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## Phytochemical analysis, Anti-inflammatory and Analgesic activity of *Vitex altissima* L.f. stem bark

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### ABSTRACT

*The present studies were designed to evaluate the anti-inflammatory and analgesic activity of ethanolic extract of Vitex altissima stem bark (Peacock Chaste tree). The anti-inflammatory activity was evaluated by in vivo models like carrageenan-induced rat hind paw edema and Acetic acid induced vascular permeability in mice using dose levels of 150 and 450 mg/kg body weight (orally) for ethanolic extract of Vitex altissima and compared to reference drugs indomethacin (10 mg/kg body weight, orally). The analgesic activity was evaluated by in vivo models like an acetic acid-induced writhing assay in mice using dose levels of 150 and 450 mg/kg body weight (orally) for ethanolic extract of Vitex altissima and compared to reference drugs acetylsalicylic acid (25 mg/kg body weight, orally). Acute toxicity study of the extract was performed prior to studies. The ethanolic extract of the stem bark of Vitex altissima showed significant dose-dependent anti-inflammatory and analgesic properties. The acute toxicity study indicated that the plant was non-toxic up to the dose level (<5g/kg). On the basis of obtained results, it was concluded that the ethanolic extract of the stem bark of Vitex altissima can be marked as a potent candidate for the management of inflammatory diseases and pain.*

**Keywords**— *Vitex Altissima, Stem Bark, Anti-Inflammatory, Analgesic, Toxicity, Ethanolic Extract*

### 1. INTRODUCTION

India is well-known for its biodiversity and has one of the richest plant medicinal cultures in the world. Currently, medicinal plants are used as therapeutic agents in most developing countries, as 80% of the human population is dependent on plant resources for health care. Modern medicine now leans towards the use of active ingredients from plants rather than the whole plants. Inflammation is a physiologic response to a variety of stimuli such as infections and tissue injury. When acute inflammation persists for a longer period it leads to chronic inflammation and causes allergies, hypersensitive reactions, autoimmune diseases etc. Mostly steroids like glucocorticoid cortisol, Non-Steroidal Anti Inflammatory Drugs (NSAIDs) like aspirin and ibuprofen were used to treat the inappropriate inflammation. NSAIDs achieve their effects by blocking the activity of cyclooxygenases (COX — 1 & 2). The COX 1 & 2 are involved in pain produced by inflammation and promotes clotting. NSAIDs inhibit clotting by interfering with the synthesis of thromboxane A<sub>2</sub> in platelets. Thus, NSAIDs exerts an anti-inflammatory response by inhibiting the cyclooxygenase pathway that produces prostaglandins and thromboxanes resulting in the reduction of vascular permeabilization and neutrophil chemotaxis [3]. Acute pain is common amongst hospitalized patients, particularly following surgery. Postoperative pain, if not treated properly, can lead to chronic pain and can be associated with other organ dysfunction as well. Pain and inflammatory stimuli result in a series of diverse effects, including pain transduction, sensitization of the central nervous system and peripheral nerve endings. Paracetamol (acetaminophen), when combined with NSAIDs (non-steroidal anti-inflammatory drugs), provide an additive analgesic effect in mild to moderate acute pain. Epidural analgesia with a combination of local anesthetics and opioids is an excellent multi-modal method for

better analgesia and enhanced recovery [10]. The *Vitex altissima* is a medium-sized tree up to 25-33m tall, commonly found in evergreen forest and in the moist deciduous forest. It is also found on slopes and foothills above 600m. The stem bark is grayish-yellow or blazes yellow in color, scaly, branchlets lenticellate and minutely tomentose. The thickness of the bark is found to be 10-13mm. The leaves are compound, trifoliolate, opposite, estipulate, petiole winged towards the apex and possess rachis with 35-60mm in length and slender [10]. The density of the wood is around 800-1010 kg/m cubic at 15% moisture content; the wood is hard and durable. [6] Peacock Chaste tree is native to India, Indo-China, Malaysia, Srilanka. In India, it is distributed throughout the Kerala state. It also found in Bangladesh, Indonesia, Myanmar, and Papua New Guinea. In Ayurveda medicine, the plant is believed to pacify vitiated kapha, vata, inflammation, wounds, ulcers, allergy, eczema, pruritus, worm infestations, urinary system diseases, stomatitis, emaciation and ailments after parturition [7]. The juice from the bark has been used externally against rheumatic swellings and chest pains [6].

