and 3,14- Dideoxyandrographolide (compound **D**) were evaluated for their cyclooxygenase inhibitory, antioxidant and anti-proliferative activity using different cancer cell lines.

Results: The results obtained revealed that compound **C** has maximum inhibitory potential towards COX-2 (83.24%; IC_{50} 9.0 μg/ml). The docking data was found to be in accordance with the *in vitro* experimental results, with compound **C** demonstrating the promising binding free energy (-8.1 Kcal/mol). The anti-proliferative data showed that all compounds had significant cytotoxicity towards selected (HCT-15, INT- 407 and HeLa-B75) cancer cell lines, while compounds also demonstrated effective antioxidant potential. The antioxidant profile reveals that, the compound **C** possess most promising SOR scavenging activity (91.41 ± 1.56%), while the lowest activity was recorded from compound **D** (16.30 ± 0.25). The results obtained by *in silico* molecular docking study are in accordance with the *in vitro* activities.

Conclusion: The isolated compounds showing promising anti-inflammatory and antiproliferative activity could be considered for acceleration in research for development of diterpenoids based anti-inflammatory agents.

1. Introduction

Cyclooxygenase (COX, E.C.1.14.99.1) is the enzyme that catalyzes the rate-limiting step of the prostanoid cascade. COX enzymes mediate the heme dependent oxidation of arachidonic acid to prostaglandin H2 (PGH2) [1], which then serves as the source for a variety of synthases that convert it into a number of biologically active products, the prostanoids, which include the prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2) and thromboxane (TXA_2) Fig. 1. There are two distinct isoforms of COX: COX-1 and COX-2. COX-1 displays the characteristics of a housekeeping gene and is constitutively expressed in most tissues while COX-2 expression is barely detected at a constitutive level and is markedly inducible in specialized cell types. It was observed that, COX-1 plays an important role in production of prostaglandins involved in protection of the gastric mucosal layer and thromboxanes (TX) in platelets. However, COX-2 generally mediates elevations of prostaglandins associated with inflammation, pain and pyresis [2]. The human and animal studies clearly demonstrated the association of COX-2 in gastrointestinal carcinogenesis [3]. Over-expression of COX-2 results in the overproduction of prostanoids, which inhibits apoptosis and immune surveillance and increases cancer cell proliferation, tumor angiogenesis and invasiveness of malignant cells, thereby favoring malignant growth [4]. Compared with the normal squamous esophageal epithelium, Barrett's oesophageal metaplasia, dysplasia and adenocarcinomas show over-expression of COX-2, but not COX-1 [5].

Currently the non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen, naproxen and rofecoxib are widely used for the treatment of inflammation and inflammatory disorders [6].

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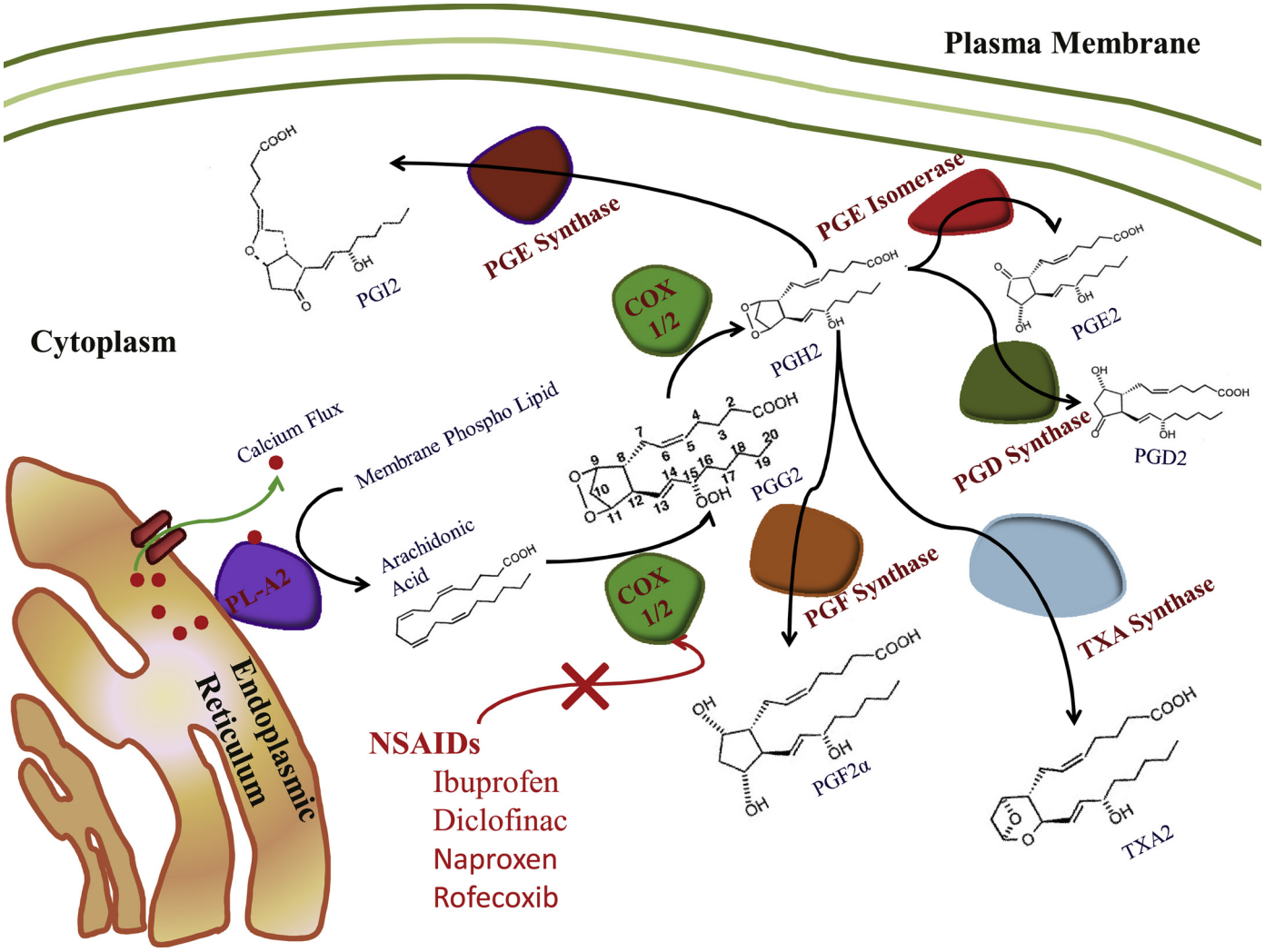

