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SIMULTANEOUS IDENTIFICATION OF GALLIC ACID, WITHAFERIN A, PIPERINE AND SPIRULINA BY HPTLC FOR STANDARDIZATION OF MULTICOMPONENT IMMUNITY BOOSTER CAPSULE

Unmesh Keshwar*, Deepmala Matey, Soumit Pimplapure, Sharad Dhurde and B. K. Shrikhande

Siddhayu Ayurvedic Research Foundation Pvt. Ltd, Nagpur, Maharashtra, India.

***Corresponding Author: Unmesh Keshwar**

Siddhayu Ayurvedic Research Foundation Pvt. Ltd, Nagpur, Maharashtra, India.

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ABSTRACT

Background: Standardization of multicomponent herbal formulation with respect to its active marker compound is still a major challenge. Siddhayu Ayurvedic Research Foundation Pvt. Ltd. has standardized multicomponent herbal capsule formulation with respect to its active marker compounds. **Objective:** Objective of the present study was to standardize multicomponent herbal capsule formulation by evaluating identification of its active marker compounds simultaneously, purity and impurity. **Methods:** The standardization parameters such as organoleptic evaluation, Simultaneous identification of active marker compounds by HPTLC, Average weight, Uniformity of weight, Disintegration, Assay (Protein content), Heavy Metals, Microbiological tests for the multicomponent herbal capsule formulation were done. Simultaneous chromatographic separation was achieved by using Toluene: Ethyl Acetate: Methanol: Glacial acetic acid, 5.0: 2.5: 0.5: 0.5 (v/v/v/v) as mobile phase followed by comparing the Rf values and overlaying the spectra with standards. The method was validated according to International Conference on Harmonization guidelines. **Results:** The presence of active marker compounds Gallic acid, Withaferin A and Piperine in multicomponent herbal capsule formulation was confirmed simultaneously by comparing the Rf values and overlaying the spectra with standards. Identification of Spirulina was confirmed in capsule by the fingerprint profile comparisons with that of reference Spirulina. All the test results of the formulated capsule evaluated were found well within the limits. **Conclusion:** The developed identification method by HPTLC was found to be simple, rapid, cost effective and able to identify the active marker compounds simultaneously, hence can be routinely employed for analysis in Quality Control Lab.

KEYWORDS: Gallic acid, Withaferin A, Piperine, Spirulina, multicomponent immunity booster capsule, HPTLC.

INTRODUCTION

The safety and efficacy of herbal medicines largely depend on the quality of herbs used for the preparation of herbal formulation. Standardisation of multicomponent herbal formulations is still a major challenge as most of the herbal formulations are not standardized with respect to its active ingredients marker molecule.

To ensure the quality, efficacy and safety of herbal products, WHO suggested finished herbal products, were primarily intended to define the quality rather than to establish full characterization during standardisation.[1] WHO has also laid down a number of resolutions to highlight the need to ensure quality control of medicinal plant products using advanced analytical techniques and applying suitable standards or active ingredients marker molecule. Standardisation involves the development of set of standards for various parameters to ensure quality, safety and efficacy of the drugs.

Multicomponent immunity booster capsule formulation contains ingredients Amala extract (*Emblica officinalis*), Aswagandha extract (*Withania somnifera*), Kalimirch powder (*Piper nigrum*), and Spirulina powder (*Spirulina platensis*).

Amala consists of pericarp of dried mature fruits of *Emblica officinalis* Gaertn. Syn. *Phyllanthus emblica* Linn (Fam. Euphorbiaceae); mostly collected in winter season after ripening and in Kashmir in summer, a small or medium sized tree, found both in natural state in mixed deciduous forests of the India ascending to 1300 m on hills; cultivated in gardens, homeyards or grown as a road side tree.[2] It contains a polyphenol compound Gallic acid which has various pharmacological activities including immune booster potential. A study results shows Gallic acid has high immunomodulatory activity evidenced by the increase of phagocytic capability, lysosomal volume, nitrite release, and intracellular calcium [$Ca^{2+}{}_i$] in macrophages.[3]

Ashwagandha consists of dried mature roots of *Withania somnifera* Dunal. (Fam. Solanaceae), a perennial shrub, found in waste land, cultivated field and open grounds throughout India, widely cultivated in certain areas of Madhya Pradesh and Rajasthan , roots collected in winter, washed and cut into short pieces.[4] The active components in *Withania somnifera* are mainly withanolide glycosides. *Withania somnfera* extract significantly improved the immune profile of healthy subjects by modulating the innate and adaptive immune systems in an experimental study.[5]

Marica (Kalimirch) consists of fully mature dried fruit of *Piper nigrum* Linn. (Fam. Piperaceae); a climber, cultivated in India, from Konkan Southwards, especially in North Konkan, Kerala, and also in Assam; fruits ripen from December to March, depending upon climatic conditions; fruits harvested from December to April.[6] It contains an alkaloid, Piperine as the major bio-active constituent which has promising immunomodulatory activity. Piperine promotes innate immunity by promoting the phagocytic activity of phagocytes and is known to inhibit LPS-induced expression of IRF-1 and IRF-7 mRNA, phosphorylation of IRF-3, type 1IFN mRNA, and down-regulation of STAT-1 activity.[7]

Spirulina is a blue-green algae enriched with high quality source of proteins, pigments, minerals and vitamins. Research suggests that spirulina has antioxidant and inflammation-fighting properties, as well as the ability to help regulate the immune system.[8]

There are several methods have been reported for analysis of Gallic acid, Withaferin A, Piperine and Spirulina in various herbal formulations by High Performance Thin Layer Chromatography (HPTLC), and High Performance Liquid Chromatography (HPLC).[9-13] But no method was found for simultaneous identification of Gallic acid, Withaferin A, Piperine and Spirulina from a multicomponent herbal formulation.

Keeping the challenges in mind for standardization of multicomponent herbal formulations, Siddhayu Ayurvedic Research Foundation Pvt. Ltd. has formulated multicomponent Immunity Booster Capsule and standardized it with respect to identification of active ingredients marker compound, purity and impurity.

In the present study, an attempt has been made to develop a simple, rapid, accurate, precise and cost effective HPTLC method which can be routinely used in Quality Control Lab for simultaneous identification of Gallic acid, Withaferin A, Piperine and Spirulina to standardize multicomponent Immunity Booster Capsule formulation. The proposed analytical method for the simultaneous identification of active marker compound is also validated for specificity as per the International Conference on Harmonization (ICH) guidelines Q2 (R1).[14]

MATERIALS AND METHODS

Ingredients used in multicomponent Immunity Booster Capsule

Multicomponent Immunity Booster Capsule formulation was formulated using Amla Extract (*Emblica officinalis)*, Ashwandha Extract (*Withania somnifera*), Kalimirch powder (*Piper nigrum*,) and Spirulina powder (*Spirulina platensis)*. All these raw materials were screened for identity, purity and strength before formulation of capsule.

Evaluation of multicomponent herbal capsule

The capsule was evaluated for organoleptic evaluation, Identification of active constituents by HPTLC, Average weight, Uniformity of weight, Disintegration, Assay (Protein content), Heavy Metals and Microbiological tests. The results are shown in Table 1.

Organoleptic evaluation

The organoleptic characters such as colour and odour were evaluated by spreading the filled content powder of capsule on a clean dry sheet and investigated through the magnifying lens by repeated observation.

Average weight

20 intact capsules were weighed on a well cleaned and calibrated balance. Average weight of capsules was calculated by dividing the weight of 20 intact capsules by 20.

