Exploring Withania somnifera L. as a Natural Remedy for Dysmenorrhea treatment via COX-2 Inhibition

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Abstract

The present work was aimed to evaluate the anti-inflammatory effect of bioactive compounds of *WithaniaSomnifera L.* against cyclooxygenase-2 (COX-2) targeting dysmenorrhea using an *in-silico* molecular docking approach. Due to the limitations and adverse effects associated with currently available anti-inflammatory drugs, the identification of safer natural COX-2 inhibitors has gained significant attention. A phytochemical library comprising 16 bioactive compounds of *Withaniasomnifera L.,*commonly known as Ashwagandha and belonging to the *Solanaceae* family,previously reported for their anti-inflammatory, antidepressant, anticancer and antianxiety activities, was selected for the study.The three-dimensional crystal structure of the target protein cyclooxygenase-2 (COX-2) was retrieved from the Protein Data Bank (PDB)(PDB ID: 1CX2), while the chemical structures of phytochemicals were obtained from the PubChem database. Active site prediction and binding pocket analysis of the target protein were carried out using CASTp (Computed Atlas of Surface Topography of Proteins). Molecular docking and virtual screening studies were performed using PyRx, with Celecoxib used as the reference inhibitor. Furthermore, protein–ligand interaction analysis was visualized using BIOVIA Discovery Studio Visualizer. The docking results revealed that 13 phytochemicals exhibited stronger binding affinities than the reference inhibitor Celecoxib (-9.2 kcal/mol) against the COX-2 active site. Among these, representative top-ranked compounds including Withaferin A (-11.5 kcal/mol), Somniferanolide(-11.4 kcal/mol), Withaoxylactone (-11.3 kcal/mol) and Withanolide A (-11.1 kcal/mol) were highlighted in the present study due to their comparatively superior binding affinities and significant interactions with key active-site residues of the COX-2 enzyme. These compounds formed multiple hydrogen bonds and other stabilizing interactions, indicating strong inhibitory potential. Overall, the findings suggest that phytochemicals from *Withaniasomnifera L.*may serve as promising natural anti-inflammatory agents for the management of dysmenorrhea. However, further *in-vitro* and *in-vivo* studies are required to validate their therapeutic efficacy and safety.

Key Words: Dysmenorrhoea, COX-2, withaferin A, Molecular docking

Introduction

Dysmenorrhea, literally meaning "painful monthly bleeding" in Greek, refers to pain arising from the uterus and is one of the most common gynaecological conditions among women during their reproductive years (Vlachou *et al.,* 2019;Iacovides *et al.*, 2015). Even with the high frequency of dysmenorrhea, it is usually underdiagnosed due to many patients not seeking medical help (Itani *et al.,* 2022). The condition causes severe menstrual lower abdominal pain usually manifesting as cramping and often radiating to the back or thighs (Serrahima & Martínez, 2023).Associated with this pain come other symptoms which are typically, vomiting, headaches, low back pains diarrhea and general body weakness (Itani *et al.,* 2022). World Health Organization (WHO) data show that dysmenorrhea is prevalent in 94% of young women between the ages of 10-20 years as well as 8.8% of women in the age group of 19-41years (Barcikowska *et al.,* 2020).The condition can however greatly affect the quality of the affected individual's lifestyle and can lead to absenteeism from school or work (Tadese *et al.,* 2021). Dysmenorrhea is described in two types; the primary and secondary types. Primary dysmenorrhea (PD) or Primary menstrual pain is defined as uncomfortable and violent contraction of the lower part of the abdomen that occurs just before or during menstruation, otherwise there is no structural pathology of the female pelvis (Itani *et al.,* 2022).It is predominantly experienced in adolescent girls and usually manifests 6 months after menarche (De Sanctis *et al.,* 2016). It starts few hours before or right at the beginning of menstrual flow and persists for 2-3 days, although its worst within the first 24 to 36 hours of menstruation (Itani *et al.,* 2022).The primary dysmenorrhea on the hand is not related to any underlying pathological state of the reproductive organs while secondary dysmenorrhea (SD) is caused by conditions that include endometriosis, chronic pelvic inflammatory disease, adenomyosis, endometrial polyps, ovarian cysts, congenital anomalies and intrauterine contraceptive device complications (Petraglia *et al.,* 2017). SD is defined by noncyclic or at least not menstruation-related pain and is seen more in the older females who are over the age of 20 years and with no history of dysmenorrhea (Ju *et al.,* 2014).There are some clinical features that differentiate between SD from PD, these include; uterine size, dyspareunia (pain during sexual intercourse) and failure to respond to indicated treatment (Bakhsh *et al.,* 2022).Chronic pelvic pain mainly arising from endometriosis, a benign disorder characterized by development of endometrial tissue outside the uterus, constitute one of the most frequent causes of SD (Cano-Herrera *et al.,* 2024). Arachidonic acid is metabolised by the cyclooxygenase 1/2 (COX 1/2) pathway and results in the formation of prostaglandins. There is evidence about the regulation of arachidonic acid synthesis by lysosomal enzyme, phospholipase A2 bound to the concentration of progesterone (Nørregaard *et al.,* 2015).

