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Evaluation of anti-inflammatory activity of *Vitex agnus castus* leaves. Quantitative analysis of flavonoids as possible active constituents.

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ABSTRACT
Vitex agnus-castus L., (Verbenaceae) traditional Chinese medicinal plant recommended for various inflammatory ailments, but its mechanism of anti-inflammatory action is still unclear. The present study reports the anti-inflammatory activities of various extracts and elucidate possible mechanism(s) with the quantification of flavonoids by RP-HPLC. The petroleum ether, ethyl acetate, methanol and aqueous extracts obtained by successive solvent extraction from *Vitex agnus castus* leaves were subjected to standard acute, subacute and chronic models of inflammation at 200 and 400 mg/kg. Further, *in vitro* cytokine release estimation (IL-6 and TNF-α) using ELISA and the identification and quantification of major constituents with the RP-HPLC was done. All extracts (200 and 400 mg/kg) produced significant inhibition while methanol extract (400 mg/kg) caused a maximum inhibition of 43% in paw edema, 75% inhibition in subacute and 59.28% inhibition in the chronic inflammation model. Methanol extract (400 mg/kg) showed 77.87% and 63.34% decline in the IL-6 and TNF-α levels respectively. Kaempferol (0.24%) and luteolin (0.12%) were quantified in a bioactive methanol extract using HPLC analysis. It was concluded that the anti-inflammatory activity might be due to flavonoids in the extracts and plant regulates the inflammation by a significant decrease of TNF-α and IL-6 by macrophages, which mediates crucial events for the initiation. Information revealed that the methanol extract of *Vitex agnus castus* leaves can be used as effective anti-inflammatory agent.

Keywords: Granuloma pouch, IL-6, TNF-α, ELISA, leaf extracts, acute toxicity, cotton pellet, inflammation, acute toxicity, carragennan, kaempferol, luteolin, p-hydroxybenzoic acid, HPLC, Cyclooxygenase, Lipooxygenase, Premenstrual syndrome.

1. Introduction

Inflammation is a process involving multiple factors acting in a complex network. The ingress of leukocytes into the site of inflammation is crucial in the pathogenesis of inflammatory conditions. Neutrophils and macrophages are known to recruit and play pivotal roles in acute and chronic inflammation, respectively [1]. Recruited cells are activated to release many inflammatory responses, causing a change from the acute phase of inflammation. Therefore, inhibition of the cellular reactions is one of the targets that are generally used as an *in vitro* model for anti-inflammatory testing. Inflammation plays an important role in a wide variety of chronic human diseases such as cardiovascular diseases and cancer. It has been demonstrated that pro-inflammatory cytokines, cyclooxygenase-2 (COX-2) and free radical species interact in a complex manner in an inflammatory environment [2]. For example, tumor necrosis factor-α (TNF-α) has been shown to be one of the major cytokines that mediates many crucial events in the initiation of both acute and chronic inflammation through regulating production of some other cytokines, up-regulation of adhesion molecule expression, and the activation of leukocyte-specific chemotactic cytokines [3]. Interleukin-6 (IL-6) is another pro-inflammatory cytokine that promotes inflammatory events through the activation and proliferation of lymphocytes, differentiation of B cells, leukocyte recruitment and the induction of the acute-phase protein response in the liver [4]. Inhibition of the expression and production of these powerful mediators by anti-inflammatory components might represent a possible preventive or therapeutic target, and may be used to develop anti-inflammatory nutraceuticals for health promotion and disease prevention.

Vitex agnus-castus L. (Verbenaceae), the chaste tree, has been an official medicinal plant in ancient medicinal art and is named in the work of Hippocrates, Dioskurides, Theophrastus and other [5]. In clinical trials, the fruit agni castus fructus was shown to relieve premenstrual syndrome (PMS) including corpus luteum insufficiency, premenstrual syndrome (PMS), menopausal symptoms, insufficient milk production and especially breast swelling and pain due to its dopaminergic effect [6, 7, 8]. It has been reported that *Vitex agnus castus* contains iridoids, flavonoids, diterpenoids, progestins, essential oils and ketosteroids [9, 10]. Recently, several secondary metabolites, artemetin, casticin, 3,3′-dihydroxy-5,6,7,4′-tetramethoxyflavon, penduletin, methyl 4-hydroxybenzoate, p-hydroxybenzoic acid, methyl 3,4-dihydroxybenzoate, 5-hydroxy-2-methoxybenzoic acid, vanillic acid and 3,4-dihydroxybenzoic acid were isolated from a folkloric medicinal plant, *Vitex agnus-castus*. Compounds were found to have significant anti-inflammatory activity in a cell-based contemporary assay [11]. A new compound trivially named vitexcarpan was isolated and the compound showed moderate *in vitro* anti-inflammatory activity [12]. The objective of the present study was to evaluate the ethnobotanical and traditional uses on well-known acute, subacute and chronic experimental models for anti-inflammatory activity and to investigate the probable mechanism of inflammation by cytokine estimation.