Fig. 1. The role of cyclooxygenase (COX) in prostaglandin (PG) synthesis. Prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2) and thromboxanes (TXA_2), which are important in inflammation are products of a biochemical cascade by which membrane phospholipids are converted to arachidonic acid, then to intermediate prostaglandins (PGG_2 and PGH_2) by cyclooxygenase, and to their final products by a series of synthases. NSAIDs = nonsteroidal anti-inflammatory drugs.

Unfortunately, besides the excellent anti-inflammatory profiles of the NSAIDs, severe side effects such as gastrointestinal (GI) ulceration, perforation, obstruction, and bleeding has limited the therapeutic usage of NSAIDs [7]. Thus, it was proposed that specific inhibitors for the COX-2 mediated reactions might be an ideal therapeutic alternative of the classical NSAIDs without causing adverse effects. In this regard, the search of COX-2 specific inhibitors remains an important thrust area in the anti-inflammatory drug discovery and development research.

Plants are considered to form the foundation of many traditional medicine systems worldwide from time immemorial and continue to provide mankind a resource for new remedies for variety of human ailments. There are several records in traditional medicine describing the importance of medicinal plants for relief from pain and inflammation [8,9]. The global renewed interest in medicinal plants has acquired a central attention on herbal cures among indigenous populations.

Andrographis paniculata Nees belonging to the Acanthaceae family is an erect herb well known in Asia. It occurs widely in the plains of India, Sri Lanka, Mainland China and Taiwan [10]. Genus *Andrographis* comprises 40 species and several members of this group find applications in traditional systems of medicine [11]. *A. paniculata*, commonly known as Kalmegh, has been used widely in India for the treatment of hepatitis and is one of the most widely used plants in Ayurvedic formulations. It is a predominant constituent of at least 26 Ayurvedic formulations used to treat liver disorders [12,13]. It is also considered to be a latent heat clearing, antipyretic, detoxicant, anti-inflammatory, detumescent, febrifugal, antiphlogistic and analgesic agent for the treatment of acute infections of the gastrointestinal tract, respiratory organs and urinary systems [14,15]. Diterpenoids, flavonoids and polyphenols are the major constituents of *A. paniculata*. It is a rich source of 20 -oxygenated flavonoids and labdane-type diterpenoids [16–18]. The active components of *A. paniculata* are very bitter diterpene lactones known as andrographolides (APs) [19].

In view of its current use, the present investigation evaluated the anti-inflammatory effect of Labdane diterpenoids isolated from *A. paniculata* targeting COX and to demonstrate their anti-proliferative properties.

2. Materials and methods

2.1. Standards and reagents

The COX-1 & 2 (human ovine) inhibitor Screening assay kit [Catalog No. 760111] was obtained from Cayman, U.S.A., DPPH (1, 1-diphenyl-2-picryl hydrazyl), MTT (3-(4,5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) were procured from Sigma-Aldrich Co. (St. Louis MO, USA). 1–10 phenanthroline, phenazine methosulphate (PMS), nitroblue tetrazolium (NBT) were obtained from S.D. Fine chem. Mumbai. Nicotinamide adenine dinucleotide (NADH) was purchased from

Table 1
COX Inhibitory activity of isolated compounds.

Compound (25 µg/ml)	IC_{50} (µg/ml)		Percent inhibition	
	COX-1	COX-2	COX-1	COX-2
A	16.0 ± 1.47	13.0 ± 1.47	47.63 ± 1.87	62.48 ± 2.94
B	11.0 ± 1.51*	12.0 ± 1.47*	48.52 ± 0.48	64.64 ± 1.76*
C	11.0 ± 1.36*	09.0 ± 0.14**	34.84 ± 1.76	83.24 ± 1.48**
D	12.0 ± 0.17*	16.0 ± 1.04	53.89 ± 1.46	61.47 ± 1.42
E	11.0 ± 1.35	10.0 ± 1.17	53.83 ± 2.07	79.74 ± 2.46

Results presented here are the mean value of $n = 3 \pm$ SD. NA: No activity under experimental condition, A = Andrographolide; B = 14-deoxy-11, 12-didehydroandrographolide; C = 14-deoxyandrographolide; D = 3,14-dideoxyandrographolide. E = Ibuprofen. *P < 0.05, **P < 0.01 *vs*. standard (One Way ANOVA for multiple comparison test followed by dunnet test).

Spectrochem, Pvt. Lit. Mumbai. Cancer cell lines were requested from National Centre for Cell Science (NCCS: a National Cell Line Facility) Pune (MS), India. All other chemicals and reagents used were of AR grade and obtained from commercial sources.

2.2. Isolation of phytochemicals

The selected plant, *Andrographis paniculata* (Acanthaceae) was collected from the nearby region of Pune district (MS). The plant was identified and authenticated by RUS, Department of Botany, Poona College, Pune- 411 001 (MS), India with the help of Flora [20]. The voucher specimen (PC-18) was deposited in departmental herbarium of host institute. The isolation process and characterization details of isolated phytochemicals (Andrographolide; 14-deoxy-11, 12-didehydroandrographolide; 14-Deoxyandrographolide and 3, 14- Dideoxyandrographolide) are published recently by our group [21]. To briefly describe the procedure, the shed dried powdered plant sample was extracted in ethanol and was evaporated under reduced pressure at room temperature. The crude extract was defatted with hexane and then fractionated into chloroform and methanol. Methanol fraction was subjected to silica gel column chromatography eluted with chloroform-methanol to yield various fractions. All fractions were further subjected to repeated column chromatography on silica gel and eluted with different concentration of chloroform-methanol solvent system to yield aforesaid compounds.

