SENRA Academic Publishers, Burnaby, British Columbia
Vol. 4, No. 3, pp. 1249-1255, October 2010
Online ISSN: 1920-3853; Print ISSN: 1715-9997

Canadian Journal of pure&applied sciences

# SCIENTIFIC EVALUATION OF AN INNOVATIVE HERBAL MEDICINE FOR RELIEF IN RESPIRATORY DISORDERS

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## ABSTRACT

The present paper describes scientific evaluation of a traditional herbal medicine for relief in cough and asthma. The formulation had been used by a practitioner of traditional medicine from Jalandhar (Punjab, India) over a period of time and found to be quite efficacious in providing relief in cough and asthma. The same comprises a powdered mixture of five herbs *viz*. *Acorus calamus* (rhizome), *Alpinia galanga* (rhizome), *Psoralea corylifolia* (fruit), *Piper longum* (fruit) and *Piper nigrum* (fruit) in equal proportions. This polyherbal mixture showed antibacterial activity against Gram-positive and Gram-negative bacteria as well as antifungal activity against *Aspergillus niger*. Analytical studies revealed absence of any externally added steroidal content in the formulation. To ensure adequate quality control of the formulation, HPLC fingerprints of different extracts prepared from this polyherbal mixture were generated and two major constituents, i.e. piperine and asarone were quantified by HPLC.

**Keywords:** herbal medicine, cough, asthma, piperine, asarone

## INTRODUCTION

Herbal preparations have been a part of the Indian system of traditional medicine. There are many traditional remedies prevalent in different parts of India. However, with the passage of time, some of the time tested formulae and recipes have been lost. We came across such an herbal formulation that is being used by a practitioner of traditional medicine Mrs. Raj Katyal from Jalandhar over a period of time. The formula was given to her by her forefathers. She is at the third generation of this 100 years old formulation. Nobody in her family was interested in practicing this further. There was a fear of losing this traditional knowledge for providing relief in cough and asthma. Her quest to protect this valuable traditional knowledge led her to IPR cell of NIPER, SAS Nagar, which after a thorough search of various databases concluded that the formulation was novel and innovative. The formula, which the traditional healer had been using, offered societal benefit. However, there was no proven clinical data on its efficacy and safety. The formulation consists of a powdered mixture of five herbs *viz*. *Acorus calamus, Alpinia galanga, Psoralea corylifolia, Piper longum* and *Piper nigrum* in equal amount, which is to be taken orally with honey. These herbs are quite safe and well documented in Indian systems of medicines for their medicinal properties (Anonymous, 1986). The dose of the herbal preparation is described in table 1.

Thereafter, scientific inputs were initiated under TePP program (Department of Scientific and Industrial Research, Ministry of Science and Technology, Govt. of India) to develop this proven herbal medicine. The present paper describes standardization and scientific evaluation of this patented (1922/DEL/2005, dated: 27/07/2005) herbal formulation for providing relief in cough and asthma.

Table 1. Dosages of the herbal preparation.

| S. No. | Age of Patients (years) | Herbal mixture to be taken with honey |
|---|---|---|
| 1 | >18 (Adults) | 1.5 g (750 mg twice a day, morning and evening after meals) |
| 2 | 12-17 | 0.75 g |
| 3 | 5-11 | 0.35 g |
| 4 | 3-4 | 0.17 g |
| 5 | < 3 years (Infants) | 0.25 g |

## MATERIALS AND METHODS

### Methodology

### Evaluation for the presence of steroids

The formulation has been tested by TLC for any external steroidal contents presence.

***Sample preparation for TLC***: 1g each of the individual plant drugs used in the formulation i.e. *Acorus calamus* (rhizome), *Alpinia galanga* (rhizome), *Psoralea*

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*corylifolia* (fruit), *Piper longum* (fruit), *Piper nigrum* (fruit) powders were refluxed in chloroform (50ml) for 1hr. The extract was filtered and concentrated *in vacuo*. Same methodology was adopted for the sample preparation of the herbal formulation provided by traditional healer. Each extract was diluted to a volume of 10 ml with chloroform. 10µl of the sample was applied on TLC Plate.