Uniformity of weight

1 intact capsule was weighed and opened without losing any part of the shell. The content was removed as completely as possible. The empty shell was weighed. The difference between the weighing gives the weight of the content. This procedure was repeated with another 19 capsules.

Disintegration

1 capsule was introduced into each tube of disintegration test apparatus. Disc was added to each tube and the assembly was suspended in the beaker containing water at 37˚ C. The instrument was set for 30 minutes time period. The time was noted down at which the capsule was disintegrated and no residue of the capsule under test remains on the screen of the apparatus.

Protein content

0.5 gm of capsule fill content was weighed and transferred into the Kjeldahl's flask. To this 20 ml of concentrated Sulphuric acid, 10 gm of anhydrous potassium sulphate and 1 g copper sulphate was added. This reaction mixture was digested in a heating mantle until a green coloured clear digest was obtained. The digestion was continued for additional 30 minutes. The digestion flask was cooled and quantitatively transferred the digested solution to 250 ml volumetric flask. 150 ml distilled water was added and mixed it thoroughly. 50 ml 0.1N sulphuric acid was taken into a beaker and few drops of methyl red indicator solution was added. This

beaker was placed in such a way that the delivery end of the distillation tube was below the acid level. To the distillation flask, 100 ml of sodium hydroxide solution [40 %] was poured into the top funnel and drop wise added it into the distillation flask by opening the tap of the funnel. The tap was closed after delivering the entire quantity of sodium hydroxide. Heating to the distillation flask was started and water was circulated in the condenser. After distilling about 100 ml into the receiver beaker, the beaker was removed and the excess acid was titrated against 0.1N sodium hydroxide solution. The end point was red to yellow. Similarly the blank run was carried out using 50 ml of 0.1N sulphuric acid and was titrated it against 0.1N sodium hydroxide using Methyl red as an indicator, the end point was red to yellow.

Heavy Metals

The detection of presence of Heavy Metals like Lead, Arsenic, Cadmium and Mercury in ppm level was quantified in formulated capsule by using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Microbiological tests

The formulated capsule was subjected for microbiological tests Total microbial plate count (TPC), Total yeast &mould, and pathogens like *Staphylococcus aureus, Salmonella sp., Pseudomonas aeruginosa, Escherchia coli*.

Simultaneous Identification of active marker compounds Gallic acid, Withaferin A, Piperine and Spirulina in multicomponent Immunity Booster Capsule by HPTLC

Preparation of Standard solutions

The standard solution of Gallic acid was prepared by dissolving 2.5 mg in 25 ml volumetric flask containing HPLC grade methanol.

The standard solution of Withaferin A was prepared by dissolving 2.5 mg in 25 ml volumetric flask containing HPLC grade methanol.

The standard solution of Piperine was prepared by dissolving 1.5 mg in 25 ml volumetric flask containing HPLC grade methanol.

The reference solution of Spirulina was prepared by weighing 50 mg of the reference Spirulina and then transferred to 25 ml volumetric flask and volume was adjusted with HPLC grade methanol.

Preparation of Sample Solution

The fill content powder of twenty capsules were removed and an amount of 500 mg powder was transferred to 25 ml volumetric flask containing 20 ml HPLC grade methanol and sonicated for 15 min. The solution was diluted up to the mark with HPLC grade methanol and filtered through Whatman filter paper. The resulting solution was used as sample solution for the study.

Stationary Phase: Silica Gel 60 F_{254} TLC Plate

Mobile phase: Toluene: Ethyl Acetate: Methanol: Glacial acetic acid (5.0: 2.5: 0.5: 0.5)

Sample application: Apply 10 µl of standard, reference sample and test solution on TLC plate.

Developing distance: 7 cm

Saturation Time: 20 min.

Relative Humidity: 33%, Saturated $MgCl_2$

Temperature: $22 \pm 5^{\circ}C$

Detection/Photograph:

a) Underivatised, UV 254 nm
b) Underivatised, UV 366 nm

Scanning

a) At 270 nm, for Gallic acid
b) At 220 nm, for Withaferin A
c) At 330 nm, for Piperine

Validation of the method

The developed analytical method was validated for the parameter specificity as per the International Conference on Harmonization (ICH) guidelines Q2 (R1). Specificity of the method was demonstrated by applying the Blank as diluent, Standard solutions, Capsule formulation solution and Placebo solution on the TLC plate.

RESULTS AND DISCUSSION

The multicomponent Immunity Booster Capsule formulation was evaluated for the parameters like organoleptic evaluation, Identification of active constituents by HPTLC, Average weight, Uniformity of weight, Disintegration, Assay (Protein content), Heavy Metals and Microbiological analysis.

Appearance of capsules was green coloured hard gelatin capsule shells filled with greenish coloured granular powder. Average weight of capsule was 14.1602 g. In uniformity of weight, not more than two of the individual weights deviate from the average weight by more than 7.5% and none deviate by more than twice that percentage. Capsule was disintegrated in 7 minutes. The capsule was assayed for the protein content and which was found to be 37.28%.

Identification of active marker compounds in formulated capsule was done by simultaneous estimation of Gallic acid, Withaferin A, Piperine and Spirulina by HPTLC. The mixtures of several mobile phases were tried on silica gel TLC plates for simultaneous estimation of Gallic acid, Piperine, Withaferin A and Spirulina. A mobile phase consisting Toluene: Ethyl Acetate: Methanol: Glacial acetic acid (5.0: 2.5: 0.5: 0.5 v/v/v/v) gave good separation. Standard Gallic acid (*R*f = 0.28), Withaferin A (*R*f = 0.35) and Piperine (*R*f = 0.63) showed single peak in HPTLC chromatogram in Figures

4, 7, 10 and HPTLC chromatogram of formulated capsule in "Fig. 5, 8, 11".

The presence of Gallic acid, Withaferin A and Piperine in formulated capsule was identified by comparing the Rf values as that of obtained for standard at Rf 0.28, 0.35 and 0.63 at 270 nm, 220 nm, 330 nm respectively and confirmed by overlaying the spectra with standards. The results are shown in "Fig. 1-12".

Identification of Spirulina was confirmed in capsule by the fingerprint profile comparisons. At 366 nm, the chromatographic profile of the capsule formulation solutions was similar to that of reference Spirulina sample solution with respect to principle bands. The results are shown in "Fig. 2".