2.3. COX inhibition assay

The assay was performed by using Colorimetric COX (human ovine) inhibitor screening assay kit [22]. Briefly, the reaction cocktail contained, 150 µl of assay buffer, 10 µl of heme, 10 µl of enzyme (either COX-1 or COX-2), and 10 µl of compounds (25 µg/ml, in 0.5% DMSO). The assay utilizes the peroxidase component of the COX catalytic domain. The peroxidase activity can be assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramthyl-*p*-phenylenediamine (TMPD) at 590 nm. Ibuprofen (25 µg/ml) was used as a standard drug. The percent COX inhibition was calculated using following equation:

$$\text{COX inhibition activity}\,(\%) = 1 - \frac{T}{C} \times 100$$

Where T = Absorbance of the test sample and
C = Absorbance of the control sample.

2.4. Antioxidant study

2.4.1. DPPH radical scavenging assay

DPPH radical scavenging assay was carried out as per reported method [23]. Briefly, 1 ml of compound (25 µg/ml) was added to equal quantity of 0.1 mM solution of DPPH in ethanol. After 20 min of incubation at room temperature, the DPPH reduction was measured by reading the absorbance at 517 nm. Ascorbic acid (1 mM) was used as reference compound. The ability of compounds to scavenge DPPH radical was calculated by the equation.

$$\%\,\text{activity} = 1 - \frac{T}{C} \times 100$$

Where T = Absorbance of the test sample and
C = Absorbance of the control sample.

2.4.2. Hydroxyl (OH) radical scavenging assay

The OH radical scavenging activity has been demonstrated by methods previously published [24]. The reaction mixture contained, 60 µl of $FeCl_2$ (1 mM), 90 µl of 1–10 phenanthroline (1 mM), 2.4 ml of phosphate buffer (0.2 M, pH 7.8), 150 µl of H_2O_2 (0.17 M) and 1.5 ml of compounds (25 µg/ml). The reaction was started by adding H_2O_2 after 5 min incubation at room temperature, the absorbance was recorded at 560 nm. Ascorbic acid (1 mM) was used as reference compound.

2.4.3. Superoxide radical (SOR) scavenging assay

The superoxide anion scavenging assay was performed by the reported method [25]. Superoxide anion radicals were generated in a non-enzymatic phenazine methosulphate - nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, superoxide anion was generated in 3 ml of tris HCL buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 µM), 0.75 ml of NADH (936 µM), and 0.3 ml of compounds (25 µg/ml). The reaction was initiated by adding 0.75 ml of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature the absorbance at 560 nm was measured in spectrophotometer. Ascorbic acid (1 mM) was used as reference compound.

2.4.4. Reducing activity assay

The $Fe3^+$ reducing power of the extract was determined by the reported method [26] with slight modification. 0.5 ml of compounds (25 µg/ml) were mixed with 0.5 ml phosphate buffer (0.2 M, pH 6.6)

Table 2
Antioxidant activity profile of isolated phytochemicals.

Compounds (25 µg/ml)	Percent Radical scavenging activity				
	DPPH	OH	SOR	RA	NO
A	58.47 ± 1.15	47.35 ± 2.41	83.22 ± 1.87*	58.67 ± 2.22	54.35 ± 1.64
B	69.52 ± 1.21	49.10 ± 2.29	76.34 ± 1.52	59.62 ± 1.47	69.59 ± 2.31
C	77.86 ± 1.25	50.13 ± 1.51	91.41 ± 1.56**	81.36 ± 1.89*	86.41 ± 1.56**
D	78.40 ± 2.61*	80.78 ± 0.27*	16.30 ± 0.25	58.67 ± 1.48	53.22 ± 1.87
AA (1 mM)	67.33 ± 1.73	21.93 ± 1.49	45.56 ± 0.60	87.23 ± 0.80	78.04 ± 2.39

Results summarized here are the mean value of $n = 3 \pm$ SD. A = Andrographolide; B = 14-deoxy-11, 12-didehydroandrographolide; C = 14-deoxyandrographolide; D = 3,14-dideoxyandrographolide. AA = Ascorbic acid. *P < 0.05, **P < 0.01 vs. standard (One Way ANOVA for multiple comparison test followed by dunnet test).