## 2. RESULTS

### 2.1 Behavioral and Toxic effects

In this study, the ethanolic extract of *Vitex altissima* (VA) showed that a dose of up to 5000 mg/kg did not cause death in animals.

### 2.2 Anti-inflammatory activity

**2.2.1 Carrageenan- induced paw edema model:** The anti-inflammatory activity of the extract bark *V. altissima* against acute pedal edema has been shown in Table 1 which showed significant anti-inflammatory activity and the results were comparable to that of control. At 125 mg/kg dose, there was 74.23% inhibition, at 450 mg/kg dose 86.35% inhibition was obtained, after three hours of carrageenan injection.

**2.2.2 Acetic acid induced vascular permeability:** In order to evaluate the action of ethanolic extract of VA in the release of vasoactive amines (histamine, bradykinin, and serotonin) and edema formation, we evaluated its effect on vascular permeability induced by acetic acid as shown in Table 2. Ethanolic extract of VA (450 mg/kg) significantly inhibited vascular permeability in 64.86% compared to control.

### 2.3 Acetic acid induced writhing in mice

*Vitex altissima* at the two doses in the study (150 & 450 mg/kg) significantly inhibited the writhing induced by acetic acid in 67.56 and 70% respectively, when compared to control as shown in table 3. This response was dose-dependent. Acetyl salicylic acid was used as positive control for its potent analgesic properties.

### 2.4 Effect of *Vitex altissima* ethanolic extract in DPPH assay

The ethanolic extract of stem bark of *V.altissima* exhibited the significant free radical scavenging activity of DPPH at 400 µg/ml (80.87%) and highest at 500 & 600 µg/ml (91.24% & 92.14%) respectively, as shown in Table 4. The IC 50 value was found to be 207 µg/ml of ethanolic extract of stem bark of VA.

### 2.5 Effect of *Vitex altissima* ethanolic extract in Nitric oxide scavenging activity

The % of inhibition was increased with increasing concentration of the extract and showed an increased level of scavenging activity at 600 µg/ml (66.96%) as shown in Table 5. The IC 50 value was found to be 394 µg/ml of ethanolic extract of stem bark of VA.

### 2.6 Phytochemical studies

The ethanolic extract of stem bark of VA was subjected to preliminary phytochemical analysis and the test results showed the presence of phenols, flavonoids, steroids, tannins, iridoids & alkaloids in the stem bark of VA are confirmed with preliminary phytochemical studies (Table 6).

### 2.7 Determination of Total Phenolic content

The total phenolic content in the ethanolic extract of stem bark of *Vitex altissima* was determined by using Folin Ciocalteu's method and it showed 1.071 mg GAE/ gram dry extract (Figure 1).

### 2.8 Determination of Total Flavonoid content

The total flavonoid content in the ethanolic extract of stem bark of *Vitex altissima* was determined method described by Ordonez et al. and it showed 78.84 mg QE/ g dry extract (Figure 2).

### 2.9 Heavy metal analysis of an ethanolic extract of stem bark of *Vitex altissima*

The concentration of heavy metals copper and lead in the sample (VA) were analyzed using atomic absorption spectrophotometry and the results are presented in table 7. The levels of heavy metals copper (0.3318 ppm) and lead (0.7518 ppm) present in the ethanolic extract of VA is found less than the permissible limits.

