Standardization of Phytomarker for Determining Immunomodulatory Activities in *Ocimum sanctum* and *Curcuma longa*

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Abstract-Medicinal plants have been used for centuries and have become part of complementary medicine worldwide because of their health benefits. Some plants used directly in the treatment and prevention of infectious diseases or indirectly by stimulating the immune system. In recent years there is a great demand throughout the world to use herbal products in healthcare system. Plant materials and their products are becoming popular because of their wide biological activities. Standardization of herbal products is essential for their acceptance as remedies for various diseases and ailments. Marker based standardization is one of the widely accepted methods which is based on the analysis of phytochemical markers using HPTLC, HPLC etc. Identification of major and unique compounds in herbs as markers and development of analytical methodologies for monitoring them are the key steps involved in marker based standardization. Marker compounds are chemically defined constituents of the herbal drug with or without therapeutic activity and can be used for the quality assurance of finished product. In nature, there are various medicinal plants which are used as immunomodulator agents. In this paper we standardize the phytomarkers of some selected plants which are very essential for our daily life.

Keywords: Phytomarker, Immunomodulatory activities, *Ocimum sanctum, Curcuma longa*

Introduction

Human survival is dependent upon their defensive immune mechanisms against External harms, pathogenic microbes and cancer. It is well known that the immune system helps the host to control microbes, allergic or toxic molecules and prevent cancer development[1]. Herbs with immunomodulatory properties are a moderately recent concept in phytomedicine. In addition to the enhancement of the humoral and cell-mediated immunity, the concept of immunomodulation initiates the activation of the "non-specific" immune responses which include the activation of the complement system, granulocytes, macrophages and natural killer cells. Hence activation of these essential immune cells initiates the production of various effect or molecules that take part in the modulation and enhancement of the immune responses[2,3]. The extreme manifestations of immunomodulating action of biologically active substances are immunosuppression and immunostimulation, hence both immunostimulating agents and immunosuppressing agents have their own standing and search for better agents exerting these activities is becoming the field of major interest all over the world [4]. There are several medicinal plants employed in different system of medicine throughout the world to improve the immunological disorders. In India, use of plants as remedy can be traced back to 6000 BC[5,6]. In recent times modulation of immune response to cure various diseases has been a very interesting concept and the concept of rasayana in Ayurveda deals with the same. Ayurvedic system of medicine describes this concept of rasayana under which plants with rejuvenating activity have been described

which the emphasis on promotion of health by strengthening host defenses against different diseases. In addition, phytomarkers from natural sources have always been of great interest to scientists working on infectious diseases[7]or to improve immune function. *Ocimum sanctum* commonly known as 'Tulsi' has been extensively used in Ayurvedic system of medicine for various ailments.The compounds present in tulsi like ursolic acid exhibited potent effect against virus and has been shown to increase immunity, which may help fight viral infections. In a 4-week study in 24 healthy adults, supplementing with 300 mg of holy basil extract significantly increased levels of helper T cells and natural killer cells, both of which are immune cells that help protect and defend our body from viral infections [8]

Curcuma longa Linn. belonging to family *Zingiberaceae*, is a perennial herb extensively cultivated in all parts of the country, India is a largest country which produces the *curcuma longa linn* (about 90% of the total output of the world). It gives color and taste to the food and is well known for its health promoting effects. In ancient time, it was used as an anti-inflammatory agents to treat gas, colic, toothaches, chest pain etc. This spice was also used to help with stomach and liver problems, to heal wounds and lighten scar and as a cosmetic. The most active component of turmeric is curcumin which makes up to 2-5% of the spice, and is responsible for most of the therapeutic effects. Turmeric contains a wide variety of phytochemical including curcumin, demethoxycurcumin, bisdemethoxycurcumin, zingiberene, curcumenol, eugenol.The characteristic yellow color of turmeric is due to Curcuminoid first isolated by Vogel in 1842.[9]

In this paper, The standardization of phytomarkers through HPTLC and HPLC present in two plants Ocimum sanctum and curcuma longa is the major objective of this paper which are reported for their immunomodulatory activity. Two commonly used plants are *Ocimum sanctum* and *curcuma longa* used for this study which is easily available for everyone and everywhere. These plants possess immunomodulatory effects as reported earlier in various research papers.