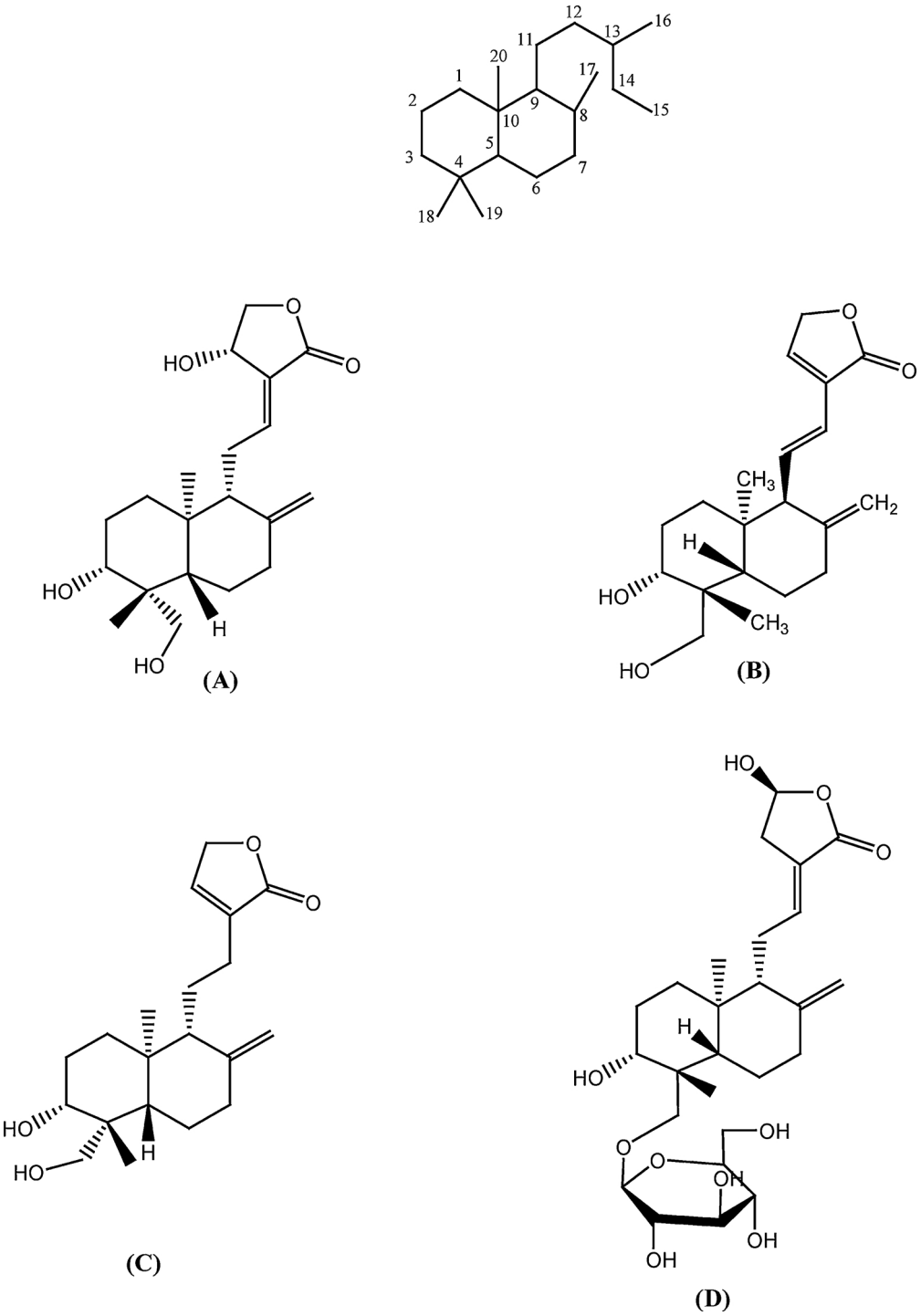

Fig. 2. Structures of isolated phytochemicals from *A. paniculata*. (A) Andrographolide; (B) 14-deoxy-11, 12-didehydroandrographolide; (C) 14-deoxyandrographolide; and (D) 3,14-dideoxyandrographolide.

and 0.5 ml potassium hexacyanoferrate (0.1%), followed by incubation at 50 °C in a water bath for 20 min. After incubation, 0.5 ml of (Trichloro acetic acid, TCA) (10%) was added to terminate the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml distilled water, and 0.1 ml $FeCl_3$ solution (0.01%) was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. Ascorbic acid (1 mM) was used as a positive control.

2.4.5. Nitric oxide radical scavenging assay

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions, which may be quantified by the GriessIllosvoy reaction [27]. The reaction mixture contained 10 mM SNP, phosphate buffered saline (Ph 7.4) and concentration of the compound solution in a final volume of 3 ml (25 μg/ml). After incubation for 150 min at 25 °C, 1 ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Then 1 ml of napthyl ethylenediamine dihydrochloride (NED) (0.1% w/v) was added and the mixture was incubated for 30 min at 25 °C. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at

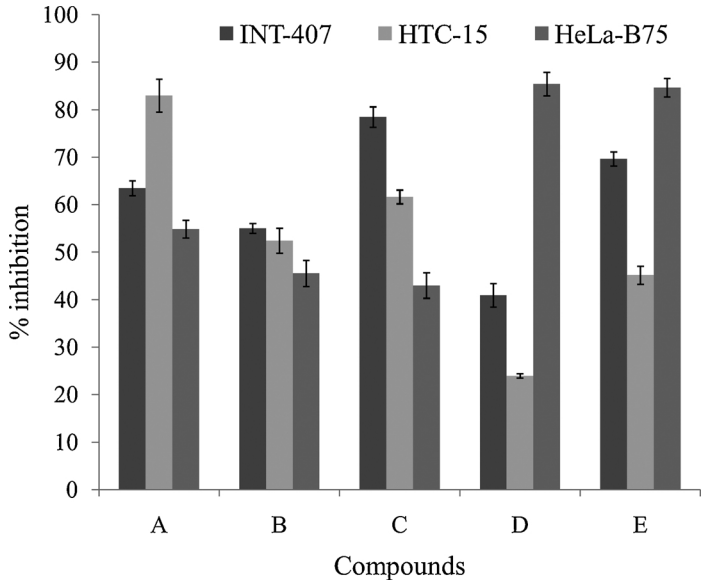

Fig. 3. Anti-proliferative activity profile of isolated phytochemicals. Results presented here are the mean value of $n = 3 \pm$ SD. E = Suramin.

540 nm against a blank sample. Ascorbic acid (1 mM) was used as a standard.

2.5. Cell lines and culturing

Cancer cell lines namely, human colon adenocarcinoma (HCT-15), human intestine (INT- 407) and HeLa-B75 cells were obtained from the National Centre for Cell Science, Pune (Maharashtra, India). All cell lines were propagated in Minimum Essential Medium (Eagle) with 2 mM l-glutamine and Earle's BSS (balanced salt solution) adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate 90%, and 10% fetal calf serum. All cell lines were grown in a humidified incubator at 37 °C.