**Table 1: Effect of ethanolic extract of *Vitex altissima* on carrageenan-induced paw edema in rats**

Treatment groups	The difference in paw volume at 3 hours (ml)	Percentage inhibition
Carrageenan (Control)	1.1 ± 0.01	-
VA (150 mg/kg)	0.28 ± 0.10	74.23%
VA (450 mg/kg)	0.15 ± 0.05	86.35%
Indomethacin(10 mg/kg p.o)	0.05 ± 0.00	94.54%

\* Values are the mean ±S. D n = 3

**Table 2: Effect of ethanolic extract of *Vitex altissima* in vascular permeability induced by acetic acid**

Treatment groups	Absorbance at 610 nm	Percentage inhibition
Vehicle control	0.122 ± 0.048	-
VA (150 mg/kg)	0.1414 ± 0.020	20.92%
VA (450 mg/kg)	0.1161 ± 0.060	64.86%
Indomethacin (10 mg/kg)	0.0371 ± 0.009	79%

\* Values are the mean ±S. D n = 3

**Table 3: Effect of ethanolic extract of *Vitex altissima* on Acetic acid induced writhing in mice**

Treatment groups	Mean no. of writhes in 30 minutes	Percentage inhibition
Acetic acid control	69 ± 3.74	-
VA (150 mg/ml)	24 ± 2.94	67.56%
VA (450 mg/ml)	22.3 ± 0.843	70%
Sodium salicylate	18.3 ± 2.027	75%

\* Values are the mean ±S. D n = 3

**Table 4: Effect of *Vitex altissima* on DPPH radicals scavenging activity**

Groups	Concentration (µg/ml)	Absorbance at 517 nm	Scavenging activity in %
Control (DPPH)	-	2.2148±0.001	-
VA	200	1.1688 ± 0.001	47.38%
VA	300	0.7674 ± 0.0002	65.45%
VA	400	0.4249 ± 0.004	80.87%
VA	500	0.1946 ± 0.002	91.24%
VA	600	0.1745 ± 0.0007	92.14%

**Table 5: Effect of *Vitex altissima* on Nitric oxide scavenging activity**

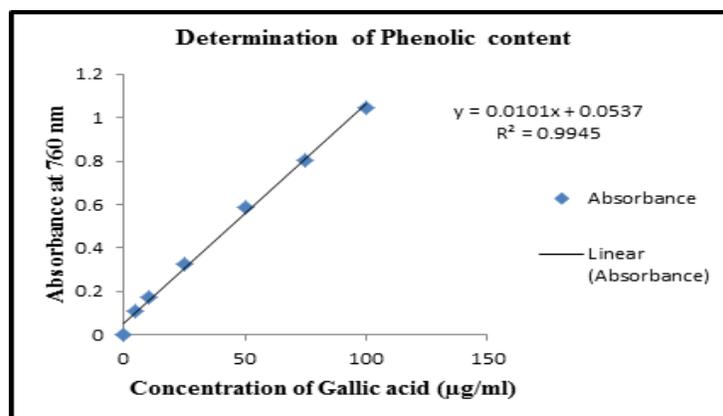
Groups	Concentration (µg/ml)	Absorbance at 540 nm	Scavenging activity in %
Control	-	3.6263 ± 0.9	-
VA	200	3.244 ± 0.09	10.54%
VA	300	2.2644 ± 0.001	33.91%
VA	400	1.4460 ± 0.166	60.12%
VA	500	1.2833 ± 0.017	64.46%
VA	600	1.2311± 0.038	66.09%

**Table 6: Phytochemical compounds present in the ethanolic extract of stem bark of *Vitex altissima***

Compounds	Positive/Negative
Alkaloids	+ve
Iridoids	+ve
Triterpenoids	+ve
Tannin & Phenols	+ve
Steroids	+ve
Flavonoids	+ve

**Table 7: Heavy metal analysis of an ethanolic extract of stem bark of *Vitex altissima***

Heavy metal	Permissible limit (ppm)	Observed values (ppm)
Copper	Not more than 2 ppm	0.3318
Lead	Not more than 3 ppm	0.7518



**Fig. 1: Determination of total phenolic content in the ethanolic extract of stem bark of *Vitex altissima***

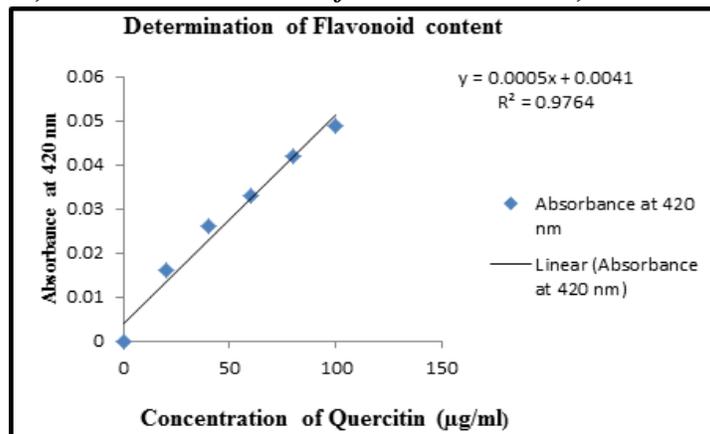


Fig. 2: Determination of total flavonoid content in the ethanolic extract of stem bark of *Vitex altissima*

