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Evaluation of antioxidant potential and quantitative analysis of Key flavonoids and bioactive compound by RP-HPLC and HPTLC in the leaves of *Vitex agnus castus*

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Abstract

Vitex agnus-castus L., of the Verbenaceae family is mentioned in ancient medicinal art as an official medicinal plant. It is used traditionally to treat premenstrual syndrome, inflammatory disorders such as pain of rheumatism etc so the objective is to evaluat the *in-vitro* and *in-vivo* antioxidant activities of its various extract of leaves part and elucidate possible mechanism(s) with the quantification of bioactive compounds by RP-HPLC and HPTLC**.** Petroleum ether, ethyl acetate, methanolic and aqueous extract of the leaves of *Vitex agnus castus* L were prepared and assessed for *in vitro* antioxidant activity by various in vitro assay models. The extracts were also evaluated for *in-vivo* antioxidant activity by estimating level of Glutathione, SOD., Catalase and Lipid peroxidase and identification and quantification of major bioactive compounds by RP-HPLC and HPTLC were done. Methanolic extract found to be having more in vitro and in vivo antioxidant activity. Further, the quantification of constituents present in the extract was carried out by RP- HPLC and HPTLC which has shown kaempferol (0.24%), Luteolin (0.12%) and Aucubin (0.43625 µg) in the methanolic extract.The significant antioxidant activity which may be due to the presence of polyphenolics and flavones in the vitex.. The antioxidant property makes it suitable for chronic and prolonged administration required for treatment of chronic inflammatory process.

Keywords: Anti–lipid peroxidation, antioxidant activity, DPPH, flavonoids, phenolic contents, Vitex agnus castus.

INTRODUCTION

Oxygen is present in the atmosphere as a stable triplet biradical ($^3O_2$) in the ground state and a vital component for the survival of the human.Once inhaled, it undergoes a gradual reduction process and ultimately gets metabolized into water.In this process,a small amount of reactive intermediates,such as super oxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), nonfree radical species (such as H_2O_2), and the single oxygen ($^1O_2$) are formed[1]. Those reactive intermediates are collectively termed as reactive oxygen species (ROS). [2,3]These primary derivatives of oxygen play an important role in mediating ROS-related effects. [4] ROS can easily initiate the peroxidation of the membrane lipids, leading to the accumulation of lipid peroxides.The peroxidation products by themselves and their secondary oxidation products, such as malondialdehyde (MDA) and 4-hydroxinonenal (4-HNE) are highly reactive; they react with biological substrates, such as protein, amines, and deoxyribonucleic acid (DNA).[5] In living organisms various ROS can be formed by different ways.In normal aerobic respiration, stimulated polymorph nuclear leukocytes and macrophages and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by cells.Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides [6]. Most living species have an efficient defense systems to protect themselves against the oxidative stress induced by ROS. Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human diseases including cancer, atherosclerosis and the aging processes. [7] Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating free catalytic metals and also by acting as oxygen scavengers. Phenolic antioxidants functions are free radical terminators and sometimes also metal chelators[8]. Thus, antioxidant defense systems have co-evolved with aerobic metabolism to counteract oxidative damage from ROS. The antioxidants may be used to preserve food quality from oxidative deterioration of lipid. Therefore,antioxidants play a very important role in the food industry. Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) are widely used in the food industry, but BHA and BHT have suspected of being responsible for liver damage and carcinogenesis. Therefore, the development and utilization of more effective antioxidants of natural origin are desired.

Vitex agnus castus was already in ancient medicinal art as an official medicinal plant and is named in the work of Hippocrates, Dioskurides, Theophrastus and others.[9] In clinical trials, the fruit agni castus fructus was shown to relieve premenstrual syndrome (PMS) including corpus luteum insufficiency, premenstrual syndrome (PMS),menopausal symptoms and insufficient milk production and especially breast swelling and pain due to its dopaminergic effect. [10,11,12] It has been reported that *Vitex agnus castus* contains iridoids, flavonoids,diterpenoids, progestins, essential oils and ketosteroids. [13,14] Considering the traditional uses of this Ayurvedic drug, the preliminary phytochemical studies, and total polyphenolic determination, it was predicted to have antioxidant activity. In this study, we wanted to determine the antioxidant effects of *Vitex agnus castus* and compare their antioxidant activities with those commonly used as natural antioxidants such as ascorbic acid and synthetic antioxidants such as BHA and BHT. The aim of this study was to investigate the antioxidant properties of *Vitex agnus castus* in order to evaluate its medicinal value and to point an easily accessible source of natural

antioxidants that could be used as a possible food supplement or in pharmaceutical industry.

