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Open Access Research Article

Physicochemical evaluation, *in vitro* anti-inflammatory, *in vitro* anti-arthritic activities and GC-MS analysis of the oil from the leaves of *Gaultheria fragrantissima* Wall of Meghalaya

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ABSTRACT

The aim of our present study is to assess the *in vitro* anti-inflammatory activity against denaturation of proteins whereby the test oil sample at different concentrations was incubated with egg albumin and the absorbance was determined at 660 nm. The anti-arthritic activity was investigated by two methods; firstly, *in-vitro* method by bovine serum protein denaturation and the absorbance was measured at 255 nm and secondly *in vitro* method by egg albumin denaturation where the absorbance was measured at 660 nm. In all the studies diclofenac sodium was used as thestandard drug. The physicochemical parameters like colour, solubility, refractive index, boiling point, specific gravity, carbon residue, iodine value, acid value, ester value were evaluated. The different components of the volatile oil were determined by GC-MS analysis. Ten organic compounds were identified out of which Methyl salicylate $C_8H_8O_6$ was found to be the most dominant organic compound of *Gaultheria fragrantissima* oil (97.7%). The present results exhibited a concentration dependent inhibition of protein (albumin) denaturation by the test oil. The study reveals that diclofenac sodium was less effective when compared with the test oil. From the present findings it can be concluded that the essential oil of *Gaultheria fragrantissima* from Meghalaya possessed significant anti-inflammatory and anti- arthritic effects against the denaturation of protein *in vitro*. This effect could be due to the high content of methyl salicylate (97.7%) which is an inflammation-fighting compound. The high altitude and conducive climatic conditions of Meghalaya make wintergreen from this region far more superior in comparison to the ones grown in other parts of the world.

Keywords: *Gaultheria fragrantissima*, anti-inflammatory, anti-arthritic, GC-MS, methyl salicylate.

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1. INTRODUCTION

The aromatic plants had been used since ancient times for a large number of purposes, for example, they are used for their preservative and medicinal properties or to impart flavour and aroma to food. In the past, 'the father of modern medicine', Hippocrates, already prescribed perfume fumigations. The presence of essential oils is one of the main causes of the pharmaceutical properties of plants[1].

Essential oils that are named as volatile oil, aromatic oil, ethereal oil, essence oil or spirit are one of the important components of plant chemicals. These oils originated from aromatic plants may be found in all plant organs or leaves, fruits, peel, fruit stem[2].

It has been reported that "essential oil" was utilized for the first time in the 16th century by Paracelsus von Hohenheim, who referred to the effective component of a drug as "quinta essential"[1, 3].

Gaultheria is a large genus belonging to family Ericaceae which comprises of about 200 species and is native to the Andes, North America, Australia, and nearby islands, eastern Asia and Himalayas. The species which is widely used for the production of essential oils includes *Gaultheria procumbens* and *Gaultheria fragrantissima*. A total of 14 species are found in various parts of India. The plant's distribution includes the eastern Himalayas, north eastern region, and southern part at high elevation. Two species occur in Meghalaya, *vis. Gaultheria fragrantissima* and *Gaultheria griffithiana* of which *Gaultheria fragrantissima* is abundantly found in the north eastern part of India particularly in Meghalaya. The plant is confined at a higher altitude from 1300-1600m of Shillong plateau and Jaintia hills. It is widely distributed in Shillong Peak, Smit, Nongkrem, Laitkor, Mawphlang,

Cherrapunji, Laitlyngkot, Lyngdoh Nongpyiur, Niangbari forest, Jakrem, etc. The plant occurs in the pine forests, slopes of the hills, edges of the forest, open places and the road sides[4].

The leaves are the most important part of the plant which gives on steam distillation an essential oil popularly known as 'Oil of Indian wintergreen'. The oil contains methyl salicylate as the chief constituent, which is prescribed for rheumatic arthritis, sciatica, and neuralgia and is also used in most of the proprietary balms, liniments or ointments. In Meghalaya it is locally known in Khasi language as *lathynrait, jirhap, jirhapiong, dienglashyrhap, jathrait.* The Khasi local practitioners use the leaves for fomentation in rheumatic pain, body ache and menstrual pain. Leaves are boiled in water and bath is taken splashing this water all over the body to get relief from rickets and body ache. Tender leaves are crushed and inhaled to get relief from headache. Fruits are slightly bitter and eaten to cure diarrhoea and gastric problems[5].

The aim of the present study is to obtain the essential oils from the leaves of *Gaultheria fragrantissima* Wall, to determine the physicochemical parameters of the oil, *in vitro* anti-inflammatory activity on egg albumin, and *in vitro* anti-arthritic activity on egg albumin and bovine serum albumin and to evaluate the components of the essential oil by GC-MS.

2. MATERIALS AND METHODS

2.1. COLLECTION, IDENTIFICATION AND AUTHENTICATION OF PLANT

The leaves of the plant *Gaultheria fragrantissima* were collected from Smit, East Khasi hills, Meghalaya. The plant was identified and confirmed by standard literature.

2.2. ESSENTIAL OIL EXTRACTION

The extraction of essential oil from the leaves of *Gaultheria fragrantissima* was done by hydro-steam distillation using Clevenger apparatus. The leaves were thoroughly washed, cut into small pieces using scissors, placed in distillation flask and subjected to hydro-steam distillation for about 3 hours. The steam and vaporized oil were condensed into liquid by a vertical condenser and collected in measuring tube. Being immiscible and lighter than water, the volatile oil separated out as an upper layer. The oil was then separated from water and collected in small bottles, dried with anhydrous sodium sulphate or separated using separating funnel, then sealed, labelled and stored in light resistant vials at 4-6°C for further use[6].

2.3. PHYSICOCHEMICAL PARAMETERS

2.3.1. PERCENTAGE YIELD OF OIL

Percentage yield of oil was calculated using this formula[7].

$$\% \text{ yield} = \frac{weight\ of\ oil}{weight\ of\ sample} \times 100$$

2.3.2. COLOUR DETERMIATION

Determination of colour of the oil is to be determined by using ultra violet chamber under ultraviolet radiation of 254 and 366 nm by physical observation in day light[8].

2.3.3. DETERMINATION OF SOLUBILITY OF ESSENTIAL OIL

The solubility of the essential oil was determined by taking two liquids i.e. solvent and the oil in the ratio of 3:1. Approximately 3ml water was taken in a test tube containing 1ml of essential oil and then stirred thoroughly; the same was followed using chloroform, diethyl ether, methanol, separately[9].