Estrogen levels are at their highest during the follicular phase of the menstrual cycle while the progesterone levels are elevated and reach their peak in the mid-secretory phase after ovulation. If conception does not occur then the corpus luteum regresses which causes a significant decrease in progesterone concentrations in blood. Progesterone drops within two hours of shedding of the endometrial tissue, menstrual break and the release of lysosomal enzymes which in return stimulate the formation of arachidonic acid from

where prostaglandins are manufactured (Itani *et al.,* 2022). COX-2 is involved in the modulation of the inflammatory pain response since it is responsible for the production of prostaglandins in inflamed cells (Desai *et al.,* 2018). COX-2 activity is frequently found to be up-regulated in such areas or tissues which are inflamed (Ahmadi *et al.,* 2022). Although there are several COX-2 selective inhibitors like celecoxib and rofecoxib, these drugs have been taken off the market after it was realised that they posed a potential threat of causing cardiovascular adverse effects in the long-term such as heart attacks and strokes (Chen *et al.,* 2021). In view of this, there has been a call forbetter and safer COX-2 inhibitors from natural sources. Ashwagandha or *Withaniasomnifera L.* belonging to Solanaceae family is distributed across India (Saleem *et al.,* 2020).In Ayurvedic traditional medicine of India it has been mainly applied as anti-inflammatory compound (Mikulska *et al.,* 2023).Since *Withaniasomnifera L.* possesses the medicinal value the purpose of the present investigation is to evaluate the molecular docking of bioactive compounds of *Withaniasomnifera L.* with COX-2 using the Autodock Vina software integrated within the PyRx tool.

Material and Methods

The details of material and methods are described under following sub-headings-

Retrieval and Preparation of protein structure

The X-ray crystallographic structure of the COX-2 protein (PDB ID: 1CX2), resolved at a 3.0 Å resolution, was obtained from the Protein Data Bank(Helen *et al.,*2021), (URL:https://www.rcsb.org/) **(Figure 1)**. Following acquisition, water molecules, ligands, and other heteroatoms were removed. Hydrogen atoms were then added to the protein structure using the CHARMm force field (Joseph *et al.,* 2017).