### 3. DISCUSSION

Our study analyzed the insight of various pharmacological properties of a plant such as antioxidant, anti-inflammatory and analgesic effects of stem bark of *Vitex altissima*. Our result indicated that the ethanol extract of VA was non-toxic up to the dose level studied (<5gm/kg). This could be the reason where in the kurichiya tribal people use this plant for their ailments. In order to provide a scientific explanation for the folk use of *Vitex altissima*, we have investigated the biological effects of ethanolic extract of stem bark, related to the inflammatory process. The present data clearly showed that extracts of dried bark have anti-inflammatory activity by inhibiting the edema formation after carrageenan sub plantar injection. Carrageenan-induced rat paw edema is a suitable experimental animal model for evaluating the anti-oedematous effect of natural products and this is believed to be triphasic, the first phase (1hr after carrageenan challenge) involves the release of serotonin and histamine from mast cells, the second phase (2hr) is provided by kinins and the third phase (3hr) is mediated by prostaglandins, the cyclooxygenase products, and lipoxygenase products [3]. The iridoid glucosides from the leaf extract of *Vitex altissima* such as 6'-O-trans feruloylne gundoside, 6'-O-trans-caffeoylnegundoside were already been evaluated as anti-inflammatory compounds from the rat paw edema study [15]. The presence of iridoid compounds namely Gardoside mono and diesters in stem bark of *Vitex altissima* might be responsible for the Anti-inflammatory action. Based on these reports, it can be inferred that the inhibitory effect of VA plant on carrageenan-induced inflammation in rats could be due to the inhibition of prostaglandin synthesis. In acetic acid induced vascular permeability, acetic acid causes dilation of arterioles and venules and increased vascular permeability by releasing inflammatory mediators such as histamine and prostaglandins. Leukotrienes are released following stimulation of mast cells. As a consequence, fluid and plasma protein are extravasated and edemas are formed. The ethanolic extract of the stem bark of VA showed 64.86% of inhibition at 450 mg/kg against increased vascular permeability which is induced by acetic acid. Pain and inflammatory stimuli result in a series of diverse effects including pain transduction, sensitization of central nervous system and peripheral nerve endings [16]. Acetic acid induced the writhing response in mice is a simple and reliable model to rapidly evaluate the peripheral type of analgesic action of herbal and other drugs. DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors. The present study showed that the plant possesses potent analgesic property. 150 mg/kg of VA showed significant writhing inhibition of 67.56% and maximum inhibition of writhing in mice was observed at 450 mg/kg dose of VA of about 70% inhibition. The DPPH radical scavenging activity of VA extract indicating that some of the compounds present in the extracts were electron donors and could react with free radicals to terminate radical chain reactions and, therefore, were able to boost the natural antioxidant defense mechanism [13]. In addition to reactive oxygen species, nitric oxide was also implicated in inflammation, cancer and other pathological conditions [7]. The ethanolic effect of stem bark of *V. altissima* showed significant nitric oxide scavenging activity at 500 µg/ml (65.48%) by competing with oxygen which leads to reduced production of nitrite ions. The presence of iridoid compounds of any one of the class namely asperulin, aucubin and monotropein supported the study strongly that the stem bark of VA possesses antioxidant properties [17]. The concentration of heavy metals copper and lead in the sample (VA) were analyzed using atomic absorption spectrophotometry and the results are presented in table 7. The levels of heavy metals copper (0.3318 ppm) and lead (0.7518 ppm) present in the ethanolic extract of VA was found to be less than the permissible limits. So the ethanolic extract of VA is considered as safe for consumption.