2.6. MTT assay for in vitro anti-proliferative activity evaluation

The MTT based cytotoxicity assay was performed using an earlier reported method with slight modifications [28]. Cells were harvested and inoculated (2.5×10^4 cells/well) in 96-well microtiter plates. Cells were washed with phosphate-buffered saline (PBS) and then inoculated with and without the individual compound (25 µg/ml). After 72 h of incubation, the medium was aspirated followed by addition of 150 µl of MTT solution (5 mg/ml in PBS, pH 7.2) to each well and the plates were incubated for 4 h at 37 °C. After incubation, 800 µl of DMSO was added to the wells followed by gentle shaking to solubilize the formazan crystal for 15 min. The absorbance of the mixture was read at 540 nm. Suramin (100 µM) was used as a reference compound. The inhibition of cell viability was calculated as follows.

$$\%\,\text{cytotoxicity} = 1 - \frac{T}{C} \times 100$$

Where T = Absorbance of the test sample and
C = Absorbance of the control sample.

2.7. Molecular docking study

Molecular docking was performed as per already established protocol described elsewhere [29,30]. Briefly, the structures of isolated Labdane diterpenes were initially drawn in ChemDraw 8.0 (Cambridge Soft, Cambridge, MA, USA) followed by generation of 3D conformation using Frog2 Server [31]. The compounds were then imported in python prescription 0.8 (PyRx) using Open Babel facility for energy minimization and finally converted to pdbqt format.

Crystallographic structures of COX-1 and COX-2 enzymes were downloaded from protein data bank database (PDB) with PDB ID 1EQG and 3NT1 respectively [32]. Formal charges were assigned to co-ordinate files and receptor molecules were prepared in pdbqt format using PyRx 'make macromolecule' command.

Prior to docking the diterpenoid compounds, the validity of docking system was checked by docking the crystallographic pose of inhibitor Ibuprofen and Naproxen into structure of COX-1 and COX-2 respectively. In validation experiment, the ability of docking software to predict the experimental pose is checked. Root-mean-square deviation (RMSD) between experimental and predicted pose is generally used as measure to infer structural resemblance. The system is said to be validated if the value of RMSD between predicted and experimental poses is found to be less than 2 Å [33]. AutoDock 4.2 suite was used for docking purpose in this investigation [34,35]. AutoGrid program was initially used to obtain grid file, followed by setting up the grid box of $50 \times 50 \times 50$ points with spacing of 0.375 Å to ensure coverage of entire active site. Lamarckian genetic algorithm was used for conformational search. The resulting structures were clustered as per the AutoDock scoring function. Only the top ranked confirmations of ligands were selected. RMSD values of 0.50 Å and 0.36 Å were obtained in validation experiment for COX-1 and COX-2 systems. These values clearly indicate that predicted poses are nearly identical to crystallographic poses (Supplementary Fig. 1) and thereby validating the system. Same parameters were used for docking Labdane diterpenes in COX-1 and COX-2 structures.

Finally, PyMolv0.99 (http://www.pymol.org) and LigPlot + [36,37] programs were used for generating images and 2D plots of ligand-protein interactions respectively.

3. Results

3.1. COX inhibitory activity of compounds

In the present investigation, the COX inhibitory potential of isolated compounds were evaluated. The results obtained are summarized in Table 1. It was observed that compound **C** possesses selective inhibition to COX-2 (83.24%, IC_{50} 9.0 µg/ml) as compared to COX-1 (34.84%, IC_{50} 11.0 µg/ml), while the rest of the compounds had moderate inhibitory potential towards COX-2 in the range of 61–64%. The results obtained were found in accordance with *in silico* docking studies of

Table 3
Docking results for isolated labdane diterpenoids compoundsin active site of COX isoforms.

Target	Compound code	Binding Free energy (Kcal/mol)	Interacting Amino acids (HBD Å)	HD::HA	Residues in hydrophobic interaction
COX-2	A	−7.7	Tyr355 (3.06) Arg120 (2.94)	Tyr355 (OH^{sc}): H:: Lig(O):O Arg120 ($NH2^{sc}$): H:: Lig(O):O	Val116, Val349, Leu352, Ser353, Leu359, Phe381, Leu384, Trp387, Phe518, Met522, Val523, Gly526, Ala527, Ser530, Leu531
	B	−8.0	Tyr355 (2.57) Arg120 (3.18)	Tyr355 (OH^{sc}):H:: Lig(O2):O Arg120 ($NH2^{sc}$):H::Lig(O2):O	Val116, Val349, Leu352, Ser353, Leu359, Leu384, Trp387, Phe518, Met522, Val523, Gly526, Ala527, Ser530, Leu531
	C	−8.1[a]	Tyr355 (2.94) Arg120 (3.17) Tyr385 (2.34)	Tyr355 (OH^{sc}):H:: Lig(O2):O Arg120 ($NH2^{sc}$):H::Lig(O2):O Tyr385 (OH^{sc}):H:: Lig(O4):O	Val116, Tyr348, Val349, Leu352, Leu359, Phe381, Trp387, Met522, Val523, Gly526, Ala527, Ser530,
	D	0.08	Tyr355 (2.95) Arg120 (3.15)	Tyr355 (OH^{sc}):H:: Lig(O6):O Arg120 ($NH2^{sc}$):H::Lig(O6):O	Val89, Leu93, Ile112, Met113, Val116, Val349, Leu352, Ser353, Leu359, Phe381, Tyr385, Trp387, Phe518, Val523, Gly526, Ala527, Ser530, Leu531
	Nap	−8.0	Tyr355 (2.63) Arg120 (2.77)	Tyr355 (OH^{sc}):H:: Nap(O) Arg120 ($NH2^{sc}$):H::Nap(O)	Val116, Val349, Leu352, Tyr385, Trp387, Met522, Val523, Gly526, Ala527, Leu531
COX-1	A	−7.0	Tyr355 (2.76) Arg120 (3.05) Ser530 (2.89)	Tyr355 (OH^{sc}):H:: Lig(O3):O Arg120 ($NH2^{sc}$):H::Lig(O3):O Ser530 (OG^{sc}) :H::Lig(O4):O	Val116, Val349, Leu352, Ser353, Trp387, Phe518, Met522, Ile523, Gly526, Ala527, Leu531
	B	−7.7	Arg120 (2.97)	Arg120 (NE^{sc}):H::Lig(O2):O	Val116, Val349, Leu352, Ser353, Tyr355, Leu359, Leu384, Trp387, Phe518, Met522, Ile523, Gly526, Ala527, Ser530, Leu531
	C	−7.0	Arg120 (3.05)Tyr385 (3.11)	Arg120 ($NH2^{sc}$):H::Lig(O2):O Tyr385 (OH^{sc}):H:: Lig(O3):O	Val116, Val349, Leu352, Tyr355, Leu359, Trp387, Phe518, Met522, Ile523, Gly526, Ala527, Ser530, Leu531
	D	1.37	Tyr355 (3.30) Tyr385 (2.58)	Tyr355(OH^{sc}):H:: Lig(O10):O Tyr385(OH^{sc}):H:: Lig(O5):O	Ile89, Leu93, Met113, Val116, Arg120, Tyr348, Val349, Leu352, Ser353, Leu357, Leu359, Leu384, Trp387, Met522, Ile523, Gly526, Ala527, Ser530, Leu531
	Ibp	−7.30	Arg120 (2.82) Tyr355 (2.85)	Arg120 (NE^{sc}):H::Ibp(O) Tyr355(OH^{sc}):H:: Ibp(O)	Val116, Val349, Met522, Ile523, Gly526, Ala527, Leu531