2. Materials and methods

2.1 Chemicals

All solvents and reagents used were of analytical grades and procured from commercial sources. Double distilled water was used throughout the work and was produced by the double distillation unit.

2.1.1 IL-6 and TNF-α estimation

IL-6 and TNF-α were quantified with rat enzyme linked immuno-sorbent assay (ELISA) kits produced by Ray-Biotech, Inc. U. S. A.

2.2. Plant material

Leaves of *Vitex agnus castus* were collected from the University of Madras, Maduravoyal (Chennai, Tamil Nadu, India) botanical garden in May 2008 from, A specimen (61/July/2007) was deposited and authenticated by the Senior Botanist, Dr. M. Ayannar, Post Graduate and Research Department of Botany, Pachaiyappa's College, Chennai which further confirms its identity.

2.3. Preparation of extract

The plant material was air dried at room temperature. The dried plant material was grounded into a fine powder. The powdered leaves were used to prepare the petroleum ether, ethyl acetate, methanol and aqueous extract by the successive solvent extraction method. The extracts were subjected to qualitative chemical tests for the detection of various Phytoconstituents [13]. The preliminary phytochemical studies showed the presence of flavonoids in methanol extracts.

2.4. Experimental animals

Albino male/ female mice body weight (B.W. 60-90 g) and Wistar rats (B.W. 140-190 g) were used. They were housed in standard microlon boxes and were given a standard laboratory diet and water *ad libitum.* The animals were maintained in polypropylene cages in the National Toxicological Center, Pune. The temperature in the experimental room was maintained at 23±2 °C. The relative humidity was maintained at 75±5%. Lighting was artificial with the sequence of 12 h light, 12 h dark. For feeding a conventional laboratory diet (Amrut Feeds, Maharashtra) was used with an unlimited supply of drinking water. The animals were randomly selected, marked to permit individual identification and kept in their cages for at least 7 days prior to dosing to allow for acclimatization to the laboratory condition. They were further segregated into groups of 6 for different experimental schedule (acute, sub-acute and chronic). All animal experiments were carried out according to the institutional animal ethical committee (Approval letter no.) - 047.

2.5. Acute oral toxicity study

The acute oral toxicity assay was performed in healthy adult female albino Swiss mice (20–35 g) divided into different groups as per the Organization for Economic cooperation and development (OECD) guidelines-423. *Vitex agnus castus* leaves crude powder were taken for the study. Three animals were used for each step. As there was no information on extracts to be tested, for animal welfare reasons, 300 mg/kg body weight was selected as the starting dose for the study. The next two doses selected for the studies were 2000 and 5000 mg/kg. The test substances were administered in a single dose by using oral feeding needle. Animals were fasted for 3-4 h prior to dosing. Following the period of fasting, the animals were weighed and the test substance was administered. After administration of the substance, the food was withheld for 1-2 h, and observed for acute toxicity.

2.6. Carrageenan-induced rat paw edema

Albino mice (six per group), 60- 90 g, were fasted for 24 h before the experiment with free access to water. Fifty microlitres of a 1% suspension of carrageenan (Sigma Co., USA) in saline was prepared 1 h before each experiment and was injected into the plantar side of both hind paws of the rats. The (petroleum ether, ethyl acetate, methanol and aqueous) extracts at the doses of 200 and 400 mg/kg was suspended in Tween 80 plus 0.9% (w/v) saline solution and administered orally. The standard drug diclofenac at the dose of 10 mg/kg were administered orally. Drugs or drugless vehicle were injected 1 h before the carrageenan treatment [14]. Paw volume was measured immediately after carrageenan injection and at 1, 2, 3, 4, 5 and 6 h after the administration of the edematogenic agent using a plethysmometer, the degree of swelling induced was evaluated by the ratio a/b, where a and b are total volumes of both hind paws after and before carrageenan treatment, respectively. The difference of average values between treated animals and controlled group was calculated for each time interval and evaluated statistically.

