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# Pharmaco-chemical characterization of terpenoid fraction of *Artemisia nilagirica* (Clarke) Pamp. from western ghats of Wayanad region of Kerala, India

**Panicker Devyani Ramachandran, Sanis Juliet, Mahesh DM, Drisya K, Adarsh Krishna TP, Sunil AR, Suresh N Nair, Ajith Kumar KG, Reghu Ravindran, Suja Rani S, Sujith S, Nisha AR and Ranjith D**

**Abstract**
**Objective**: Nowadays a lot of herbal formulations are widely being used in Ayurveda. However, it is not easy to analyse their pharmacological activity unless a phytochemical investigation is conducted of that particular plant extract. *Artemisia nilagirica* (Clarke) Pamp, a herb belonging to family Asteraceae is reported for its mosquito larvicidal and insect repellent activities. The present study was undertaken to characterize the ethanolic extract, hexane fraction and terpenoid fraction of *Artemisia nilagirica* using chromatographic techniques.
**Results:** The prepared ethanolic extracts and its fractions were subjected to phytochemical screening which showed presence of terpenoids, flavonoids, saponins, tannins, fixed oils and fats and steroids. HPTLC was used as a tool to detect the presence of polyvalent compounds present in the ethanolic extract and its fractions. The chromatogram showed presence of polyvalent compounds in ethanolic extract and hexane fraction which depicted varied composition present in the plant The terpenoid fraction of *A. nilagirica* showed presence 9 polyvalent compounds at 366 nm with Rf ranging from 0.14 to 0.96 with the highest peak intensity at 0.56 Rf.
**Conclusion:** A detailed investigations of the terpenoid fraction should be carried out so as to study its pharmacological activity as well as for developing new drugs so that they can be used in treatment of various diseases.

**Keywords:** *Artemisia nilagirica*, terpenoid fraction, phytoconstituents, HPTLC, phytochemical screening

## 1. Introduction
Plants are rich sources of secondary metabolites such as alkaloids, steroids, terpenoids, flavonoids and phenolic compounds with complex chemical configurations [1]. Among them, the terpenoid are the most abundant and play a significant role in providing the plants and flowers their fragrance. They are mostly produced in leaves, fruits and flowers such as citrus, eucalyptus and conifers [2]. More than 20,000 naturally occurring varieties of terpenoids has been isolated from various plants and its chemical characterization has been done. Their functions in plants include pest repellant, as pheromone and semiochemical, attractants for pollenating insects, plant growth hormones, phytoalexins, allelochemicals and also as deadly toxins in some species of plants. Therapeutically, they are used as anticancer, anti-inflammatory, analgesics, neuro protective and wound healing agents. In addition, they have a significant economic value which may help in combatting diseases and also for development of new drugs that can increase the economical foothold for the farmers and there can be a significant growth in pharmaceutical sector as well as various drug industries [3].
*Artemisia nilagirica* (Clarke) Pamp. is a herb found in the hilly regions of India belonging to family Asteraceae and is commonly known as Indian wormwood [4]. It has been used traditionally in the treatment of epilepsy, diuresis, anti-inflammatory and various skin diseases. It has been reported in the literature that the aerial parts of *A. nilagirica* possess antimicrobial, anthelminthic, antispasmodic, antifungal, and insecticidal activities [5, 6, 7]. Further, the leaf extracts of *A. nilagirica* possessed notable repellent as well as cidal action against different lifecycle stages of *Anopheles stephensi* and *Aedes aegypti* vector mosquitoes [8] and potential acaricidal properties [9]. The crude ethanolic extract of *Artemisia nilagirica* was found to contain terpenoids, saponins, tannins, flavonoids, steroids, phenols and fixed oils [4]. There exists quite a few studies where the antifeedant, larvicidal, pupicidal and acaricidal properties of plant extracts [10, 11, 12, 13] have been attributed to terpenoids.

Therefore, in the present study an attempt has been made to characterize the terpenoid fraction of *A. nilagirica* by HPTLC profiling.

## 2. Materials and Methods

### 2.1 Collection of plant material

The aerial parts of plant *Artemisia nilagirica* (Clarke) Pamp. were collected from Kunnathidavaka village of Vythiri Tehsil, Wayanad, Kerala, India (Figure 1) prior to the flowering season between March to June 2016. The collected plants were identified, authenticated by a botanist in the Department of Botany, University of Calicut. A voucher specimen of the plant was produced in the University Herbarium CALI, University of Calicut (Accession no. 88647).

