Nepal Journal of Science and Technology Vol. 14, No. 2 (2013) 59-64

Phytochemical and Biological Studies on Essential Oil and Leaf Extracts of *Gaultheria fragrantissima* Wall

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Abstract

Essential oil of *Gaultheria fragrantissima* Wall was isolated by hydrodistillation and GC/MS was used to find out the active constituents present in the oil. Methyl salicylate was the major compound (94.16%) and 4-hydroxy-4-methyl-2-pentanone as minor (5.84%) of oil. Phytochemical screening of methanol, ethyl acetate, chloroform and hexane extracts of leaves showed the presence of volatile oils, alkaloids, carotenoids, steroids, triterpenes, fatty acids, phenolic compounds and glycosides. Brine Shrimp Bioassay showed the oil was cytotoxic having LC50 value of 81.3 mg/l. Oils having 50% and 100% were found to be biologically active towards *Staphylococcus* and *Kelbesia* species but no positive activity was noted against *Proteus* and *Escherichia* species. Both concentrations (50% & 100%) of ethyl acetate and hexane extracts were biologically active only towards Proteus species. Further, chloroform extract was biologically inactive among the tested species. *E. coli* and *S. aureus* showed greater zone of inhibition towards methanol extracts for both concentrations.

Key words: antibacterial, cytotoxic, physiochemical parameter

Introduction

Gaultheria fragrantissima Wall belongs to family Ericaceae, is a genus of about 170-180 species of shrubs and seven species of the *Gaultheria* are found in Nepal. This species (Dhasingare in Nepali language) is commonly found as wild vegetation in Nepal at altitude of 1500 to 2700m. It is one of the important medicinal shrubs of Nepal having maximum 1.75 m height. The most useful and valuable parts of the plant are leaves which are dark green in color and on steam distillation provides essential oil known as wintergreen oil. The leaves are rich in essential oil and great economic value has many medicinal and perfumery applications. It has been used to treat numerous ailments including back pain, cold symptoms, colic, headaches, fever, skin diseases, sore throats, and tooth decay (Chevallier 1996). It is used as a flavoring agent in the confectionery industry, in the manufacture of soft drinks and also used as constituent in insect repellents. The oil contains methyl salicylate as the chief constituent which is prescribed for rheumatic arthritis, sciatica and neuralgia (Apte *et al.* 2006).

An overdose of oil may cause degenerative changes in liver and kidneys and may cause death of the patients (Shirreff & Pearlman 1940). The Oil is a pale yellow liquid heavier than water and strongly aromatic with a sweet characteristic odor. Methyl salicylate was found more than 95% of the water distillable oil. Beside this benzyl benzoate, menthyl acetate, β-asarone, hexanal, α-pinene, myrcene, δ-3-carene limonene, 3,7-guaiadiene, δ-cadinene are also found in small amount in distillable oil (Baruah 1976).

Methyl salicylate along with glucose and xylose are formed by enzymatic hydrolysis or by mild hydrolysis of the gaultherin present in leaves of *G. fragrantssima.*

Methodology

Specimen collection and plant identification

Plant specimens (leaves) were collected from the Godawari forest at altitude of about 1900m during February 2010 and identified by Professor Dr. Mohan Siwakoti, a taxonomist from Central Department of Botany, Tribhuwan University, Kathmandu.

Extraction of leaves

The essential oil content in the crude material (45g) was extracted by hydrodistillation using the Clevenger apparatus and repeated 5 times taking fresh pieces of leaves. Upper water layer was removed by dropper and the oil thus obtained was mixed with ether. Then the upper ether with oil layer was separated using separating funnel. The separated oil was dried over anhydrous sodium sulphate (Sigma-Aldrich, USA) and 6ml of pure oil was obtained. Likewise, 30 gm of powdered leaves were macerated with each 100ml hexane, ethyl acetate, chloroform and methanol separately for 15 days and were used for photochemical screening as well as biological studies.

GC/MS analysis

Gas chromatography-mass spectrometry (GC-MS) technique was used for analysis of the bioactive compounds present in the oil.

