Antioxidant activity of isolated phytoconstituents from the leaves of *Hyptis suaveolens* (L.) Poit.

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Abstract. *Hyptis suaveolens* (L.) Poit (Family Lamiaceae) is a medicinal plant used traditionally for antifertility, anti-inflammatory and antiplasmodial properties. Therefore, this study was designed to isolate bioactive compounds and to evaluate the antioxidant activity of isolated compounds from *H. suaveolens* by silica gel Column Chromatographic separation method and gallic acid (10 mg) was mainly isolated. Among them, the isolated compound was structurally characterized by physicochemical determination and spectroscopic techniques such as UV, FTIR and \(^1\)H-NMR by comparing with the reported data. Moreover, the essential oil was extracted from the fresh leaves of *H. suaveolens* by the hydro-distillation method and analyzed by GC-MS. It was found that carophyllene, sabinene, and naphthalene were predominant compounds in the essential oil of *H. suaveolens*. The antioxidant activity of isolated compounds and essential oil was determined by DPPH free radical scavenging assay method. The results suggest that the major compounds gallic acid from *H. suaveolens* possess effective antioxidant activity.

1. Introduction

Traditional medicine is widely used by the majority of the population in Myanmar either as an alternative or as a supplement to modern medicine [1]. Nowadays, even in Western countries, more people prefer medicines from natural herbs rather than modern drugs based on chemical compounds, which can have a side effect. Reactive oxygen species (ROS) play an important role in oxidative damage to the cellular compartment which leads to tissue injury and has been implicated in disease progression and oxidative damage of nucleic acid and protein [2]. Consequently, increased antioxidant intake in humans through diet is an important way to minimize such oxidative damage. The commercially available synthetic antioxidants are known to exhibit severe toxicity. Hence there are efforts to search for natural, economical, and effective antioxidants. The genus *Hyptis* including *Hyptis suaveolens* (L.) Poit in diverse regions of the world has been screened for its antioxidant activity [3]. Due to its biodiversity, the active principles differ from plant to plant, and it produces definite physiological actions on the human body. The plant *Hyptis suaveolens* (L.) Poit is commonly known as “Taw-Pin-Sein” in Myanmar. It belongs to the family Lamiaceae and is also an ethnobotanical important medicinal plant. In Myanmar Traditional Medicine, the whole plants are used as various skin diseases, carminative, and in treatment of stomach ache. Chemically, the plants contain

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glycosides, flavonoids, terpenoids, steroids, phenolic compounds that are responsible for the medicinal activities. The chemical point of view from *H. suaveolens* had reported the occurrence of several chemotypes. β-caryophyllene was a major component of the oil from Brazil [4]. Although some researchers have previously conducted the phytochemical and pharmacological studies on *H. suaveolens*, the scientific study on that plant from Myanmar still lacks. Thus, the objective of the present study is to isolate and characterize the phytoconstituents and to evaluate the antioxidant activity free radical scavenger from *H. suaveolens*.

2. Materials and methods

2.1. Collecting of plants materials

The specimen used in this research was collected from Loelem Township (North Latitude 20° 55’ 30” and East Longitude 97° 33’ 15”), the Shan State of Myanmar during the flowering period from July to December 2017. Field notes were made of detail for plant description, habitat types and precise location by using GPS. The identification of specimens was carried out by referring to the books [5,6,7,8]. The final verification was made by examination of the herbarium specimens in the Department of Botany, University of Yangon.

2.2. Preparation of plant extracts from dry leaves of *H. suaveolens* (L.) Poit.

2.2.1. Extraction. In preparation of ethyl acetate extract, the air-dried leaves powdered (100 g) were mixed with petroleum ether (60-80°C). Then, it was placed into the ultrasonic extractor at room temperature for 45 min. The filtrate was concentrated by using a Rotatory evaporator (60°C, for 15min). Then, the defatted residue was mixed with ethyl acetate and performed the above procedure to obtain defatted ethyl acetate extract.

2.3. Separation and isolation of organic compounds from the ethyl acetate extract

The defatted ethyl acetate extract (3.0 g) of the leaves of *H. suaveolens* was column chromatographically separated by eluting with PE:EtoAc (30:1, 20:1, 25:1, 9:1, 3:1, 1:1) solvent systems. It is shown in Figure 1. From this separation, a total of 167 fractions were collected and checked by TLC. From F-I (f1-f30), compound A was directly isolated as solid materials. The compound A (0.20 mg) was obtained as colorless crystal, whereas compound B from FII (f16-f170) was obtained as colorless needle-shaped crystals. Moreover, from fraction F-IV (f161-f187), Compound C as pale yellow needle-shaped crystals (10.01mg) was obtained.

2.4. Characterization and Identification of an isolated compound

The physicochemical properties such as Rf value, melting point and some chemical properties of isolated compound C were determined by UV and FTIR, 1H-NMR spectroscopic analysis.

