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ORIGINAL ARTICLE

High-Performance Thin-Layer Chromatography Marker-Based Standardization of *Piperine, Asiaticoside,* and *Withanolide-A* in the Developed Polyherbal Formulation and *in vitro* Evaluation of Acetylcholinesterase Inhibition

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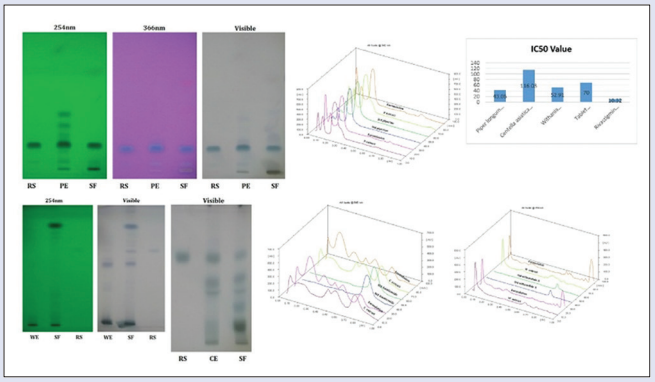
ABSTRACT

Background: Preparation of highly standardized polyherbal formulation with its chief active chemical constituents supported by therapeutic efficacy *in vitro* is a valuable approach in the field of pharmaceutical sciences. **Objective:** The present work aims to develop the high-performance thin-layer chromatography (HPTLC) marker-based standardization of polyherbal formulation using *Piperine, Asiaticoside,* and *Withanolide-A* and *in vitro* acetylcholinesterase inhibition activity. **Materials and Methods:** For successful standardization, the HPTLC quantification of *Piperine, Asiaticoside, and Withanolide-A* was carried out. Suitable solvent systems were optimized to achieve the better resolution of the marker compounds, extracts, and sample formulation. The reproducibility of the methods was also confirmed by repeating the procedure twice. The identity of the bands in the sample formulation was confirmed by comparing the R_f value with those of their respective reference standards. *In vitro* acetylcholinesterase inhibition assay was done on the different crude extracts as well as tablet formulation. **Results:** HPTLC quantification of formulation for *Piperine* content showed 1.97% content w/w, whereas *Piper longum* extract showed 2.44% w/w. Similarly, *Asiaticoside* showed 0.71% in formulation and in *Centella asiatica* extract, it was 1.33% w/w. Contrary to the above, *Withanolide-A* content was 2.12% w/w in formulation and in *Withania somnifera* extract, it was only 0.81% w/w. *In vitro* acetylcholinesterase inhibition assay exhibited significant inhibition with IC_{50} value of 70 µg for tablet formulation. **Conclusion:** The presence of *Piperine, Asiaticoside, and Withanolide-A* in formulation was identified by rapid HPTLC quantification method. The method is very precise, reproducible, and accurate. The developed method can be used to quantify the marker compounds in the formulations available in the market. The formulation also evaluated for its acetylcholinesterase inhibition assay and showed significant activity. Hence, it could be used in the treatment of memory-related disorders such as Alzheimer's disease.

Key words: Acetylcholinesterase inhibition, Alzheimer's disease, *Asiaticoside* and *Withanolide-A*, high-performance thin-layer chromatography quantification, *Piperine*

SUMMARY

- The therapeutic effect of polyherbal formulation is mainly because of the presence of their chief active constituents, the quantification of the active compounds present in the formulation was performed by rapid HPTLC method using marker compounds like *Piperine, Asiaticoside* and *Withanolide-A*. The HPTLC method is very precise, accurate and also reproducible. The formulation was also evaluated by *in vitro* acetylcholinesterase inhibition assay and showed a promising inhibition. Hence it proves that the formulation could be used in the management of Alzheimer's disease.



Abbreviations used: HPTLC: High-performance thin-layer chromatography; AD: Alzheimer's disease; DC: Direct compression; AchE: Acetylcholinesterase enzyme; RS: Reference standard; PE: *Piper* extract, SF: Sample formulation; CE: *Centella* extract; WE: *Withania* extract; ICH: International Council for Harmonization.

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INTRODUCTION

Alzheimer's disease (AD) is now selected as a major health problem which is affecting thousands of elderly people and their families worldwide. The incidence rates range from 1% to 5% of the population every year and are adding to the group of elderly people. In the USA, AD is the sixth leading cause of death, and about 5 million elderly people are suffering and living with AD. One in three seniors lose their life because of AD and other forms of dementia. Alzheimer's organization 2016 shows that about 5.4 millions of Americans are having AD and was estimated that around

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5.2 million elderly people are of the 65 years' age group. Approximately 0.2 million individuals are under age group 65 who are having high risk of young-onset Alzheimer's. These numbers are rapidly increasing in the upcoming years. By 2050, the number of people in the 65 years' age group having AD may be nearly 5.3 million to 13.9 million, respectively.[1-3]

Herbal product standardization supports and encourages marketing opportunities for polyherbal formulations. Standardization includes many steps and is starting from raw material to its end products. The main chemical composition present in the formulation in optimum level is responsible for biopotency. Hence, it is very important to estimate such active constituents using marker compounds and developing the analytical methodology for its identification. The marker-based standardization using high-performance thin-layer chromatography (HPTLC) is one of the best analytical methods to standardize most of the herbal formulation, which gives an idea about the required constituent in that formulation.[4,5]

The polyherbal formulation consists of hydroalcoholic extracts of *Piper longum*, *Centella asiatica*, and *Withania somnifera*. The polyherbal formulation is developed in the form of tablet formulation and is evaluated for its acetylcholinesterase inhibition assay. The present work was aimed to standardize the formulation using marker compound *Piperine*, *Asiaticoside,* and *Withanolide-A* by the HPTLC method.