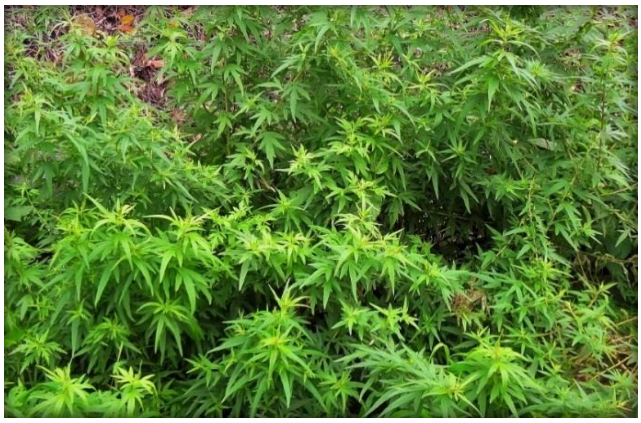

**Fig 1:** *Artemisia nilagirica* (Clarke). Pamp in wild

The protocol for isolation of terpenoid fraction of *A. nilagirica* and HPTLC fingerprinting has been depicted below.

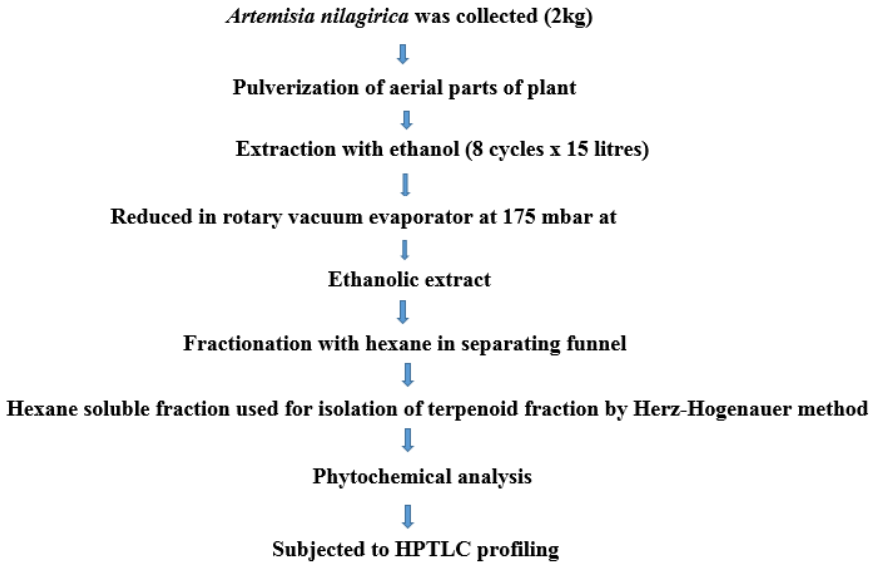


**Fig 2:** Protocol for terpenoid fraction of *A. nilagirica*

### 2.2 Preparation of plant material

Aerial parts of plant were cleaned and kept for shade drying for two weeks at room temperature to remove moisture. The dried plant material was powdered using temperature controlled electrically operated plant sampler grinder (M/s Rotek, India) and it was stored in an air tight container for further extraction.

### 2.3 Preparation of test material

#### 2.3.1 Ethanolic extract of *A. nilagirica*

Plant material (2 kg) was powdered, weighed and kept in thimbles (Whatman filter paper No. 1) in an extraction chamber of Soxhlet's apparatus and extracted using ethanol for eight to nine refluxes. The extract was reduced and concentrated under pressure of 175 mbar at a temperature range of 65°C in rotary vacuum evaporator (M/s Buchi, Switzerland). The concentrated extract was kept for air drying at room temperature. After reduction, the extract was kept for air drying at room temperature and then stored in refrigerator until further use. The recorded weight of the dried extract was used for the calculation of extractive yield:

$$\text{Extractive value (\%)} = \frac{\text{Weight of the extract x 100}}{\text{Weight of the powdered plant sample taken}}$$