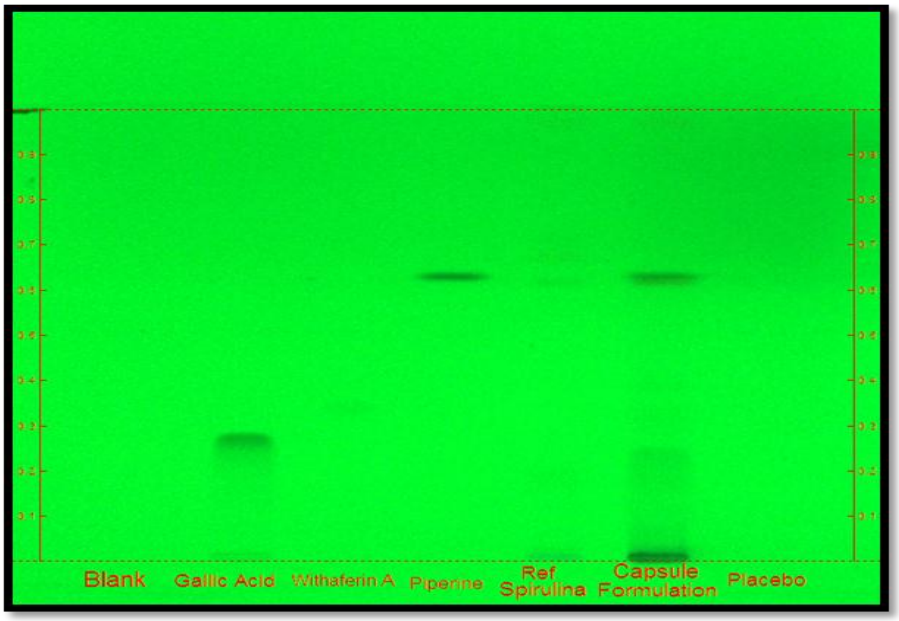

Figure 1: HPTLC Fingerprint Photo-documentation at 254 nm; Track number 1 – Blank, Track number 2 - Standard Gallic acid, Track number 3 - Standard Withaferin A, Track number 4 - Standard Piperine, Track number 5 – Reference Spirulina, Track number 6 - multicomponent herbal capsule formulation and Track number 7 – Placebo.

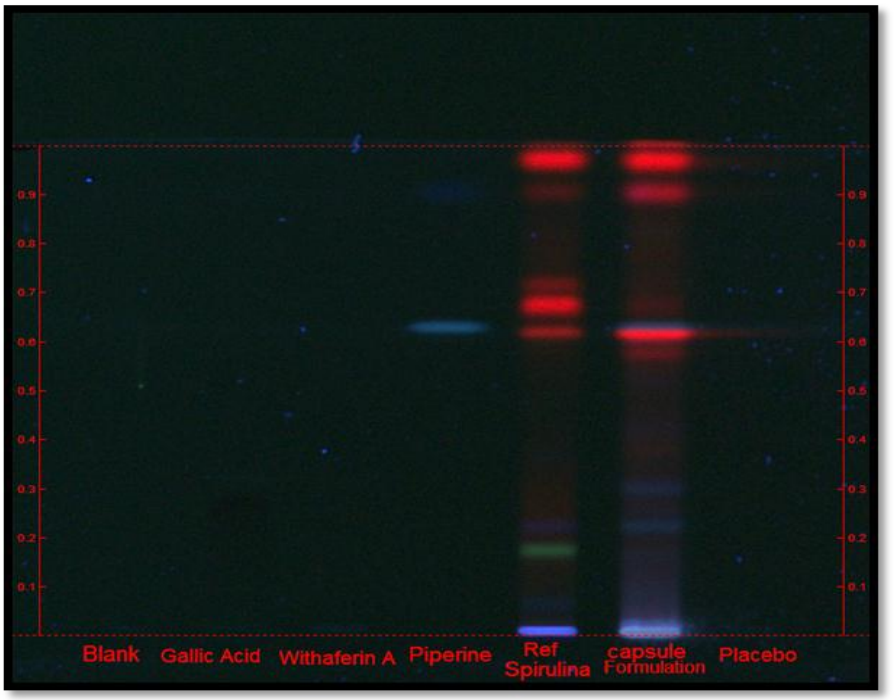

Figure 2: HPTLC Fingerprint Photo-documentation at 366 nm; Track number 1 – Blank, Track number 2 - Standard Gallic acid, Track number 3 - Standard Withaferin A, Track number 4 - Standard Piperine, Track

number 5 – Reference Spirulina, Track number 6 - multicomponent herbal capsule formulation and Track number 7 – Placebo.

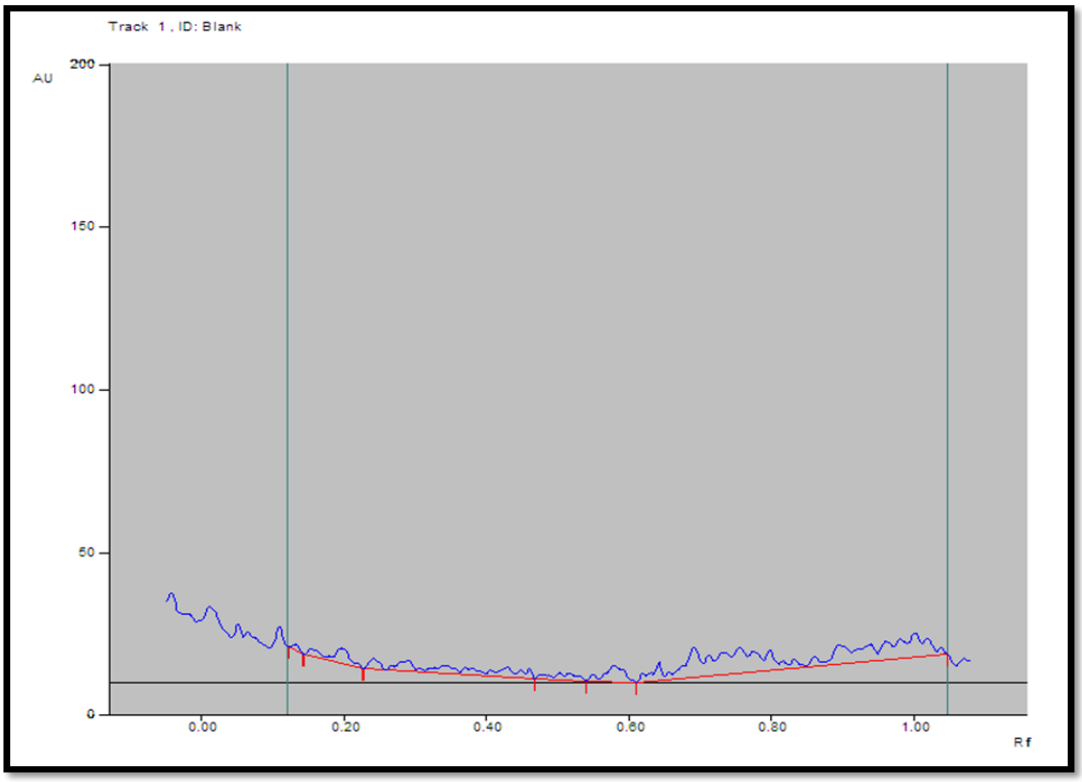

Figure 3: HPTLC Chromatogram of Blank Solution.