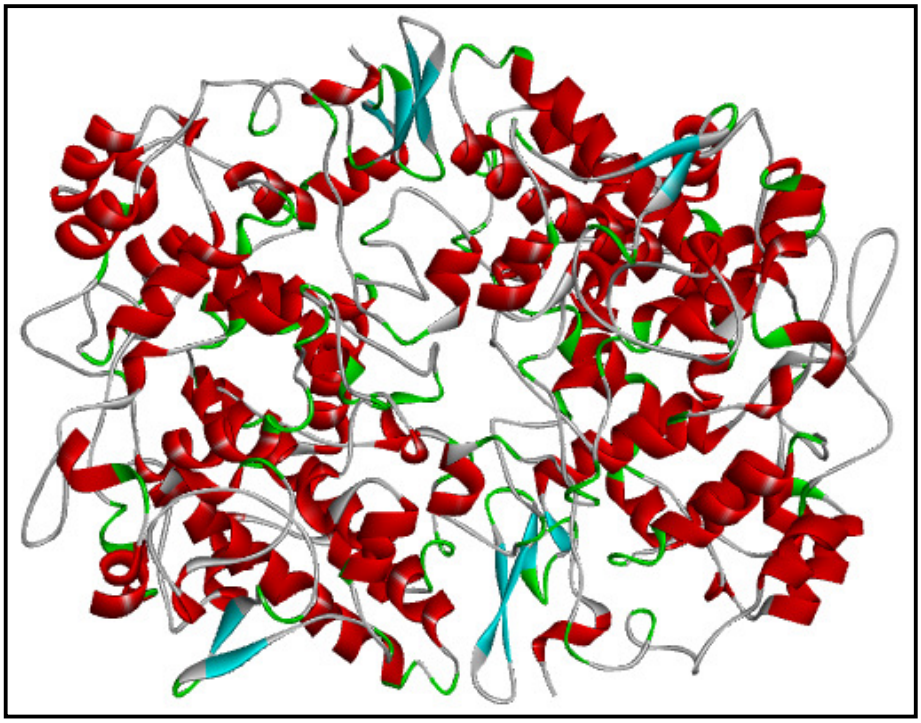


Figure 1: Three-dimensional (3D) structure of COX-2 protein (PDB ID: 1CX2)

Ligand Preparation

The three-dimensional structures of each phytochemical compound of *Withaniasomnifera L.*and the reference drug,Celecoxib,(**Table 1**), were retrieved from the PubChem database (Kim *et al.,* 2016), (URL:https://pubchem.ncbi.nlm.nih.gov/) in SDF format. These structures were subsequently converted into PDB format using the Open Babel software (O'Boyle *et al.,* 2011).

Table 1: Selected Phytochemicals of *Withaniasomnifera L.*and Reference Drug Celecoxib

S.No.	Compound Name
1	Withaferin A_CID_265237
2	Somniferanolide_CID_ 102066415
3	Withaoxylactone_CID_101687981
4	Withanolide A_CID_11294368
5	Withasomniferanolide_CID_102066414
6	Somniferawithanolide_CID_102066416
7	Withanolide B_CID_14236711
8	Somnifericin_CID_101687980
9	Withanone_CID_21679027
10	Physagulin D_CID_10100412
11	Somniwithanolide_CID_102066417
12	Withanolide D_CID_161671
13	Sominone_CID_44249449
14	Anaferine_CID_443143
15	Cuscohygrine_CID_1201543
16	Pseudotropine_CID_449293
Reference drug	**Celecoxib_CID_2662**

Active site prediction

$CAST_P$ (Computed Atlas of Surface Topography of proteins) software (URL: http://sts.bioe.uic.edu/) was used to predict the active site of COX-2. This was used to find the functioning site of COX-2 protein *(*Binkowski *et al.,* 2003).

Molecular Docking and Visualization

Molecular docking is a technique designed to estimate the binding energy of an intermolecular interaction between ligand and protein. Molecular docking was performed by PyRx virtual screening tool (Dallakyan & Olson, 2015)**,**(URL:https://pyrx.sourceforge.io/) a virtual screening software developed for *in-silico* drug discovery. The grid box used for docking was centered at x = 44.188, y = 25.200; z=37.994 and grid size of X-69.533 Å, Y-65.264Å and Z -56.389 Å.

Results and discussion

Before the virtual screening, the active site of COX-2 protein was predicted by using Computed Atlas of Surface Topography of Proteins (CASTp) for determine the active site of COX-2 protein, meanwhile the molecular docking was conducted by using PyRx virtual screening tool docked with control drugs and the phytochemicals of the plants studied. The more negative value of the docking score of phytochemical means the better docking affinity (Ralte *et al.,* 2022).Data shows that 13 compounds showed good binding energy than reference drug,celecoxib. (**Table 2)**. The molecular docking study evaluated the binding affinities and interactions of various compounds from *Withaniasomnifera L.* with the COX-2 enzyme, comparing these with the reference drug celecoxib. Celecoxib, a well-known COX-2 inhibitor, showed a binding affinity of -9.2 kcal/mol. It interacted with residues Gly A:235, Gln A:241, and Lys A:333 through conventional hydrogen bonds, with Ser B:143 via a carbon hydrogen bond, and with Leu A:224 through a halogen (fluorine) interaction. Among the phytochemicals tested, Withaferin A exhibited the highest binding affinity at -11.5 kcal/mol, forming conventional hydrogen bonds with Cys A:41 and Gly A:45, a carbon hydrogen bond with Gln B:327, and an unfavorable acceptor-acceptor bond. Somniferanolide also showed a strong binding affinity of -11.4 kcal/mol, interacting with residues Asn A:34, Cys A:47, Tyr A:136, and Gly A:135 through conventional hydrogen bonds, a Pi-alkyl bond, and a carbon hydrogen bond. Withaoxylactone, with a binding affinity of -11.3 kcal/mol, formed conventional hydrogen bonds with Cys B:41 and Gly B:135, and a carbon hydrogen bond with Gln A:327. Withanolide A, which had a binding affinity of -11.1 kcal/mol, interacted with residues Arg A:61, Lys B:546, Ser A:126, Lys A:532, and Gln A:372 through conventional hydrogen bonds and a carbon hydrogen bond. Withasomniferanolide and Somniferawithanolide both displayed a binding affinity of -11.0 kcal/mol. Withasomniferanolide formed alkyl bonds with Val B:291 and Leu B:294, a conventional hydrogen bond with Gln B:203, and an unfavorable acceptor-acceptor bond with Phe B:210 and His B:207. Somniferawithanolide interacted similarly, forming alkyl bonds with Val A:291 and Leu A:294, a conventional hydrogen bond with Gln A:203, and an unfavorable acceptor-acceptor bond with His A:207. Withanolide B, with a binding affinity of -10.9 kcal/mol, formed conventional hydrogen bonds with Pro B:153, Tyr B:130, Asn B:34, and Gln B:461, along with alkyl bonds with Cys B:36. Somnifericin, exhibiting a binding affinity of -10.8 kcal/mol, interacted