2.7. Granuloma pouch model

Male and female Wistar rats with a body weight between 150 and 200 g were used. Six animals were taken for controls and for test groups. The back of the animals was shaved and disinfected. With a very thin needle a pneumoderma was made in the middle of the dorsal skin by injection of 6 ml of air under ether anesthesia. Into the resulting oval air pouch 4 ml of a 2% solution of Croton oil in sesame oil was injected avoiding any leakage of air [15]. 48 h later the air is withdrawn from the pouch and 72 h later any resulting adhesions are broken starting with the formation of the pouch, the animals were treated every day orally with the test compound (bioactive petroleum ether and bioactive methanol extract at a dose of 200 and 400 mg/kg) and the standard (1.5 mg/kg indomethacin). On the 7th day the animals were sacrificed under anesthesia. The pouch was opened and the exudate was collected in glass cylinders. The average value of the exudate of the controls and the test groups

was calculated. Comparison was made by statistical means.

2.8. Histology
The paw was fixed in formalin and paraffin-embedded. Serial sections 6 µthick were carried out perpendicularly to the skin surface and stained with hematoxylin– eosin, to show the common morphology of the tissues.

2.9. Cotton pellet induced granuloma model
Male Wistar rats with an average weight of 200 g are anaesthetized with ether. The back skin was shaved and disinfected with 70% ethanol. An incision was made in the lumbar region. By a blunted forceps subcutaneous tunnels were formed and a sterilized cotton pellet weighing 50 mg was placed on both sides in the scapular region. Animals were divided into six groups (n = 6). Bioactive methanol and petroleum ether extracts (200 and 400 mg/kg of each), diclofenac sodium and control vehicle were administered daily for 10 days. On the 11th day the pellets were dissected out, dried at 60°C, and the dry weight was determined [16]. The weight of the cotton pellet before implantation is subtracted from the weight of the dried, granuloma pellets.

2.10. Serum interleukin–6 and TNF–α estimation for sub-acute croton oil granuloma pouch model
The serum level of IL-6 and TNF–α was estimated by *in vitro* enzyme linked immunosorbent assay (ELISA) kit, using colorimetric reaction method. This assay employed an antibody specific for Rat TNF-α coated on a 96-well plate and antibody specific for rat IL-6 coated on a 96-well plate. Standards and samples were pipetted into the wells and TNF-α present in a sample is bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-Rat TNF-α antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells.
The wells were again washed, a TMB substrate solution was added to the wells and color developed in proportion to the amount of TNF-α bound. The Stop Solution changed the color from blue to yellow, and the intensity of the color was measured at 450 nm.

2.11. Statistical analysis
Results were expressed as mean ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test. A value of $P \geq 0.05$ was considered to be statistically significant.

2.12. Quantitative analysis by HPLC
2.12.1. Preparation of standard solution
Accurately weighed quantity 5 mg of p-hydroxybenzoic acid was dissolved in a 10 ml mobile phase that gives 500 µg/ml, dissolved 1.5 mg kaempferol in 1 ml of mobile phase and 1.0 mg luteolin in 1 ml of mobile phase. The stock solution was diluted in the ratio of 1:3.

2.12.2. Preparation of sample solution
Accurately weighed quantity 100 mg of methanol extract and was dissolved in a mobile phase and volume was made up to 100 ml that gives 1000 µg/ml solution.

2.12.3. Chromatographic conditions
The Reverse phase high performance liquid chromatography (RP-HPLC) analysis was done using Shimadzu Instrument with UV-VIS detector at a Wavelength of 254 nm using Merck RP-C-18, 250 x 4.6 mm ID, 5 µ Column at a Flow rate of 0.6 ml/min. The Mobile phase used was- water: acetic acid (99.0:1.0 v/v) as solvent A and acetonitrile as solvent B using a gradient elution in 0-14 min with 20-35% of solvent B, 14-40 min with 35-50% of solvent B, 40-80 min with 50-20% of solvent B injecting 20 µl volume.

3. Results
There was no mortality or morbidity observed in animals through the 14 day period following single oral administration at all selected dose levels of crude extracts of *Vitex agnus castus.* No observed adverse effect limit (NOAEL) was >5000 mg/kg when administered orally.