### 4. CONCLUSION

The present study focused on the key medicinal plant *Vitex altissima*, stem bark and explored the beneficial effects of it as a safe and efficacious herbal drug. The ethanolic extract of the stem bark of VA showed significant anti-inflammatory and analgesic potential. Results pertaining to acute inflammation indicates that the presence of iridoid compounds namely Gardoside mono and diesters in stem bark might be responsible for the anti-inflammatory action in rats, which could be due to the inhibition of prostaglandin synthesis. Moreover, it is also observed that at 150 mg/kg of VA showed significant writhing inhibition of 67.56% in mice which implies that plant possesses potent analgesic property. The medicinal plant *V. altissima* also showed a significant increase in free radical scavenging activity of DPPH at 400 µg/ml (80.87%) and scavenges the nitric oxide at 500 µg/ml (65.48%). The presence of phenols, flavonoids, steroids, tannins, iridoids & alkaloids in the stem bark of *Vitex altissima* is confirmed with preliminary phytochemical studies. These phyto-compounds possess antioxidant effects. From the results obtained it was concluded that levels of heavy metal copper and lead fall within the permissible range and therefore the ethanolic extract of stem bark of VA can be consumed by mankind as it is safe and non-toxic. The present study thus substantiates the tribal claim of its therapeutic potential. Further

research has to be carried out to extrapolate the exact inhibition mechanism of inflammatory mediators by the compounds present in the ethanolic extract of stem bark of *Vitex altissima*.

## 5. MATERIALS AND METHODS

### 5.1 Plant material

The stem bark of *Vitex altissima* was collected from the campus of Jawaharlal Nehru Tropical Botanical Garden and Research Institute, Palode. A voucher specimen was prepared and stored at the institute herbarium under the number TBGT 57063.

### 5.2 Preparation of extract

The stem bark of *Vitex altissima* (VA) was washed in tap water, shade dried and powdered. The powder (150 gm) was extracted with 1500 ml of ethanol overnight, at room temperature with thorough shaking. The ethanol extract was finely filtered using filter paper. The filtrate was then concentrated and the solvent was evaporated completely at low temperature under reduced pressure in a rotary evaporator. The yield of the extract was found to be 21.2 gm. The extract was then completely dried in a desiccator and stored in an airtight container for further use. For administration orally, the crude extract was suspended in 0.5% Tween 80 to appropriate concentrations.

### 5.3 Behavioural and Toxic effects

Wistar rats (175 to 250 gm) and Swiss albino mice (45 to 50 gm), of either sex, were obtained from the Animal House of the Jawaharlal Nehru Tropical Botanical Garden Research Institute, Palode. They were grouped and housed in poly-acrylic cages (three animals per cage) and maintained under standard laboratory conditions (temperature 24-28°C, relative humidity 60-70% and 12 hours dark-light cycles). They were fed commercial rat feed (Lipton India Ltd, Mumbai, India) and boiled water. All animal experiments were carried out after getting the approval of the Institute's Animal Ethics Committee (B-01/12/2011/13) and followed according to NIH. Six groups of 2 mice were administered orally 50, 150, 450, 1350, 2500, 5000 mg/kg of VA. They were observed continuously for 1 hour for any gross behavioural changes, symptoms of toxicity and mortality, if any and intermittently for the next 6 hours and then again, 24 hours after dosing with *Vitex altissima* (VA) extract.

### 5.4 Anti-inflammatory activity

**5.4.1 Carrageenan – induced paw oedema model [8]:** Four groups of 3 animals each were used for the study. Group 1 served as control i.e: without the drug, while groups II, III were administered 150 and 450 mg/kg of VA extract. Group IV was administered with standard drug indomethacin (10 mg/kg) orally. Briefly, 0.1 ml of 1% carrageenan was injected into the right hind paw, under the plantar aponeurosis (carrageenan controls). A similar volume of 0.5% Tween 80 was injected into the left hind paw of the animals in group 1 (vehicle controls). The hind paw volume of the vehicle controls and the carrageenan controls and the drug treatment groups was measured before carrageenan injection plethysmographically and the time course of oedema formation was followed for 3 hours. The volume of increase of the inflamed paw was estimated by subtracting the volume of the control hind paw. The anti-inflammatory activity of the plant extract was estimated as the degree of oedema inhibition. The percentage inhibition of paw edema was calculated by following formula: % Inhibition =  $(1 - V_t/V_c) \times 100$ , Where,  $V_t$  - paw volume of the treated group and  $V_c$  - paw volume of control group respectively.