2.3.4. DETERMINATION OF REFRACTIVE INDEX

The refractive index of the oil samples was determined by using Abbe refractometer model A 80251 (BS).First the prism is cleaned by using cotton and acetone, then the apparatus was left aside for 5 min for the purpose of drying the prism, then two drops of particular oil samples was placed on the prism and the prism was firmly closed, after that reading was recorded from the display screen[10].

2.3.5. DETERMIATION OF SPECIFIC GRAVITY

Determination of the specific gravity of the essential oil was done using a 50ml specific gravity bottle, a clean 50 ml specific gravity bottle was weighted (W_0). Then the bottle (W_0) was filled to the brim with water and stopper was inserted. Extra water on the stopper and bottle were carefully wiped off and reweighed (W_1). In the same manner, the oil samples were used instead of water it was weighed (W_2). The specific gravity of the essential oil were calculated by using the following formula given below[11]:

$$\text{Specific gravity} = \frac{W2 - W0}{W1 - W0}$$

W_0 = Weight of empty specific gravity bottle 0

W_1 = Weight of water + specific gravity bottle 1

W_2 = Weight of test sample + specific gravity bottle.

2.3.6. DETERMINATION OF pH OF ESSENTIAL OIL

pH meter was used to record the pH value of the oil sample . This was repeated for three times and then the average was recorded[12].

2.3.7. DETERMINATION OF BOILING POINT OF OIL

Determination of boiling point of the oil sample was done by using Thiele's apparatus which has the liquid paraffin bath inside it, which was heated slowly and stirring was done gently keeping an eye on the liquid and the fusion tube and also on the thread of the mercury in the thermometer. At first a bubble or two will be seen escaping at the end of the capillary dipping in the liquid, but soon a rapid and continuous stream of air bubbles escapes from it. This is the stage when the vapour pressure of the liquid in the sealed capillary just exceeds the atmospheric pressure. The temperature when continuous stream of bubbles starts coming out was noted. Finally, the flame was removed and the temperature was noted when the evolution of bubbles from the end of the capillary tube stops[13].

2.3.8. IODINE TEST OF OIL

Iodine test is used to determine the presence of unsaturated bonds in a compound or molecule. 2-3 drops of extracted oil was taken in a test tube. Few crystals of iodine was added. The presence of limonene or aromatic compounds in the oil sample shows brown colour precipitate after the addition of few crystals of iodine[14].

2.3.9. CARBON RESIDUE

An empty crucible was taken and weighed (W_1), the sample oil was transferred in the crucible and reweighed (W_2), and then placed inside the muffle furnace andheated strongly at 450°C till the vapour and smoke disappeared for 3 hours. The sample was then cooled down in desiccators. Carbon residue was then calculated by the following formula[15]:

$$\text{Carbon Residue (\%)} = \frac{W3}{W4} \times 100$$

Weight of carbon residue in crucible = (W_3)

Weight of sample taken = (W_4)

2.3.10. DETERMINATION OF SAPONIFICATION VALUE

2 g of the oil sample was weighted into a clean dried round bottom flask, followed by the addition of 25 ml of alcoholic potassium hydroxide (KOH). Then the flask was heated for an hour with a reflux condenser attached to it with periodic shaking. The completion of saponification was indicated by appearance of a clear solution. Then 1 ml of 1 % phenolphthalein indicator was added to the hot excess alkali which was titrated with 0.5 M hydrochloric acid (HCl) until it reached the end point where it turned colourless. A blank titration was carried out at the same time and under the same condition. The Saponification value was calculated as[16]:

Where,

Titre value of blank = 0.5 N HCl required (ml) by the blank

Titre value of sample= 0.5 N HCl required (ml) by the sample.

Saponification value =

$$\left[\frac{titer\ value\ of\ blank - titer\ value\ of\ sample}{weight\ of\ sample}\right] \times 28.05$$

2.3.11. TOTAL ACID NUMBER (TAN)

50 ml of methylated spirit was added to the flask and 2.5g of oil was transferred in the same flask, then the mixture was mixed properly and then titrated against 0.1N KOH solution using phenolphthalein as indicator. Alkali was added till a pink colour was established for few seconds. The TAN was then calculated using the following formula[17].

Where,

V = volume of potassium hydroxide used

N = normality of Potassium hydroxide

W = weight in g of the sample

$$Acid\ value = \frac{V \times N \times 56.1}{W}$$

2.3.12. DETERMINATION OF ESTER VALUE

ESTER VALUE (EV) = Saponification Value (SV) – Acid value (AV)

METHOD:

1.5 grams of essential oil was weighed, and was introduced into a glass flask. 25ml of ethanol solution of KOH (0.5mol/L) was added through a burette. Then the condenser was adapted and the ball was placed on the heating mantle and allowed to heat for one hour. After cooling, 20ml of distilled water and 5 drops of 0.2% PP. were added to it. Finally, as the excess of KOH solution with hydrochloric acid 0.5mol/L. alongside the operation cited, blank was made under the same conditions and with the same reagents[16].

2.4. GAS CHROMATOGRAPHY–MASS SPECTROMETRY (GC-MS) ANALYSIS

Gas chromatography–mass spectrometry (GC-MS) is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample. GC-MS has been regarded as a "gold standard" for forensic substance identification because it is used to perform a 100% specific test, which positively identifies the presence of a particular substance. For GCMS analysis of *Gaultheria fragrantissima* oil the sample was send to DIYA Labs in Mumbai. Pure compounds and essential oil constituents were verified by GC/MS. A Finnigan (San Jose, Calif.) GC (9610) and MS (4000) hooked on-line to a Data General Nova/4 data processing system, used electron impact analysis[18].

2.5. *IN VITRO* ANTI-IFLAMMATORY ACTIVITY ANALYSIS

2.5.1. Egg Albumin Denaturation Method

The total reaction mixture is of 5 ml, which consists of 2 ml of varying concentrations of the essential oil of *Gaultheria fragrantissima* oil so that final concentrations become 50, 100, 250 µg/ml respectively, followed by 2.8 ml of phosphate buffered saline and 0.2 ml of egg albumin (from fresh hen's egg). Dimethyl sulfoxide was served as a control.

