Isolation of chemical constituents and In-Vitro screening of *Plectranthus mollis* Spreng for anthelmintic activity

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**Article History:**
Received on: 28.08.2019
Revised on: 05.11.2019
Accepted on: 19.11.2019

**Keywords:**
Anti-helminthic activity, Antioxidant activity, GC-MS analysis, Indian earthworm, *Plectranthus mollis*, Total phenol content

**ABSTRACT**
The human being are the most affected with diseases than any other animal species. For a long time, almost all the medicines were extracted from plants, and many herbal drugs were used by different civilization and many of them are still used. Tannins and flavonoids are the chemical constituents in the plants that shows the anti-helminthic activity. In traditional medicine, *Plectranthus mollis* has been used against snakebites, respiratory stimulant and cure for hemorrhage, antimicrobial, treatment of mental retardation and rheumatism. Moreover, *Plectranthus mollis* is reported to have a high content of tannins and flavonoids. Dried aerial parts of the hydroalcoholic extract of *Plectranthus mollis* are subjected to preliminary tests for the identification of various active constituents present in the extract. Estimation of total phenolic content were determined calorimetrically using Folin-Ciocalteu method and total flavonoid content were determined by aluminum chloride colorimetric technique for the hydroalcoholic extract of *Plectranthus mollis*. The antioxidant activity of the hydroalcoholic extract of the plant were carried out by DPPH radical scavenging assay. The anthelmintic activity of dried aerial parts of the hydroalcoholic extract of *Plectranthus mollis* are evaluated on adult *Pheretima Posthuma* (Indian earthworm). The chemical compound present in dried aerial parts of hydroalcoholic extract of *Plectranthus mollis* were isolated by flash chromatography. The isolated compound were detected by HPTLC technique and confirmed by GC-MS analysis.

**INTRODUCTION**
*Helminthiasis* are the worm infection, any part of a body in humans and other animals is infected with parasitic worms, known as helminths. Gastrointestinal parasite becomes a serious threat to the livestock production in the developing nations (Manke \textit{et al.}, 2015). Helminth infections causes nutritional deficiencies, after deworming, there is an increased status in nutritional absorption (Albonico \textit{et al.}, 2006).

Helminthiasis are caused by the parasitic worms of three classes,

1. Cestodes (Tapeworms) Beef tapeworm/fish tapeworm.
2. Nematodes (Round worms) Ascaris, pinworm, whipworm.

3. Trematodes (Flukes) Schistosoma haematomium (bilharziasis)

Based on the external and internal morphology of helminths, the definitive classifications are made (World Health Organization, 2011). Natural herbs are used as an anthelmintic such as, Indian mallow (Abutilon Indicum), Moringa, Drumstick Tree (Moringa Oleifera), Indian Oleander (Nerium Indicum), Indian Podophyllum (Podophyllum Emodi), Bhringaraj (Eclipta Alba). The plant Plectranthus mollis Spreng leaves locally used for the treatment of microbial infection in The Nilgiris, India. Plectranthus mollis is a soft-stem mint leaf is a small, erect, fleshy, annual herb, growing up to 30-50 cm tall. Flowers are purple. Flowering: July-August. The compounds such as flavones, fatty acids, triterpenoids and related compounds, fenchone, α-humulene, piperitenone oxide, cis-piperitone oxide, have been identified in the essential oil of P. mollis from South India while fenchone, piperitone oxide, piperitenone, piperitenone oxide, cis-piperitone oxide have been reported from Northern India (Waldia et al., 2011). The future scope for the anthelmintic drug discovery are also ongoing and providing a potent information, hence not only aim on a core signaling pathway information which translated a marketable drug (Yadav and Singh, 2011).