Determination of physical parameters (Guenther 1960)

Specific Gravity determination

An ignition tube, previously cleaned and dried, was weighed as W and was filled with the oil and was weighted as w_1. The same procedure was performed using the same tube containing water and its weight was noted as w_2. Specific gravity (d_t) was calculated using following equation

$$d_t = (w_1\text{-}w)/(w_2\text{-}w)$$

Where, dt = Specific gravity

Refractive index determination

The refractive index of the oil was measured using Abbe's refractometer.

Optical rotation determination

Oil solution (1%) was prepared by dissolving 1gm of the sample in 100ml methyl alcohol in volumetric flask. From this solution, 0.5% and 0.25% oil solution were prepared by proper dilution. The Polarimeter was switch on and the left for five minutes. Then the reading of Polarimeter was set to zero using distilled water in the Polarimeter tube. The Polarimeter tube was rinsed and fitted with the oil solution, fixing the bubble at the center i. e. opposite of the hole. Then the reading was noted as angle of rotation in degree by adjusting the pointer at equilibrium. Same procedure was repeated for other solutions as well. Then the specific rotation

$$[\alpha]_D^t = \frac{\alpha}{l \cdot c}$$

Where, t = the temperature at the time of measurement, D = D line of Sodium as light source, α = Angle of rotation of the plane of plane polarized light (mm), l=Length of Polarimeter tube and c = Concentration of the oil solution.

Determination of chemical parameters

Saponification value determination

Saponification value was determined by using standard procedure given by N. K.Visnoi (Visnoi 1990).

Acid value determination

Oil (0.5 gm) was accurately weighted into a 250 ml conical flask. To this 15 ml of neutral 95% alcohol and 2-3 drops of 1% phenolphthalein solutions were added. The free acid was then titrated with a standard 0.1N aqueous Sodium hydroxide (NaOH) solution adding the alkali drop wise at a uniform rate of about 30 drops per min. The content of the flask was continuously agitated. The first appearance of the red coloration that did not fade within 10 seconds was considered the end point. Then the acid value (A.V.) was calculated using the following equation,

$$\text{A. V.} = \frac{5.61 \text{ x number of ml of 0.1 N NaOH}}{\text{Weight of sample in gram}}$$

Phytochemical screening of leaf extracts

Concentrated leaves extracts were used for Identification of various chemical constituents, presence in leaves. The method employed for phytochemical screening was based on the procedure given by Prof. I. Ciulei (Ciulei, 1953). The different types of natural compound present in the extract are identified by their colour reaction with the different specific reagents.

Brine- shrimp bioassay of essential oil

The brine- shrimp Bioassay, was carried out to determine LC_{50} (mg/ml) of oil as mentioned by J.L.Mc.Laughlin *et al.* (Laughlin *et al.*1998) .

Antibacterial screening of extracts and essential oil

Inhibition of bacterial growth was tested by using the paper disc diffusion method with slight modifications.

Collection of test organisms

Employed microbial strains were obtained from the Central Department of Microbiology, T.U. and these

strains include four different bacteria two gram-positive (*Klebsiela pneumonia* and *Staphylococcus aureus*) and two gram-negative (*Escherichia coli* and *Proteus vulgaris*).

Observation of result

After 24 hours of incubation results were recorded as the presence or absence of inhibition zones. Resulting zones of inhibition were observed and recorded as "+" and "–". The diameter of zone of inhibition (ZOI) produced by plant oil on particular bacteria was also measured with the help of millimeter ruler. The inhibitory zone around test paper discs indicates absence of bacterial growth and that was recorded as positive and absence of zone as negative. Tests were repeated three times to insure the reliability of the result.

Results and Discussion

The essential oil was founded as pale yellow fluid liquid and strongly aromatic with a sweet characteristic odor. Analysis of components presents in the oil was based on the instrumental conditions set on the GC-MS and identified by direct comparison of mass spectra with literature data (Jayasekara *et al.* 2002). The GC spectra of the oil given in figure 1 showed only two distinct peaks at retention times 13.51 minutes for methyl salicylate as major chemical constituents (94.16%) and 5.75 minutes for 4-hydroxy-4-methyl-2-pentanone as minor (5.84%). Mass spectra of methyl salicylate show molecular ion at m/z 152 with the ions corresponding to molecular formula $C_8H_8O_3$ and the base peak was at m/z 120 with loss of methanol (M-32) followed by other peak at m/z 92 with loss of carbonyl group (M-32-28).