Material and Methods

Plant materials

The leaves of *Ocimum sanctum* and *rhizome of Curcuma longa* were collected from the herbal garden of The Himalaya Drug company, Dehradun. Selected leaves/rhyzomes were washed thoroughly with water, and then air dried under shade and grounded using a pestle and mortar.

Experimental

The instrument used in the present study was CAMAG-HPTLC system with Wincat software and SHIMAZDU-HPLC system with LC-solution software.

Method of HPTLC

Sample preparation: Measure accurately 5gm of samples of *Ocimum sanctum and Curcuma longa* separately in a 250ml flat bottomed flask add Methanol and reflux it by immersing in a water bath at 80^0C for 30 minutes . Filter the extract through whatman no.1 filter paper into an conical flask.

Standard: 1) Oleanolic acid- 10mg in 10ml methanol
2) Curcuminoid 10mg in 100ml methanol

Application-Applied the sample and standard solution as 10-12mm band, in a distance of 12mm from the bottom of a pre coated thin layer silica plate of uniform thickness, made a mark up to a distance of 8.5 cm from the application point as a development mark using pencil.

Mobile phase

1 *Ocimum sanctum*- Toluene:ethyl acetate:acetic acid(5.5:4.5:0.2)[10]
2 *Curcuma longa* - Chloroform: Methanol : glacial acetic acid (94:5:1)[11]

Used camag twin trough development tank (10×10cm). Visualized the dried plate under UV 254 nm and 366nm using reprostar-3. Exposed the plate of tulasi extract at white light after derivatized with vanillin sulphuric acid reagent.

Estimation of Total oleanolic acid by HPLC

HPLC Conditions required for analysis

1.0 Column : C18
2.0 Mobile Phase: Methanol: Sodium phosphate buffer (89:11)
3.0 Flow Rate: 1.0ml/min
4.0 Detection: 210nm
5.0 Volume of injection: 20µl of standard and sample solution

Standard oleanolic acid Solution (0.101mg/ml)

Weighed accurately 10.1mg of standard in 10ml of volumetric flask. Added 7-8 ml of methanol and dissolved. Made the volume up to the mark with methanol. Took 1 ml of this solution in 10 ml of volumetric flask and made up the mark with methanol.

Sample solution (10.03 mg/ml) : Weighed accurately 1g of the Tulsi powder in a 250ml flat bottom flask and add 80ml of methanol. Refluxed on a water bath at 80^0C for 30 minute. Transferd the extract into a 100ml volumetric flask without filtering.

Method of Analysis

Stabilized the instrument with the mobile phase and injected 20µl of working standard solutions. Recorded the chromatogram. Injected 20µl of the sample solution and recorded the chromatogram. Calculated the AUC of the of the two major adjacent out of which the first peak corresponds to oleanolic acid and second peak corresponds to ursolic acid.

Calculation: % total oleanolic acids w/w content can be calculated using the formula

$$\frac{\text{Total area of both peaks in Sample}}{\text{Total area of both peaks in Standard}} \times \frac{\text{Standard concentration}}{\text{Standard concentration}} \times \%\ \text{Purity of standard}$$

Estimation of Curcumin in *Curcuma longa*

HPLC Conditions required for analysis:

1.0 Column : C18
2.0 Mobile Phase : Acetonitrile: 0.1% orthophosphoric acid in purified water (50:50)
3.0 Flow Rate: 1.0ml/min
4.0 Detection: 420nm
5.0 Volume of injection: 20µl of standard and sample solution

Standard Solution

(0.0105mg/ml) Weighed about 10.5 mg of standard of Curcumin in a 10ml of volumetric flask, and dissolved by sonication in methanol. Made the volume up to the mark with methanol. Took 1 ml of the above stock solution into a 100ml volumetic flask, and made up the volume up to the mark with methanol and filtered the solution through 0.20 µm syringe filter.

Sample solution

(1.0mg/ml) Weighed about 100mg of the sample in a 250ml flat bottom flask and add 80ml of methanol. Refluxed on a water bath at 80^0C for 30 minute. Transfered the extract into a 100ml volumetric flask without filtering. Made the volume up to the mark with methanol and shook well allow the residue to settle to settle for 5 minutes. Filtered the solution through 0.20 µm syringe filter.