The mixtures were incubated at 37±2ºC in an incubator for 15minutes and then heated at 70ºC for 5 minutes. After cooling down, their absorbance was measured at 660 nm by using vehicle as blank.

Diclofenac sodium at the final concentration of (50, 100, 250 µg/ml) was used as reference drug and treated similarly and then the absorbance of mixture was taken.

The percentage inhibition of protein denaturation was calculated by using the following formula[19]:

The experiment was done in triplicate and the average was taken.

$$\%\ \text{inhibition} = 100 \times \left[\frac{abst}{absc} - 1\right]$$

Where, abst = absorbance of test sample,

absc = absorbance of control.

2.6. *IN VITRO* ANTI ARTHRITIC ACTIVITY

2.6.1. Bovine Serum Protein Denaturation Method:

0.05 ml various concentrations (50, 100, 250 µg/ml) of test sample and standard drug diclofenac sodium (50, 100, 250 µg/ml) were taken respectively and 0.45 ml (0.5% w/v) BSA were mixed.

The samples were incubated at 37°c for 20 minutes and the temperature was increased to keep the samples at 57°C for 3 minutes. After cooling, add 2.5 ml of phosphate buffer was added to the above solutions.

The absorbance was measured using UV-Visible spectrophotometer at 255 nm. The same procedure was followed using Diclofenac sodium and the results were compared.

The percentage inhibition of protein denaturation can be calculated as[19]:

$$\%\ \text{inhibition} = 100 - \left[\left\{\frac{abs\ test - abs\ control}{abs\ test}\right\}\right] \times (100):$$

The experiment was done in triplicate and the average was taken.

$$\%\ \text{INHIBHITION} = \frac{\text{Abs control} - \text{Abs treated}}{\text{Abs control}} \times 100$$

2.6.2. Egg Albumin Denaturation Method

The total reaction mixture of 5 ml, consists of 2 ml of varying concentrations of the essential oil of *Gaultheria fragrantissima* oil so that final concentrations become 50, 100, 250 µg/ml respectively, followed by 2.8 ml of phosphate buffered saline and 0.2 ml of egg albumin (from fresh hen's egg). Dimethyl sulfoxide was served as a control.

The mixtures were incubated at 37±2ºC in an incubator for 15 minutes and then heated at 70ºC for 5 minutes. After cooling down, their absorbance was measured at 660 nm by using vehicle as blank.

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The percentage inhibition of protein denaturation was calculated by using the following formula[19]:

The experiment was done in triplicate and the average was taken.

$$\% \text{ inhibition} = 100 \times \left[\frac{abst}{absc} - 1\right]$$

Where, abst = absorbance of test sample,

absc = absorbance of control.

3. RESULTS

The leaves of *Gaultheria fragrantissima* were subjected to extraction by using Clevenger apparatus through hydro distillation and subjected to physicochemical analysis. In this study we have analysed the various physicochemical parameters such as % yield, colour, solubility, iodine test, pH test, iodine value, refractive index, boiling point, specific gravity, carbon residue, saponification value, acid value and ester value. The results are presented below.

3.1. PHYSICOCHEMICAL PARAMETERS

Table 1: Physicochemical parameters of oil of *Gaultheria fragrantissima*

Physicochemical parameters	Results
% yield	0.5%
Colour of extracted oil	Colourless at visible light, 254nm and 366nm.
Solubility	Soluble in methanol, diethyl ether and chloroform. Insoluble in water.
pH of extracted oil at 22°C	8.808 ± 0.05
Iodine test	Reddish brown, presence of limonene or aromatic compound.
Specific gravity	0.633 ± 0.04
Boiling point	220°C
Refractive index	0.472 ± 0.002
Carbon residue	0.33%
Saponification value	780
Acid value	5.61
Ester value	774.39

3.2. RESULT FOR GCMS

Ten organic compounds were identified by GCMS i.e. Methyl salicylate $C_8H_8O_3$ was found to be the most dominant organic compound of Gaultheria fragrantissima oil (97.7%) , Benzoic acid, 2-hydroxy-, ethyl ester $C_9H_{10}O_3$; (0.97%), 3-Aminobenzhydrazide $C_7H_9N_{30}$; (0.56%) , 4-Nitrophenyl anthranilate $C_{13}H_{10}N_2O_4$ (0.11%), Salicylic acid $C_7H_6O_3$;(0.10%) , Benzoic acid, 4-amino-, hydrazide $C_7H_9N_{30}$; (0.10%) , Benzoic acid, 2-ethoxy $C_9H_{10}O_3$; (0.08%), Benzoic acid, 2-hydroxy-, 1-methylethyl ester $C_{10}H_{12}O_3$;(0.07%) , Salicylamide $C_7H_7NO_2$; (0.04%) ,Aspirin $C_9H_8O_4$; (0.03%).

Compound 1:

Methyl salicylate

Chemical formula: $C_8H_8O_3$

MF: 943; RMF: 946; Prob 97.7%; CAS: 119-36-8; Lib: nistdemo; ID: 678.

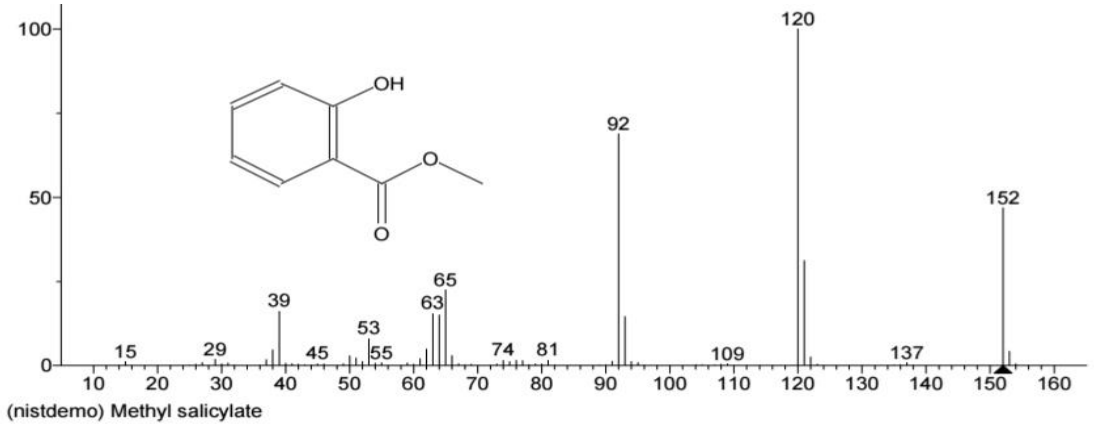


Fig.1: Mass Spectrum and structure of Methyl salicylate

MW: 152 CAS#: 119-36-8 NIST#: 291552 ID#: 678 DB: nistdemo Other DBs: None Comment: NIST Mass Spectrometry Data Center, 1998. 10 largest peaks: 120 999 | 92 688 | 152 468 | 121 311 | 65 225 | 39 161 | 63 155 | 64 149 | 93 145 | 53 81 |

Compound 2:
Benzoic acid, 2-hydroxy-, ethyl ester
Chemical formula: $C_9H_{10}O_3$
MF: 734; RMF: 819; Prob 0.97%; CAS: 118-61-6; Lib: nistdemo; ID: 826.