[sc] represent side chain, Nap: Naproxen, Ibp: Ibuprofen. Interaction *Arg120 ($NH2^{sc}$):H::Lig(O2):O* is to be read as *Arg120 donates the hydrogen bond via side chain NH2 atom to the O2 oxygen from Ligand that acts as hydrogen bond acceptor*.

[a] Represent compounds with best binding free energy values towards COX-2.

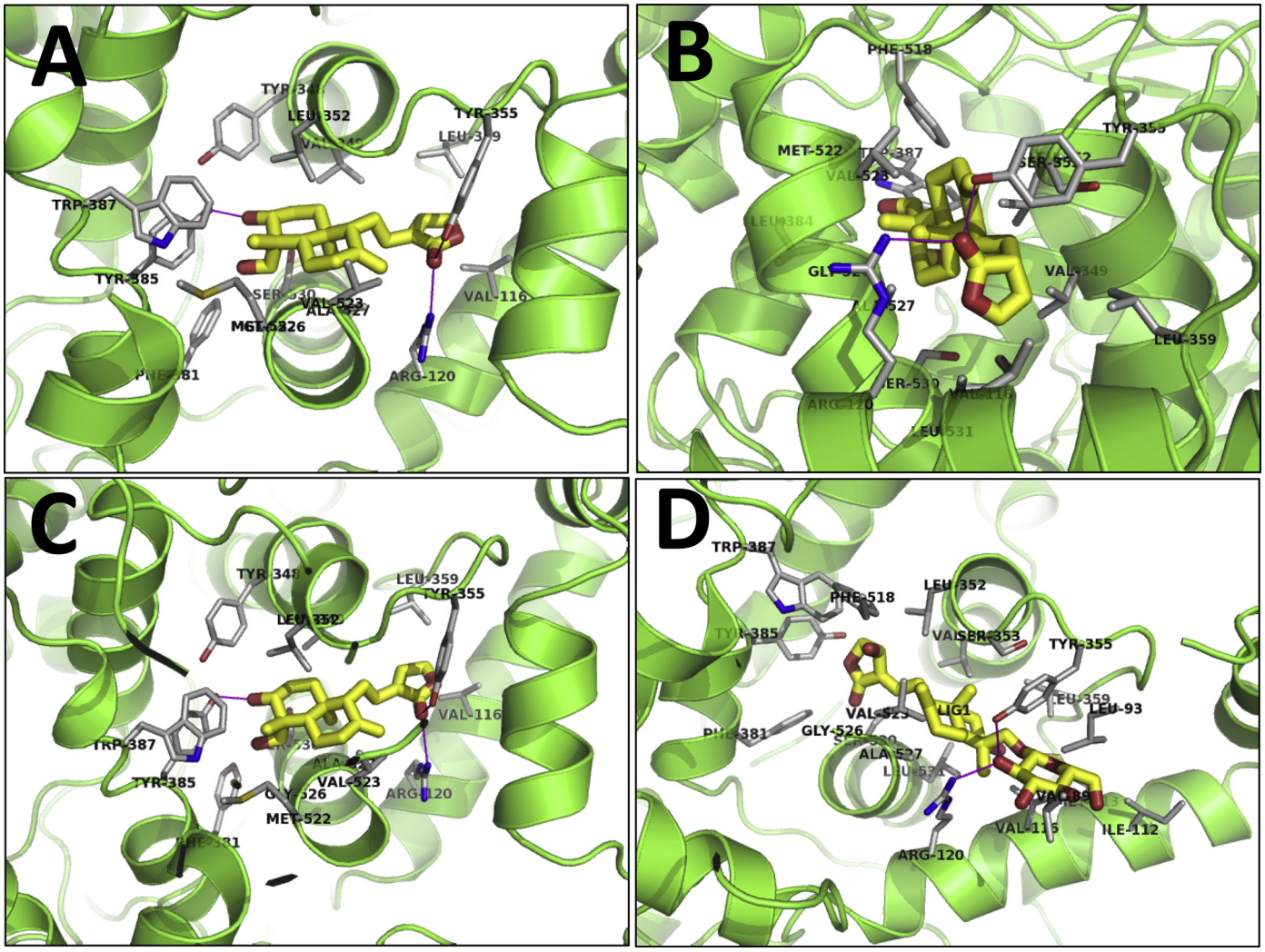

Fig. 4. Docking complexes of isolated phytochemicals (Yellow sticks) in COX-2 (Green cartoons). Hydrogen bonds are shown as Magenta lines and residues at the binding site are depicted as white sticks). **A:** Compound-A – COX-2 complex. **B:** Compound-B – COX-2 complex. **C:** Compound-C – COX-2 complex. **D:** Compound-D – COX-2 complex (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

compounds.