MATERIALS AND METHODS

Chemicals

Chemicals used in this study were 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma no D 9132), potassium ferricyanide, sodium nitrite, trichloroacetic acid, folin-Ciocalteu reagent, sodium nitroprusside, sulfanilamide, N-1-naphthylethylenediamine dihydrochloride, butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), ascorbic acid (Merck [India], thiobarbituric acid, tocopheryl acetate. All reagents used for the experiments were of analytical grade.

Plant Material

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

Preparation of extract

The plant material was then air dried at room temperature.The dried plant material was grounded into a fine powder. The powdered leaves was used to prepare the petroleum ether, ethyl acetate, methanolic and aqueous extract. The extracts were subjected to qualitative chemical tests for the detection of various Phytoconstituents.[15] . The preliminary phytochemical studies showed the presence of flavonoids in methanolic extracts.

Estimation of total phenolics

The total phenolic contents of ethanol extracts were determined with Folin-Ciocalteu reagent according to Sadashivam & Manickam [16] and slightly modified. The stock solution of extract 1mg/1ml in water was prepared. From the stock solution, 5 ml of solution was transferred to a 25-ml volumetric flask, the volume was made up with distilled water, and out of this sample 5 ml or standard 2 ml was taken in 25 ml volumetric flask, to this 10 ml of distilled water, and 2 ml of phenol reagent (20%) was added, and then the volume was made up with 29% sodium bicarbonate.The mixture was kept in dark for 20 min, after which the absorbance at 760 nm was noted. Standard used was gallic acid, and the total polyphenolic content was calculated as gallic acid, equivalents and expressed in % as gallic acid.

Estimation of total Flavonoids

Aluminium chloride colorimetric technique was used for flavonoids estimation. [17] Each extract (0.5 ml of 1:10 g ml^{-1}) in methanol was separately mixed with 1.5 ml of methanol, 0.1 ml of 10 % aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. It was left at room temperature for 30 min after which the absorbance of the reaction mixture was measured at 415 nm with a double beam UV/Visible spectrophotometer, Perkin Elmer (Japan).The calibration curve was plotted by preparing the quercetin solutions at concentrations 12.5 to 100 g ml^{-1} in methanol.

DPPH radical scavenging activity

The free-radical scavenging activities of leaves of *Vitex agnus castus* extracts were measured by decrease in the absorbance of methanol solution of DPPH according to Blois *et al* [18] and slightly modified. A stock solution of DPPH (33 mg in 1l) was prepared in methanol, which gave initial absorbance of 0.8, and 5ml of this stock solution was added to 1ml of *Vitex agnus castus* extract solution at different concentrations (100–1200 µg/mL). After 30 min, absorbance was measured at 517 nm. Ascorbic acid was used as reference antioxidant compound. Antiradical activity was calculated as % inhibition from the given formula:

$$\% \text{ antiradical activity} = 1 - \left(\frac{As}{Ac}\right) \text{ X } 100$$

Where; $A_{Control}$: Control absorbance (DPPH).
A_{Sample} : Sample/Standard absorbance.

Determination of reducing power assay

The reducing power of *Vitex agnus castus* was determined as per the reported method. [19] Different concentrations of *Vitex agnus castus* extracts (100–1200 µg) in 1ml of ethanol were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium Ferrocyanide (2.5 ml, 1%).The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

ABTS radical scavenging assay:

ABTS radical scavenging activity of *Vitex agnus castus* extracts was determined according to Re *et al* [20] ABTS radical was freshly prepared by adding 5 ml of a 4.9 mM potassium per sulfate solution to 5 ml of a 14mM ABTS solution and kept for 16 hr in dark. This solution was diluted with distilled water to yield an absorbance of 0.70 at 734nm and the same was used for the antioxidant assay. The final reaction mixture of standard group was made upto 1 ml with 950 µl of ABTS solution and 50 µl of vitamin-C. Similarly, in the test group 1 ml reaction mixture comprised 950 µl of ABTS solution and 50 µl of the extract solutions. The reaction mixture was vortexed for 10 s and after 6 min absorbance was recorded at 734 nm against distilled water by using an Perkin-Elmer UV-Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.