2.5. Extraction of essential oil from the fresh leaves of *H. suaveolens* (L.) poit

The extraction of essential oil from *H. suaveolens* was performed by hydro-distillation method [9]. Then, the obtained essential oils were collected and dried on anhydrous sodium sulfate. After that, the obtained oil was weighed and kept in airtight container. The percentage of essential oil in the seeds was calculated using the relationships [10].

\[
% \text{ Yield} = \frac{W_1}{W_2} \times 100 \tag{1}
\]

Where

- \( W_1 \) = Weight of essential oil obtained in gram
- \( W_2 \) = Weight of sample extracted
Figure 1. Flow diagram of isolation of phytoconstituents from ethyl acetate extract by using column chromatography.

2.6. GC-MS analysis of essential oil
The essential oil of leaves of *H. suaveolens* was subjected to GC-MS analysis on an Agilent system, which consists of a model 6890N gas chromatograph, a model 5973N mass selective detector (EIMS, electron energy, 7-eV), and an HP-5ms fused silica capillary with a 5% phenyl methylpolysiloxane stationary phase, the film thickness of 0.25µm, a length of 30m, and an internal diameter of 0.25mm. The GC settings were as follows: the initial oven temperature was held at 60°C for 1 min and then heated at 180°C at a rate of 10°C/min, held for 1 min, and then heated at 280°C at 20°C/min and held for 15 min. The injector temperature was maintained at 270°C. The sample (1µL, diluted 100:1 in acetone) was injected, with a split ratio of 1:10. The carrier gas was helium at a flow rate of 1.0 mL min⁻¹. Spectra were scanned from 20 to 550 m/z at 2 scans⁻¹. Most constituents were identified by gas chromatography by comparison of their retention indices with those of authentic compounds available in our laboratories. The retention indices were determined about a homologous series of n-alkanes (C₈-C₂₄) under the same operating conditions. Further identification was made by comparison of their mass spectra with those stored in NIST 08.

2.7. DPPH free radical scavenging activity

2.7.1. Preparation of watery and 95% ethanol extract. The samples of dry powdered leaves from *H. suaveolens* were mixed with different solvents, distilled water and 95% ethanol, then placed into the ultrasound-assisted extractors at room temperature for 45 minutes at 70W output power. The extracts were then filtered through the whatman No.2 filter paper. The solvents were evaporated using a rotatory vacuum-evaporator at 65°C to obtain watery and 95% ethanol extract.

2.7.2. Preparation of sample solutions. The stock solution (400 µg/ml) of the samples, such as watery extract, 95% ethanol extract, isolated gallic acid compound, and essential oil from the leaves of *H. suaveolens* were prepared by dissolving 4mg of respective samples in 10 ml of 95% ethanol. This stock solution was two-fold diluted serially with 95% ethanol to get the sample solution with a concentration of 100, 50, 25, 12.5, 6.25µg/ml, respectively. Then, the control solution was also prepared by mixing 1.5ml of 0.002% DPPH solution and 1.5 ml of the test sample solution in different concentrations of 0.625, 1.25, 2.5, 5.0 and 10 µg/ml. The sample containing bottles was incubated at room temperature and was shaken on a shaker for 30 min; after 30 min, the absorbance of these solutions was measured at 517nm by using UV-visible spectrophotometer. Standard BHT was used as a standard. The percentage of radical scavenging activity was calculated by the following equation.
The IC50 (50% inhibition concentration) value was calculated by a linear regressive excel program [11,12].

\[
\%RSA = \frac{A_{DPPH} - (A_{sample} - A_{Blank})}{A_{DPPH}} \times 100
\]  

(2)

Where

\%RSA = % radical scavenging activity

\(A_{DPPH}\) = absorbance of DPPH in EtOH solution

\(A_{sample}\) = absorbance of sample + DPPH solution

\(A_{Blank}\) = absorbance of sample + EtOH solution

3. Results and discussion

3.1. Isolation of bioactive compounds

The ethyl acetate extract of \(H\) suaveolens was fractionated by column chromatography using PE: EtoAc – 30:1 v/v, 15:1v/v, 1:1v/v as eluents. Three compounds A, B, C at \(R_f\) value of 0.43, 0.63, and 0.42, were isolated. Among them, the isolated compound C (10.0 mg) was selected and identified by spectroscopic methods such as UV and FT-IR, 1HNMR due to high content in the ethyl acetate extract of \(H.\) suaveolens.