#### 2.3.2 Fractionation of ethanolic extract of *A. nilagirica* using hexane

The ethanolic extract of aerial parts of *A. nilgaricia* was fractionated in a separating funnel using hexane (polarity index: 0.1). The crude ethanolic extract (~ 200 g) was transferred to a separating funnel and the fractionation was done with hexane solvent. Two fractions were obtained, hexane soluble as well as hexane insoluble fractions. The hexane soluble fraction was further reduced using rotary vacuum evaporator (M/s Buchi, Switzerland) to remove the solvent. The reduced fraction was dried, weighed, packed into containers and stored at room temperature for isolation of terpenoids.

### 2.4 Isolation of terpenoids

After fractionation with hexane solvent the dried fraction was subjected for isolation of terpenoids by Hertz-Hogenauer method [14, 15]. Briefly, 20g of the dried hexane fraction was soaked overnight in (approx.100ml) dichloromethane and sonicated. The slurry product was filtered and the green filtrate was evaporated in rotary vacuum evaporator (M/s Buchi, Switzerland). The residue was then dissolved in 95% ethanol (approx. 70 mL) and warmed to enhance solubility in water bath (M/s Thermo Scientific, USA) at 70-80ºC. Then 5% lead acetate aqueous solution was added in drop wise

manner (approx. 10mL) for precipitation of fatty acids, phenolic and chlorophyll. The precipitate was then removed by filtration using a pad of silica gel (230-400 mesh, M/s Merck, India). The filtrate was kept in a water bath at 40-50ºC, until the viscous mass remained. By this method fats, chlorophyll and phenolics were removed and the fraction obtained was subjected for HPTLC fingerprinting.

### 2.5 Phytochemical Screening

The phytochemical analysis was conducted according to standard protocol as detailed below (Table 1) and were subjected to screening for secondary metabolites in ethanolic extract of *A. nilagirica*, its hexane fraction and terpenoid fraction as per the method by Raaman [16].

**Table 1:** Tests for Phytochemical Analysis

| S. No. | Phytochemical constituents | Test |
|---|---|---|
| 1 | Alkaloids | a) Mayer's test<br>b) Wagner's test<br>c) Dragendorff's test<br>d) Hager's test |
| 2 | Carbohydrates | a) Fehling's test<br>b) Benedict's test |
| 3 | Glycosides | Borntrager's test |
| 4 | Saponins | Foam test |
| 5 | Proteins and amino acids | a) Biuret test<br>b) Ninhydrin test |
| 6 | Fixed oils and fats | Spot test |
| 7 | Phenolics and tannins | a) Ferric chloride test<br>b) Lead acetate test |
| 8 | Terpenoids | Salkowski test |
| 9 | Flavonoids | Ammonium test |
| 10 | Steroids | Liebermann Burchard test |
| 11 | Gums and mucilages | Absolute alcohol test |

#### 2.5.1 Salkowski test for terpenoid fraction

Small quantity of terpenoid fractions were taken separately in a test tube and added in 2ml of chloroform and to that 3ml of concentrated sulphuric acid was added. The tube was allowed to stand for some time. Presence of a brown ring formation at the interface was taken as an indication for the presence of terpenoids.

### 2.6 High performance thin layer chromatography (HPTLC) analysis

#### 2.6.1 Preparation of test samples

The ethanolic extract (approx.1 mg) was solubilised in HPLC grade methanol whereas hexane and its terpenoid fraction (approx. 1 mg) were solubilised in hexane solvent. All the samples prepared were stored in refrigerator at 4˚C.

#### 2.6.2 Instrumentation

Chromatographic separation was performed on TLC plates precoated with silica gel 60 $F_{254}$ (Merck 10 cm ×10 cm with 200 $\mu$m layer thickness). The samples were spotted on a 10 x 10 cm plate, approximately 1cm above the base. The spotting device was a CAMAG Linomat V Automatic Sample Spotter (M/s Camag Muttenz, Switzerland); the syringe, 100µL (Hamilton); the developing chamber was a CAMAG glass twin trough chamber (20 × 10 cm); the densitometer consisted of a CAMAG TLC scanner 3 linked to CAMAG Reprostar 3.