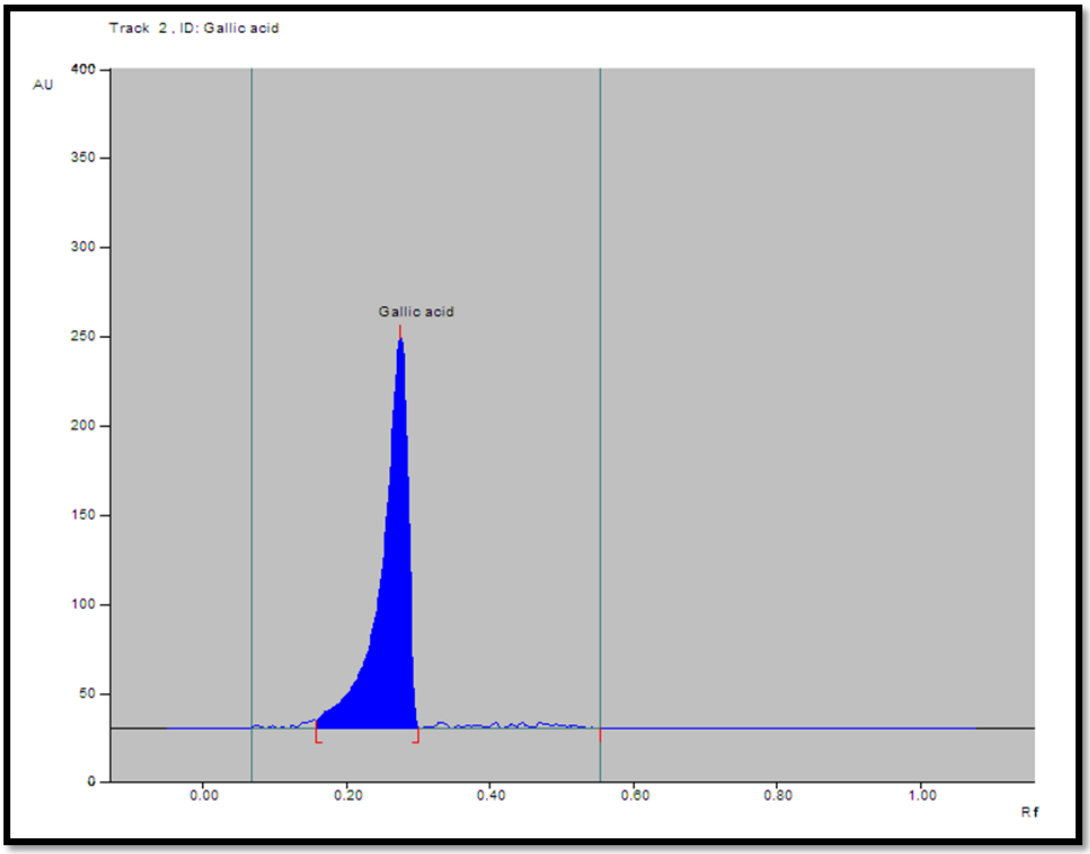

Figure 4: HPTLC Chromatogram of Standard Gallic Acid Solution.

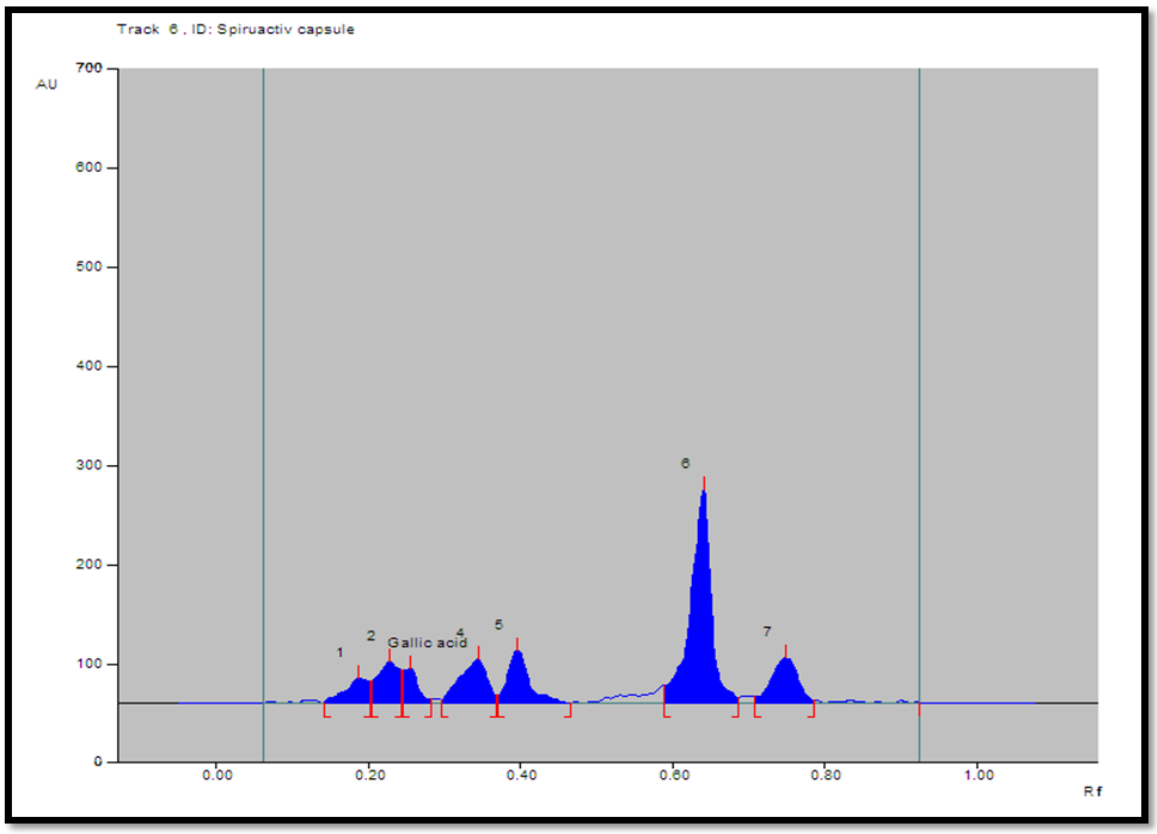

Figure 5: HPTLC chromatogram of multicomponent herbal capsule formulation solution.

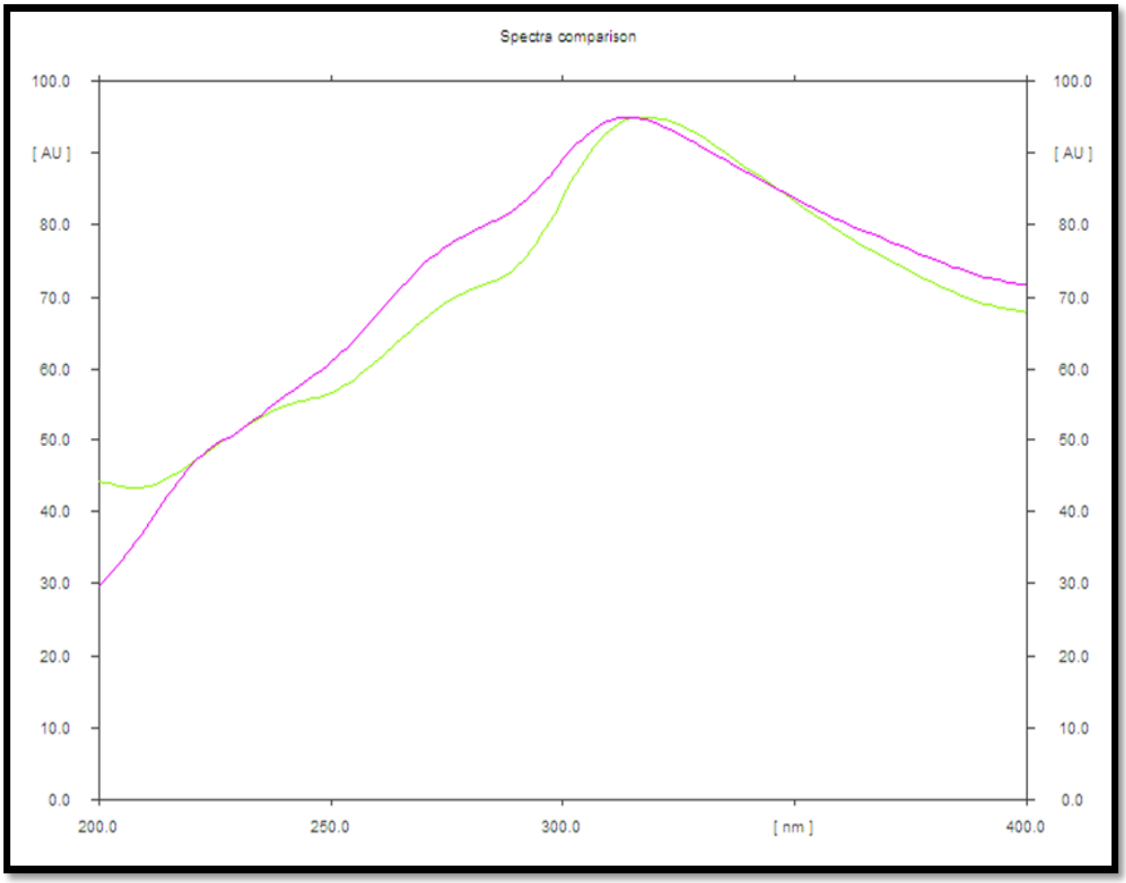

Figure 6: Overlain spectrum of standard Gallic acid solution and multicomponent herbal capsule formulation solution.