MATERIALS AND METHODS

Plant collection and extraction

The aerial parts of plant Plectranthus mollis Spreng, the drug of interest, was collected from Dhavanai village Udhamandalam, The Nilgiris, TamilNadu. Authentified by Dr. B. Duraiswamy Professor and Head, Department of Pharmacognosy and Phytopharmacy, JSS College of Pharmacy, Udhamandalam, the Nilgiris, Tamil Nadu. Aerial parts of the plant Plectranthus mollis were shade dried and powdered. The 300 grams of the coarse powder of plant Plectranthus mollis were cold macerated using hydro alcohol (ethanol and water (aqueous) 70:30) for 72 hours. After a complete extraction of the plant, it is filtered and concentrated in a rotary evaporator (Buchi, Switzerland) vacuum is applied at 40°C under reduced pressure. The concentrated extract were dried. The yield contents are calculated in terms of percentage (Das, 2013).

Qualitative screening

Preliminary phytochemical test

The hydroalcoholic crude dried leaf extracts of Plectranthus mollis Spreng aerial parts of the plant were subjected for the preliminary Phytochemical test for the identification of various active constituents present in the extract. The extract were subjected for the Test for Alkaloids, Flavonoids, Tannins & phenolic compounds, Terpenoids, Steroids and Saponins (Boufalous et al., 2017; Kumar et al., 2015).

Figure 1: Graphical representation of the total phenol content of hydroalcoholic extract of Plectranthus mollis

Figure 2: Graphical representation of the total flavonoid content of hydroalcoholic extract of Plectranthus mollis.

Figure 3: Graphical representation of concentration & percentage of inhibition of absorbance of the standard & hydroalcoholic extract of Plectranthus mollis.
Determination of Total Phenol Content (TPC)
The total phenols in the extracts was quantified by folin-ciocalteu colorimetric assay. In the test, the extract of 0.3 ml (1 mg/ml), 10% aqueous folin-ciocalteu reagent (1.5 ml) are added and allow to stand for 5 minutes and then 7.5% sodium bicarbonate (1.2 ml) were added and makeup for a total volume of 3 ml. Standard were prepared using gallic acid monohydrate, and linearity is observed. After incubating for 30 min, the absorbance was recorded at 765 nm. Ethanol was used to prepare a blank. The absorbance was calculated in terms of mg gallic acid equivalent (mg of GaA/g of extract) (Aziz et al., 2014).

Determination of Total Flavonoid Content (TFC)
The total flavonoid content was determined by the aluminum chloride colorimetric assay. In the sample test, the extract of 0.5 ml (20 mg/ml) and 5% sodium nitrate (300 μl) were taken in a test tube and mixed well with distilled water (2.5 ml). After incubating for 5 min to the test tube, 10% aluminum chloride (300 μl) and 1M sodium hydroxide (2 ml) were added to it and finally with distilled water (1 ml). At 510 nm, the absorbance was recorded. Standard were prepared using Quercetin (10-100 μg/ml) and a blank was also maintained with the same using methanol. The absorbance was calculated as Quercetin equivalent/g of dry extract (Piluzza and Bullitta, 2011).

Pharmacological screening

In-vitro antioxidant activity
10 mg of ascorbic acid was dissolved in 10 ml of methanol. From the standard solution of ascorbic acid, various concentrations like 10, 25, 50, 100, 150, 200 & 400 μg/ml were added to the 10 ml volumetric flask. 0.4 ml of 40 μg/ml solution of DPPH was added to all the above solution and volume was made up to 10 ml with methanol. In dark conditions, it is incubated at room temperature for 30 minutes and the absorbance of solution was measured at 517 nm using a UV spectrophotometer (Khomdram and Singh, 2011).

