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IN VIVO, IN VITRO AND IN SILICO ANTI-DYSMENORRHEA STUDIES OF A. BRACTEOLATA LAM. LEAF EXTRACT AND ITS MOLECULAR DOCKING

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ABSTRACT

Menstruation is the periodic discharge of blood and the endometrium from the uterus and vagina with a painful experience. The pain can vary from woman to woman, for some women the pain is debilitating. Painful menstruation is known as Dysmenorrhea which literally means "difficult menstruation". Women with menstrual cramps had high levels of Prostaglandin F2 alpha (PGF2α) receptor. Prostaglandin F2 alpha is made by the uterus to stop Progesterone production. NSAIDs have been found to decrease menstrual flow, which may reduce menstrual pain. Herbal or natural remedies are more effective than NSAIDs for reducing menstrual disorders. *Aristolochia bracteolata* Lam. is a shrub distributed throughout India, belongs to the family Aristolochiaceae. *A.bracteolata* Lam. is proved to have antioxidant, potent antimicrobial, antidiabetic and antihelminthus properties. The results indicates that, regular treatment of normal rats with *A.bracteolata* Lam. leaf extracts improve hematological parameters. *In-vitro* studies revealed that it has got relaxation property. *In- silico* screening (Docking analysis) proved that the leaf extract has got four bioactive compounds act against PGF2α receptor.

Present study shows the potential for replacement of synthetic drugs by the use of natural extract.

KEYWORDS: Menstruation, Dysmenorrhea, PGF2α receptor, *A. bracteolata* Lam. leaf extract.

INTRODUCTION

The menstrual cycle is controlled by a number of glands and a series of hormonal changes beginning in the brain. Gonadotropins stimulate the ovaries to secrete the sex hormones, Estrogen and Progesterone. The four phases of the menstrual cycle are menstruation, the follicular phase, ovulation and the luteal phase. Menstruation occurs when the broken down lining of the uterus and flows from the body through the vagina. Menstruation generally lasts from 3 to 7 days. The length of a period can differ between women, and between cycles in individuals. Common problems are include heavy or painful periods and premenstrual syndrome (PMS).[1] In women with dysmenorrhea the contractions of uterus is very painful and the uterus may even spasm. The pain can be localized to the lower abdomen, but it can also be in the lower back, in the vulva, as well as radiating down the thighs.[2, 3]

Scientists discovered that women with menstrual cramps had high levels of Prostaglandin F2 alpha (PGF2α). Women with Dysmennorhea have been shown to produce seven times more PGF2α than women who do not.[4] This helps and explain why non-steroidal anti-inflammatory drugs (NSAIDs) reduce the production of prostaglandins and works for menstrual pain.[5]

Some studies have reported that the relief from pelvic pain is achieved after acupuncture or acupressure.[6] Yoga and meditative techniques that promote relaxation that may help relieve menstrual cramps.[7] Drugs can affect the body's chemistry, and therefore have the potential to produce side effects and may be harmful. Studies have generally found herbal or natural remedies to be effective for reducing menstrual disorders.[8]

Black cohosh (also known as *Cimicifuga racemosa* or squawroot) contains a plant estrogen and is the most studied herbal remedy for treating menopausal symptoms, including dysmenorrhea. Headaches and gastrointestinal tract problems are common side effects. It should not be taken more than 6 months.[4] Pycnogenol, an extract from the bark of the French maritime pine tree, may help to reduce menstrual pain and discomfort.[9]

Aristolochia bracteolata Lam. is a shrub, distributed throughout India, belongs to the family Aristolochiaceae. *A. bracteolata* Lam. commonly called as Worm killer in English and Aadutheendaapaalai in Tamil. *Aristolochia* is a large plant genus with over 500 species. Its members are commonly known as birthwort, pipe vine or Dutchman's pipe and are widespread and occur in the most diverse climates. Some species, like *A. utriformis* and *A.westlandii*, are threatened with extinction. This plant is chosen because it is used in traditional medicines as a gastric stimulant and in the treatment of cancer, lung inflammation, dysentery and snakebites. In the indigenous system of medicine, the plant was used as purgative, antipyretic & anti-inflammatory agents.[10] Its leaves are bitter and antihelminthic, antiulcer, antiplasmodial and are medicinally important. Almost every part of the plant has medicinal usage. The plant contain Aristolochic acid, has many medicinal properties in various disease condition. This study shows the potential for replacement of synthetic drugs by the use of natural extract.