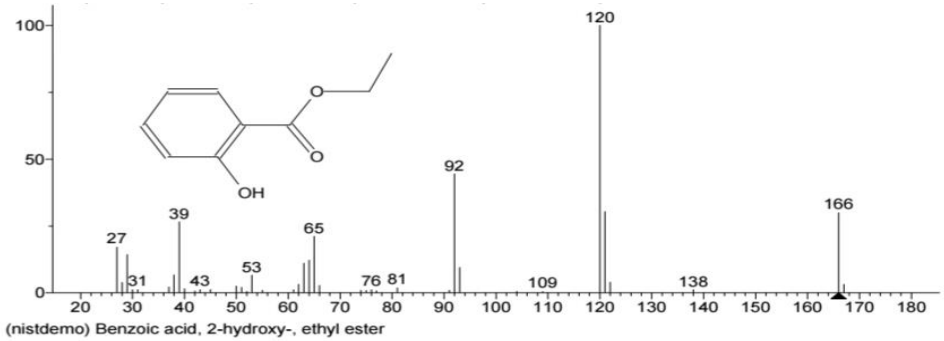


Fig.2: Mass Spectrum and structure of Benzoic acid, 2-hydroxy-, ethyl ester

MW: 166 CAS#: 118-61-6 NIST#: 6291 ID#: 826 DB: nistdemo Other DBs: None
10 largest peaks: 120 999 | 92 444 | 121 303 | 166 301 | 39 265 | 65 211 | 27 172 | 29 142 | 64 121 | 63 110 |
Compound 3:
3-Aminobenzhydrazide
Chemical formula: $C_7H_9N_3O$
MF: 718; RMF: 727; Prob 0.56%; CAS: 14062-34-1; Lib: nistdemo; ID: 60.

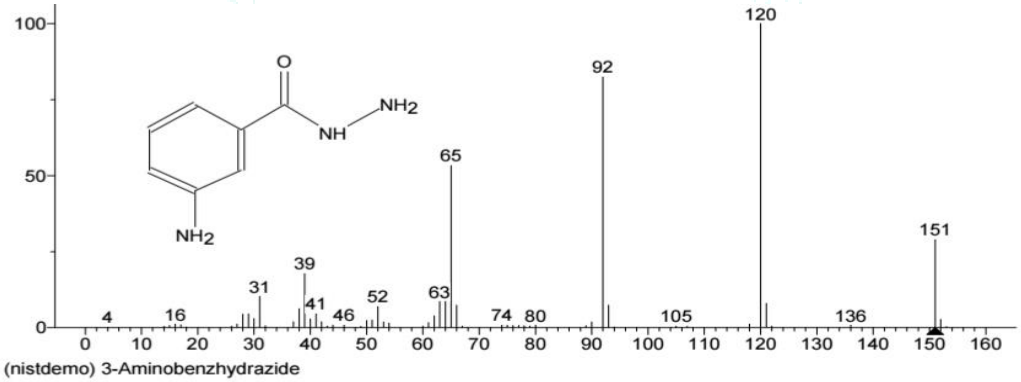


Fig.3: Mass Spectrum and structure of 3-Aminobenzhydrazide

MW: 151 CAS#: 14062-34-1 NIST#: 133949 ID#: 60 DB: nistdemo Other DBs: None Comment: NIST Mass Spectrometry Data Center, 1994
10 largest peaks: 120 999 | 92 824 | 65 534 | 151 291 | 39 180 | 31 103 | 63 86 | 64 86 | 121 79
Compound 4:
4-Nitrophenylanthranilate
Chemical formula: $C_{13}H_{10}N_2O_4$
MF: 672; RMF: 749; Prob 0.11%; CAS: 19176-60-4; Lib: nistdemo; ID: 1462.

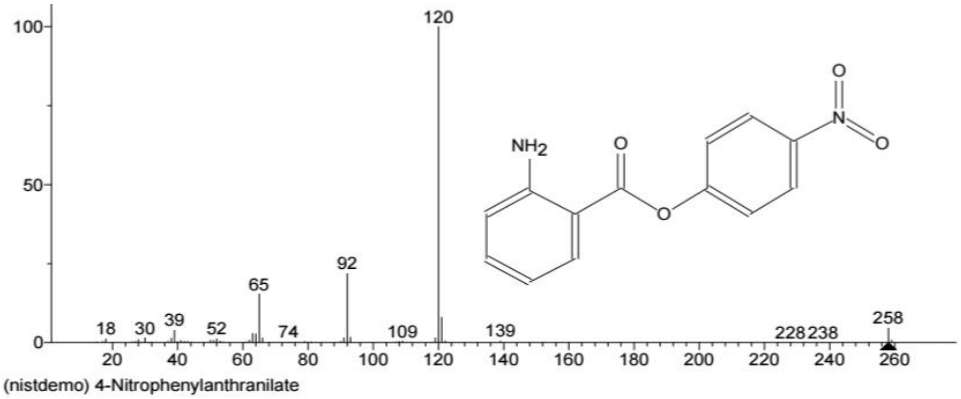


Fig.4: Mass Spectrum and structure of 4-Nitrophenylanthranilate

MW: 258 CAS#: 19176-60-4 NIST#: 234155 ID#: 1462 DB: nistdemo Other DBs: None Comment: Japan AIST/NIMC Database-Spectrum MS-NW-2359
10 largest peaks: 120 999 | 92 221 | 65 154 | 121 79 | 258 48 | 39 40 | 63 28 | 64 27 | 93 16 | 30 15 |
Compound 5:
Salicylic acid
Chemical formula: $C_7H_6O_3$
MF: 670; RMF: 740; Prob 0.10%; CAS: 69-72-7; Lib: nistdemo; ID: 888.