The mass spectra of 4-hydroxy-4-methyl-2-pentanone show molecular ion at m/z 117 with the ions corresponding to molecular formula $C_6H_{13}O_2$ that is protonated parent molecules. On basis of collisionally activated dissociation studies, the base peak in the electron impact mass spectrum was due to CH_3CO^+ (M/Z = 43). This primary ion reacts rapidly along with other minor primary ions giving protonated parent molecule (M/Z = 117). The ion (M/Z = 101) is due to loss of methane from protonated parent molecules. The ion (M/Z = 99) was formed due to protonated mesityl oxide by loss of water from protonated parent molecule. The excited protonated mesityl oxide may either be collisionly deactivated or lose CH_4 to produce an ion (M/Z = 83). Another peak of ion (M/Z=59) is observed due to protonated diacetone for reversible reaction of protonated parent molecules. The mass spectra of both methyl salicylate and 4-hydroxy-4-methyl-2-pentanone alcohol were compared with their standard previously published mass spectra and all are identical which are shown in figures 3 and 4. The structures of these two components are given figure 4.

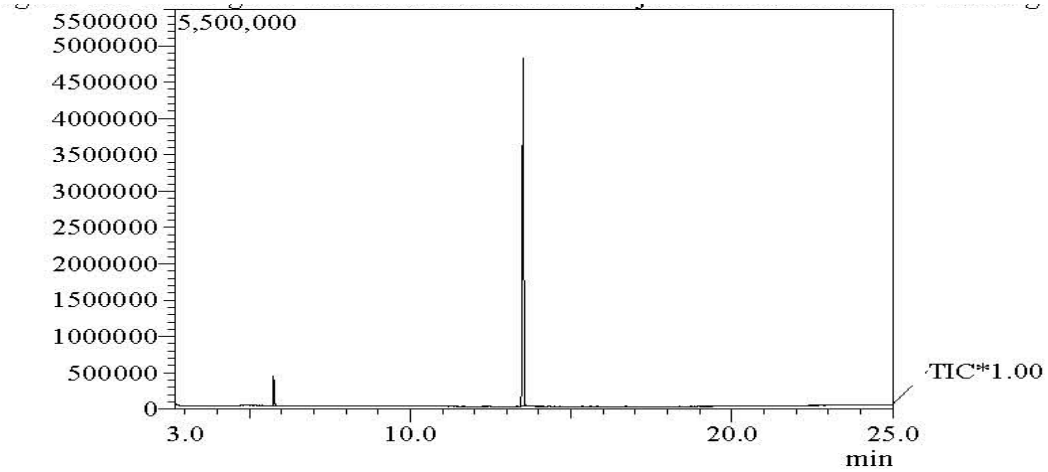


Fig. 1 . Gas chromatography for essential oil

Fig. 2. Comparison of mass spectra of methyl salicylate of oil (a) with literature spectra (b