***Thin layer chromatographic (TLC) analysis***

TLC Plates: Pre coated plates of silica gel $F_{254}$ (E. Merck) of 0.2mm thickness
Solvent system: Hexane - Ethyl acetate (8:2)
Spray reagent: Liebermann -Burchard Reagent.
***Liebermann- Burchard Reagent***: A solution of 5ml acetic anhydride and 5ml concentrated sulphuric acid was added carefully to 50ml of absolute ethanol while cooling in ice (Wagner and Bladt, 1996).

**Evaluation of antimicrobial activity**

Various microorganisms associated with respiratory tract infections were selected for the study. The antimicrobial activity of the formulation was determined by agar well-diffusion method as recommended by the National Committee for Clinical Laboratory Standards (NCCL 1992, 1997, 1998) at a concentration of 0.1mg/ml, using dimethyl sulfoxide (DMSO) as the diluent/solvent. The antibacterial activity was evaluated against Gram positive bacteria *viz. Staphylococcus aureus* MTCC 96 and Gram negative bacteria such as *Esherichia coli* MTCC 739, *Klebsiella pneumoniae* MTCC 109 and *Pseudomonas aeruginosa* MTCC 1688. Ciprofloxacin was used as standard for antibacterial activity. The antifungal activity was determined against *Aspergillus niger* MTCC 1344. Griseofulvin was used as standard antifungal drug.

**HPLC fingerprinting**

To ensure adequate quality control of the formulation, HPLC fingerprinting of the polyherbal formulation was done as detailed below.

***Sample preparation***

10g of powdered plant material was taken in a beaker, and 50ml of *n*-Hexane added in to it. Thereafter, it was sonicated at room temperature for 10minutes. The solvent was then decanted and concentrated under vacuum. The marc obtained after preparation of hexane extract was extracted sequentially with ethyl acetate and methanol under same conditions. A sample of 10mg/ml of extracts in HPLC grade Acetonitrile was prepared and filtered through 0.22µ syringe filter and injected into HPLC to obtain the fingerprints.

***Chromatographic conditions***

Instrument: Shimadzu SCL-10 AVP with PDA detector, Column: Kromasil (250X4.6 mm, 5µ, Phenomenex, USA) Injection volume: 10µl, Flow rate: 1 ml/min, Detection: 254nm, Solvent composition: A: Water, B: ACN.

For Hexane extract: 0-10min: 40-50% B, 10-20min: 50-70% B, 20-30min: 70-80% B, 30-40min: 80-90% B, 40-50min: 90-100% B, 50-65min: 100% B, Runtime: 65min
For Ethyl acetate extract: 0-10min: 45-55% B, 10-30min: 55-65% B, 30-40min: 65-75% B, 40-50min: 75-100% B, 50-65min: 100% B, Runtime: 65min

For Methanol extract: 0-10min: 20% B, 10-20min: 20-50% B, 20-30min: 50-70% B, 30-40min: 70-90% B, 40-50min: 90-100% B, 50-55min: 100% B, Runtime: 55min

**Quantification of asarone and piperine**

Quantification of the two major constituents, i.e. piperine and asarone in the formulation was performed using following methodology.

***Standards***

Piperine (Pip) and Asarone (Asa) were isolated and characterized as reported from *Piper nigrum* (Ikan, 1991) and *Acorus calamus* (Yingjuan *et al*., 2008).

***Preparation of standard and test solutions***

Standard stock solutions of Piperine (1000µg/mL) and Asarone (1000µg/mL) were prepared in Methanol. Working standard solutions were prepared by diluting appropriately the stock solutions with the same solvent. Both the calibrants were prepared over the range of 0.1-0.5µg/ml. For the preparation of test solution, 2.5g of formulation was accurately weighed into a 25mL beaker and 10mL of methanol was added in to it. It was sonicated for 20min at room temperature. After this period, the solution was filtered and dried using rotary evaporator under vacuum. A stock solution of 10 mg/mL was prepared and the test solution was prepared after adequate dilution with methanol (1mg/mL). Finally, before injecting 20µL on chromatograph, the standard and test solutions were filtered through a 0.22-µm filter.

***Chromatographic conditions***

The chromatographic conditions were same as of used for fingerprinting analysis. The quantitative analysis was also carried out in a gradient elution mode with 50% ACN at 0 min gradually increased to 60% at 15min, then back to 50% from 17min.