with residues Phe B:142, Leu B:145, Arg A:376, Glu A:236, Trp B:139, and Asn B:375 through conventional hydrogen bonds, a Pi-alkyl bond, and an alkyl bond. Withanone showed a binding affinity of -10.7 kcal/mol, forming conventional hydrogen bonds with Arg B:61 and Gln A:543, and a carbon hydrogen bond with Lys A:546. Physagulin D, with a similar binding affinity of -10.7 kcal/mol, interacted with residues Ser B:143, Glu A:236, Phe B:142, Trp B:139, Gly A:235, Asp A:229, and Leu B:224 through conventional hydrogen bonds, carbon hydrogen bonds, and a Pi-alkyl bond. Somniwithanolide, with a binding affinity of -10.6 kcal/mol, formed conventional hydrogen bonds with Cys B:47, Asn B:34, Asn B:39, and Asp B:157. Withanolide D exhibited a binding affinity of -10.4 kcal/mol, interacting with Pro B:154, Ala B:156, and Cys B:36 through conventional hydrogen bonds and a carbon hydrogen bond. Sominone, with a binding affinity of -10.1 kcal/mol, formed conventional hydrogen bonds with Cys A:36, Glu B:326, and Asn A:39, and a carbon hydrogen bond with Ala A:156. In contrast, Anaferine and Cuscohygrine displayed lower binding affinities of -6.8 and -6.3 kcal/mol, respectively. Anaferine interacted with Val A:291, Leu A:294, and His A:207 through conventional hydrogen bonds and an alkyl bond, while Cuscohygrine formed conventional hydrogen bonds with Cys A:47 and carbon hydrogen bonds with Glu A:465 and Arg A:244, as well as alkyl bonds with Leu A:152, Pro A:153, and Cys A:41. Lastly, Pseudotropine showed the lowest binding affinity at -5.6 kcal/mol, forming conventional hydrogen bonds with Glu B:236 and Gln B:241, carbon hydrogen bonds with Gly B:235 and Gln B:330, and an unfavorable donor-donor bond. These results suggest that several compounds from *Withaniasomnifera L.* may serve as potent COX-2 inhibitors, with binding affinities and interactions that are often superior to those of the reference drug, celecoxib.The 2D molecular interaction analysis of selected top-hit compounds with significant binding affinities towards COX-2 was carried out using BIOVIA Discovery Studio Visualizer. Among the identified hit compounds, selected representative top-ranked compounds were presented in **figure 2** to provide a clear and concise visualization of the protein–ligand interactions.