Calculation: % content of Curcumin

$$\frac{\text{AUC of the major peak in the Sample}}{\text{AUC of the major peak of standard}} \times \frac{\text{Std conc(mg/ml)}}{\text{Sample conc(mg/ml)}} \times \%\ \text{Purity of standard}$$

Results and Discussion

Oleanolic acid and ursolic acid are closely related isomers occurring in Tulsi. Since they are structurally related, their retention times are very close and often appear as less resoluted peaks. Hence, they can be collectively estimated as total oleanolic acids comprising both oleanolic acid and ursolic acid peaks. HPTLC methods were developed for estimation of oleanolic acid in *Ocimum sanctum* using different solvent system. The proper solvent system selected for estimation of oleanolic acid was toluene:ethyl acetate:glacial acetic acid in the ratio of 5.5:4.5:0.2.(Fig. 1).

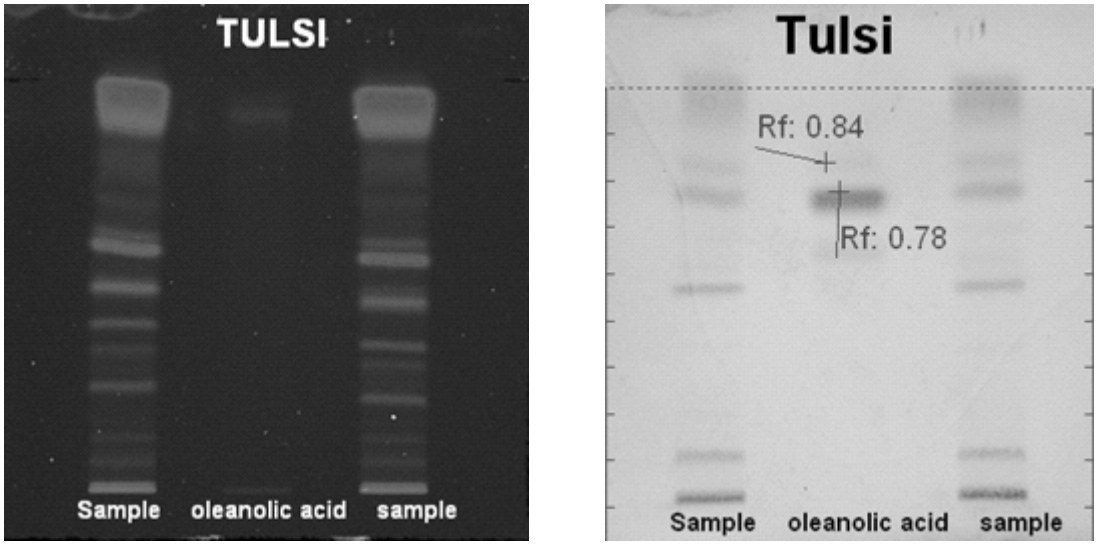

Figure 1 HPTLC chromatogram of *Ocimum sanctum*.

The validated HPLC method was applied to quantify the markers in methanolic extracts of *Ocimum sanctum*. The methanolic extract of the sample was found to have more amounts of active constituents. The amount of total oleanolic acid in methanolic extract was found 2.13% w/w. The method developed was specific, sensitive, precise and accurate. Therefore, this validated method can be used for routine quality control analysis for *Ocimum sanctum* extracts and formulations.