Petroleum ether and acetone extracts of *A.bracteolata* Lam. was investigated for their anti-pyretic activity[11]**.** Antiallergic activity of *A.bracteolata* Lam. was evaluated by using compound 48/80 induced anaphylaxis, dermatitis rhinitis and pruritis, as a preclinical model for acute phase of hypersensitivity reactions.[12,13] The ethanolic extract of the shade dried leaves of *A.bracteolata* Lam. was evaluated anti inflammatory activities in Wistar rats by using the Carrageenan induced left hind paw edema method.[14] Anti arthritic activity was demonstrated using Freund's complete adjuvant in rats. The results of a study shows that regular treatment of adjuvant induced arthritic rats with *A.bracteolata* Lam. extracts improves ESR, Hb value and also restores body weight. The aqueous extract of leaves of *A. bracteolata* Lam. exhibited antiulcer activity in rats.[15] *In-vitro* antiplasmodial activity against *Plasmodium falciparum* 3D7 (chloroquine sensitive) and Dd2 (chloroquine resistant and pyrimethamine sensitive) was investigated by Ramasubramania raja R.[16] A study reveals that the 50% hydroethanolic leaf extract of *A. bracteolate* Lam. possess potent antioxidant and antidiabetic activity in Streptozotocin-induced diabetic rats.[17]

MATERIALS AND METHODS

Collection and Preparation of Leaf Extract: Fresh leaves of *A. bracteolata* Lam. used in this study were collected from the Peelamedu, Coimbatore, Tamil Nadu during the month of January at the flowering stage. The plant samples were identified and authenticated by a Siddha medical practitioner, MR.B.GANESH residing at the same place. The leaves of *A.*

bracteolata Lam. were gently cleaned from any of the foreign materials. The leaves were shade dried for several days. The dried leaves were pulverized by a mechanical grinder and stored. About 100 gm of the powdered sample was taken in a clean flask and soaked in 500 mL of 95% ethanol. This mixture was kept at room temperature with occasional shaking and stirring for about 72 hrs. The whole mixture was then filtered through cotton followed by Whatmann No.1 filter paper. The filtrate was allowed to dry and the dried powder was kept in air-tight container at room temperature.

Experimental Animal

Albino Wistar rats of female weighing 120-150 g were used for the present study. They were maintained in the animal house of KMCH COLLEGE OF PHARMACY, Coimbatore, Tamil Nadu for experimental purpose. The animals were maintained under controlled conditions of temperature 23°C, humidity 50% and 12 hrs. light-dark cycles. All the animals were acclimatized for seven days before the study. The animals were randomized into experimental and control groups and housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellets as basal diet and water *add libitum*. Animals were habituated to laboratory conditions for 48 hrs. prior to experimental protocol to minimize if any of non-specific stress. All experimental protocols were in compliance with Ethical committee on Research in Animals as well as internationally accepted principles for laboratory animal use and care.

Acute Toxicity Studies

The acute toxicity of *A. bracteolata* Lam. leaves extract was determined in female Wistar rats by HILAY method. Rats fasted for 16 hrs and were divided into groups. Graded doses of the extract (400, 800 and 1,200 mg/kg bw) were separately administered to the rats orally. All the rats were then allowed free access to feed and water and observed over a period of 48 hrs for signs of acute toxicity.