Super oxide radical Scavenging assay

This assay was based on the capacity of extract to inhibit the photochemical reduction of nitro blue tetrazolinium (NBT) in the presence of riboflavin-light-NBT system with slight modifications. [21]. In brief, each 3 ml reaction mixture contained 50 mM phosphate buffer (pH-7.8, 13 mM methionine, 2µM riboflavin,100 µM EDTA, NBT (75 µM) and 1 ml test sample solution. It was kept in front

of fluorescent light and a absorbance was taken after 6 min at 560 nm by using an Perkin-Elmer UV-Vis Spectrophotometer. Identical tubes with reaction mixture were kept in the dark and served as blanks. The percentage inhibition of superoxide generation was measured by comparing the absorbance of the control and those of the reaction mixture containing test sample solution.

***In vivo* antioxidant study**

Animals were divided into following groups comprising of six rats in each group. Group-1 served as normal control and was given the vehicle alone (DMSO).The second group served as Disease control and was treated with CCl_4 only. The third, fourth, fifth and sixth groups received Petroleum ether, ethyl acetate, methanolic and aqueous extract of *Vitex agnus castus* leaves orally at 200 mg/kg body weight along with CCl_4. The treatments were given for 7 days and on the 8th day of the experiment, all the animals were sacrificed by decapitation. The liver and kidneys were removed, weighed and homogenised immediately with Elvenjan homoginizer fitted with teflon plunger, in ice chilled 10% KCl solution (10 mg/g of tissue). The suspension was centrifuged at 2000 rpm at 4°C for 10 min and the clear supernatant was used for the following estimations.

Catalase was estimated by following the breakdown of hydrogen peroxide according to the method of Usoh *et al* [22] Superoxide dismutase (SOD) activity was assayed according to the method of Marklund and Marklund. [23] Lipid peroxidation was measured in terms of MDA content following the thiobarbituric acid (TBA) method of Ohkawa *et al* . [24]

Reduced glutathione (GSH) activity was assayed according to the method of Ellman [25] Statistical analysis was carried out using the Student's t-test and the results were judged significant, if $P < 0.05$.

Quantitative analysis by HPTLC

Chromatographic conditions

The 10 mg of methanolic extract was dissolved in 2ml of methanol to give concentration 5 µg/ml.The Methanolic extract was quantified for aucubin by taking its standard using Linomat 5 Applicator (Camag) by.injecting 4 µl volume and the optimized solvent System (Ethyl acetate: water: acetic acid: formic acid) with the visualizing agent (Vanillin: MeOH: Glacial acetic acid: Sulphuric acid in a ratio of 0.5 g: 170: 20:10 in ml. The TLC plate Development-using Presaturated Camag Twin Trough Chamber

Sample application

Sample application in bands provides high resolution as compared to spot application; desaga allows sample application in narrow by a spray technique. Sample volume of more than 2 µl to 100 µl can be applied on HPTLC plates. The precoated aluminium plates of 10x 10 cm were taken and samples were applied.

Chromatographic development and evaluation

The plates was developed with the mobile phase in Twin trough chamber with lid upto 8 cm. after development plates were air-dried and subjected to scanning. All plates were scanned at different wavelengths. Retention factor R_f and area under curve (AUC) for sample and standard were determined using integration software.

Quantitative analysis by HPLC

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 1.5mg 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.

Preparation of sample solution

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

Chromatographic conditions-

The 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, 5µ 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

RESULTS

From preliminary phytochemical evaluation of the extracts of leaves of *Vitex agnus castus* it was confirmed that the major groups were flavonoids, phenolic compounds and triterpenoids. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups [26]. Total phenolic content in the *Vitex agnus castus* leaves was found to be 10.76% w/w.The phenolic compounds may contribute directly to antioxidative action [27].It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g daily ingested from a diet rich in fruits and vegetables.[28]

It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process.Total flavonoid content in the *Vitex agnus castus* leaves was found to be 2.60% w/w.

DPPH can be used in determining radical scavenging activity as it forms a stable molecule on accepting an electron or hydrogen atom. Table-1 illustrate that the four extracts of the leaves showed free radical scavenging property in a dose dependant manner. There was a reduction in the concentration of DPPH due to scavenging ability of the extract of leaf. Maximum inhibition was observed with the methanol extract and minimum inhibition was observed with petroleum ether extract. Based on % inhibition, the order of scavenging activity of different fraction was found to be-Methanolic extract > aqueous extract > ethyl acetate extract > petroleum ether extract.IC_{50} value of methanolic, aqueous, ethyl acetate, petroleum ether extract and ascorbic acid were 321.82, .409.82, 705.64, >1200 and 64.82 respectively. These results indicated that all extracts have a noticeable effect

on scavenging the free radicals. Free-radical scavenging activity of all the extracts were concentration dependent.