3.1. Carrageenan induced paw edema in rat
Extracts showed the dose dependant response for anti-inflammatory activity in a carrageenan induced paw edema. Methanol extract at a dose of 400 mg/kg was found to be most active amongst the extracts and activity was comparable with standard anti-inflammatory drug diclofenac as shown in Table-1

3.2. Croton oil granuloma pouch model
In the sub-acute croton-oil induced granuloma pouch test, the anti-inflammatory activity of extracts was profound and found to be highly significant as shown in Table-2. Since granuloma represents the late exudative and proliferative phases of inflammation. Based on the data, among all the extracts, the methanol extract was found to be more potent at a dose of 400 mg/kg. The activity was found to be dose-dependant.

3.3. Histology
Specimens from histological paw sections of treated animal, according to the experimental procedure showed a significant reduction of the inflammatory process.
The histology as shown in Fig1 (a-d) revealed the science of healing in the form of crust formation and epithelization at some sites, there was healing in the form of fibrosis. The tissue was densely perforated with densely populated infiltration of polymorphs nuclear leucocytes and lymphocytes. This confirms that various extracts have anti-inflammatory activity in terms of facilitation of healing in chronic granulomatous inflammation. This is also correlated with the effects of the extracts on the levels of various inflammatory cytokines. The healing process was almost equivocal with all the extracts.

3.4. Cotton pellet induced granuloma in rats
In the chronic cotton pellet-induced granuloma model, all the extracts showed inhibition the methanol extract significantly more effective. The results showed that the methanol extract was most inhibitory in its action and is proportional to the doses employed (Table-3); thus proving its activity in the proliferative phase of inflammation.

3.5. Cytokines Estimation
The methanol extract was found to be more potent as it had the highest % inhibition for IL-6 and TNF-α amongst all the extracts. (Tables 4a & 4b)

3.6. HPLC Analysis
Quantitative analysis of *Vitex agnus castus* leaves with the methanol extract were carried out using reverse phase HPLC and the chromatographic profile were compared with the retention times of the reference standard. From the chromatographic profile shown in Fig 2 and 3. It was observed that kaempferol having retention time of 49 min was found to be 0.24% in a methanol

extract and luteolin having retention time of 52 min was found to be 0.12% in a methanol extract. The selection of these standards is due to their medicinal properties stated in the literature.

Table 1: Carrageenan induced paw volume

		Before drug admini-stration	30 min	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
Group-1	Carrageenan 0.1 ml	2.26± 0.08	3.98± 0.14	4.08± 0.12	4.58± 0.14	5.00± 0.16	4.38± 0.16	3.89± 0.16	3.49± 0.12
Group-2	Control	2.26± 0.08	2.25± 0.04	2.27± 0.06	2.24± 0.07	2.25± 0.08	2.26± 0.08	2.26± 0.08	2.26± 0.08
Group-3	Diclofenac-10 mg	2.25± 0.10**	2.78± 0.08**	2.62± 0.07**	2.32± 0.08**	2.01± 0.07**	2.34± 0.06**	2.30± 0.06**	2.16± 0.06**
Group-4	Petroleum ether 200 mg	2.24± 0.06**	3.5± 0.08**	3.19± 0.12**	3.26± 0.12**	3.42± 0.14**	2.86± 0.08**	2.65± 0.14**	2.28± 0.14**
Group-5	Petroleum ether-400 mg	2.23± 0.08**	3.10± 0.15**	3.19± 0.16**	3.18± 0.14**	3.26± 0.16**	2.74± 0.14**	2.48± 0.16**	2.16± 0.24**
Group-6	Ethyl acetate-200 mg	2.23± 0.06*	3.42± 0.08*	3.48± 0.08*	3.12± 0.06*	4.21± 0.08*	3.64± 0.08*	3.26± 0.08*	2.85± 0.06*
Group-7	Ethyl acetate-400 mg	2.23± 0.07**	3.32± 0.07**	3.42± 0.07**	3.79± 0.14**	4.12± 0.20**	3.54± 0.14**	3.12± 0.31**	2.68± 0.36**
Group-8	Methanol-200 mg	2.20± 0.06**	2.96± 0.06**	2.79± 0.06**	3.10± 0.08**	3.36± 0.08**	2.12± 0.06**	2.45± 0.07**	2.21± 0.08**
Group-9	Methanol-400 mg	2.16± 0.06**	2.86± 0.07**	2.89± 0.07**	2.94± 0.06**	3.02± 0.09**	2.64± 0.16**	2.24± 0.18**	2.02± 0.18**
Group-10	Aqueous -200 mg	2.24± 0.08 ns	3.64± 0.08 ns	3.72± 0.08 ns	4.28± 0.16 ns	4.29± 0.14 ns	3.72± 0.12 ns	3.25± 0.14 ns	2.84± 0.14 ns
Group-11	Aqueous -400 mg	2.25± 0.06 ns	3.58± 0.08 ns	3.74± 0.0 ns 7	4.16± 0.18 ns	4.06± 0.16 ns	3.58± 0.32 ns	3.18± 0.14 ns	2.82± 0.14 ns

Value are mean ± S.E.M.;** $P < 0.01$; * $P<0.05$; ns $P>0.05$; as compared to the control, $n=7$.