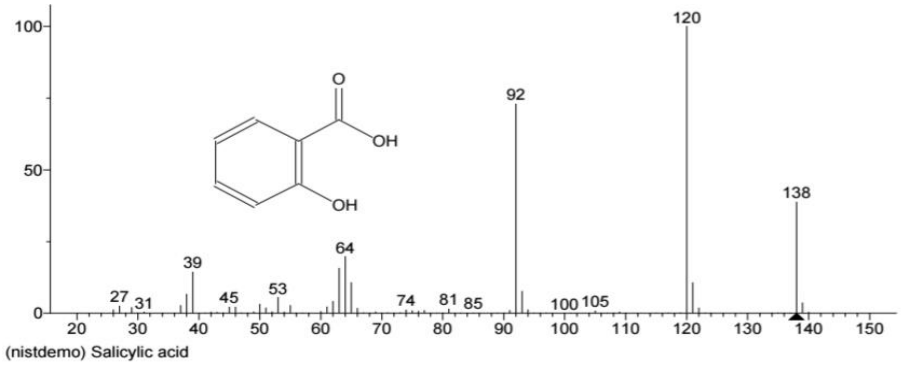


Fig.5: Mass Spectrum and structure of Salicylic acid

MW: 138 CAS#: 69-72-7 NIST#: 156389 ID#: 888 DB: nistdemo Other DBs: None Comment: Chemical Concepts
10 largest peaks: 120 999 | 92 731 | 138 389 | 64 197 | 63 156 | 39 145 | 65 106 | 121 106 | 93 76 | 38 66 |
Compound 6:
Benzoic acid, 4-amino-, hydrazide
Chemical formula: $C_7H_9N_3O$
MF: 670; RMF: 676; Prob 0.10%; CAS: 5351-17-7; Lib: nistdemo; ID: 113.

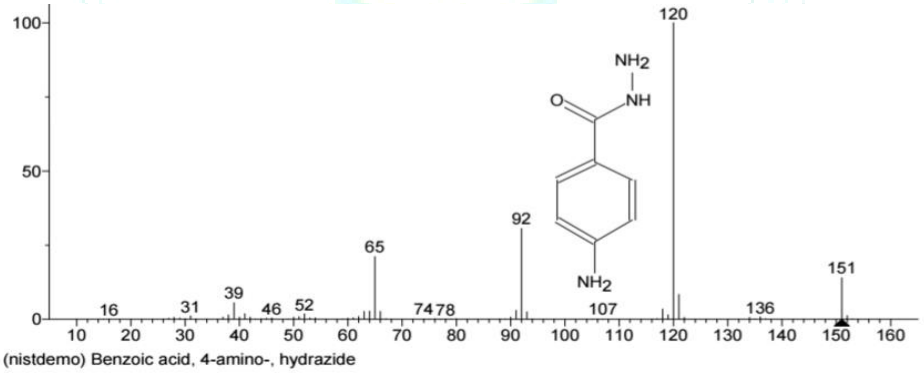


Fig.6: Mass Spectrum and structure of Benzoic acid, 4-amino-, hydrazide

MW: 151 CAS#: 5351-17-7 NIST#: 234692 ID#: 113 DB: nistdemo Other DBs: None Comment: Japan AIST/NIMC Database-Spectrum MS-NW-6185
10 largest peaks: 120 999 | 92 307 | 65 213 | 151 142 | 121 83 | 39 57 | 118 34 | 91 30 | 64 27 | 63 26 |
Compound 7:
Benzoic acid, 2-ethoxy
Chemical Formula: $C_9H_{10}O_3$
MF: 665; RMF: 666; Prob 0.08%; CAS: 134-11-2; Lib: nistdemo; ID: 2074

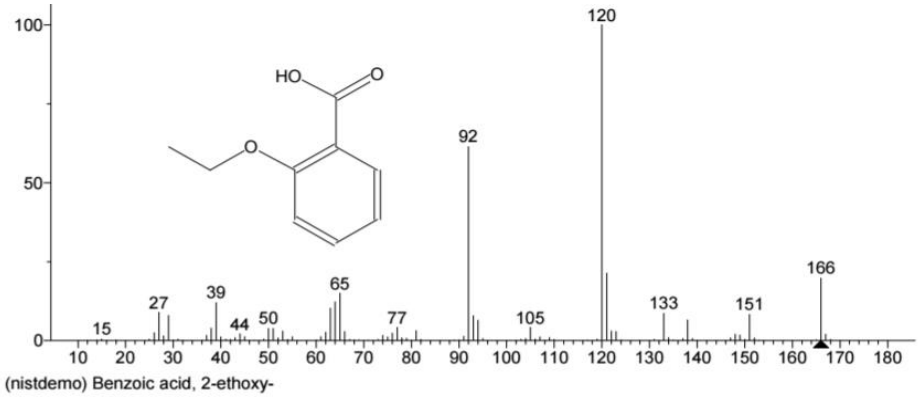


Fig.7: Mass Spectrum and structure of Benzoic acid, 2-ethoxy

MW: 166 CAS#: 134-11-2 NIST#: 231989 ID#: 2074 DB: nistdemo Other DBs: None Comment: Japan AIST/NIMC Database-Spectrum MS-NW-3420
10 largest peaks: 120 999 | 92 614 | 121 213 | 166 199 | 65 150 | 39 122 | 64 122 | 63 102 | 27 90 | 133 88 |
Compound 8:
Benzoic acid, 2-hydroxy-, 1-methylethyl ester
Chemical Formula: $C_{10}H_{12}O_3$
MF: 661; RMF: 724; Prob 0.07%; CAS: 607-85-2; Lib: nistdemo; ID: 1012.