**RESULTS AND DISCUSSION**

**Evaluation for the presence of the steroids**

It is generally believed that traditional herbal practitioner use steroidal drugs like dexamethasone, prednisolone in the formulations for relief in pain and inflammation etc. Liebermann -Burchard reagent is used to detect steroids, terpenoids, lignans and saponins. Initial comparison of the chloroform extract of the individual plant drugs by TLC

suggested an identical profile for the formulation with respect to its plant drugs. The spots that could be detected with Liebermann- Burchard (LB) reagent in the formulation were also observed in the individual drugs. It suggested complete absence of any externally added steroids in the formulation. Literature reports the presence of terpenoids and lignans in these plant drugs and the spots shown with LB reagent can be ascribed to individual chemical constituents of these drugs. For example, dihydrostigmasterol is present in *Piper longum*; Sesquiterpenes (acoranedione, shyobunone, alpiniol) are present in *Acorus calamus* and *Alpinia galanga.* Lignans are present in *Piper* species. All these classes of compounds give positive test with LB reagent.

**Evaluation of *in vitro* anti-microbial activity**

Results reveal that the herbal formulation has significant activity against Gram positive and Gram-negative microorganisms, which cause respiratory tract infections. This formulation exhibited comparable activity against *K. pneumoniae* as that of standard drug ciprofloxacin. It showed moderate activity against *P. aeruginosa.* The activity against *S. aureus* and *E.coli* is less in comparison to the standard drug. In case of antifungal screening, the formulation showed moderate activity against *A. niger*. The results are shown in table 2.

**HPLC fingerprinting**

The HPLC fingerprint analysis of all the three extracts *viz*. hexane; ethyl acetate and methanol of the formulation are given in figures 1, 2 and 3, respectively. From the fingerprints obtained, it is clear that the extraction process significantly influences the molecular composition of the extract. However, in the present composition the powdered herb is given as a 'whole', which ensures that no molecules are lost during extraction and the 'holistic therapeutic potential' if any is duly preserved.

**Quantification of asarone and piperine**

There were HPLC methods reported for piperine and asarone individually. Piperine was analyzed using a Symmetry C18 column (150X4.6mm, 5µm) and isocratic elution with 25 mM $KH_2PO_4$ (pH 4.5)–acetonitrile (50:50) with a flow-rate of 1 ml/min (Bajad *et al*., 2002). While, asarone was analyzed using Hypersil ODS C18 column (150X4.6mm, 5µm) and isocratic elution with acetonitrile-water (60:40) at 1mL/min flow rate (Wu and Fang, 2004). So, a method was developed using acetonitrile-water gradient at 1ml/min on Kromasil C18 Column (250×4.6, 5µm) for simultaneous determination of Asarone and Piperine. Calibration curves were obtained by plotting peak area against the concentrations of respective substances as shown in figures 4 and 5. In both cases, straight regression lines with correlation coefficients ($r^2$) of 0.99 were obtained. Data are summarized in table 3.

Table 3. Linearity parameters for the proposed RP-HPLC method.

| Parameter | Piperine | Asarone |
|---|---|---|
| Concentration (µg/ml) | 0.1-0.5 | 0.1-0.5 |
| Regression equation | Y= 2038868*X-17818 | Y= 2743932*X-16739 |
| Correlation coefficient ($r^2$) | 0.9982 | 0.9984 |

The precision of analysis was examined by performing the intra-day and inter-day assays by replicate injections of the sample solutions used above. The intra-assay precision was performed with the interval of 4 h in a day, while the inter-assay precision was performed over 3days. The precision result of the solution are presented in table 4, and it has been shown that the RSD values of concentration from peak area are lower than 0.11% both for the intra-assay and inter-assay precision for both calibrants.

Piperine and Asarone were identified in sample by the comparison of their retention times ($t_R$ 10.6 and 11.3min, respectively in Figs. 6 and 7) and UV spectra with the corresponding pure compounds. The Piperine and Asarone were calculated using calibration curves results. Piperine was found to be 0.2674 ± 0.0014 µg/ml (0.4479 %w/w) and asarone was found to be 0.1901 ± 0.0029 µg/ml (0.3183 %w/w) in the formulation on dry powder basis.