Assay of Biochemical and hematological parameters

Animals were grouped in to six groups, six in each. One group (Group I) is for control whereas other for experimental purpose. Picric acid is used to differentiate the rats present in the same group in a single cage. 1% DMSO solution was prepared and mixed with the powdered sample of *A. bracteolata* Lam. leaves extract. At the doses of 200, 400, 600, 800 and 1000 µg/kg bw/day were administered to the experimental animals orally for about 5

days. On 6th day morning, blood was collected from the normal and experimental rats with EDTA. The blood samples were subjected to check hematological parameters.

ESTIMATION OF HEMATOLOGICAL PARAMETERS

Enumeration of white blood cells: John method (1972)

The total white blood cells were enumerated according to the method of John (1972)[18] using Turk's fluid (WBC diluting fluid) and using a white blood cell pipette of haemocytometer.

Enumeration of red blood cells: John method (1972)

Using a red blood cell pipette and the haemocytometer, enumerated the RBC.

Differential Leukocyte Count: John method (1972)

Differential Leukocyte count was done by the method of John (1972) using Leishmann's stain.

Estimation of Hemoglobin: (Sahli's acid haematin method)

Hemoglobin is converted into acid haematin by the action of HCl. The acid haematin solution is further diluted with distilled water until its colour matches with exactly that of permanent standard of comparator block. The Hb concentration is read directly from the calibration tube.

EFFECT OF *A. bracteolata* Lam. LEAVES EXTRACT ON THE ISOLATED UTERUS

Studies were conducted on female albino wistar rat uterus by the methodology developed by Parker and Schimmer (2001).[19] Adult female wistar rats were pre-treated with Stilboestrol (1 mg/kg s.c.) 48 hrs. before the experiment to induce oestrous. Each rat was then sacrificed following a blow on the head and the abdomen cut open to reveal the fallopian tubes (uterine horns). The horns were dissected free of the adhering tissues. About 2 cm strip was cut out and mounted in a 25 ml organ bath containing De Jalon's solution. The effect of *A. bracteolata* Lam. leaves extract was studied with oxytocin.

GC-MS ANALYSIS of *A. bracteolata* Lam. LEAVES EXTRACT

GC-MS analysis of *A. bracteolata* Lam. leaf was performed using a Perkin-Elmer GC Clarus 500 system and Gas chromatograph interfaced to a Mass spectrometer (GC-MS). It is equipped with a Elite-I, fused silica capillary column (30mmX0.25mm 1D X1 µMdf, composed of 100% Dimethyl poly siloxane). For GC-MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the

carrier gas at constant flow rate 1ml/min and an injection volume of 2µl was employed (split ratio of 10:1); Injector temperature is 250°C; Ion-source temperature is 280°C.

IN-SILICO SCREENING (DOCKING ANALYSIS)

Phytochemical compounds identified by GC-MS analysis were used as ligands for docking on Prostaglndin f2 alpha receptor. Glide docking uses the assumption of a rigid receptor, although scaling of van der Waals radii of nonpolar atoms, which decreases penalties for close contacts, can be used to model a slight "give" in the receptor and/or ligand. Docking studies of designed compounds were carried out using GLIDE (Grid-based Ligand Docking with Energetics) module version 5.9. Schrödinger, LLC, New York, 2013. The software package running on multi-processor is Linux PC. GLIDE has previously been validated & applied successfully to predict the binding orientation of many ligands. The steps involved in docking are as follows:

Ligand structure and preparation

The chemical structure of each ligand was drawn using build module**.** 3D structures of all-atom for large numbers of drug-like molecules, starting with the 3D structures in SD Maestro format, LigPrep.

Preparation of protein

A typical PDB structure file consists only of heavy atoms and may include a co-crystallized ligand, water molecules, metal ions, and cofactors for immediate use in molecular modeling calculations.

Receptor Grid Generation

This requires a "prepared" structure: multi atom structure with appropriate bond orders and formal charges.