#### 2.6.3 Selection of mobile phase

Mobile phase in which the extract/ fractions showed better separation was selected. The mobile phase selected was Hexane: Ethyl acetate in the ratio 8:2.

#### 2.6.4 Procedure

The TLC plate was dried using hot air for activation of silica in the plate. The ethanolic extract/hexane fraction/terpenoid fraction (7.2 µL each) was applied on TLC plate. The plate was developed in Hexane: Ethyl acetate (8:2) solvent system. The chamber was previously saturated with mobile phase vapor for 25 minutes at room temperature (25 ± 2ºC) and plates were developed at distance of approximately 90 mm from the point of application. The plates were dried at room temperature in air. Scanning was performed using Camag TLC scanner 3 (at 254 nm and 366 nm) through fluorescence mode and operated by win CATS software (version 1.4.1, Camag). Extracts deposited on the silica plates were visualized under ultraviolet (254nm and 366 nm) and visible light. The retardation factor ($R_f$) of the resolved bands were noted.

#### 2.6.5 Chromatographic conditions

Application mode: CAMAG (Switzerland) Linomat 5
Lamp: CAMAG UV cabinet with range of 254-366 nm
Chamber saturation: 10 min
Technique: one way ascending
Band width: 8 mm
Distance between tracks: 23.3 mm
Development time: 30 min.
Data system: win CATS software

## 3. Results

### 3.1 Extractive values of aerial parts of *A. nilagirica* crude extract, hexane fraction and its terpenoid fraction

The extractive values of ethanolic extract, hexane fraction and its terpenoid fraction of *A. nilagirica* are 20.98%, 22.70% and 7.29% respectively. The extractive values of the above extract and fraction are depicted in Table 2.

**Table 2**: Extractive yields of ethanolic extract and fractions

| Plant | Extractive value (%) | | |
|---|---|---|---|
| | Ethanolic extract | Fraction of ethanolic extract | |
| | 20.98% | n-Hexane fraction | Terpenoid fraction |
| *Artemisia nilagirica* | | 22.70% | 7.29% |

### 3.2 Phytochemical analysis

Ethanolic extract of aerial parts of *A. nilagirica* showed presence of flavonoids, terpenoids, fixed oils and fats, saponins, glycosides, steroids, phenolic compounds and tannins. Further on fractionation of the ethanolic extract of aerial parts of *A. nilagirica* with hexane showed the presence of saponins, terpenoids, flavonoids, fixed oils and fats. Terpenoid fraction isolated from hexane fraction showed presence of terpenoids and minute quantities of fixed oils and fats. However, alkaloids, carbohydrates, proteins and amino acids, gums and mucilages were not detected any of the extract/fractions. The results of phytochemical screening are depicted in table 3 and result for salkowski's test is shown in figure 2.

**Table 3:** Results of preliminary phytochemical screening of ethanolic extract of aerial parts, hexane fraction and terpenoid fraction of *A. nilagirica*

| Bioactive components | Ethanolic extract of aerial parts of *A. nilagirica* | Hexane fraction of ethanolic extract of aerial parts of *A. nilagirica* | Terpenoid fraction isolated from hexane fraction of *A. nilagirica* |
|---|---|---|---|
| Alkaloids | - | - | - |
| Carbohydrate | - | - | - |
| Saponin | ++ | ++ | - |
| Steroids | + | - | - |
| Phenolics and tannins | + | - | - |
| Terpenoids | + | ++ | +++ |
| Flavonoids | + | + | - |
| Protein and amino acids | - | - | - |
| Fixed oils and fats | + | + | + |
| Glycosides | + | - | - |
| Gums and mucilages | - | - | - |

(Symbol (+) indicates presence and (-) indicates absence of phytoconstituents)

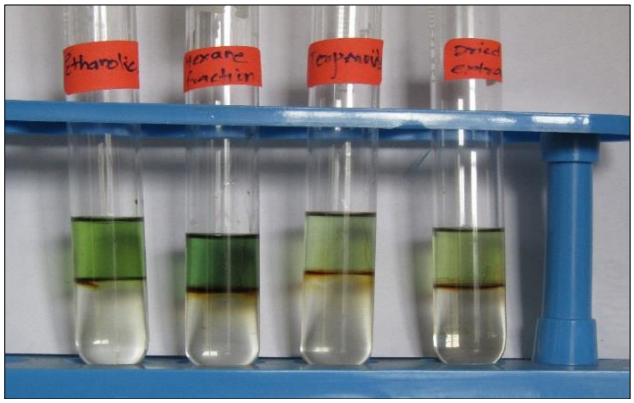


**Fig 2:** The ethanolic extract of aerial parts, hexane fraction and terpenoid fraction of *A. nilagirica* each showing brown ring at the interface (Salkowski test positive for presence of terpenoids)