The reducing capacity of a compound Fe^{3+}/ ferricyanide complex to the ferrous form may serve as significant indicator of antioxidant capacity.The existence of redutones are the key of the reducing power which exhibit their antioxidant activity through the action of breaking free radicals chain by donating H atom.The reduction of Fe^{3+}/ ferricyanide complex to the ferrous form occurs due to the presence of reductants in the solution. Absorbance of Fe^{2+} can be observed by measuring O.D. value at 700 nm.The reducing power of the extracts increase with increase in the concentration as shown in Table-2 . Based on % inhibition, the order of scavenging activity of different fraction was found to be-Methanolic extract > Aqueous extract > Ethyl acetate extract > Petroleum ether extract

Table No. 1- DPPH radical scavenging assay

Concentration (µg/mL)	% inhibition[a] Petroleum ether	Ethyl acetate	Methanol	Aqueous
100	----	10.4 ± 0.8	29.5 ± 0.8	17.2 ± 0.8
300	----	26.2 ± 0.6	46.6±0.9	36.6±0.7
500	----	34.5 ± 0.7	62.4 ± 1.4	57.4 ± 0.7
700	----	49.6 ± 1.2	79.8 ± 0.8	66.2 ± 1.6
1000	----	62.4 ± 1.4	84.6 ± 0.7	82.4 ± 0.7
1200	----	76.2 ± 0.8	85.6 ± 0.6	83.2 ± 0.8

Here n = 5, a = mean ± S.E.

Table No. 2 - Reducing Power assay.

S.No	Concentration (µg/mL)	Absorbance[a] Petroleum ether	Ethyl acetate	Methanolic	Aqueous	Ascorbic acid
1	100	0.189±0.04	0.192±0.01	0.224±0.02	0.217±0.04	0.246±0.02
2	300	0.223±0.02	0.234±0.02	0.278±0.04	0.292±0.02	0.312±0.05
3	500	0.269±0.08	0.278±0.09	0.362±0.06	0.336±0.05	0.396±0.04
4	700	0.311±0.06	0.317±0.05	0.410±0.05	0.382±0.04	0.434±0.08
5	1000	0.368± 0.09	0.374±0.05	0.478±0.04	0.446±0.03	0.502±0.09
6	1200	0.410± 0.04	0.416± 0.05	0.524±0.06	0.496±.0.04	0.548±0.05

(Here a = mean ± S.E.M., n=5, all the values showed significant relationship)

Table No. 3 ABTS radical scavenging assay

Concentration (µg/mL)	% inhibition[a]-Petroleum ether	% inhibition Ethyl acetate	%inhibition-methanol	%inhibition Aqueous
100	---	11.6 ± 0.8	29.5 ± 0.8	24.2 ± 1.3
300	---	36.2 ± 0.9	46.6 ± 0.8	45.7 ± 1.4
500	---	39.7 ± 1.0	74.0 ± 1.0	66.4 ± 0.8
700	---	54.3 ± 1.0	84.2 ± 0.8	76.2 ± 1.1
1000	---	69.6 ± 1.0	96.2 ± 0.9	89.6 ± 0.6
1200	---	84.8 ± 1.2	95.8 ± 0.7	83.2 ± 0.7

Here n=5 all the observation in different groups showed significant relationship between concentration and % inhibition (pearson correlation analysis) a=mean ±SEM.