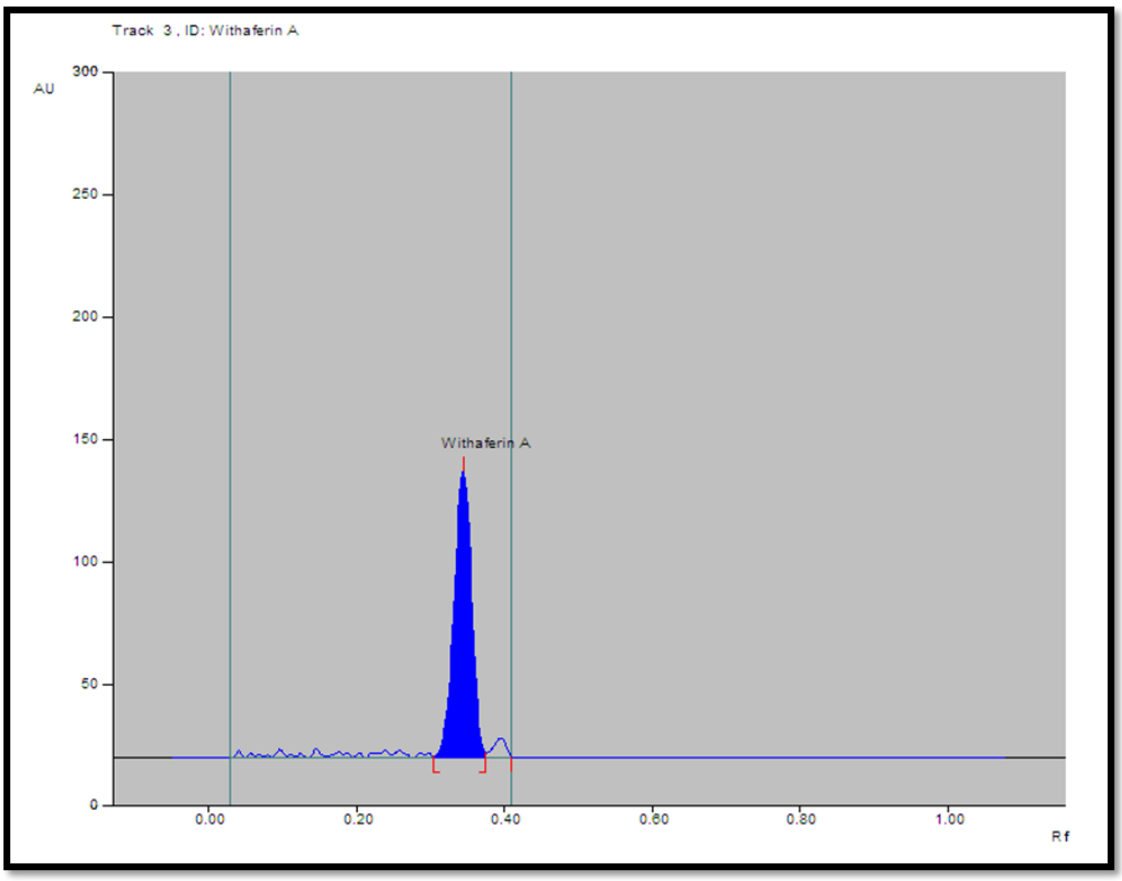

Figure 7: HPTLC chromatogram of standard Withaferin A solution.

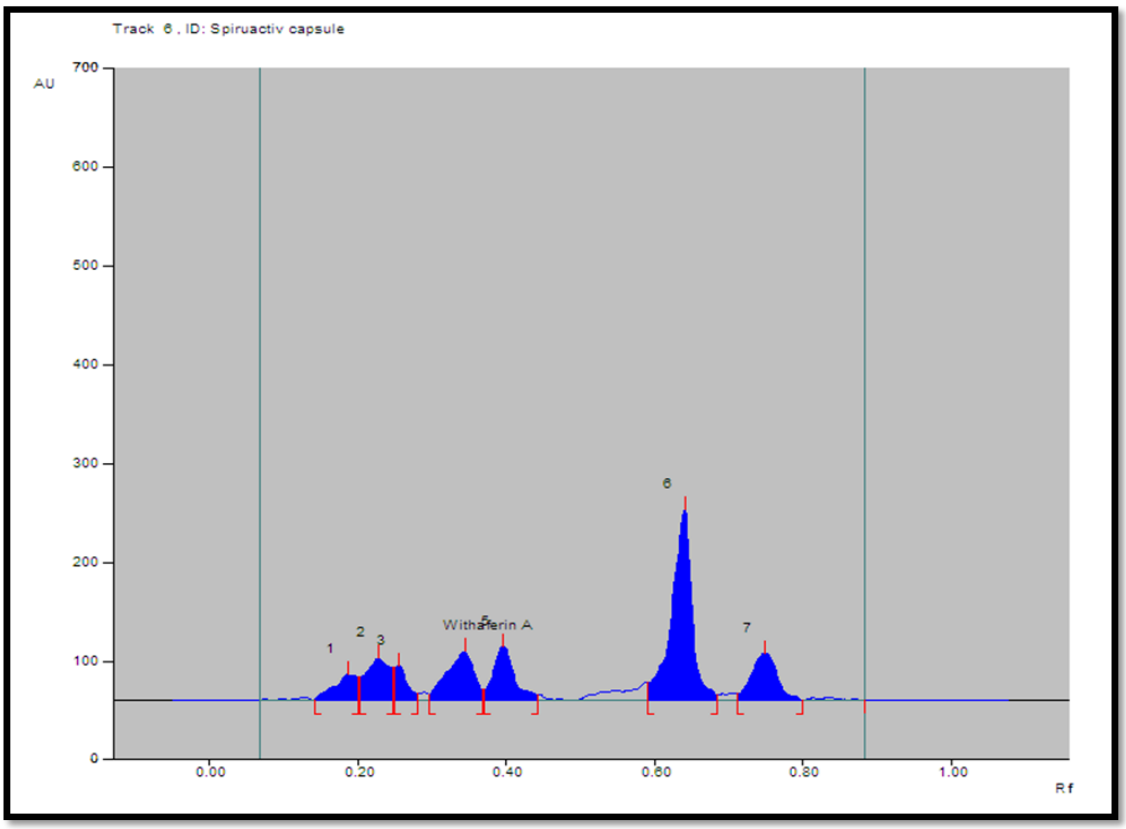

Figure 8: HPTLC chromatogram of multicomponent herbal capsule formulation solution.