### 3.3 HPTLC fingerprint profiling of extract/fractions of aerial parts of *A. nilagirica*

The HPTLC fingerprint profile of ethanolic extract of aerial parts of *A. nilagirica*, at 366 nm revealed the presence of three, ten and nine polyvalent compounds in ethanolic extract of aerial parts of *A. nilagirica*, its hexane and terpenoid fractions respectively. Whereas, at 254 nm wavelength, the ethanolic extract of aerial parts of *A. nilagirica*, its hexane and terpenoid fractions revealed the presence of four, six and nine polyvalent compounds respectively. The retention factor (Rf) values of all major peaks has been depicted in table 4 and 5.

**Table 4:** Retention factor (Rf) values of all major peaks in ethanolic extract, hexane fraction and terpenoid fraction at absorbance 366 nm.

| Extracts/fractions | Major Peaks | Rf Value | Area |
|---|---|---|---|
| Ethanolic extract | 02 | 0.64 | 425.7 AU |
| Hexane fraction | 06 | 0.69 | 2801.5 AU |
| | 07 | 0.85 | 2425.4 AU |
| Terpenoid fraction | 05 | 0.56 | 2146.3 AU |
| | 06 | 0.68 | 975.4 AU |
| | 07 | 0.84 | 1108.1 AU |

**Table 5:** Retention factor (Rf) values of all major peaks in ethanolic extract, hexane fraction and terpenoid fraction at absorbance 254 nm.

| Extracts/fractions | Major Peaks | Rf value | Area |
|---|---|---|---|
| Ethanolic extract | 01 | 0.08 | 1202.2 AU |
| | 02 | 0.13 | 1191.6 AU |
| Hexane fraction | 01 | 0.14 | 5231.1 AU |
| | 02 | 0.18 | 1109.7 AU |
| | 03 | 0.31 | 2084.9 AU |
| Terpenoid fraction | 01 | 0.14 | 8159.0 AU |
| | 02 | 0.23 | 2624.4 AU |
| | 03 | 0.30 | 3034.8 AU |
| | 05 | 0.49 | 2136.2 AU |