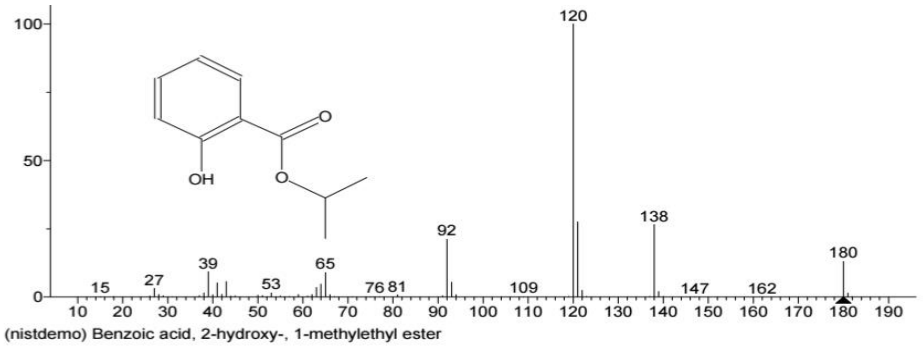


Fig.8: Mass Spectrum and structure of Benzoic acid, 2-hydroxy-, 1-methylethyl ester

MW: 180 CAS#: 607-85-2 NIST#: 229507 ID#: 1012 DB: nistdemo Other DBs: None Comment: Japan AIST/NIMC Database-Spectrum MS-NW- 803
10 largest peaks: 120 999 | 121 274 | 138 266 | 92 213 | 180 133 | 39 93 | 65 91 | 43 56 | 93 54 | 41 51 |
Compound 9:
Salicylamide
Chemical Formula: $C_7H_7NO_2$
MF: 644; RMF: 708; Prob 0.04%; CAS: 65-45-2; Lib: nistdemo; ID: 1619.

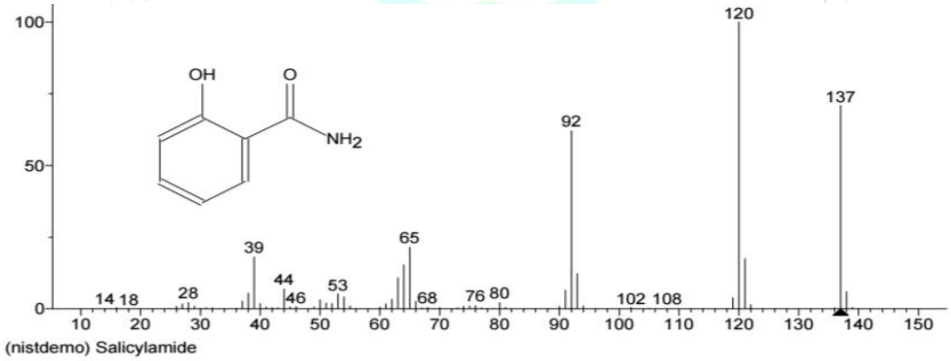


Fig.9: Mass Spectrum and structure of Salicylamide

MW: 137 CAS#: 65-45-2 NIST#: 229916 ID#: 1619 DB: nistdemo, Other DBs: None Comment: Japan AIST/NIMC Database-Spectrum MS-NW-4798
10 largest peaks: 120 999 | 137 708 | 92 622 | 65 217 | 39 182 | 121 174 | 64 153 | 93 123 | 63 108 | 44 72 |
Compound 10:
Aspirin
Chemical Formula: $C_9H_8O_4$
MF: 642; RMF: 697; Prob 0.03%; CAS: 50-78-2; Lib: nistdemo; ID: 885.

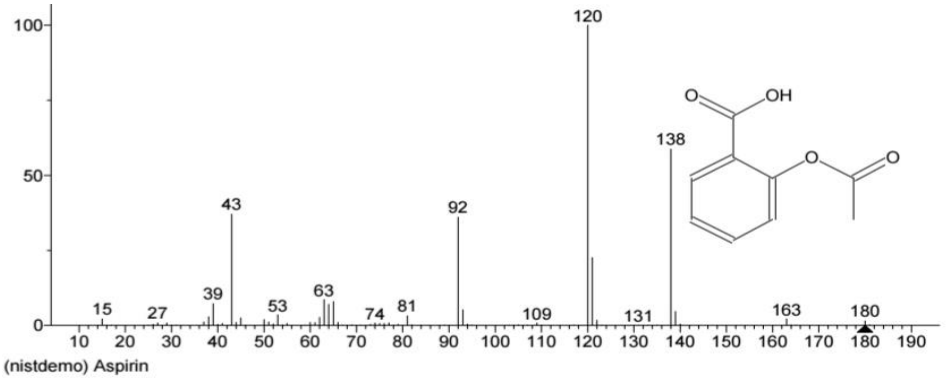


Fig.10: Mass Spectrum and structure of Aspirin

MW: 180 CAS#: 50-78-2 NIST#: 250572 ID#: 885 DB: nistdemo Other DBs: None Comment: Virginia Division of Forensic Science 10 largest peaks: 120 999 | 138 588 | 43 372 | 92 361 | 121 225 | 63 86 | 65 79 | 39 75 | 64 70 | 93 52 |

3.3. RESULT FOR *IN VITRO* ANTI-INFLAMMATORY ACTIVITY

3.3.1. Denaturation of egg albumin

Table 2: *In vitro* studies of anti-inflammatory activity

Drug	Concentration	Test Absorbance	% inhibition
Test (1)	50µg/ml	0.019	11.16%
Test (2)	100µg/ml	0.021	23.52%
Test (3)	250µg/ml	0.028	64.70%
Diclofenac (1)	50µg/ml	0.018	5.88%
Diclofenac (2)	100µg/ml	0.019	11.76%
Diclofenac (3)	250µg/ml	0.026	52.94%
Control	-	0.017	-