Table No. 4 - Superoxide anion scavenging activity

Concentration (µg/mL)	% inhibition[a] Petroleum ether	% inhibition-Ethyl acetate	%inhibition-Methanol	% inhibition Aqueous
100	----	12.6 ± 0.9	39.5 ± 0.8**	26.2 ± 1.3**
300	----	29.4 ± 0.9	47.6 ± 1.2**	43.7 ± 1.4**
500	----	39.7 ± 1.0	64.0 ± 1.0**	56.8 ± 0.8**
700	----	48.3 ± 1.0	74.2 ± 1.0**	66.2 ± 1.1**
1000	----	56.4 ± 0.8	76.2 ± 0.7**	69.6 ± 0.9
1200	----	64.8 ± 0.9	79.8 ± 0.8	70.2 ± 1.0

Here n=5 all the observation in different groups showed significant relationship between concentration and % inhibition (pearson correlation analysis) a=mean ±SEM

Table No. 5. *In-vivo* antioxidant activity -

Treatment Group	In-vivo antioxidant enzymes			
	SOD	GSH	Catalase	LPO
Vehicle treated (DMSO)	8.95± 0.51	37.27 ± 6.7	29.5 ±2.98	3.98 ± 1.69
CCl_4	3.96± 0.52	12.11 ± 0.64	15.38 ±0.75	12.98 ± 1.73
Petroleum ether Extract + CCl_4	4.57± 0.51	13.63 ± 0.53	17 ± 0.87	11.09 ± 0.71
Ethyl Acetate Extract + CCl_4	6.71± 0.52	30.67 ± 1.85	24.92 ± 0.94	7.13 ±0.51
Methanolic Extract + CCl_4	7.05± 0.22	33.19 ±1.91	28.73 ±2.51	4.22 ± 0.31
Aqueous Extract + CCl_4	5.13± 0.36	16.79 ± 0.88	23.9 ±1.27	8.96 ±0.61

Here n=3 all the observation in different groups showed significant relationship between various groups as compared to untreated rats. a=mean ±SD.

In ABTS radical scavenging assay as shown in Table-3, all the observations in different groups showed significant (P <0.01) relationship between the concentration and percentage inhibition.(Pearsons correlation analysis)Maximum inhibition was observed with the methanol extract and minimum inhibition was observed with petroleum ether extract .Based on IC_{50} value, the order of scavenging activity of different fraction was found to be-Methanolic extract > Aqueous extract > Ethyl acetate extract > Petroleum ether extract

In the PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen, by PMS-NADH coupling reaction reduces NBT (yellow dye) to blue-colored product called formazan. Drugs' possessing super oxide scavenging activity decreases the reduction of NBT, which is a measure of super oxide anion scavenging activity that is indicated by reduction in absorbance at 560 nm**.** All the observations in different groups showed significant (P < 0.01) relationship between the concentration and percentage inhibition (Pearson correlation analysis**).**Maximum inhibition was observed with the methanol extract and minimum inhibition was observed with petroleum ether extract (Table-4).Based on IC_{50} value,the order of scavenging activity of different fraction was found to be-Methanolic extract > Aqueous extract > Ethyl acetate extract> Petroleum ether extract

SOD activity

Results, summarized in Table-5 showed a significant increase ($p<0.05$) in serum activity of SOD in the rats treated with petroleum ether, ethyl acetate ,methanolic and aqueous extracts of *Vitex agnus castus leaves* as compared to the untreated rats. This increase was more significant ($p<0.05$) in the methanolic extract.The order for increase in the level of SOD is Methanolic > Ethyl acetate > Aqueous > Petroleum ether

Catalase activity

Results, summarized in Table 5 showed a significant increase ($p<0.05$) in serum activity of Catalase in the rats treated with petroleum ether, ethyl acetate ,methanolic and aqueous extracts of *Vitex agnus castus leaves* as compared to the untreated rats. This increase was more significant ($p<0.05$) in the methanolic extract.The order for increase in the level of catalase is Methanolic > Ethyl acetate > Aqueous > Petroleum ether

GSH activity

Results, summarized in Table 5 showed a significant increase ($p<0.05$) in serum activity of GSH in the rats treated with petroleum ether, ethyl acetate ,methanolic and aqueous extracts of *Vitex agnus castus leaves* as compared to the untreated rats. This increase was more significant ($p<0.05$) in the methanolic extract.The order for increase in the level of GSH is Methanolic > Ethyl acetate > Aqueous > Petroleum ether