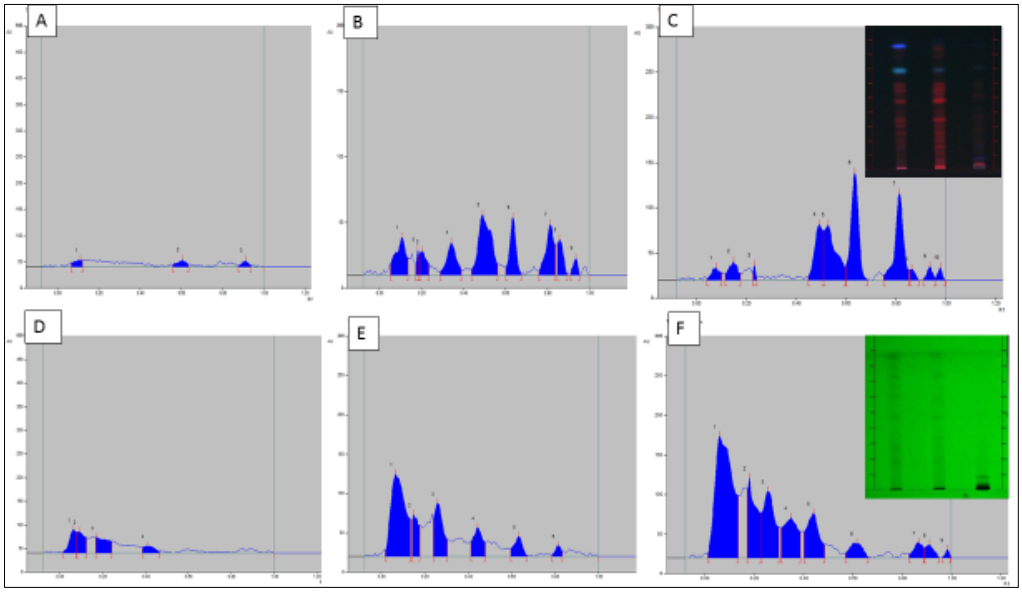


**Fig 3:** HPTLC fingerprint profiles of (A: Ethanolic extract of *A. nilagirica*; B: Hexane fraction of *A. nilagirica*, C: Terpenoid fraction of *A. nilagirica*) eluted with mobile phase hexane: ethyl acetate (8:2) at 366nm HPTLC fingerprint profiles of D: Ethanolic extract of *A. nilagirica*; E: Hexane fraction of *A. nilagirica*, F: Terpenoid fraction of *A. nilagirica* eluted with mobile phase hexane: ethyl acetate (8:2) at 254nm.

## 5. Discussions

*Artemisia nilagirica* is a tall, aromatic, herbaceous perennial plant belonging to Asteracea Family which grows in the hilly regions of India. Recent studies have demonstrated the activity of *Artemisia* species against pests and many other activities [17, 18, 19, 20, 5, 21, 9, 15]. Many authors have reported the role of terpenoids in insect repellant and cidal activities. Hence, the terpenoid fraction was isolated from *A. nilagirica* and explored in the present study.

The extractive yield of ethanolic extract of aerial parts of *A. nilagirica*, its hexane fraction was found to be higher than that reported by Darsana [9]. However, the yield of terpenoid fraction was 7.29 per cent. The increase in the yield percentage could be attributed to the difference in the period during which the plants were collected. The chemical constituents of the plants can vary depending on the climatic condition of that geographical region [22]. In the present study, phytochemical analysis of the ethanolic extract of aerial parts of *A. nilagirica* showed the presence of terpenoids, flavonoids, steroids, saponins, fixed oils and fats, tannins and glycosides which were in accordance with the findings cited in many literatures previously [7, 23, 24, 25, 9]. The hexane fraction of the crude extract of *A. nilagirica* also showed presence of saponins, terpenoids, flavonoids, fixed oils and fats indicating that the hexane fraction was enriched with terpenoids. And therefore, the hexane fraction was subsequently used for the isolation of terpenoids by Herz-Hogenauer method, which gave satisfactory separation of terpenoids as in other *Artemisia* species [14]. Terpenoid fraction of *A. nilagirica* on phytochemical analysis also has shown the presence of terpenoids besides fixed oils and fats in the present study. Presence of higher content of terpenoids in *A. nilagirica* collected from higher altitudes are also well supported in these literatures [5, 26, 27]. HPTLC was used as a tool in addition to the phytochemical screening for fingerprint profiling of the ethanolic extracts of the aerial parts of *A. nilagrica*, its hexane and terpenoid fractions to detect the presence of chemical constituents in them. The results revealed the presence of three polyvalent components at 366 nm with Rf ranging from 0.12 to 0.94 in the ethanolic extract of aerial parts of *A. nilagirica*. The florescent band (366 nm) at Rf 0.64 showed the highest peak intensity. The hexane fraction and terpenoid fraction of *A. nilagirica* showed presence of 10 and 9 polyvalent compounds at 366 nm with Rf ranging from 0.10 to 1.00 and 0.14 to 0.96 respectively. The fluorescent band (366 nm) of hexane fraction and terpenoid fraction showed highest peak intensity at Rf 0.69 and 0.56 respectively. At 254 nm the ethanolic extract and hexane fraction showed more elution in (8:2) hexane: ethyl acetate mobile phase than terpenoid fraction. However, the separation of more polyvalent compounds in the present study when compared with the findings of Darsana [9] and Drisya [15] could be due to the variation in climate and collection period.

In conclusion, the present study has revealed that the plant *Artemisia nilagirica* has a novel source for development of botanicals. Further characterization of terpenoid fractions has to be done by applying more sophisticated separation and purification techniques. Through these necessary techniques we can find out the chemical compounds and their different pharmacological activity.

### Conflict to Interests

The authors declare that there is no conflict of interest regarding the publication of this research paper.

## 6. Acknowledgment

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