Table 2: Molecular Docking Results of Phytochemicals from *Withaniasomnifera L.* and Reference Compound Celecoxib against COX-2

Compounds	Binding affinity (kcal/mol)	Residues in contact	Types of interaction
Reference (Celecoxib)	-9.2	(Gly A:235) (Gln A:241) (Lys A:333) (Ser B:143) (Leu A:224)	Conventional hydrogen bond Conventional hydrogen bond Conventional hydrogen bond Carbon hydrogen bond Halogen (fluorine)
Withaferin A	-11.5	(Cys A:41) (Gln B:327) (Gly A:45)	Conventional hydrogen bond Carbon hydrogen bond Unfavourable acceptor-acceptor bond
Somniferanolide	-11.4	(Asn A:34) (Cys A:47) (Tyr A:136)	Conventional hydrogen bond Conventional hydrogen bond Pi-Alkyl bond

		(Gly A:135)	Carbon hydrogen bond
Withaoxylactone	-11.3	(Cys B:41) (Gly B:135) (Gln A:327)	Conventional hydrogen bond Conventional hydrogen bond Carbon hydrogen bond
Withanolide A	-11.1	(Arg A:61) (Lys B:546) (Ser A:126) (Lys A:532) (Gln A:372)	Carbon hydrogen bond Conventional hydrogen bond Conventional hydrogen bond Conventional hydrogen bond Conventional hydrogen bond
Withasomniferanolide	-11	(Val B:291) (Leu B:294) (Gln B:203) (Phe B:210) (His B:207)	Alkyl bond Alkyl bond Unfavourable acceptor-acceptor bond Conventional hydrogen bond Conventional hydrogen bond
Somniferawithanolide	-11	(Val A:291) (Leu A:294) (Gln A:203) (His A:207)	Alkyl bond Alkyl bond Unfavourable acceptor-acceptor bond Conventional hydrogen bond
Withanolide B	-10.9	(Pro B:153) (Cys B:36) (Tyr B:130) (Asn B:34) (Gln B:461)	Alkyl bond Alkyl bond Conventional hydrogen bond Conventional hydrogen bond Conventional hydrogen bond
Somnifericin	-10.8	(Phe B:142) (Leu B:145) (Arg A:376) (Glu A:236) (Trp B:139) (Asn B:375)	Alkyl bond Pi-Alkyl bond Conventional hydrogen bond Conventional hydrogen bond Conventional hydrogen bond Conventional hydrogen bond
Withanone	-10.7	(Arg B:61) (Lys A:546) (Gln A:543)	Carbon hydrogen bond Conventional hydrogen bond Conventional hydrogen bond
Physagulin D	-10.7	(Ser B:143) (Glu A:236) (Phe B:142) (Trp B:139) (Gly A:235) (Asp A:229) (Leu B:224)	Carbon hydrogen bond Carbon hydrogen bond Pi-Alkyl bond Conventional hydrogen bond Conventional hydrogen bond Conventional hydrogen bond Conventional hydrogen bond
Somniwithanolide	-10.6	(Cys B:47) (Asn B:34) (Asn B:39) (Asp B:157)	Conventional hydrogen bond Conventional hydrogen bond Conventional hydrogen bond Conventional hydrogen bond
Withanolide D	-10.4	(Pro B:154) (Ala B:156)	Carbon hydrogen bond Conventional hydrogen bond

		(Cys B:36)	Conventional hydrogen bond
Sominone	-10.1	(Cys A:36)	Carbon hydrogen bond
		(Glu B:326)	Conventional hydrogen bond
		(Ala A:156)	Conventional hydrogen bond
		(Asn A:39)	Conventional hydrogen bond
Anaferine	-6.8	(Val A:291)	Alkyl bond
		(Leu A:294)	Conventional hydrogen bond
		(His A:207)	Conventional hydrogen bond
Cuscohygrine	-6.3	(Cys A:47)	Carbon hydrogen bond
		(Glu A:465)	Carbon hydrogen bond
		(Arg A:244)	Alkyl bond
		(Leu A:152)	Alkyl bond
		(Pro A:153)	Alkyl bond
		(Cys A:41)	Conventional hydrogen bond
Pseudotropine	-5.6	(Glu B:236)	Carbon hydrogen bond
		(Gly B:235)	Carbon hydrogen bond
		(Gln B:330)	Unfavourable donor-donor bond
		(Gln B:241)	Conventional hydrogen bond