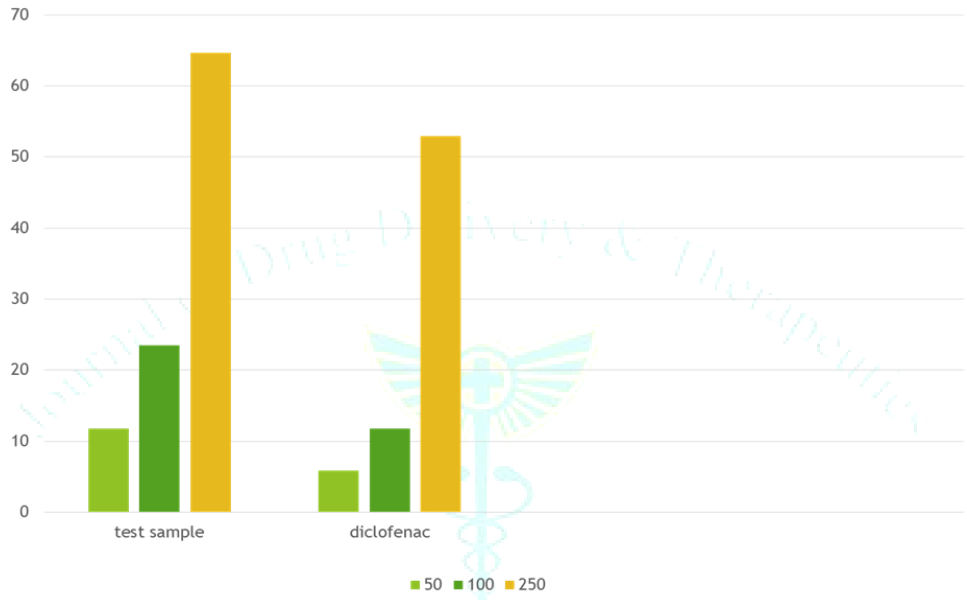


Figure 11: Comparison studies of anti-inflammatory activity

The report of the present investigation on *in vitro* activity of anti-inflammatory of *Gaultheria fragrantissima* essential oil against denaturation of egg albumin is summarized in Table 2. However, diclofenac sodium was found to be less effective when compared with the test oil sample. This was consequently confirmed by comparing their inhibiting effect, where the oil of *Gaultheria fragrantissima* inhibiting effect possessed 99.98 in total for three different concentrations of oil which was more when compared to diclofenac sodium inhibiting effect with only 69.7 in total. The graph (fig. 11) also shows that the *Gaultheria fragrantissima* oil (as test sample) shows significant inhibiting effect than diclofenac sodium.

3.4. RESULT FOR *IN VITRO* ANTI-ARTHRITIC ACTIVITY

3.4.1. Denaturation of bovine serum albumin

Table 3: *In vitro* studies of anti-arthritic activity analysis with bovine serum albumin

Sl no.	Drug	Concentration	Absorbance	Product control	% inhibition
1.	Test (1)	50µg/ml	0.375	0.149	44.47%
2.	Test (2)	100µg/ml	0.346	0.160	46.24%
3.	Test (3)	250µg/ml	0.371	0.185	49.86%
4.	Diclofenac (1)	50µg/ml	0.359	0.133	37.04%
5.	Diclofenac (2)	100µg/ml	0.418	0.166	39.71%
6.	Diclofenac (3)	250µg/ml	0.462	0.185	40.04%

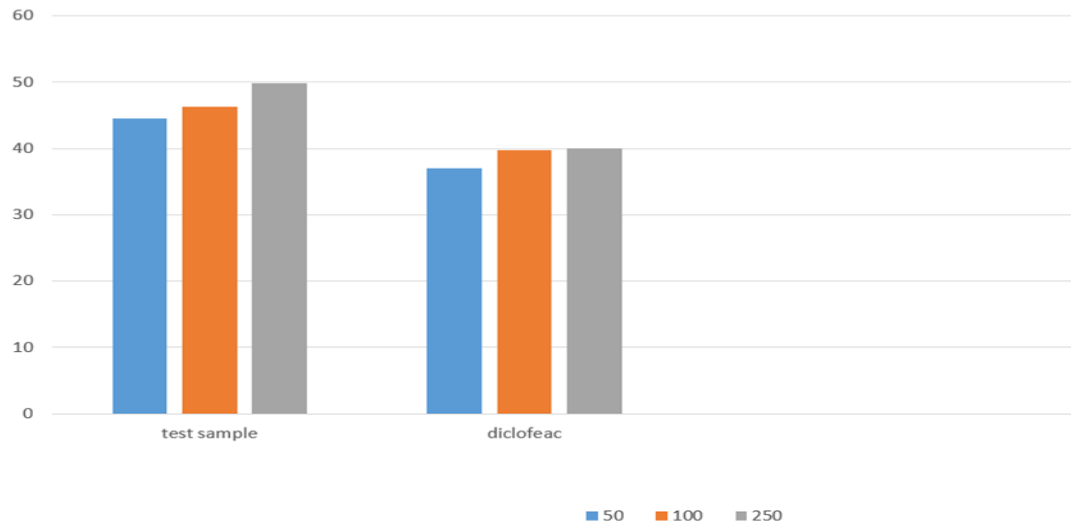


Figure 12: Comparison studies of anti-arthritic activity with bovine serum albumin

The present investigation reports the *in vitro* anti-arthritic effect of *Gaultheria fragrantissima* essential oil against bovine serum albumin. The results were summarized in Table 3. However, diclofenac sodium was found to be less effective when compared with the test oil sample. This was consequently confirmed by comparing their inhibiting effect, where the oil of *Gaultheria fragrantissima* inhibiting effect possessed 140.57 in total for three different concentrations of oil which was more when compared with diclofenac sodium inhibiting effect with only 116.79 in total. The graph (fig. 12) also shows that the *Gaultheria fragrantissima* oil (as test sample) shows significant inhibiting effect than diclofenac sodium.

3.4.2. Denaturation of egg albumin

Table 4: *In vitro* studies of anti-arthritic activity analysis with egg albumin

Drug	Concentration	Test Absorbance	% inhibition
Test (1)	50µg/ml	0.019	11.16%
Test (2)	100µg/ml	0.021	23.52%
Test (3)	250µg/ml	0.028	64.70%
Diclofenac (1)	50µg/ml	0.018	5.88%
Diclofenac (2)	100µg/ml	0.019	11.76%
Diclofenac (3)	250µg/ml	0.026	52.94%
Control	-	0.017	-