**5.4.2 Acetic acid induced Vascular Permeability [18]:** Four groups of 3 animals each were used for the study. Group 1 served as control that is without the drug, while groups II, III was administered 150 and 450 mg/kg of VA extract. Group IV was administered with standard drug indomethacin (10 mg/kg) orally. Group I normal control that is it was administered with 0.5% Tween 80. After 1 hour, 0.2 ml of 0.25% Evan's blue was administered through the tail vein of mice. After 30 minutes, each mouse was injected with 0.5 ml of 0.6% acetic acid intraperitoneally. Then 30 minutes later each mouse was anesthetized and 5 ml of normal saline is injected intraperitoneally. The peritoneal cavity was massaged well and the peritoneal fluid was collected after killing the animal. The peritoneal fluid was then centrifuged at 1000 rpm for 20 minutes. The OD was read at 610 nm using a UV-Visible spectrophotometer.

### 5.5 Analgesic activity- Acetic acid induced writhing assay [1]

Analgesic responses were assessed by counting the number of writhes (constriction of the abdomen, turning of the trunk (twist) and extension of hind limbs induced by 0.5% acetic acid solution in mice. It was administered by injecting acetic acid solution intraperitoneally. Healthy albino mice were divided into four groups, each group containing 3 animals. Four groups of 3 animals each were used for the study. Group 1 served as control that is without the drug, while groups II, III was administered 150 and 450 mg/kg of VA extract. Group IV was administered with standard drug acetylsalicylic acid (25 mg/kg) orally. Group I normal control that is it was administered with 0.5% Tween 80. The number of writhes per animal was counted for 30 minutes exactly after 5 minutes of treatment with 0.5% acetic acid.

### 5.6 Antioxidant Studies

**5.6.1 DPPH radical scavenging activity:** The DPPH assay was done by following the Blois et al., (1958) method. To 2 ml of a methanolic solution of 0.2 mM DPPH, 0.2 ml of VA extract dissolved in methanol was added at varying concentrations 25, 50, 75, 100, 150 and 250 µg/ml. the reaction mixture was mixed well and incubated for 20 minutes at 28°C under dark condition. The control contains 0.2 ml methanol and 2.8 ml DPPH. The DPPH radical scavenging activity was determined by measuring absorbance at 517 nm using Cary 100 UV-Vis spectroscopy. The percentage of radical scavenging activity of the sample was calculated using the formula as given below:

$$\text{Radical Scavenging Activity \%} = 1 - (\text{absorbance of sample}) / (\text{absorbance of control}) \times 100$$

**5.6.2 Nitric oxide radical scavenging activity [5]:** The compound Sodium nitroprusside SNP is known to decompose in aqueous solution at physiological pH (7.4) producing nitric oxide radicals. Under aerobic conditions, Nitric oxide reacts with oxygen to produce stable products (nitrate and nitrite), the quantity of which can be determined using the Griess reagent. The phosphate buffer is taken as blank and the control contains 1 ml phosphate buffer and 1 ml griess reagent. The scavenging effect of the plant extract according to the modified method of Marcocci et al. 1 ml of extract solution at different concentrations was added in the test tubes. Then 1 ml of Sodium nitroprusside solution (100 mM) was added to all the tubes and incubated at 29C for 2.5 hours. An aliquot of 1 ml of the incubated solution was removed and diluted with 1 ml of Griess reagent (1% sulfanilamide in 2% H<sub>3</sub>PO<sub>4</sub> and 0.1% N-1-Naphthylene diamine dihydrochloride). The absorbance of the chromophore that formed with naphthylethylene diamine dihydrochloride was immediately read at 540 nm. The percentage inhibition was calculated as follows.

$$\% \text{ inhibition} = 1 - \text{Absorbance of sample} / \text{Absorbance of control} \times 100$$

### 5.7 Statistical Analysis

The results were expressed as mean  $\pm$  SD for each experimental group.