Table 2. Antimicrobial activity (zones of inhibition in mm, cup diameter 6mm).

| | *K. pneumoniae* | *S. aureus* | *P. aeruginosa* | *E. coli* | *A. niger* |
|---|---|---|---|---|---|
| Herbal Formulation (0.1mg/ml) | 22 | 20 | 24 | 8 | 12 |
| Ciprofloxacin (50µg/ml) | 22 | 30 | 28 | 16 | - |
| Griseofulvin (50µg/ml) | - | - | - | - | 11 |
| DMSO | - | - | - | - | - |

Table 4. Precision parameters for the proposed RP-HPLC method.

| | Interday precision (Conc µg/ml) | Interday precision (%RSD) | Intraday precision (Conc µg/ml) | Interday precision (%RSD) |
|---|---|---|---|---|
| Piperine | 0.2680 ± 0.0011 | 0.04 | 0.2668 ± 0.0016 | 0.08 |
| Asarone | 0.1895 ± 0.0033 | 0.11 | 0.1906 ± 0.0015 | 0.07 |

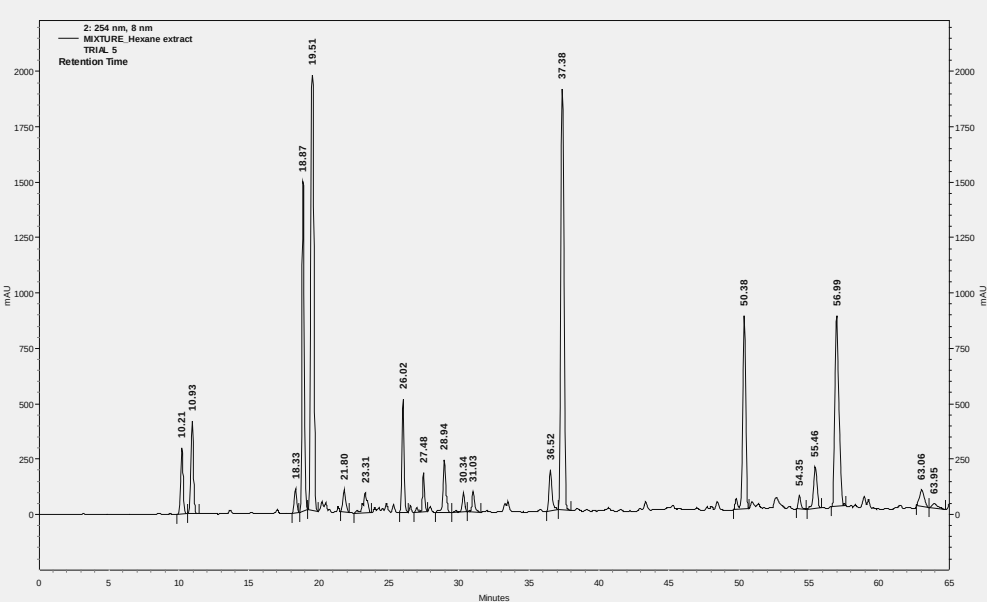


Fig. 1. HPLC fingerprint of hexane extract of the polyherbal mixture.