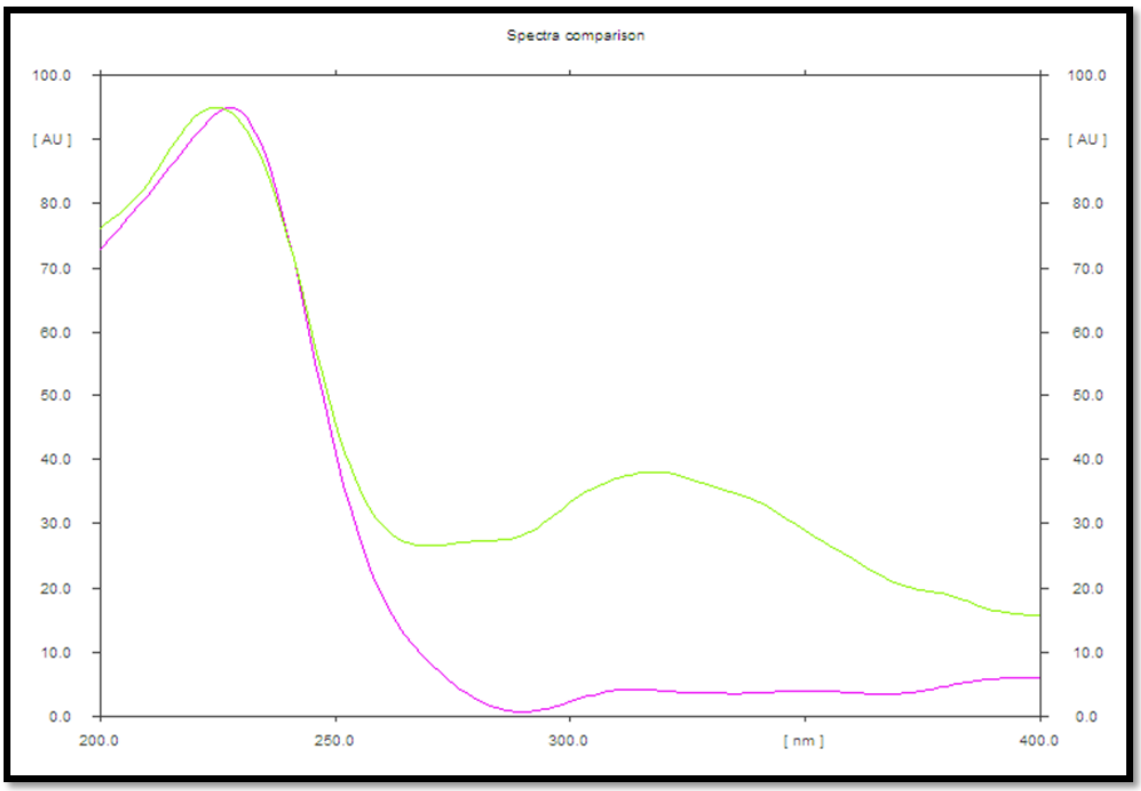

Figure 9: Overlain spectrum of standard Withaferin A solution and multicomponent herbal capsule formulation solution.

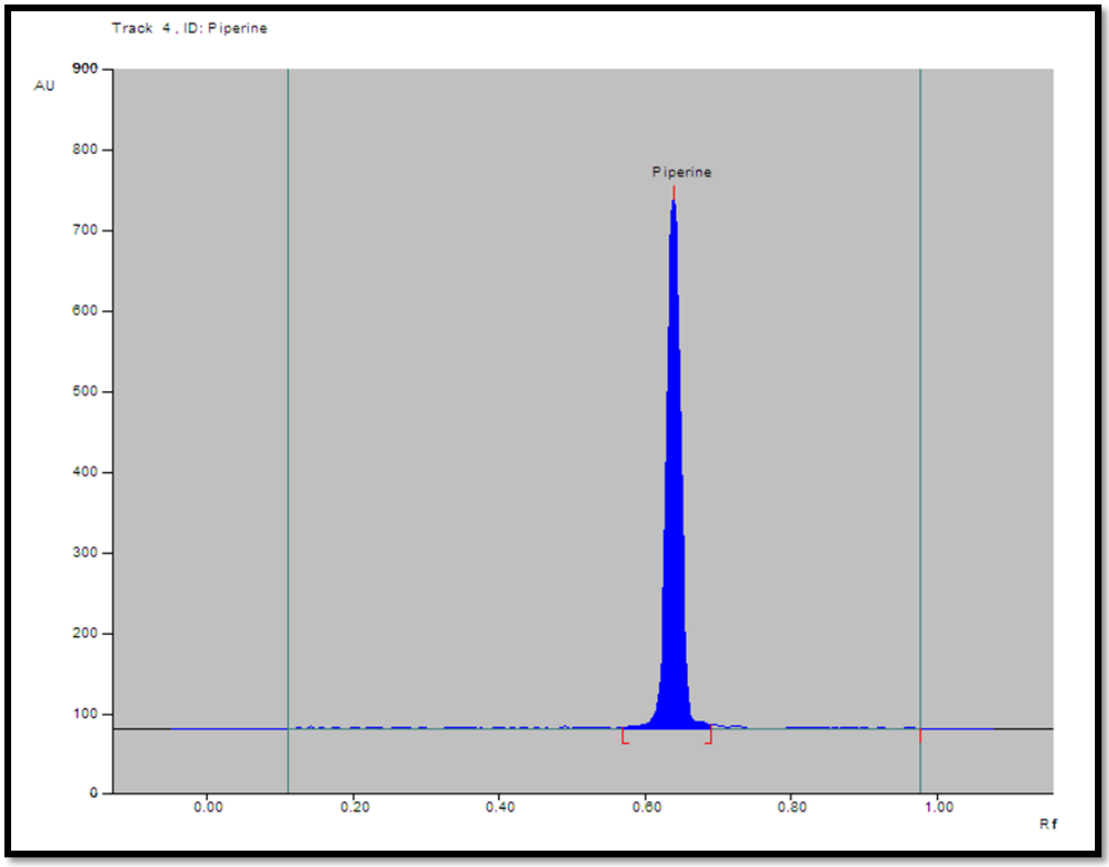

Figure 10: HPTLC chromatogram of standard Piperine solution.