In-Vitro Anthelmintic studies
Approximately equal size earthworms in five groups (consist of six worms in each group) were released in 10 ml of each formulation. The concentrations (25, 50 and 100 mg/ml) of each standard drugs and extract were tested by bioassay. It determines the time of paralysis and the time of death of the worms. Albendazole and fenbendazole and Piperazine citrate were used as the standard reference because each drug possesses a different mode of action and saline water as a control. The anatomy and phys-
iology of Indian earthworm (*Pheretima Posthuma*) resembles the intestinal round worm parasite of human beings. Thus the bioassay were carried out using the adult Indian earthworm. Time taken to paralyse and death of worms is the two parameters considered for the observations. Unless the worms were shaken vigorously, when there is no movement, it is considered as the time for paralysis. After worms lose their motility, it is then dipped in warm water (50°C) followed by fading away of their body colors; it is considered as the time of death (*Vennila and Nivetha, 2015*).

**Thin-layer chromatography**

TLC is used in separating, identifying and estimation analytical tool of different classes of natural products. The mobile phase (Toluene: Ethyl acetate (8:2)) of 10ml were poured in the twin trough chamber. The chamber is allowed for saturation. The test and reference were spotted on the starting line using capillary tubes. The mobile phase is intended to elute through the stationary phase. The RF values were then calculated with the formula of elution distance of the sample by the elution distance of the solvent. The different spots developed were detected by the UV –TLC visualization chamber.

**Flash chromatography**

It was carried out by RF value guided technique. In the solvent system, binary solvents are employed; one of the solvents having a higher polarity than the other allows for the availability of easy adjustment of the average polarity of the eluent. In a glass column, directly above the stopcock glass frit or a plug of cotton wool is fitted to prevent the silica gel escaping from the column through the stopcock. A flat surface is maintained. Drain down the silica with a solvent until a complete flush with the surface of the silica. By applying the prescribed pressure, the solvent forcefully enters through the column. The solvent system used for the sample were Toluene: ethyl acetate of ratio 95:5. The fractions to be collected should be one-tenth of the column volume to maximize the efficiency of chromatography. Then TLC is performed to determine the compounds present in each fractions. Based on the TLC report, fractions are combined together and then concentrate the sample using a rotary evaporator (*Andrews, 1986*).

**High-performance thin-layer chromatography**

The samples are applied onto the plate as bands, the sample solvent used is methanol and spray gas as an inert gas. The respective mobile phase (Toluene: ethyl acetate (16:4)) is taken about 10ml in twin trough chamber and preconditioned for 30 minutes, the solvent front position is taken up to 80 mm and after development appropriate drying is done in an oven. Then before evaluation, the plates are sprayed by the solution of 10% ethanolic sulphuric acid by using a chromatographic sprayer, a volume of 50 ml was used and then it is dried. Detection was done using the CAMAG TLC Scanner and the analysis was done using the novel software WinCATS Planar chromatography manager. The measurements are taken in visualizer in two wavelengths that is 254nm and 366nm, and the image information was done by digital camera DXA252. The analysis gave a detailed report regarding each samples along with their images and chromatograms (*Senguttuvan and Subramaniam, 2016*).

**Gas chromatography-mass spectrometry analysis(GC-MS)**

GC-MS analysis is an effective chemical analysis and a common confirmation test. In this analysis, the compounds are separated from the sample by providing a representative spectral output. A compound present in the sample was analyzed by Thermo Gas Chromatography TRACE ULTRA VER: 5.0. The oven temperature of gas chromatography was maintained at 220°C at a rate of 6°C/min. A flow rate of the carrier gas is maintained at 1 ml/min. The sample is injected in the ratio of 1:10 by the split sampling technique. Compare the retention times of a series and identification of each component, which determines the retention indices (RI) of the compounds and confirms by comparing the retention index with data in the GC-MS library (*Rukshana et al., 2017*). The identification of the unknown component spectrum was compared with NIST library.

**RESULTS AND DISCUSSION**

The dried aerial parts of the plant *Plectranthus mollis* extracts have been significantly proved for the presence of terpenoids, steroids, flavonoids and tannins (Table 1). The quantitative phytochemical analysis for the plant extract of *Plectranthus mollis* revealed that the total phenols contained 137mg/ml and total flavonoid content were found to be 258 mg/ml (Figure 1 &Figure 2). The percentage of inhibition of absorbance of the hydroalcoholic extract of *Plectranthus mollis* were found to be 600µg/ml (Figure 3).