3.2. Antioxidant profile of isolated compounds

3.2.1. DPPH radical scavenging activity

The results clearly demonstrate the fact that, compound **D** possesses highest DPPH radical scavenging ability (78.40 ± 2.61%), while the lowest activity is shown by Compound **A** (58.47 ± 1.15%). The compound **B** and **C** shows a moderate radical scavenging potential (69.52 ± 1.21 and 77.86 ± 1.25%) respectively. The results are summarized in Table 2.

3.2.2. OH radical scavenging activity

As per the OH radical scavenging activities it was observed that about all isolated compounds showed effective OH radical stabilizing potential. Compound **D** was found to be significant radical stabilizing compound (80.78 ± 0.27%) as compared to Ascorbic acid (21.93 ± 1.49%), while rest of the compounds have good OH radical scavenging ability. The results are shown in Table 2.

3.2.3. SOR scavenging activity

The compound **C** was observed to possess promising SOR scavenging activity (91.41 ± 1.56%), while the lowest SOR activity was detected in the case of compound **D** (16.30 ± 0.25%). The ascorbic acid was ascertained to have moderate SO radical scavenging activity (45.56 ± 0.60%). The results are provided in Table 2.

3.2.4. Reducing activity profile

The isolated compounds exhibit prominent reducing ability. Among all tested compounds, Compound **C** had maximum reducing property (81.36 ± 1.89%). However Compound **A, B** and **D** exhibited moderate reducing activity in the range of (58.67–59.62 %). The results are summarized in Table 2.

3.2.5. NO radical scavenging activity

The results of NO radical scavenging activity shows that, Compound **C** possesses better activity (86.41 ± 1.56%) as compared to standard ascorbic acid (78.04 ± 2.39%). While the other compound **A** (54.35 ± 1.64%), **B** (69.59 ± 2.31%) and **D** (53.22 ± 1.87%) had moderate NO radical scavenging ability. The results are summarized in Table 2.

3.3. Antiproliferative activity of compounds

The role of COX-2 in carcinogenesis has been recently described. COX-2 activity is frequently related to the formation of carcinogens, tumor promotion and inhibition of apoptosis, angiogenesis and the metastatic process. Considering this fact, three different cancer cell

lines were used to evaluate anticancer property of isolated phyto-constituents. The results obtained are summarized in Fig. 3. It was observed that, all the tested samples exhibited promising anti-proliferative activity toward the selected cancer cell lines. Compound **D** possessed significant activity by inhibiting HeLa-B75 cell line (85.36 ± 2.48%), while compound **C** showed moderate inhibition (42.98 ± 2.69%). All the compounds under investigation were found to reveal moderate cytotoxic activity towards INT- 407 and HCT-15 cell lines in the range of (23.95–82.95 %). The cytotoxic activity was compared with Suramin (100 μM), which was used as the standard anticancer drug that inhibited INT-407 (69.64%), HCT-15 (45.13%) and HeLa-B75 (84.63%).

3.4. Results of docking analysis

The labdane diterpenes family of compounds possesses a typical bicyclic fused ring skeleton (carbon number 1–10 in Fig. 2) along with a six carbon extension (carbon number 11–16, referred to as C6 moiety here onwards) that may or may not be fused oxygen containing heterocyclic ring. The chemical structures of diterpenes isolated in present study are shown in Fig. 2. Table 3 summarizes the binding free energy values recorded by isolated phytochemicals from *A. paniculata* in active site of COX-1 as well as COX-2 enzymes. The values obtained from docking investigation clearly indicate that the selected Labdane diterpenes are more suited for COX-2 inhibition. Compounds **B** and **C** demonstrated comparable binding free energy values -8.0 and -8.1 Kcal/mol in COX-2 enzyme; interestingly matching the experimental *in vitro* percent inhibition data (64.64 and 83.24 μg/ml respectively). Carefully observing Table 3 clarifies the fact that all the hydrogen bond donor atoms are from active site COX residues and acceptors are from phytochemical compounds. This data can provide an essential feature for designing novel pharmacophore model for diterpenes based COX inhibition.

4. Discussion

Traditional systems of medicine are intricate systems of healing that originated in the world thousands of years ago. But the fact is there exists a wide gap between traditional and modern medicine. Modern medicine has no replacement, but traditional healthcare providers still form the basis of rural healthcare in India and abroad. The present study is an attempt to highlight importance and need for integrating modern medicine with Traditional medicine systems. The results obtained provide the scientific basis of a plant which is traditionally used in the management of inflammatory disorders. Medicinal plants are the enormous source of bioactive molecules; thus the practice of using plants for developing the novel anti-inflammatory and anticancer agents has been running for many years.

Several studies have demonstrated a variety of diterpenoids structures presenting anti-inflammatory capacity [38]. It was reported that, Briarane diterpenoids inhibits COX-2 expression induced by LPS in macrophages [39]. Several marine drugs as, eunicellin-based diterpenes include krempfielins, hirsutalins, klymollins, klysimplexin, klysimplexin sulfoxide, simplexin, and cladieunicellin have been isolated and identified from soft corals belonging to the genera *Cladiella* +or *Klyxum*. These phytochemicals have shown the capacity to inhibit the upregulation of COX-2, or IL-6 proteins in RAW 246.7 macrophages stimulated with lipopolysaccharide [40,41].