Table 2: Sub-acute granuloma pouch model

Each group	Extract	Weight of granuloma in (mg)	% inhibition
Group-1	Control	148.42 ± 24.36	
Group-2	Diclofenac-5 mg	28.62 ± 4.62**	80.72
Group-3	Petroleum ether-200 mg	58.62 ± 9.62**	60.5
Group-4	Petroleum ether-400 mg	54.86 ± 8.69**	63.03
Group-5	Methanol-200 mg	39.68 ± 8.42**	73.06
Group-6	Methanol -400 mg	36.68 ± 4.94**	75.32

Value are mean ± S.E.M.; ** $P < 0.01$; as compared to the control, $n=7$.

Table 3: Cotton pellet induced granuloma in rats-

Groups	Extracts	Weight of cotton pellets (mg) (B.D.A)	A.D.A Wet weight(mg) (mean ± S.E.M)	Dry weight (mg)(mean ± S.E.M)	% change in granuloma weight
Group-I	Control	50	536.66 ± 30.48**	286.00 ± 26.28**	
Group-II	Positive control (diclofenac 5 mg)	50	198.64 ± 4.98**	87.64 ± 5.86**	69.5
Group-III	Petroleum ether-200 mg/kg	50	333.33 ± 29.87**	178.36 ± 21.86**	37.0
Group-IV	Petroleum ether-400 mg/kg	50	298.64 ± 27.86**	156.64 ± 24.28**	45.2
Group-V	Methanol 200 mg/kg	50	265.00 ± 26.28**	129.16 ± 16.10**	54.8
Group-VI	Methanol 400 mg/kg	50	236.64 ± 18.11**	116.68 ± 5.86**	59.2

Value are mean ± S.E.M.; ** $P < 0.01$; as compared to the control, $n=7$.

Table 4a: Estimation of Interleukins-6 (IL-6)

Treatment Group	IL-6 (pg/ml)		
	Absorbance	Concentration	% Inhibition
Vehicle	0.341±0.02*	33.325	0
Granuloma Control	0.358±0.03*	470.825	0
Ethyl acetate Extract	0.357±0.02*	445.825	5.31
Petroleum ether Extract	0.35±0.04*	270.825	42.48
Methanol Extract	0.344±0.03*	104.175	77.87
Aqueous Extract	0.357±0.02*	425	9.73
Diclofenac-5 mg	0.342±0.02*	86.840	81.56
Value are mean ± S.E.M.;	*P<0.01; as compared.	to the control,	n=7

Table 4b: Estimation of TNF-α (pg/ml)

Treatment Group	TNF-α (pg/ml)		
	Absorbance	Concentration	% Inhibition
Vehicle	0.385±0.02*	41.65	0
Granuloma Control	0.39±0.04*	250	0
Ethyl acetate Extract	0.389±0.03*	233.35	6.66
Petroleum ether Extract	0.388±0.02*	150	40
Methanol Extract	0.386±0.04*	91.65	63.34
Aqueous Extract	0.388±0.03*	175	30
Diclofenac-5mg	0.386±0.03*	74.25	70.3
Value are mean ± S.E.M.;	*P < 0.01.	as compared to the control	, n=7

4. Discussion

This study revealed an anti-inflammatory effect of the petroleum ether, ethyl acetate, methanol and aqueous extract of *Vitex agnus castus* leaves. All the extracts were effective anti-inflammatory in nature, however, methanol extract at a dose of 400 mg/kg was found to be most potent. It was found to be comparable with that of diclofenac-10 mg for prevention of edema at the later phase in the fourth hour after carrageenan injection with major reduction in paw volume. It has been reported that various mediators are released by carrageenan in the rat paw. The initial phase is attributed to the release of histamine and 5-hydroxytryptamine (5-HT). A second phase is mediated by kinins and finally in a third phase, the mediator is suspected to be prostaglandin [17, 18]. Again, our results indicated inhibition of TNF-α and IL-6by the methanol extract.