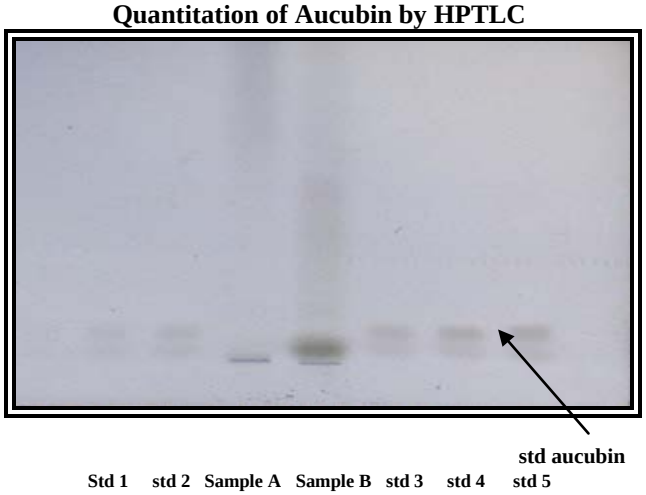
Lipid peroxidase (LPO) activity

Results, summarized in Table 5 showed a significant increase ($p<0.05$) in serum activity of LPO in the rats treated with petroleum ether, ethyl acetate, methanolic and aqueous extracts of *Vitex agnus castus leaves* as compared to the untreated rats. This increase was more significant ($p<0.05$) in the Petroleum ether extract .The order for increase in the level of LPO is Petroleum ether > Aqueous > Ethyl acetate > Methanol

HPTLC Analysis

Quantitative analysis of *vitex agnus castus* leaves with the methanolic extract were carried out using reverse phase HPTLC and the chromatographic profile were compared with the retention times of the reference standard. From the chromatographic profile shown in Fig.1-3, it was observed that the Aucubin was found to be 0.15495 µg in a Methanolic extract.

Quantitation of Aucubin by HPTLC



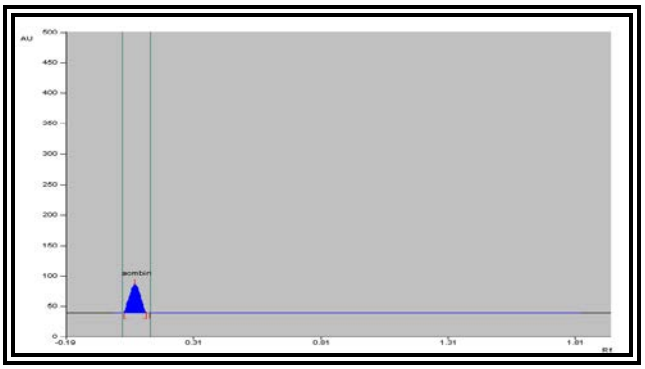


Fig.-1 & 2 HPTLC chromatogram of standard aucubin (sigma make)

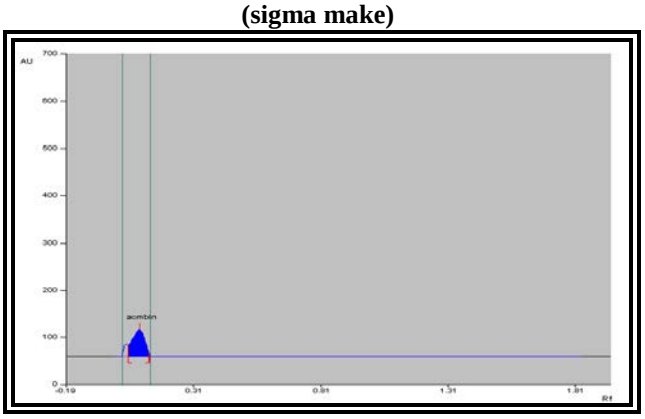


Fig.-3 HPTLC chromatogram of methanolic extract

Details of HPLC chromatogram of Methanolic extract

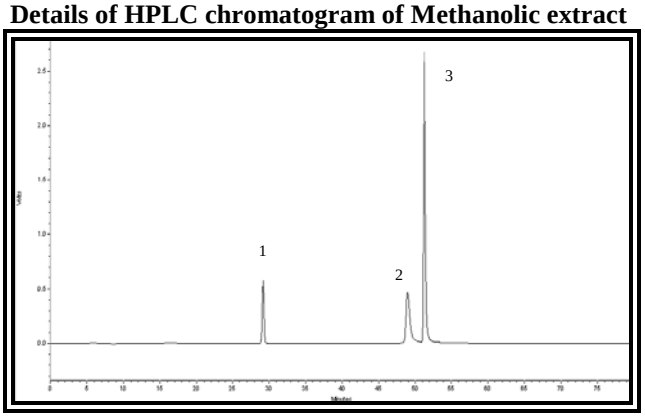


Fig. No.4 HPLC chromatogram of standards: 1. p-hydroxy benzoic acid, 2.kaempferol and 3. Luteolin