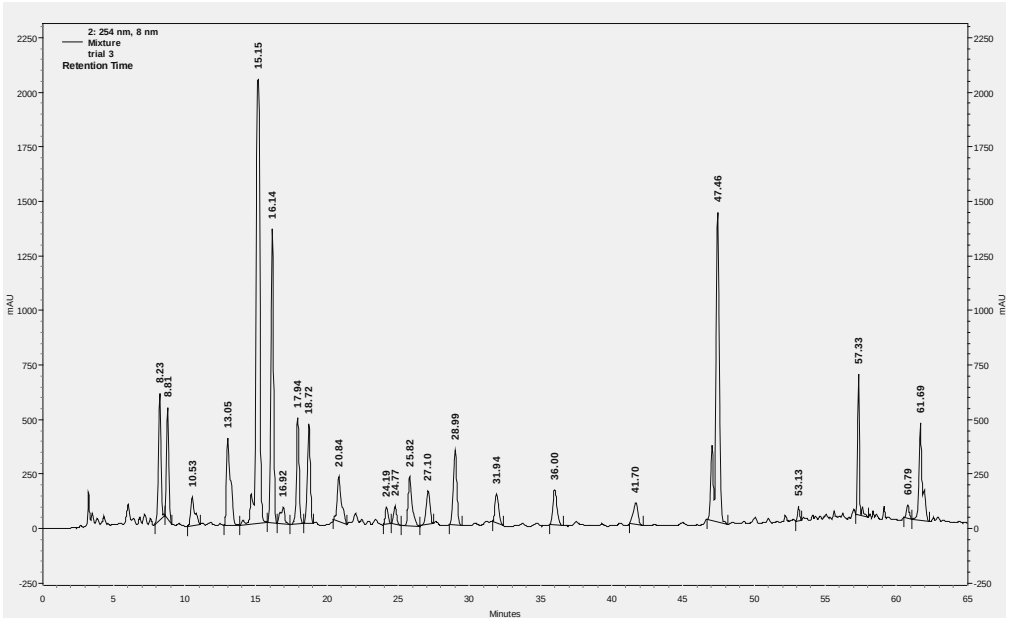


Fig. 2. HPLC fingerprint of ethyl acetate extract of the polyherbal mixture.

***Significance of asarone estimation***

The beta form of pure asarone, which is present in *Acorus calamus* oil, has been associated with toxicity and there are concerns in the western scientific world regarding possible carcinogenic effect of pure asarone. However, the powdered root as well as rhizome of *Acorus calamus* has been used in India for thousands of years without any reports of toxicity or cancer. The pharmacological profile

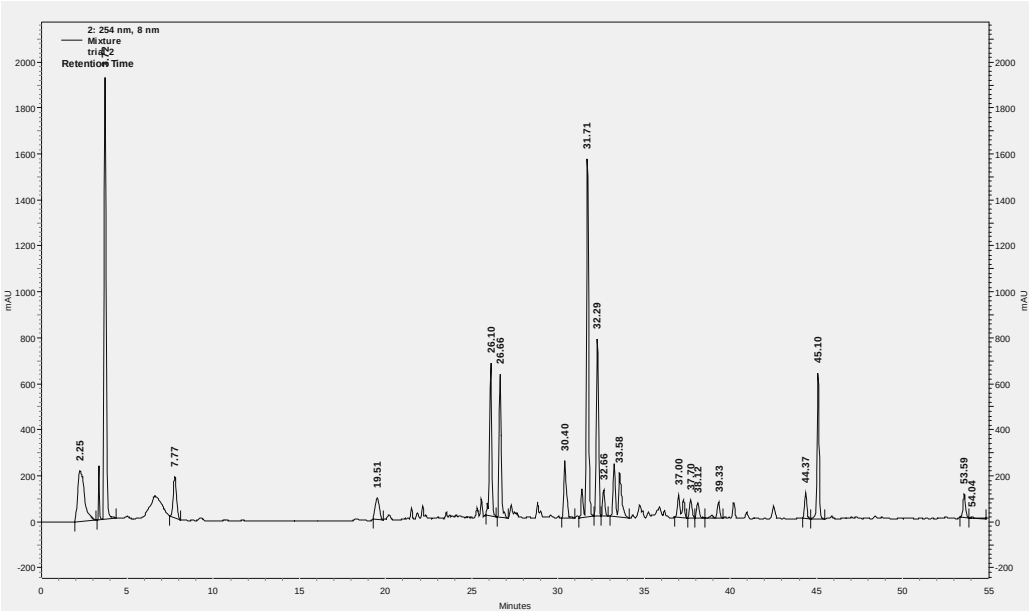


Fig. 3. HPLC fingerprint of methanol extract of the polyherbal mixture.