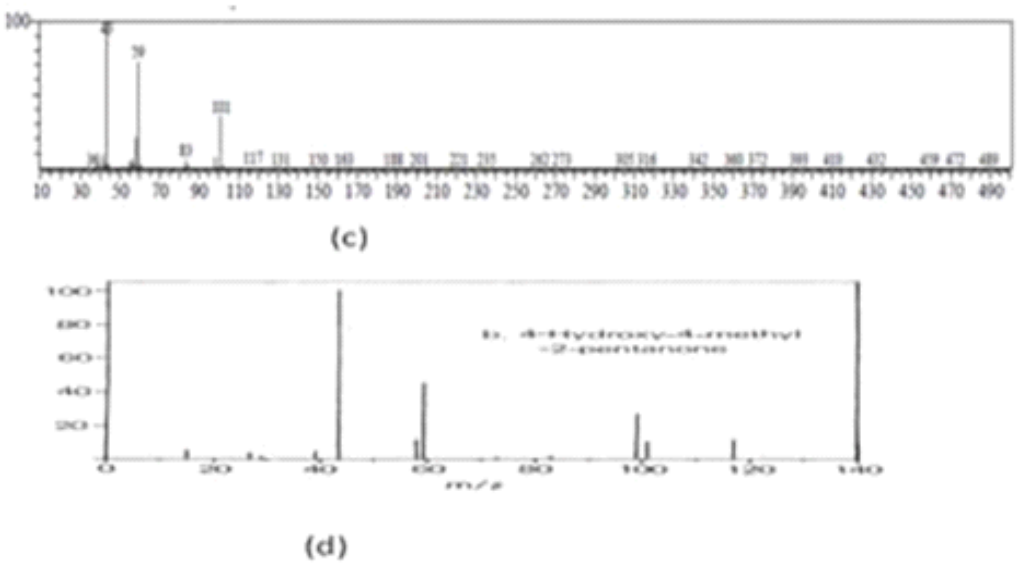
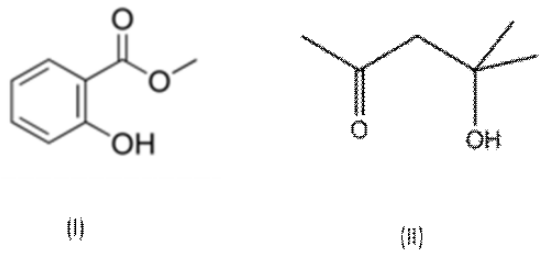


Fig. 3. Comparison of mass spectra of 4-hydroxy-4-methyl-2-pentanone of oil (c) with literature spectra (d)



(I) (II)

Fig. 4. Structure of methyl salicylate (I) and 4-hydroxy-4-methyl-2-pentanone (II)

Refractive index obtained for essential oil of *Gaultheria fragrantissima* was 1.529 and the specific rotation value was – 10.5^0. Likewise, the saponification value was found to be 353 and the specific gravity of the oil was found to be 1.17618, while the acid value was found to be 21. Essential oil was taken for the study of biological activity, which was evaluated on the basis of their toxicity towards the freshly prepared

brine shrimps nauplii. Bioassay showed lethal concentration (LC_{50}) of the oil was found to be 81.3mg/l, which indicated cytotoxicity.

The phytochemical screening of various extracts were identified by the colour reaction using different the reagent and the results are presented in Table 1.

Table 1. The results of phytochemical analyses of different fractions of leaf extracts

Constituents	Leaf extracts			
	Ethyl acetate	**Methanol**	**Chloroform**	**Hexane**
Volatile Oils	+	+	-	-
Alkaloids	+	+	+	-
Carotinoids	+	+	+	-
Steroids & Triterpenes	+(steroids)	-	-	+(triterpenes)
Fatty acids	+	+	+	-
Coumarins	-	-	-	-
Flavone aglycones	-	-	-	-
Emodins	-	-	-	-
Quinones	-	-	-	-
Phenolic compounds	+	+	+	-
Glycosides	+	+	+	-

Note: + present, - absent

Phytochemical screening of the leaf extract showed that volatile oils, alkaloids, carotinoids, steroids, triterpenes and fatty acids were present in the plant.

The result obtained from screening of different solvent extracts and essential oil of *G. fragrantissima* against tested bacteria are given Table no.2.

Table 2. Zone of inhibition of oil and leaves extracts

S.N	Oil/Extracts	% of extract solution	Tested Micro-Organisms(Bacteria)			
			Staphylococcus aureus	Proteus	Klebsiella species	*Escherichia coli*
1.	Oil	100%	8mm	-	8mm	-
2.	Oil	50%	8mm	-	8mm	-
3.	Ethyl acetate	100%	-	8mm	-	-
4.	Ethyl acetatae	50%	--	8mm	-	-
5.	Methanol	100%	13mm	-	8	12
6.	Methanol	50%	9mm	-	8	11
7.	Chloroform	100%	-	-	-	-
8.	Chloroform	50%	-	-	-	-
9.	Hexane	100%	-	9mm	-	-
10.	Hexane	50%	-	8mm	-	-

Oil and different solvents extracts of leaves were found to be biologically active towards tested bacteria except chloroform extract of leaves.

Acknowledgements

The authors are very much indebted to the Central Department of Chemistry, Kirtipur, Kathmandu, for providing opportunity to conduct this research in the laboratory.

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