The hydroalcoholic extract of *Plectranthus mollis* and the standard drugs in various concentrations were screened for the anthelmintic activity on Indian earthworm (*Pheretima Posthuma*) (Table 2); it showed a significant results on the time of paralysis followed by the time of death caused at the
Table 1: Phytochemical screening of hydroalcoholic extract of *Plectranthus mollis*

| S.No | Constituents              | Hydroalcoholic extract of *P.mollis* |
|------|---------------------------|-------------------------------------|
| 1    | Alkaloids                 | (-)                                 |
| 2    | Saponins                  | (-)                                 |
| 3    | Terpenoids and steroids   | (+)                                 |
| 4    | Tannins                   | (+)                                 |
| 5    | Flavonoids                | (+)                                 |
Table 2: Anthelmintic activity of hydroalcoholic extract of *Plectranthus mollis*

| Sl.no | Treatment                          | Concentration (mg/ml) | Paralysis Time (in Minutes) | Death Time (In Minutes) |
|-------|------------------------------------|-----------------------|-----------------------------|-------------------------|
| 1.    | Control                            | -                     | -                           | -                       |
| 2.    | Standard Albendazole 25 mg/ml      | 21 ± 0.32             | 33 ± 4.12                   |
|       | 50 mg/ml                           | 09 ± 1.22             | 31 ± 0.6                    |
|       | 100 mg/ml                          | 06 ± 0.33             | 26 ± 0.4                    |
| 3.    | Standard Fenbendazole 25 mg/ml     | 28 ± 0.23             | 39 ± 0.57                   |
|       | 50 mg/ml                           | 21 ± 0.15             | 31 ± 0.26                   |
|       | 100 mg/ml                          | 16 ± 0.49             | 28 ± 0.31                   |
| 4.    | Standard Piperazine citrate 25 mg/ml| 30 ± 0.3              | 55 ± 0.3                    |
|       | 50 mg/ml                           | 15 ± 0.6              | 49 ± 0.64                   |
|       | 100 mg/ml                          | 09 ± 0.5              | 43 ± 0.42                   |
| 5.    | Hydro alcoholic extract of P. mollis | 16 ± 0.3              | 33 ± 0.36                   |
|       | 25 mg/ml                           | 09 ± 0.84             | 29 ± 0.25                   |
|       | 100 mg/ml                          | 05 ± 0.28             | 26 ± 0.16                   |

The chemical constituents of dried aerial parts of the plant *Plectranthus mollis* were isolated using flash chromatography and detected by HPTLC (Figure 6). Based on the GC-MS spectral analysis, the isolated compound were found to be a polyphenolic, a class of phenols, which is named by Phenol 3, 5-BIS (1, 1-dimethylethyl) (Figure 7 & Figure 8).

CONCLUSIONS

The traditional healers of The Nilgiris District of Tamil Nadu are practicing for the treatment of antihelminthic activity by using locally available weed plant *Plectranthus mollis* spreng for their people and their domestic animals. The present study scientifically proved the anthelmintic activity of the dried aerial parts of *Plectranthus mollis* spreng. The in-vitro antioxidant study potentially confirmed that the dried aerial parts of the hydroalcoholic extract of *Plectranthus mollis* spreng was having free radical scavenging activity. The GC-MS spectral analysis of the isolated compound revealed the medicinally valued bioactive compound called Phenol, 3, 5-BIS (1, 1-dimethylethyl). Hence the anthelmintic activity may be due to the presence of phenols present in the dried aerial parts of hydroalcoholic extract of *Plectranthus mollis* spreng. Further studies for the anthelmintic activity of the isolated compound and their related activity are in progress.

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