Ligand Docking

This is carried out using GLIDE DOCK. Glide searches for favorable interactions between one or more ligand molecules and a receptor molecule, usually a protein. Poses that passed these initial screens entered the final stage of the algorithm, which involves evaluation and minimization of a grid approximation to the OPLS-AA non bonded ligand-receptor interaction energy. Final scoring is then carried out on the energy-minimized poses.

Glide Extra-Precision Mode (XP)

The extra-precision (XP) mode of Glide combines a powerful sampling protocol with the use of a custom scoring function designed to identify ligand poses that would be expected to have unfavorable energies, based on well-known principles of physical chemistry. GlideScore is based on ChemScore, but includes a stearic-clash term and adds buried polar terms devised by Schrodinger to penalize electrostatic mismatches: Glide Score = 0.065*vdW + 0.130*Coul + Lipo + Hbond + Metal + BuryP + RotB + Site.

Docking Procedure

Docking studies of phytochemical compounds were performed using prostaglandin f2 alpha protein obtained from the RCSB Protein Data Bank, http://www.rcsb.org/pdb.

Experiments were performed using the program GLIDE (Grid-based Ligand Docking with Energetics) module version 5.9, Schrödinger, LLC, New York, NY, 2013 (Schrodinger Inc.). Coordinates of the full-length substrate-complexed dimmer were prepared for Glide 5.9 calculations by running the protein preparation wizard. The p-prep script produces a new receptor file in which all residues are neutralized except those that are relatively close to the ligand (if the protein is complexed with a ligand) or form salt bridges. The improved script runs a series of restrained impact energy minimizations using the Impact utility. Minimizations were run until the average root mean square deviation (RMSD) of the non-hydrogen atoms reached 0.3Å.

Glide uses two boxes that share a common centre to organize its calculations: a larger enclosing box and a smaller binding box. The grids themselves are calculated within the space defined by the enclosing box. The binding box defines the space through which the centre of the defined ligand will be allowed to move during docking calculations. It provides a measure of the effective size of the search space. The only requirement on the enclosing box is that it be large enough to contain all ligand atoms, even when the ligand centre is placed at an edge or vertex of the binding box. Grid files were generated using the co-crystallized ligand at the centre of the two boxes.

The size of the binding box was set at 20 Å in order to explore a large region of the protein. The 3D structures of the compounds were constructed using the Maestro interface. The initial geometry of the structures was optimized using the OPLS-2005 force field performing 1000 steps of conjugate gradient minimization. The compounds were subjected to flexible docking

using the pre-computed grid files. For each compound the 100 top-scored poses were saved and analyzed.

Qik Prop analysis

Qik Prop efficiently evaluates pharmaceutically relevant properties for over half a million compounds per hour, making it an indispensable lead generation and lead optimization tool. Accurate prediction of Absorption, Distribution, Metabolism, Elimination (ADME) properties prior to expensive experimental procedures, such as High Throughput Screening (HTS), can eliminate unnecessary testing on compounds that will ultimately fail; ADME prediction can also be used to focus lead optimization efforts to enhance the desired properties of a given compound.

Software used

1. GLIDE module version 5.9., mastero 9.iv

Quik prop -3.6 -Schrödinger, LLC, New York, NY, 2013 - docking

2. Swiss PDB viewer-4.04 – protein viewer
3. Pymol viewer 1.3 – image viewer
4. Marveen sketch 5.5 – drawing ligand structure

RESULT AND DISCUSSION

ACUTE TOXICITY TEST

In an acute toxicity study, rats showed no significant changes in behavior, breathing, cutaneous effects, sensory nervous system responses or gastrointestinal effects during the observation period. No mortality or any toxic reaction was recorded in animal group at 48 hrs. after administration of *A.bracteolata* Lam. leaves extract was safe up to a dose level of 1200 mg/kg body weight.