The chemical analysis of the plant extract revealed presence of a number of flavonoids. A variety of *in vitro* and *in vivo* experiments have shown that selected compounds of this large group of naturally occurring products possessed various important pharmacological activities including anticancer, anti-inflammatory and anti-allergic activities [19, 20]. Studies have shown that the anti-inflammatory activity of flavonoids is mediated by the inhibition of arachidonic acid-metabolizing enzymes, cyclooxygenase and lipoxygenase as well as by anti-oxidative properties. It has been previously demonstrated that several flavonoids such as flavones (chrysin, 3-hydroxyflavon and galangin), some chalcones (having a 3–4 hydroxyl substitution) were COX inhibitors [21, 22]. Flavonols such as kaempferol, morin and quercetin were 5-LO inhibitors [23], while most flavanols are COX-1 inhibitors [24]. According to these studies, the anti-inflammatory activity of *Vitex agnus castus* might be due to its content in flavonoids, which were detected by analytical methods.

In the chronic cotton pellet-induced granuloma test, the methanol extract significantly reduced the weight of cotton pellet-induced granuloma in rats at a dose of 200 and 400 mg/kg, however, less effective than the comparative standard used in this experimental model. The results showed that the methanol extract was inhibitory in its action in the proliferative phase of inflammation.

Sub-acute inflammation involves infiltration of macrophages, neutrophil and proliferation of fibroblasts [25]. Hence, the decrease in granuloma and the anti-inflammatory activity of methanol extract of *Vitex agnus castus* leaves was profound and highly significant. Since granuloma represents the late exudative and proliferative phases of inflammation [26], the investigations indicated that the methanol extract possesses anti-inflammatory activity against acute, sub-acute and chronic inflammatory response.

The literature has reported the *Vitex agnus castus* methanol extract interacted with opioid receptor and showed hazy analgesic activity [27]. In the present study the results demonstrated the analgesic potential, further complemented with potential anti-inflammatory. The possible reason might be the different contents and sorts of bioactive compounds, including phenolics, flavonoids and other compounds responsible for the activity.

Presence of flavonoid compound like 3,5-dihydroxy-3′,4′,6,7-tetramethoxyflavonol were reported in the leaves of *Vitex agnus castus*. In the present study, *Vitex agnus castus* leaf (crude basis) was shown to contain 2.60% flavonoid compounds. Flavonoids are known to inhibit the enzyme prostaglandin synthetase, more specifically the endoperoxidase and reported to produce significant anti-inflammatory effect [28].

In cytokine estimation using ELISA, amongst all extract including methanol extract of *Vitex agnus castus* leaves showed a significant

decrease in IL-6 and TNF-α production in a dose-dependent matter. The methanol extract produced highest % inhibition for IL-6 (pg/ml) and TNF- α (pg/ml) production followed by petroleum ether, aqueous and ethyl acetate extract. This further proves that *Vitex agnus castus* regulates the inflammation by a significant decrease of pro-inflammatory cytokines such as TNF-α and IL-6 by macrophages, which mediates many crucial events for the initiation of acute, subacute and chronic inflammation. Also, it is having an anti-proliferative activity and the potential to directly react with free radicals.

Kaempferol and luteolin has a definite anti-inflammatory role [29, 30]. In a similar study, phenolic compounds were shown potent anti-inflammatory activity [30]. The suppressive action of kaempferol on activation of proinflammatory NF-kB was recently demonstrated in young and old rats and endothelial cell lines. Luteolin has antioxidant, anti-inflammatory, anti-allergic, anticancer, and immune-modulating properties to suppress hyperactive immune systems. A diet rich in the plant compound luteolin reduces age-related inflammation in the brain and related memory deficits by directly inhibiting the release of inflammatory molecules in the brain. Luteolin inhibits TNF-α and allergic edema [31]. Furthermore the p-hydroxybenzoic acid from *Vitex agnus castus* has shown significant anti-inflammatory activity *in vitro* studies [11].

The phytochemical standardization of the extracts revealed high total flavonoids and phenolic content, which have been known for its anti-inflammatory activities. Results proved that plant regulates the inflammation by a significant decrease of TNF-α and IL-6 by macrophages, which are the mediators for initiating the crucial events. Furthermore phytochemistry of *Vitex agnus castus* has been worked out [11]. The presence of kaempferol, luteolin and p-hydroxybenzoic acid in methanol extract of *Vitex agnus castus* was phytochemically confirmed.

The present research work validates the traditional claim of *Vitex agnus castus* on modern scientific line

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