However, multiple lines of evidence *in vitro, in vivo,* observational and clinical data now confirm that selective COX-2 inhibitors reduce prostaglandin production and the risk of colorectal, skin and other neoplasias. The current trend in cancer therapeutics is based on a targeted molecular design derived from preclinical *in vitro* and *in vivo* studies. The *in vitro* studies have demonstrated the possible role of COX inhibitors as a single agent to prevent the occurrence of tumors. In addition, large epidemiological studies have shown indications that COX inhibitors could have a beneficial influence on diminishing the development and growth of malignancies. Effectively, these studies showed reduced risk of developing several malignancies including colorectal cancer with treatment of COX inhibitors [42,43].

In the light of structure activity relationship, binding free energies are often regarded to reflect the very crucial aspect of tight or loose binding of ligand to protein and provide sound biophysical insights in ligand-protein interactions [44]. The results obtained in the present study also feature details of the hydrogen bonding interactions as well as residues that are involved in hydrophobic interactions or van der Waal's contacts. Plenty of literature has accumulated in recent past that clearly demonstrates the fact that hydrogen bonds, hydrophobic interactions and van der Waal's contacts are major factors involved in the stabilization of protein-ligand complexes [45]. Compound **A** (Andrographolide) is a diterpene with a hydroxy substituted keto furan ring as its C6 moiety and two active hydroxyl groups are substituted on the fused bicyclic ring [46]. Docking investigation conducted in this study predicted differential confirmation of this compound in the two COX isoforms. The C6 moiety of Andrographolide appears to occupy the hydrophobic pocket mainly lined by residues like Ser530, Gly526, Trp387 and Val523in COX-2. In contrast, the same C6 moiety appears to from a hydroxy mediated polar interaction with Arg120 and Tyr355 in COX-1 (Fig. 4). However, the hydroxy group at C-19 position seems to interact with both the COX isoforms forming hydrogen bonds with conserved residues from the active site. For example, it makes a dual hydrogen bonding with Arg120 and Tyr355 in case of COX-2 while it hydrogen bonds with Ser530 in case of COX-1.

Compound **C** (14-deoxyandrographolide) isolated in this study is a close structural analog of Andrographolide that differ only by the absence of hydroxyl from the C6 moiety. In contrast to compound **A**, compound **C** seems to adopt similar confirmation in two COX isoforms active sites. The C6 fragment from 14-deoxyandrographolide is observed to make bipolar hydrogen bonding interaction with Arg120 and Tyr355 via its keto substituent in COX-2; while, COX-1 appears to donate a single hydrogen bond with same atom of compound **C** through Arg120. The polar groups located on the fused bicyclic skeleton of 14-deoxyandrographolide appear to be engaged in hydrogen bonding with Tyr385. The comprehensive structure activity relationship (SAR) conducted on 25 analogues of 14-deoxyandrographolide clearly indicate the crucial importance of oxygen containing substituents on the fused bicyclic frame in defining inhibitory potential of the diterpene compounds [47]. Interestingly, it is important to note that compound **C** scored comparatively better binding free energy value (-8.1 Kcal/mol) that is in accordance with the observed *in vitro* COX-2 inhibition activity

Compound **B** isolated in this study corresponds to 14-deoxy-11,12-didehydroandrographolide and is found to be forming hydrogen bonds with major active site residues from both the cyclooxygenase isozymes. The C6 moiety of compound **B** appears to form double hydrogen bonding interaction with two highly conserved and functionally important residues (Tyr355 and Arg120) in COX-2. On the other hand, compound **B** is observed to make a single hydrogen bonding interaction with Arg120 in COX-1 enzyme. Structural analysis of docking pose indicates similar orientation of this compound in COX active sites. Structure function studies conducted on enzyme inhibition potential of 14-deoxy-11,12-didehydroandrographolide clearly support the crucial interest of these polar interaction in defining its enzyme inhibitory activity [48].

Compound **D** appears to be the only major phytochemical isolated in current study that possesses a sugar moiety. The sugar group is observed to form polar interactions in form of hydrogen bonds with Tyr355 and Arg120 from COX-2; in contrast to single hydrogen bond with COX-1 (Table 3). The glucoside group of this compound is already characterized to play an important role in defining protective activity against hepatotoxicity [49] and modulation of Quinone reductase activity [50]. It is also worth to mention that despite forming numerous hydrogen bonds with active site residues, compound **D** contributed a little during *in vitro* COX inhibition. Its lowered potency can be partly

attributed to the unfavorable steric bumps that might have created in the COX active site due to bulky nature of this compound.

4.1. Limitations of study

While describing the limitations of the present study, it is important to note that this is a preliminary *in-vitro* screening of the selected plant against COX enzyme. The phytochemicals isolated from *A. paniculata* shows significant COX inhibitory results. However, *in vivo* pharmacological investigations of promising samples or lead compounds are needed to authenticate the efficiency of the isolated plant phytochemicals.

5. Conclusion

The result of the present investigation shows the anti-inflammatory, anti-proliferative and antioxidant potential of selected Labdane diterpene compounds isolated from *A. paniculata.* The results of *in vitro* study in present investigation clarify that diterpene compound **C** has promising COX inhibition potential. The compound **C** was also demonstrated to possess significant antioxidant ability. However, compound **D** exhibited effective anti-proliferative activity against selected cancer cell lines. Results from docking investigation conducted demonstrate the chances of involvement of the highly conserved residues like Arg120, Tyr355, Tyr385 and Ser530 in present diterpene dependent COX inhibition. The trends in docking results are in agreement with the *in vitro* COX inhibition data; that demonstrates the potential of compound **C** to emerge as a lead compound whose further modification may lead to formulation of an effective anti-inflammatory agent.

Authors

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Declaration of Competing Interest

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.eujim.2019.100983.

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