HEMATOLOGICAL PARAMETERS

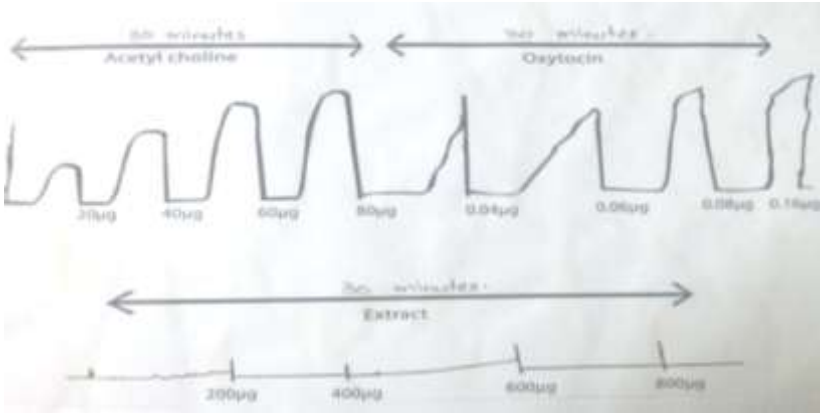
Hematological parameters report gives the values for complete blood cell count and differential count for normal and experimental rats. Values of hematological parameters were compared with the normal values and it is depicted in Table - 1. Hematological report showed that there is no any abnormal changes in the blood cells. Hence, the given extract is not toxic or unsafe for human consumption. Moreover, WBC content was increased slightly, where it indicates that there will be more immune capacity against foreign particles.

IN-VITRO STUDY OF *A. bracteolata* Lam. LEAF EXTRACT ON THE UTERUS

The rhythmic contractions of Stilboestrol pre-treated uteri were altered by the *A. bracteolata* Lam. leaf extract. This was validated by the contractile effects of oxytocin and acetylcholine on the same tissues. In addition to this, the contractile effect of oxytocin on the tissues was lightly blocked by the extract. Oxytocin is known to stimulate both the frequency and force of uterine contractions. The ability of the leaf extract to concentration-dependently reduce the contractile effect of oxytocin on rat uteri suggests that the oxytocin receptors were partially blocked by the extract at the tested concentrations. Results are shown in graph 1.

Table-1 Comparison of normal hematological parameters with experimental hematological parameters.

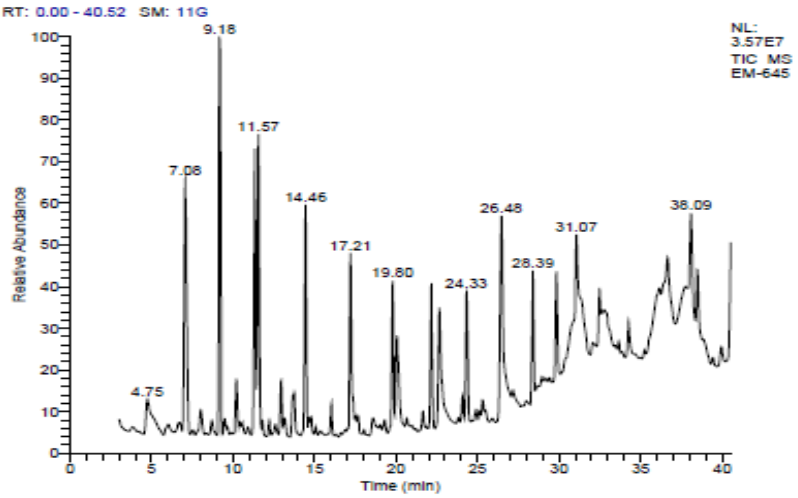
S. No	Hematological parameters	Group -I (Normal)	Group- II (200µg/ kgbw)	Group-III (400µg/ kg bw)	Group-IV (600µg/ kg bw)	Group-V (800µg/k g bw)	Group- VI (1000µg/ kg bw)
1	Total aemoglobin, g/dl	14.9±2.1	14.0±3.4	14.2±3.7	18.7±3.9	19.2±4.2	15.7±2.9
2	Packed cell volume in %	49.2±6.3	48.1±4.7	48.9±4.8	60.2±7.4	58.8±6.9	50.1±6.2
3	Total WBC count, Million/µl	8.5±0.8	9.7±0.7	10.5±1.2	11.2±1.3	13.2±0.9	11.5±1.1
4	Polymorphs in %	02±0.3	03±0.2	03±0.4	03±0.2	02±0.2	04±0.3
5	Lymphocytes in%	91±5	89±7	93±6	84±8	89±7	90±6
6	Monocytes in %	04±0.2	05±0.6	02±0.2	08±0.3	06±0.4	03±0.2
7	Eosinophils in %	03±0.3	03±0.4	02±0.3	05±0.7	03±0.4	03±0.4
8	Total RBC count, Million/µl	6.01±0.9	5.69±1.1	5.87±1.3	8.93±1.7	9.42±1.7	6.29±1.3
9	MCV, fL	81.8±7.2	84.5±9.9	83.3±8.3	67.4±7.9	62.4±7.5	79.6±11.3
10	MCH, Pg	24.7±3.0	24.6±1.8	24.2±2.4	20.9±1.1	20.3±1.8	24.9±1.8
11	MCHC, g/dl	30.2±3.6	29.1±4.8	29.0±4.1	31.0±5.6	32.6±4.9	31.3±3.7
12	RDW, %	17.0±4.0	16.8±2.0	17.2±2.4	16.8±1.9	17.3±2.0	15.6±3.1
13	Platelet count Million/µl	598±11	638±16	726±09	646±16	735±13	564±11
14	MPV, fL	8.3±0.7	7.8±1.1	8.0±1.6	9.2±2.3	8.4±1.9	7.2±0.8