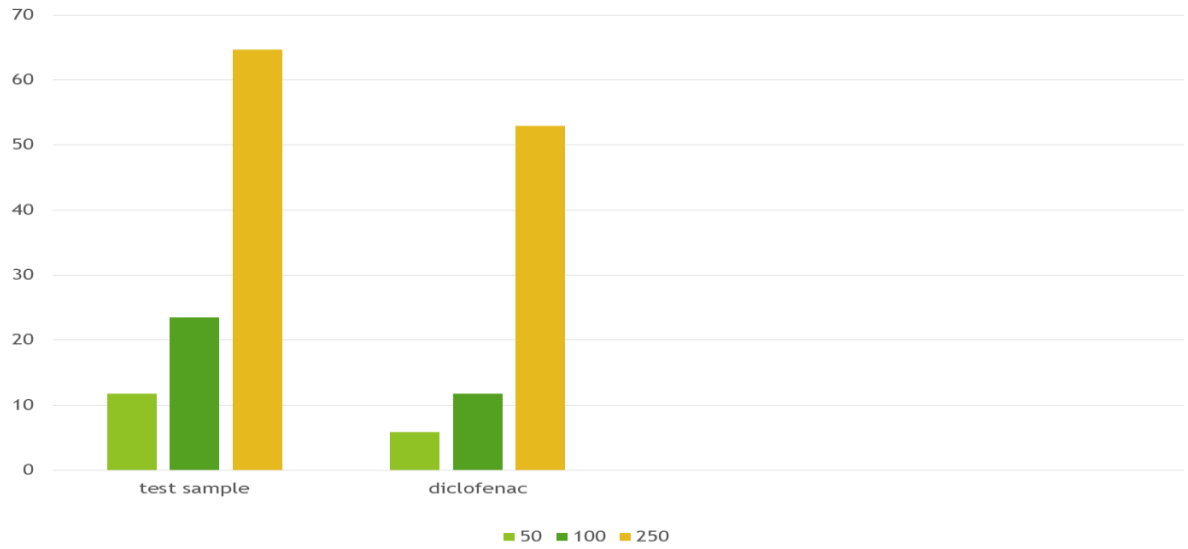


Figure 13: Comparison studies of anti-arthritic activity with egg albumin

The present investigation reports the *in vitro* anti-arthritic effect of *Gaultheria fragrantissima e*ssential oil, against denaturation of egg albumin. The results were summarized in Table 4. However, diclofenac sodium was found to be less effective when compared to the test oil sample. This was consequently confirmed by comparing their inhibiting effect, where the essential oil of Gaultheria *fragrantissima* inhibiting effect possessed 99.98 in total for three different concentrations which was more when compared with diclofenac sodium inhibiting effect with only 69.7 in total. The graph (fig. 13) also shows that *Gaultheria fragrantissima* oil (as test sample) shows significant inhibiting effect than diclofenac sodium.

4. DISCUSSION

The plant *Gaultheria fragrantissima* were collected from Smit, East Khasi hills, and the leaves were taken for extraction. The extraction was done using a Clevenger apparatus continuously for 4 hours. The oil was collected, wherein 200g of wintergreen leaves gives a yield of 1ml of oil in an average. Wintergreen oil was found to be colourless at visible light, at 254nm and 366nm using UV spectrometer. The appearance of a reddish brown colour after performing iodine test, indicates that the oil of wintergreen has limonene or aromatic compound. The pH was found to be 8.808 ± 0.05 at 22°C, which shows that the wintergreen oil is basic in nature. The specific gravity was found to be 0.633 ± 0.04 at room temperature which indicates that the wintergreen oil is lighter than water. The refractive index was found to be 0.472 ± 0.002 and this shows how light propagates when it passes through the wintergreen oil. Boiling point was found to be 220°C which proves that methyl salicylate is an active ingredient of the essential oil as the boiling point of methyl salicylate is also 220°C. Clark (1999) reported that wintergreen oil contains methyl salicylate (99.6%) as the dominant constituent of wintergreen oil. Carbon residue determines the presence of non-volatile impurities which was found very less (0.33 % w/w). Saponification value was found to be 780 which shows the average molecular weight of all the fatty acids present in the oil. Acid value was found to be 5.61, which measures the free fatty acids present in the oil and weight of potassium hydroxide in mg needed to neutralize the organic acids present in 1g of fat. Ester value was found to be 774.39, this indicates the weight of potassium hydroxide required in mg to saponify the esters in 1g of substance.

Ten organic compounds were identified by GCMS i.e Methyl salicylate $C_8H_8O_3$ was found to be the most dominant organic compound of *Gaultheria fragrantissima* oil (97.7%) , Benzoic acid, 2-hydroxy-, ethyl ester $C_9H_{10}O_3$; (0.97%), 3-Aminobenzhydrazide $C_7H_9N_3O$; (0.56%) , 4-Nitrophenyl anthranilate $C_{13}H_{10}N_2O_4$ (0.11%), Salicylic acid $C_7H_6O_3$;(0.10%) , Benzoic acid, 4-amino-, hydrazide $C_7H_9N_3O$; (0.10%) , Benzoic acid, 2-ethoxy $C_9H_{10}O_3$;(0.08%) ,Benzoic acid, 2-hydroxy-, 1-methylethyl ester $C_{10}H_{12}O_3$;(0.07%) , Salicylamide $C_7H_7NO_2$; (0.04%) ,Aspirin $C_9H_8O_4$; (0.03%).

From the *in vitro* anti-inflammatory activity carried out by denaturation of egg albumin, it was recorded that oil from the leaves of *Gaultheria fragrantissima* Wall showed higher percentage of inhibition compared to standard drug.

From the *in vitro* anti-arthritic activity carried out by denaturation of bovine serum albumin method and denaturation egg albumin method, it was revealed that the oil from the leaves of *Gaultheria fragrantissima* Wall showed higher percentage of inhibition compared to standard drug.

CONCLUSION

In conclusion, our study is the first attempt to evaluate and investigate the physiochemical parameters, chemical composition, *in vitro* anti-inflammatory activity and *in vitro* anti-arthritic activity of the essential oil from the leaves of *Gaultheria fragrantissima* Wall. The physiological parameters helped in formulating the pharmacopoeial standard for the drug. The essential oil was characterized by the high content of Methyl salicylate $C_8H_8O_3$ which was found to be the most dominant organic compound of *Gaultheria fragrantissima* Wall oil (97.7%). From the *in vitro* anti-inflammatory activity carried out by denaturation of egg albumin, it was revealed that the oil sample at different concentrations showed higher percentage of inhibition compared to the standard drug. From the *in vitro* anti-arthritic activity carried out by denaturation of bovine serum albumin method and denaturation egg albumin method, it was revealed that the oil sample at different concentrations showed higher percentage of inhibition compared to the standard drug.

This effect could be due to the high content of methyl salicylate (97.7%) which is an inflammation-fighting compound. The high altitude and conducive climatic conditions of Meghalaya make wintergreen from this region far more superior in comparison to the ones grown in other parts of the world. Though the study fulfilled the objectives of our work, further scientific investigation on the mechanism of action, safety and efficacy of this plant will support the claims made by the tribal healers of the region. Furthermore, on account of urbanization, industrialization and deforestation there is a threat of depletion of this treasure, therefore, new approaches towards conservation strategy and documentation is mandatory for the overall beneficial of humankind.

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