### 5.8 Detection of heavy metals using atomic absorption spectrophotometer

To the 2 g of plant bark sample (VA), Conc. HCl and Conc. HNO<sub>3</sub> was added in 1:1 ratio (15 ml each). The samples were heated at 100C for 30 minutes to digest the sample. Then the treated sample was cooled and filtered using whatman no.1 filter paper. Then the filtered suspension is diluted with 100 ml of deionized water. Stock standard solutions of Copper (Cu) containing 1000 ppm of metal were prepared by dissolving weighed quantities of appropriate dried analytical grade salts in deionized water. Calibration standards of 1 ppm, 2 ppm, 4 ppm, 6 ppm and 8 ppm of the element were obtained by appropriate dilution of the stock solutions. The Cu contents were measured using flame atomic absorption spectrometry. The elements were measured at 324.8 nm with air-acetylene flame [4].

### 5.9 Preliminary Phytochemical analysis

**5.9.1 Test for alkaloids [14]:** A few ml of filtrate with few drops of Wagner's reagent was added to the side of the test tube. A reddish brown precipitate confirms the presence of alkaloids. Wagner's reagent: Iodine (1.27g) and Potassium iodide (2 g) are dissolved in 5 ml of distilled water and made up to 100 ml of distilled water.

#### 5.9.2 Test for Phytosterols [14]

- **Libermann – Burchard's test:** The extract (50 mg) was dissolved in 2 ml acetic anhydride. To this, one or two drops of concentrated sulphuric acid were added slowly along the side of the test tube. An array of blue-green colour showed the presence of phytosterols.
- **Salkowski's test:** The extract were treated with chloroform and filtered separately. The filtrates were treated with few drops of concentrated sulphuric acid. Shaken and allowed to stand. The lower layer turning red indicated sterols and lower layer turning golden yellow indicated the presence of triterpenes.

**5.9.3 Test for Flavonoids Shinoda's test [2]:** The extract (50 mg) was dissolved in 5 ml of alcohol and few fragments of magnesium ribbon and concentrated hydrochloric acid (dropwise) were added. Presence of flavonol glycosides was inferred by the development of pink to crimson colour.

#### 5.9.4 Test for Phenolic compounds and Tannins

- **Ferric chloride test [14]:** The extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of phenolic compounds.
- **Test for Iridoids [2]:** To 1 gram of plant extract, 5 ml of 1% aqueous HCl was added and incubated for 3 hours. Then 0.1ml from the incubated sample was withdrawn and 1 ml of Trim – hill reagent was added (10 ml acetic acid, 1 ml of 2% copper sulphate and 0.5 ml of Conc. HCl). The tube was heated for short time in a flame, a color was produced indicating iridoids were present.

**5.9.5 Test for Saponin:** 50 mg of extract was diluted with distilled water and made upto 20 ml. The suspension was shaken in a graduated cylinder for 15 minutes. The formation of a 2 cm layer of foam indicates the presence of saponin.

### 5.10 Quantitative determination of Total phenolic contents [12]

Briefly, 0.02 ml of VA ethanolic extract (10 mg/ml) was mixed with 1 ml of 10% Folin – Ciocalteu solution and 0.8 ml of 7.5% sodium carbonate solution. The mixture was incubated for 1 hr at room temperature. The absorbance at 760 nm was measured and converted to phenolic contents according to the calibration curve of gallic acid (0.1 mg/ml)

### 5.11 Quantitative determination of Total flavonoid contents [9]

Total flavonoid contents of the extracts were determined based on the formation of a flavonoid-aluminum complex. 0.5ml of various solvent extracts (1mg/ml) was mixed with 0.5ml of aluminum chloride prepared in (2% in ethanol). The resultant mixture was incubated for 60 min at room temperature for yellow colour development which indicated the presence of flavonoid. Absorbance was measured at 420 nm using UV–VIS spectrophotometer. Total flavonoid content was calculated as quercetin equivalent (mg/g) using the following equation based on the calibration curve:  $Y = 0.217x$ ,  $R^2 = 0.9582$ , where X is the absorbance and Y is the quercetin equivalent.

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