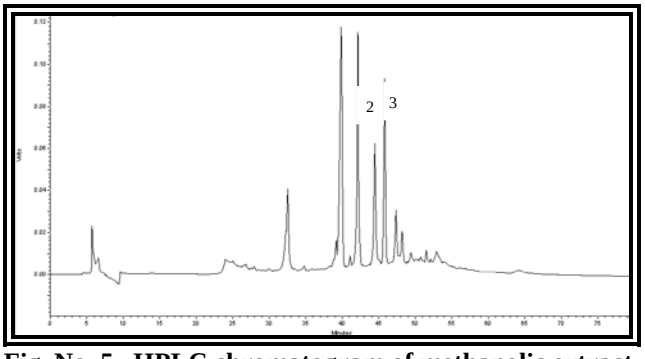


Fig. No. 5 HPLC chromatogram of methanolic extract

HPLC Analysis

Quantitative analysis of *vitex agnus castus* leaves with the methanolic 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 4 & 5 it was observed that Kaempferol was found to be 0.24% in a methanolic extract and luteolin was found to be 0.12% in a methanolic extract. The selection of these standards is due to their medicinal properties stated in the literature.

DISCUSSION

All the fractions of Vitex exhibited a potent scavenging for DPPH and ABTS radical cations in a concentration dependent manner, showing a direct role in trapping free radicals.Studies have indicated that the antioxidant effect is related to development of reductones. Reductones are reported to be terminators of free-radical chain reaction [29]; thus the antioxidant activity of *Vitex agnus castus* extracts may be related to its reductive activity. Removal of superoxide in a concentration dependant manner by *Vitex agnus castus* extracts may be attributed to the direct reaction of its phytomolecules with these radicals or inhibition of enzymes. Since here, SO radicals are being generated through non-enzymatic method, the action of *Vitex agnus castus* extracts is a clear indication of its direct reaction with this radical species. It may be suggested that leaves of *Vitex agnus castus* possess antioxidant activity. The number of antioxidant compounds like flavonoids and polyphenolic compounds which can effectively scavenge various reactive oxygen species or free radical under in vitro conditions.

This property may be attributed more to the polar fraction (methanolic extract) due to the presence of polyphenolics and flavones in the Vitex. The polar fraction may have more polyhydroxy phenolic compound which may be acting synergistically, with other compound.

In vivo antioxidant activity was assayed by estimation of level of glutathione, superoxide dismutase (SOD), and catalase and lipidperoxidase levels. CCl_4 is being used extensively to investigate hepatoprotective and antioxidant activity on various experimental animals**.** A major defense mechanism involves the antioxidant enzymes, including SOD, catalase and glutathione peroxidase which convert active oxygen molecules into non-toxic compounds. The toxic metabolite CCl_4 radical is produced which further reacts with oxygen to give trichloromethyl peroxyl radical. Cytochrome P_{450} 2El is the enzyme responsible for this conversion.This radicals bind covalently to the macromolecules and cause peroxidative degradation of lipid membrane of the adipose tissue.CCl_4 treatment decreased SOD, catalase, glutathione, and peroxidase and increased lipid peroxide level. Pretreatment with 200 mg/kg (p.o.) of *Vitex agnus castus* extract improved the SOD, catalase, glutathione, and peroxidase levels significantly and reduced lipid peroxidation. This could suggest stabilization of plasma membrane as well as repair of hepatic tissue damage cause by CCl_4. As the *Vitex agnus castus* extracts, in the dose of 200 mg/kg, p.o., has improved the SOD, catalase, glutathione, and peroxidase

levels significantly. It has been concluded that the methanolic extract, from the leaves of *Vitex agnus castus* possesses significant antioxidant activity.

Further, the quantification of constituents present in the extract was carried out by RP- HPLC and HPTLC which has shown kaempferol (0.24%), Luteolin (0.12%) and Aucubin (0.43625 µg) in the methanolic extract .

Kaempferol exerts its antioxidative and antiinflammatory activity by modulating the age related NF k B signaling cascade and its proinflammatory gene by suppressing age induced NADPH oxidase.Luteolin has antioxidant, anti-inflammatory, anti-allergic, anticancer, and immune-modulating properties to suppress hyperactive immune systems. [30]

This would further confirm the anti-oxidant role of *Vitex agnus castus* as revealed in our research study that could be used as a possible food supplement or in pharmaceutical industry.

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