Graph 1: Results of isolated uterus of rat.

PHYTOCHEMICAL SCREENING

In the phytochemical screening, ie. GC-MS analysis of the extract of *A.bracteata* Lam. demonstrated the presence of 10 compounds represented in the Graph-2 and Table-2.



Graph 2: GCMS analysis of the ethanolic extract of *A. bracteolate* Leaves.

Table-2 Report of GCMS analysis which is used for molecular docking.

S. No	Name Of Thecompounds	Molecular Weight
1	HEXADECANOIC ACID	284
2	VITAMIN-E	430
3	3-0-METHYL-d-GLUCOSE	194
4	α – D- GLUCOSE	180
5	β – SITOSTEROL	414
6	1-PHENANTHRENE CARBOXYLIC ACID	316
7	TRIMETHYL SILYL ESTER	328
8	PHYTOL	296
9	9,12,15,OCTADEOATRIENOICACID	292
10	3,7,11,15-TETRAMETHYL 2-HEXADECEN-1-OL	296

MOLECULAR DOCKING

Docking report says that, among ten phyto chemical compounds, four compounds such as β-sitosterol, Vitamin-E, Phytol, Trimethylsilyl ester are biologically active against the PGF2α-receptor. Least docking scores of compound indicate the great potential activity against painful menstrual cramps (Table-3). Literature studies of this compound also confirms that it has got a potential pain relieving activity with a very less side effects in men which can be the negligible factor in this problem.

Table- 3: Results of molecular docking in GLIDE software.

S. No	Name of Thecompounds	Docking Score	Interaction
1	HEXADECANOIC ACID	-7.729018	Weak
2	VITAMIN-E	-5.875682	Strong
3	3-0-METHYL-d-GLUCOSE	-7.925533	Weak
4	α – D- GLUCOSE	-8.220283	Weak
5	β – SITOSTEROL	-10.097247	Very Strong
6	1-PHENANTHRENE CARBOXYLIC ACID	-6.918876	Weak
7	TRIMETHYL SILYL ESTER	-8.093299	Strong
8	PHYTOL	-8.384455	Strong
9	9,12,15,OCTADEOATRIENOICACID	-7.886384	Weak
10	3,7,11,15-TETRAMETHYL 2-HEXADECEN-1-OL	-7.443515	Weak

From the literature survey and acute toxicity study, it has been found that the plant *A.bracteolata* Lam. is safe for use. From the hematological parameters analysis report it was conformed that this plant extract did not produce any toxic effect on human cells and hence it is safe for human consumption. From the biochemical analysis like ESR, CRP it is proved that this drug significantly decreases the level of prostaglandin which is the only major cause for this menstrual pain.[20] When comparing with the standard, our results have report a significant activity.