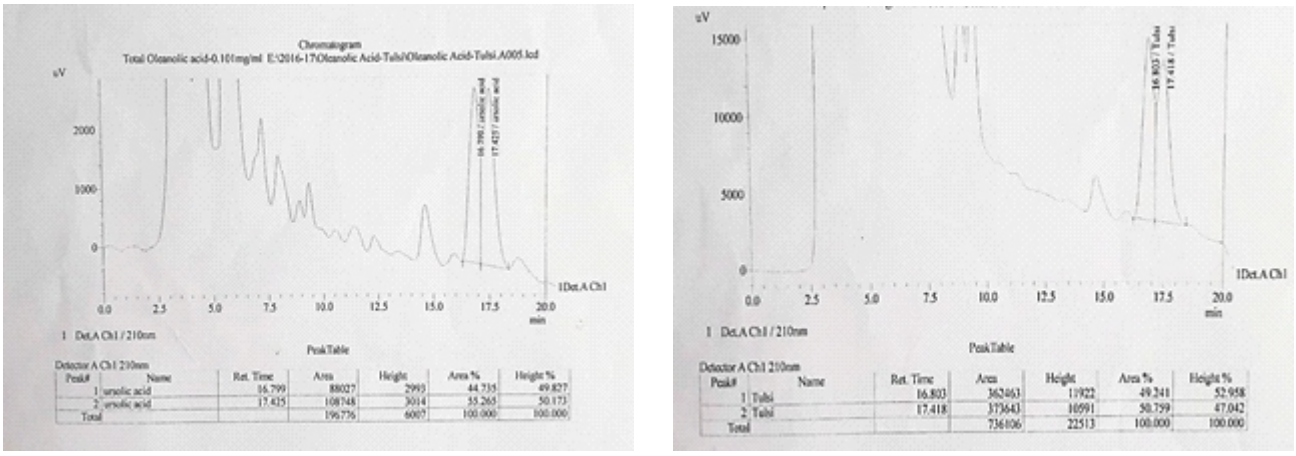

Figure 2 HPLC chromatogram of *Ocimum sanctum*.

In Figure 3 the HPLC peak for *Ocimum sanctum* at RT(16.803) and RT(17.418) corresponds to the peak of ursolic acid and oleanolic acid. Both the peak represent the total oleanolic acid.

Estimation of total oleanolic acid in *Ocimum sanctum* leaves:(0.101 /10.03) x (736106 /196776) x 56.66% = 2.13% [w/w]

Turmeric contains many plant substances, but one group, curcuminoids, has the greatest health promoting effects. Three notable curcuminoids are Curcumin(60-70%), demethoxycurcumin(20-27%) and bis-demethoxycurcumin(10-15%). Of these, Curcumin is the most active and most beneficial to health. HPTLC methods were developed for estimation of Curcumin using different solvent system. The suitable solvent system selected for the separation of curcuminoid was Chloroform: Methanol : glacial acetic acid (94:5:1) (Figure-3).

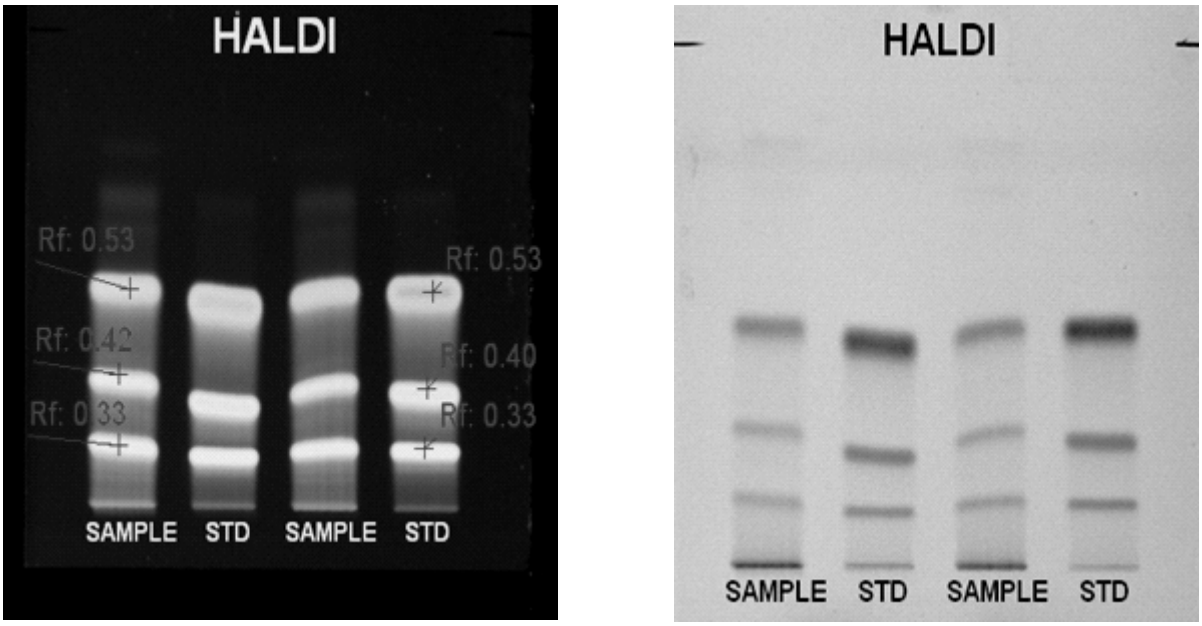

Figure 3 HPLC chromatogram of *Curcuma longa*.