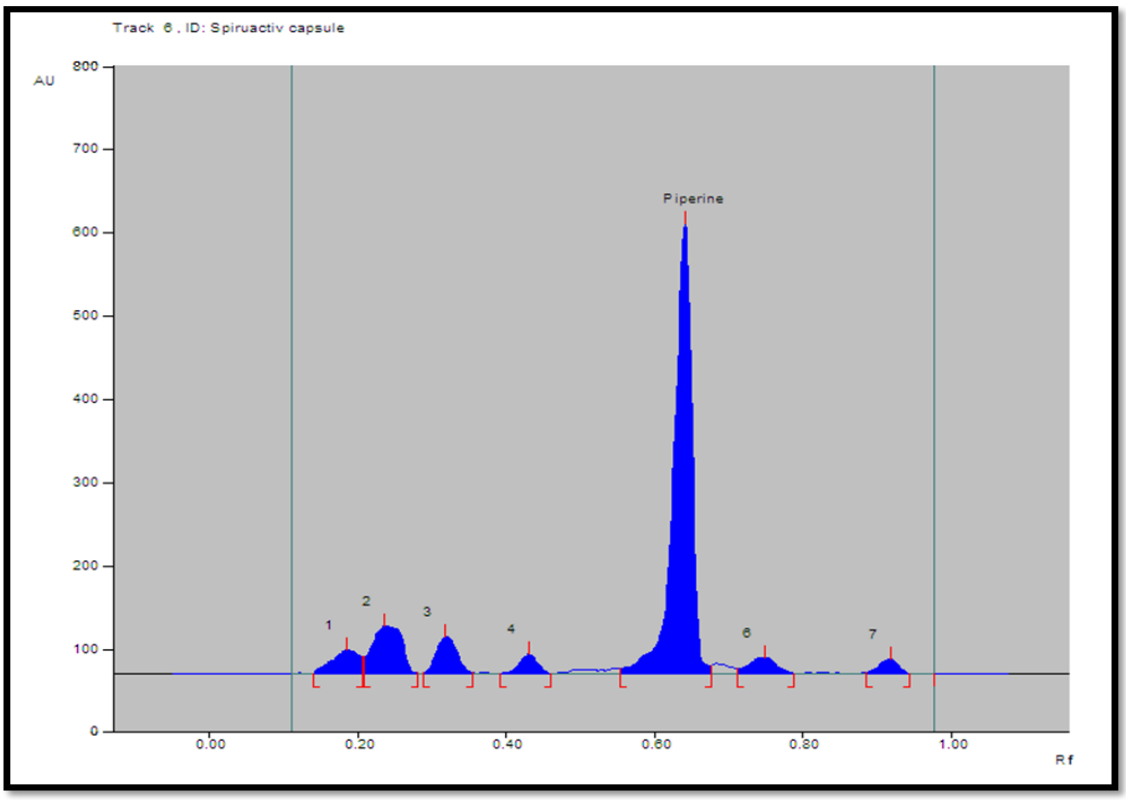

Figure 11: HPTLC chromatogram of multicomponent herbal capsule formulation solution.

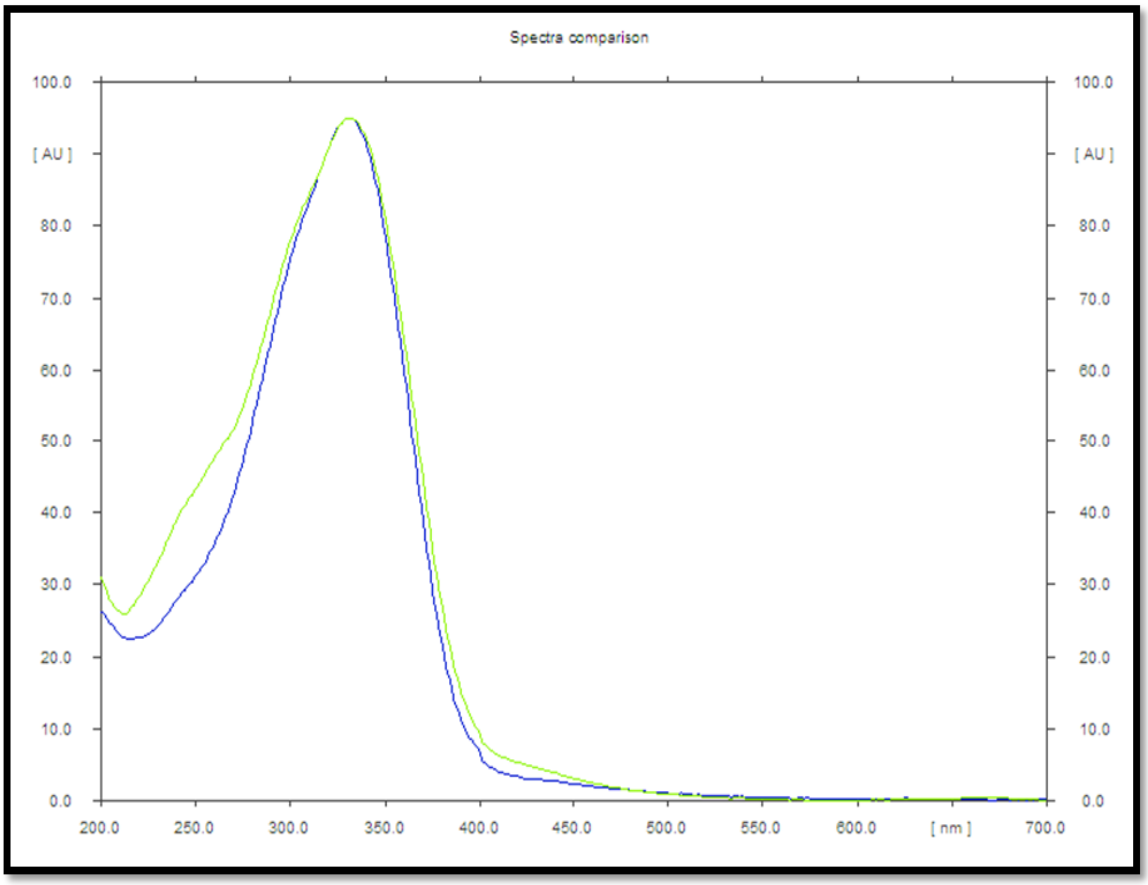

Figure 12: Overlain spectrum of standard Piperine solution and multicomponent herbal capsule formulation solution.

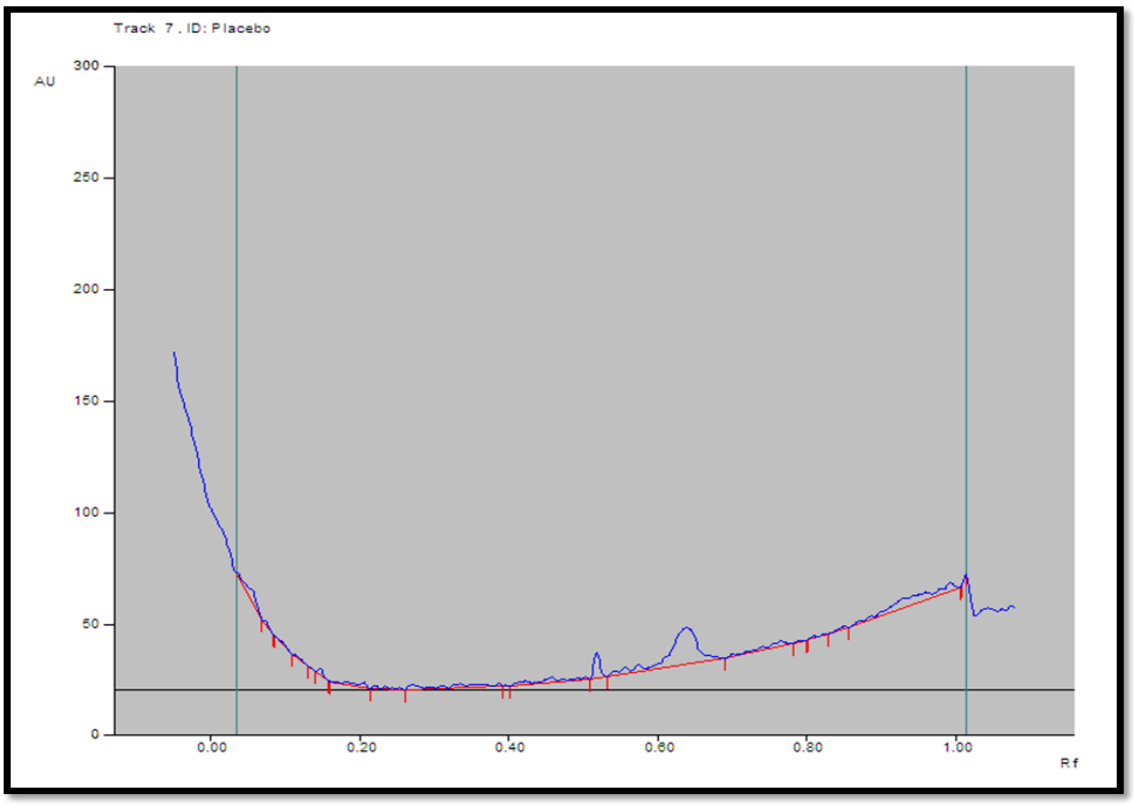

Figure 13: HPTLC chromatogram of Placebo solution.