**Figure 2.** UV spectral data of compound C.

**Figure 3.** FTIR spectral data of compound C.
Table 1. Mean (%) Inhibition and IC₅₀ values of crude extracts, isolated gallic acid essential oil and standard BHT of leaves from *H. suaveolens* extracts.

| Sample            | Mean% inhibition in different concentrations |
|-------------------|---------------------------------------------|
|                   | 6.25 (µg/ml) | 12.5 (µg/ml) | 25 (µg/ml) | 50 (µg/ml) | 100 (µg/ml) | IC₅₀ (µg/ml) |
| 95% ethanol extract | 41.78±0.001  | 45.07±0.002  | 48.74±0.001 | 51.83±0.011 | 57.83±0.000 | 3.52        |
| Aqueous extract   | 42.94±0.002  | 46.81±0.001  | 49.52±0.003 | 54.55±0.001 | 60.15±0.001 | 2.74        |
| Gallic acid       | 45.87±0.000  | 58.60±0.001  | 66.15±0.012 | 75.24±0.011 | 84.14±0.02  | 0.83        |
| Essential oil     | 43.91±0.011  | 57.45±0.002  | 63.25±0.001 | 74.85±0.012 | 86.46±0.011 | 1.21        |
| BHT               | 41.18±0.000  | 51.26±0.012  | 71.18±0.011 | 74.08±0.001 | 87.42±0.000 | 1.17        |

The isolated compound C was a pale-yellow crystal, and *R*ᵥ value was 0.42. The ultraviolet spectrum of isolated compound C was recorded in MeOH as well as in the presence of NaOH. It showed that πₘₐₓ at 221nm (π-π*) and 271 nm (n-π*) in MeOH indicated the presence of unsaturation and non-bonding electron pairs. In the presence of NaOH, the absorption band at a shorter wavelength of 209 nm was not shifted. However, a longer wavelength of 271nm was shifted to a longer wavelength of 287nm, ascribable to bathochromic shift, redshift. It indicated the presence of the phenolic OH group. The melting point of isolated compound C was 235°C, which agreed with the melting point of gallic acid, as cited in [13]. In the FTIR spectrum, the –COOH stretching bands appeared at 3550 and 2500, indicating the presence of the acid group. The absorption band of –OH stretching band was found at 3371 and 3278. The absorption band at 1701 due to C=O stretching indicated the presence of carboxylic acid, and the C=C stretching bands appeared at 1620 and 1543, indicating the presence of the aromatic ring. In ¹H-NMR spectrum of compound C, only one single signal occurred to appear at the chemical shift of 7.07 ppm related to one or two equivalent protons of the benzene ring. According to the result of melting points, UV, FTIR, and ¹H-NMR spectral data, the isolated compound C could be considered as gallic acid. The UV and FTIR, ¹H-NMR spectral data were shown in Figure 2, Figure 3, and Figure 4.

Figure 4. ¹H-NMR spectrum (CD₃OD, 400MHz) of Compound C.

3.2. GC-MS spectroscopic analysis
The extraction of essential oil from *H. suaveolens* was performed by hydro-distillation method. The yield percentage of essential oil was 0.12%. Moreover, GC-MS spectroscopic study on isolated essential oil has been carried out. It was found that *H. suaveolens* oil contained caryophyllene, sabinene, and naphthalene as the main constituents indicated by the peaks at retention times 5.66 min,
5.10 min, and 5.99 min, respectively. The MS spectra were found to be identical with the library data. The respective GC-MS spectrums are illustrated in Figure 5, Figure 6, Figure 7 and Figure 8.

3.3. DPPH radical scavenging activity

DPPH radical scavenging activity of the aqueous extract, 95% ethanolic extract, gallic acid and essential oil of *H. suaveolens* is represented in Table 1. From the results, it was observed that an increase in concentration showed an increase in percent inhibition, i.e. increased free radical scavenging activities. The IC$_{50}$ values were found to be 3.52 µg /ml for 95% ethanol extract, 2.74µg /ml for aqueous extracts, 0.73 µg /ml for isolated compound Gallic acid, 1.21µg/ml for essential oil. It is shown in Figure 9 and Figure 10. Since the lower the IC$_{50}$, the higher the free radical scavenging activity. Thus, the isolated compound gallic acid has the highest free radical scavenging activity followed by essential oil, aqueous extract, and 95% ethanol extract. The antioxidant activities of isolated compound gallic acid showed a higher potency than standard BHT (Butylated Hydroxyl Toluence). However, the essential oil, 95% ethanol extract, and aqueous extract showed lower potency than that of standard BHT. It is because the isolated compound gallic acid from *H. suaveolens* can be used as a natural antioxidant.
Figure 7. MS spectrum of standard sabinene and isolated sabinene in *H. suaveolens* essential oil.

Figure 8. MS spectrum of standard naphthalene and isolated naphthalene in *H. suaveolens* essential oil.

Figure 9. Comparison of % oxidative inhibition in various concentration of four samples and Standard BHT.
Figure 10. Comparison of IC$_{50}$ values of four samples and standard BHT.

4. Conclusion
In summary, gallic acid, a phenolic compound was the main compound extracted from leaves of $H.\ suaveolens$ and chemically characterized by spectral studies; the presence of this compound in measurable amounts in the leaves of the plant can significantly show its antioxidant activity. Therefore, it is suggested that the phytoconstituents of $H.\ suaveolens$ are a source of natural antioxidant compounds.

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