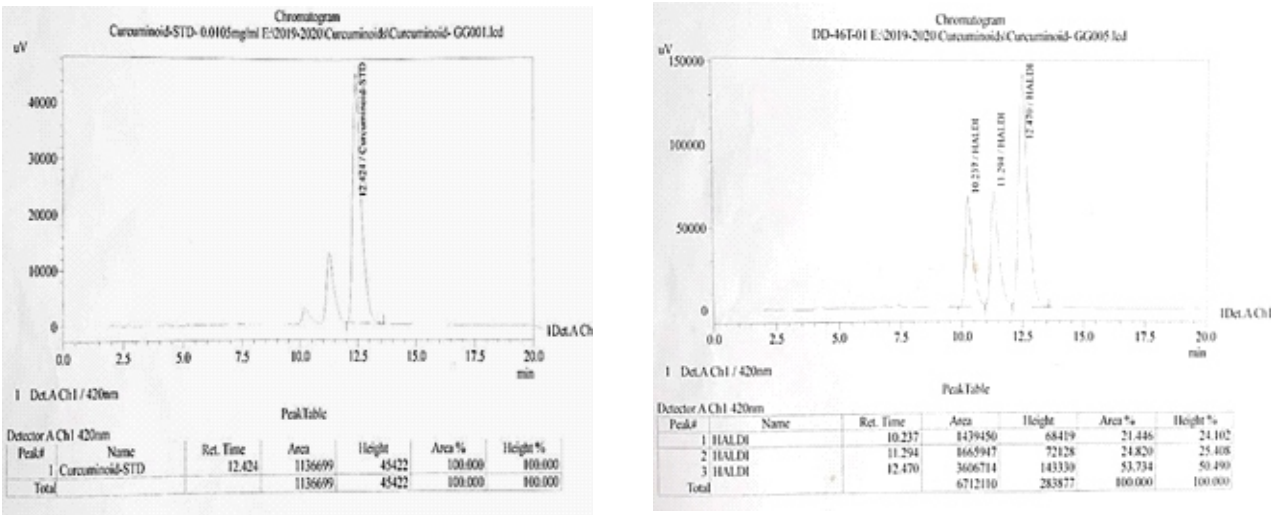

Figure 4 HPLC chromatogram of *Curcuma longa.*

In Figure 4 the HPLC chromatograph for *curmuma longa* shows the peak at RT(10.237) for Curcumin, RT (11.294) for demethoxycurcumin and RT(12.470) bis-demethoxycurcumin. **Estimation of curcumin in *Curcuma longa*: (0.0105 /1.0) x (3606714 /1136699) x 96.67% = 3.22% [w/w]**

Table-1 The % calculation of Phytomarkers

Sr. No	Plant Name	Phytomarker	% w/w	Limit of IP
1	*Ocimum sanctum*	Total oleanolic acid	2.13%	NLT- 2.0%
2	*Curcuma longa*	Curcumin	3.22%	NLT- 1.5%

Conclusion

Immunity is our body's natural defence against disease causing bacteria and virus. It can considerably reduce the odds of getting sick. Summer season is approaching and our immune system is getting compromised slightly due to the change in weather. It is only due to the weak immunity that people are getting affected with the widespread coronavirus and other such panedemics.

Ocimum sanctum and Curcuma longa aids in making our immunity stronger, the main life saving marker in Tulsi is Oleanolic acid (2.13%) and in turmeric is Curcumin(3.22%). According to IP its limit is NLT-2% for oleanolic acid and NLT- 1.5%for Curcumin. The results of this investigation revealed that the *Ocimum sanctum* and *curcuma longa* contains good quantity of immunobooster phytomarker which gives us protection against the various viral infections.

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