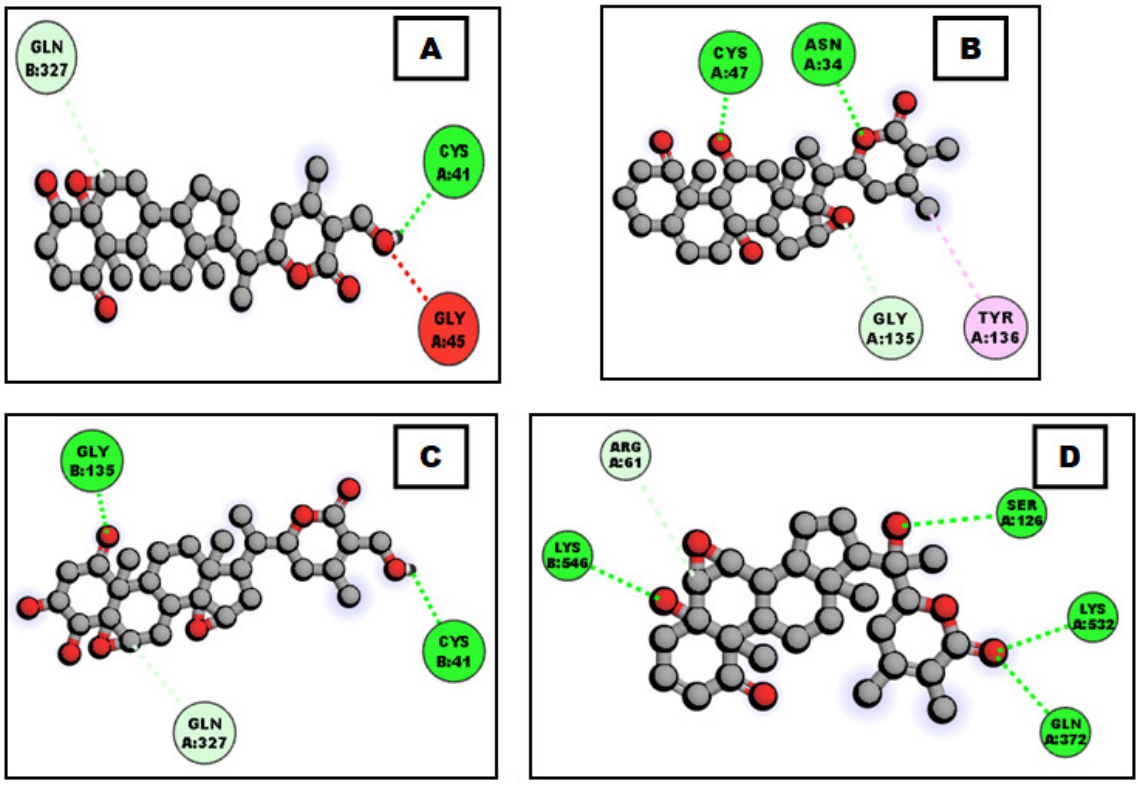


Figure 2: Molecular interaction of COX-2 with (a) Withaferin A(b) Somniferanolide (c) Withaoxylactone and (d) Withanolide A

Conclusion

Results of the present investigation demonstrated that several bioactive phytochemicals of *Withaniasomnifera L.* exhibited stronger binding affinities towards the COX-2 enzyme than the reference drug Celecoxib, suggesting their potential therapeutic significance in the management of dysmenorrhea and inflammation-associated pain. Among the screened compounds, thirteen phytochemicals exhibited stronger binding affinities than Celecoxib, indicating promising inhibitory potential against the COX-2 enzyme. Among these, representative top-ranked compounds including Withaferin A, Somniferanolide, Withaoxylactone and Withanolide A demonstrated the highest binding affinities and formed multiple conventional hydrogen bonds along with other stabilizing interactions with key active-site residues of COX-2, which may contribute to their potent inhibitory activity. These findings suggest that phytochemicals from *Withaniasomnifera L.* could serve as potential natural COX-2 selective inhibitors with possible advantages over conventional drugs associated with cardio-toxic side effects. The study provides preliminary evidence supporting the therapeutic relevance of these compounds in the treatment of inflammatory conditions, including dysmenorrhea. However, further *in-vitro*, *in-vivo* and clinical investigations are required to validate their safety, efficacy and practical therapeutic applications.

Abbreviations

PD	Primary Dysmenorrhoea
$CAST_P$	Computed Atlas of Surface Topography of Proteins
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
PDB	Protein Data Bank
SD	Secondary Dysmenorrhoea
WHO	World Health Organization

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Author contributions

Suraj Joshi performed literature search, writing the manuscript and designed the figures. **Vineeta Sharma** and **Suman Bhandari** performed literature search and contributed to the writing. Deepika Yadav, supervised the research and manuscript preparation. **Shankar Mondal** had the conceptualized the research, designed, edited and approved final manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no competing interests.

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