Validation of the method

The developed analytical method was validated for the parameter specificity. The Specificity was proved by chromatographic comparison of blank, Placebo solution, standard solutions and the test solution. As there was no interfering band/peak was observed from blank and placebo solution at the Rf of Reference standard solution of Gallic acid, Withaferin A, Piperine and Reference Spirulina solution, the method was found to be specific. The Fingerprint Photo-documentation was shown in "Fig. 1-2" and chromatograms in "Fig. 3-13".

The detection of Heavy Metals like Lead, Arsenic, Cadmium and Mercury in ppm level was quantified in formulated capsule by using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The results are much below the acceptable limits. The results are tabulated in table 1. multicomponent Immunity Booster Capsule formulation was also evaluated for microbiological test; total microbial plate count was found to be 35000 cfu/g. and total yeast and mould count was found to be less than 10 cfui/g. Pathogens like *Staphylococcus aureus*, *Salmonella sp.*, *Pseudomonas aeruginosa*, and *Escherchia coli* was absent in the formulated capsule. All these results are tabulated in table 1.

The test results obtained for the multicomponent Immunity Booster Capsule formulation shows the results well within the limits, which indicate good quality of product.

Table 1: Results for evaluation of multicomponent Immunity Booster Capsule.

Sr. No.	Test Parameters	Acceptable limits	Results
1	Appearance	Green coloured hard gelatin capsule shells filled with greenish coloured granular powder.	Complies
2	Identification by HPTLC	Positive for Gallic acid, Withaferin A, Piperine Spirulina	Complies
3	Average weight of Capsules	0.6850 g ± 7.5 % (0.6336 g – 0.7363 g)	0.7080 g
4	Uniformity of weight	Not more than two of the individual weights deviate from the average weight by more than 7.5% and none deviate by more than twice that percentage.	Complies
5	Disintegration time	NMT 30 minutes	7 minutes
6	Assay (Protein content)	NLT 20 %	37.28 %
7	Heavy Metals (by ICPMS)		

	Lead	NMT 10.0 ppm	0.247 ppm
	Arsenic	NMT 3.0 ppm	0.187 ppm
	Cadmium	NMT 0.3 ppm	Less than 0.1 ppm
	Mercury	NMT 1.0 ppm	Less than 0.1 ppm
8	Microbiological Test		
	Total microbial plate count (TPC)	NMT 10^5 cfu/g	3500 cfu/g
	Total yeast & mould count	NMT 10^3 cfu/g	Less than 10 cfu/g
	Staphylococcus aureus/g	Absent/g	Absent
	Salmonella sp./g	Absent/g	Absent
	Pseudomonas aeruginosa/g	Absent/g	Absent
	Escherchia coli/g	Absent/g	Absent

CONCLUSION

In the present work a multicomponent Immunity Booster Capsule formulation was standardized. A special approach was given to identify the active marker compounds Gallic acid, Withaferin A, Piperine and Spirulina simultaneously by HPTLC.

The developed identification method was found to be simple, rapid, cost effective and able to identify the active constituents simultaneously.

All the results in the evaluation of multicomponent Immunity Booster Capsule formulation showed that they are well within the acceptable limits hence these evaluation parameters can be used for the standardization and also can be routinely employed for analysis in Quality Control Lab.

REFERENCES

1. World Health Organisation. WHO guidelines on good manufacturing practices (GMP) for herbal medicines. Geneva, 2007.
2. The Ayurvedic Pharmacopoeia of India. Part 1, Volume I, 1st ed., Delhi; The Controller of Publications, 1999; pp 5-6.
3. Alves MMDM, Brito LM, Souza AC, Queiroz BCSH, de Carvalho TP, Batista JF, et al. Gallic and ellagic acids: two natural immunomodulator compounds solve infection of macrophages by leishmania major. Naunyn-Schmiedeberg's Arch Pharmacol, 2017; 390: 893–903.
4. The Ayurvedic Pharmacopoeia of India. Part 1, Volume I, 1st ed., Delhi; The Controller of Publications, 1999; pp 15-6.
5. Tharakan A, Shukla H, Benny IR, Tharakan M, George L, Koshy S. Immunomodulatory Effect of *Withania somnifera* (Ashwagandha) Extract—A Randomized, Double-Blind, Placebo Controlled Trial with an Open Label Extension on Healthy Participants. J. Clin. Med, 2021; 10(16): 3644.
6. The Ayurvedic Pharmacopoeia of India. Part 1, Volume III, 1st ed., Delhi; The Controller of Publications, 1999; pp 115-7.
7. Mrityunjaya M, Pavithra V, Neelam R, Janhavi P, Halami PM and Ravindra PV. Immune-Boosting, Antioxidant and Anti-inflammatory Food Supplements Targeting Pathogenesis of COVID-19. Front. Immunol, 2020; 11: 570122.
8. Khan Z, Bhadouria P, Bisen PS. Nutritional and Therapeutic Potential of Spirulina. Current Pharmaceutical Biotechnology, 2005; 6: 373-79.
9. Vyas N, Patel S. Simultaneous Estimation of Curcuminoids, Piperine, and Gallic Acid in an Ayurvedic Formulation by Validated High-Performance Thin Layer Chromatographic Method. Asian J Pharm Clin Res, 2016; 9(2): 117-22.
10. Bhondave PD, Potawale SE, Gabhe SY. A rapid and simple high performance thin layer chromatographic method for simultaneous analysis of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A in Ashvagandharishta. Journal of Applied Pharmaceutical Science, 2014; 4(7): 82-7.
11. Jeganathan NS, Kannan K, Manavalan R, Vasanthi HR. Standardization of a Siddha Formulation Amukkara Curanam by HPTLC. Afr. J. Trad. CAM, 2008; 5(2): 131-40.
12. Shaikh M. Jadhav AP. Development and Validation of RP-HPLC Method for Simultaneous Estimation of Gallic Acid, Curcumin and Piperine in an Ayurvedic Formulation. Int J Pharm Pharm Sci, 2020; 12(6): 81-6.
13. Hynstova V, Sterbova D, Klejdus B, Hedbavny J, Huska D, Adam V. Separation, identification and quantification of carotenoids and chlorophylls in dietary supplements containing *Chlorella vulgaris* and *Spirulina platensis* using High Performance Thin Layer Chromatography. Journal of Pharmaceutical and Biomedical Analysis, 2018; 148: 108-18.
14. ICH Harmonized Tripartite Guideline, Q2 (R1), Validation of Analytical Procedures: Text and Methodology. International Conference on Harmonisation, Geneva, 2005; 1-13.