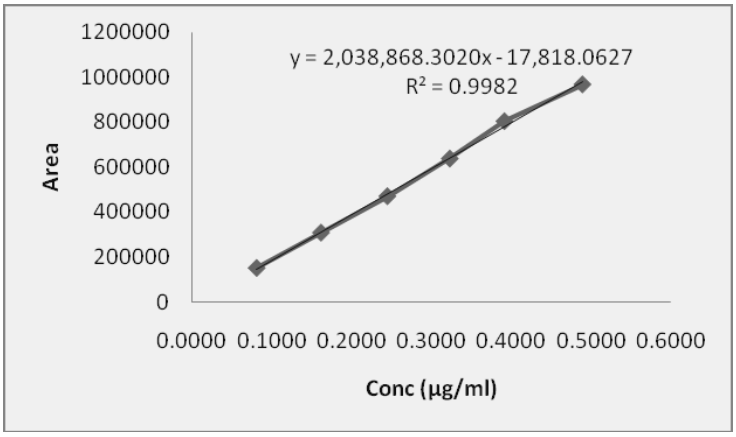


Fig. 4. Calibration curve of Piperine.

of *Acorus calamus* has been reviewed in detail (Yende *et al*., 2008).

The 'Indian Materia Medica' also lists several useful properties of *Acorus calamus* root and rhizome. The powdered oral dose of *Acorus calamus* indicated for dyspepsia (impairment of digestive function) ranges from 144mg to 433mg (20-60 grains in a mixture of seven ingredients in equal proportion). For respiratory disorders, small powdered doses of 10 grains (650mg) repeated every two or three hours till relief has been obtained are indicated. The present formulation recommends a dose of only 750mg of the mixture, which amounts to 150mg of *Acorus calamus* in powdered form- well within the safe limit. Even if the formulation is taken twice or thrice a day, the maximum intake of *Acorus calamus* amounts to only 300mg and 450mg respectively- again well within the range already documented.

***Significance of piperine estimation***

Piperine is the alkaloid responsible for the pungency of black pepper and long pepper, along with chavicine (an isomer of piperine). It has also been used in some forms of traditional medicines and as an insecticide. Piperine has also been found to inhibit human CYP3A4 and P-glycoprotein, enzymes important for the metabolism and transport of xenobiotics and metabolites (Bhardwaj *et al*., 2002). In animal studies, piperine also inhibited other enzymes important in drug metabolism (Atal *et al*., 1985) (Reen *et al*., 1993).

By inhibiting drug metabolism, piperine may increase the bioavailability of various compounds. Notably, piperine may enhance bioavailability of curcumin by 2000% in humans (Shoba *et al*., 1998). Due to its effects on drug metabolism, piperine should be taken cautiously (if at all) by individuals taking some other medications.

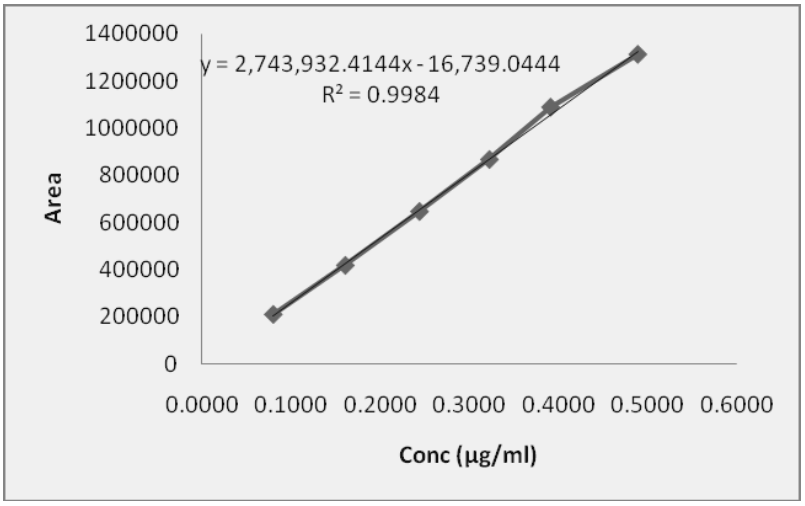


Fig. 5. Calibration curve of Asarone.