From the *in-vivo* analysis, anti-inflammatory activity of *A.bracteolata* leaves in Carrageenan induced paw edema method has shown a very good potential in concentration of 500 mg against inflammatory conditions than the standard drug diclofenac sodium.[15] Isolated rat uterus reports that it has got a good relaxation property. From the phytochemical analysis by GCMS method, it shows that this plant has got ten compounds.

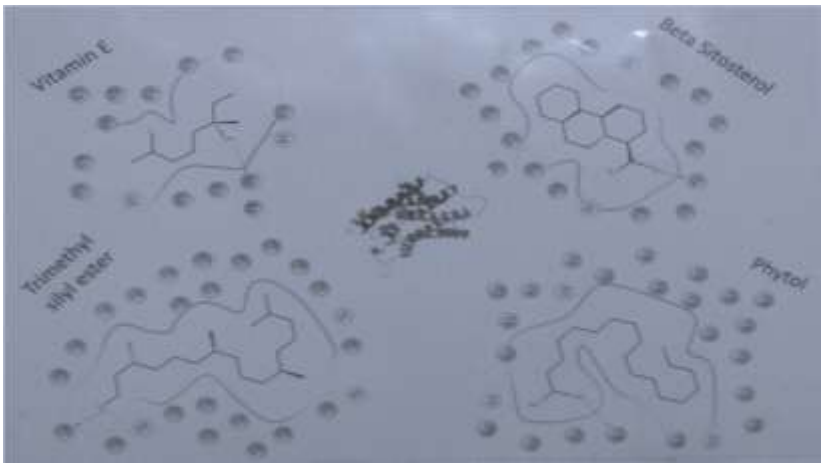


Plate-1: Results of docking using GLIDE software. Thecenter is the receptor of Prostaglandin f2 alpha docked with the ligands shown as in the picture.

In-silico study against the hormone, prostaglandin reveals that, β-Sitosterol has got a high potential activity among all the bioactive compounds. Other three bioactive compounds such as Vitamin-E, Trimethylsilyl ester, Phytol shows the similar activity against the PGF2α receptor (Plate-1). The potential compound β-sitosterol is a plant substance similar to cholesterol. It helps to reduce cholesterol levels by limiting the amount of cholesterol. Literature studies about other docked compounds says that it mainly possess pain relieving and anti-inflammatory activity other than many medicinal values. This scientific study supports and suggests that the plant has got potential drug activity on painful menstrual cramps and as an alternative to commonly used synthetic drug having various side effects.

SUMMARY AND CONCLUSION

Aristolachia bractelata Lam. has been used by many rural people traditionally in the treatment as anti-allergic, anti-arthritic, anti-pyretic, anti-inflammatory etc. The plant contain Aristolochic acid, has many medicinal properties in various disease condition. But there is no extensive pharmacological study to validate these uses, especially against this menstrual problem. Plant material was selected through various literature surveys. Acute toxicity test and biochemical assays conforms that this plant is not toxic and safe for human use. *In-vivo* report confirms that this plant has got potential activity to use as anti inflammatory drug. *In-vitro* studies revealed that it has got relaxation property. Thus, major causes of the problem can be solved using this drug. *In-silico* studies revealed that the drug has got four bioactive compounds namely β-sitosterol, Vitamin-E, Phytol and Trimethylsilyl ester. Among all the four compounds, β-sitosterol showed a very strong interaction. Literature studies of this bioactive compound conforms that it has got anti-inflammatory property and relaxation property. Hence it is concluded that the drug targets the suitable receptor and shows a good potential against painful menstrual cramps. This study also shows the potential for replacement of synthetic drugs by the use of natural extracts.

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