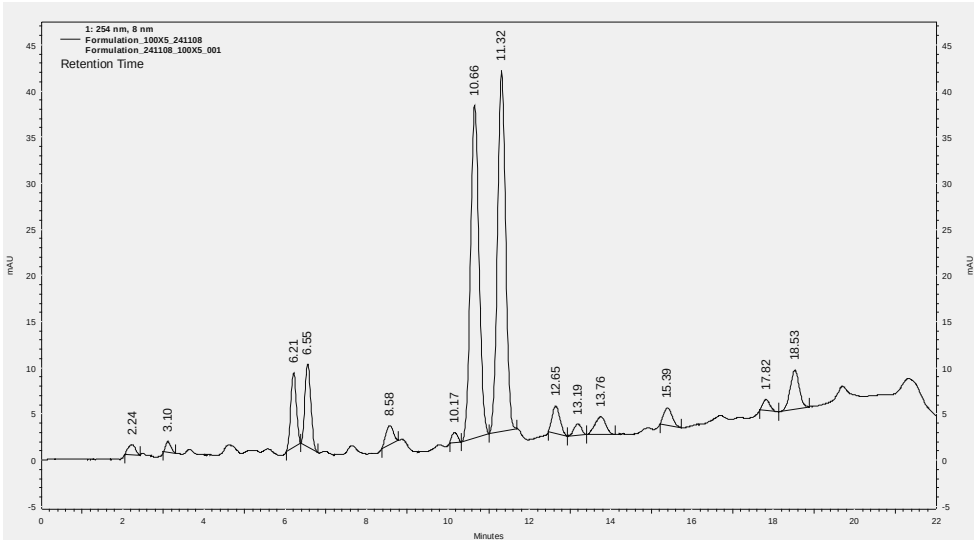


Fig. 6. HPLC Chromatogram of methanol extract for quantification.

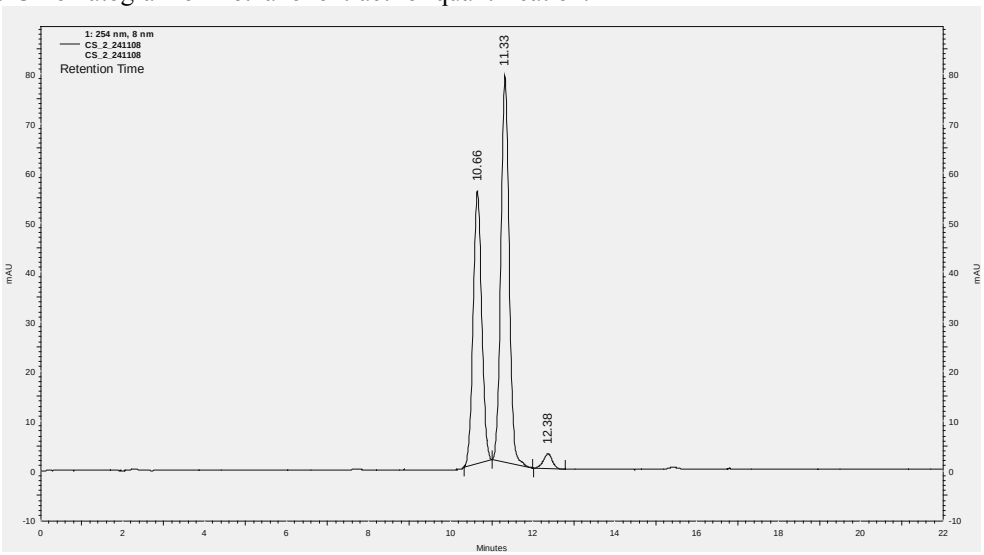


Fig. 7. HPLC Chromatogram of Piperine and Asarone.

## CONCLUSIONS

Based on the detailed scientific evaluation of the herbal formulation, it is clear that the one of the major reason for its good therapeutic efficacy is the highly potent anti-microbial activity of the formulation, which can be attributed to synergistic effect of the ingredients. The formulation holds promise as a safe, low-cost medicine for the treatment of respiratory disorders. A point worthy of mention is the use of the herbs in 'whole powdered form' and not as extracts, which might prevent loss of bioactive components that would occur during extraction.

## ACKNOWLEDGEMENTS

Financial support provided under the TePP program (Project No. DSIR/TEPP/1176/6) of Department of Scientific and Industrial Research, Ministry of Science and Technology, Govt. of India is greatly acknowledged.

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Received: March 16, 2010; Revised: Sept 6, 2010;

Accepted: Sept 20, 2010