MATERIALS AND METHODS

Methods

The hydroalcoholic extracts were used to make tablet formulation with DC grade excipients. The developed tablet is used for quantification of *Piperine*, *Asiaticoside,* and *Withanolide– A*. Extracts used in the formulation are given in Table 1.

Extraction and preparation of polyherbal formulation

The compressed tablet was powdered and extracted in methanol, and the individual drug extracts were also dissolved in methanol and dried. The dried powder in used for HPTLC quantification.

Chemicals and solvents

Asiaticoside and *Withanolide-A* were procured from Natural Remedies Pvt. Ltd, Bengaluru, and *Piperine* was procured from Sigma Aldrich Pvt. Ltd. (Steinheim, Germany; ≥97.0%). All the other solvents and chemicals were of analytical grade and were purchased from Merck, Ltd., Mumbai, and SD Fine Chemicals, Mumbai.

Development of solvent system by thin-layer chromatography study

Standard marker compound, individual crude extracts, and sample formulation were subjected for TLC study using different solvent systems, and optimization was carried out for each marker compound. The solvent system optimization was carried out to get maximum separation of phytoconstituents and the same solvent system was used for HPTLC studies.

Table 1: Crude drugs used for formulation and percentage

Crude drugs	Extract prepared	Percentage of extracts in polyherbal formulation
Piper longum	Hydroalcoholic	20
Centella asiatica	Hydroalcoholic	40
Withania somnifera	Hydroalcoholic	40

Development of high-performance thin-layer chromatography method

High-performance thin-layer chromatography instrumentation

The sample solution is applied in the pattern of bands of 6 mm with sample applicator 100 µl syringe on precoated TLC plate Silica gel $60F_{254}$ of E. Merck (20 cm × 10 cm with 250 µm thickness) using Linomat applicator. The slot dimension was kept about 10 cm × 10 cm, each track was scanned three times and baseline correction was performed. The mobile phase for *piperine* consists of toluene: ethyl acetate (9:1), the mobile phase for *Asiaticoside* was ethyl acetate: methanol: water (10:2.5:1), and the mobile phase for *Withanolide-A* consists of toluene: ethyl acetate: formic acid (5:5:1). The Camag twin trough glass chamber saturated with the respective mobile phases is used for each marker compound. The optimum time for saturation of the glass chamber was about 25–30 min at room temperature around 25°C–27°C. The migration distance was about 80 mm. The precoated TLC plates were dried using drier with hot air. Densitometric scanning is also done using Camag TLC Scanner at different wavelengths.

Preparation of solution

Preparation of standard solution of marker compounds

1. *Piperine* 100µg/ml in methanol (Sigma Aldrich with purity 97%).
2. *Asiaticoside* 100µg/ml in methanol (Natural Remedies with purity 95%).
3. *Withanolide* – A 100µg/ml in methanol (Natural Remedies with purity 95%).

Preparation of sample solutions

5 mg/ml of *Piper longum, Centella asiatica* and *Withania somnifera* extracts were taken in methanol. Similarly, sample formulation was also prepared as 5mg/ml in methanol.

Estimation of marker compounds in sample formulation

Piperine

A volume of 40 µL of standard *Piperine* solution, 40 µL of *P. longum* extract, and 40 µL of sample formulation were used for spotting. The plates were developed and dried. For *Piperine,* the plate was derivatized using 5% aqueous H_2SO_4 and heated at 110°C. The developed plates were scanned at 254 nm, 366 nm, and visible light.[6-8]

Asiaticoside

A volume of 40 µL of standard *Asiaticoside* solution, 40 µL of *C. asiatica* extract, and 40 µL of sample formulation were used for spotting. The plates were developed and dried. For *Asiaticoside,* the plate was derivatized using 5% aqueous H_2SO_4 and heated at 110°C. The developed plates were scanned in visible light.[9,10]

Withanoilde

A volume of 40 µL of standard *Withanoilde* solution, 40 µL of *W. somnifera* extract, and 40 µL of sample formulation were used for spotting. The plates were developed and dried. For *Withanolide-A,* the plate was derivatized using 5% aqueous H_2SO_4 and heated at 110°C. The developed plates were scanned in 254 nm and visible light.

In vitro acetylcholinesterase inhibition assay

Approaches to enhance cholinergic function in AD have included stimulation of cholinergic receptors or prolonging the availability of acetylcholinesterase (Ach) released into the neuronal synaptic cleft by use of agents which restore the level of acetylcholine through inhibition of ACh enzyme (AChE). AChE holds a key role not only to enhance cholinergic transmission in the brain but also to reduce the aggregation of β-amyloid and the formation of the neurotoxic fibrils in AD. Therefore, AChE inhibitors

have become remarkable alternatives in treatment of AD. Inhibiting the activity of AChE increases the concentration of the neurotransmitter with positive effect on cognitive function. Inhibition of AChE also serves as a strategy for the treatment of not only AD but also senile dementia.

The colorimetric method of Ellman *et al*. (1961) which is based on determining the amount of thiocholine released when acetylthiocholine is hydrolyzed by AChE is widely used. The thiocholine released is quantified by its reaction with 5,5'-bisdithionitrobenzoic acid (DTNB), which produces a yellow 5-thio-2-nitrobenzoate anion.

Extracts used for assay

Hydroalcoholic extracts of *P. longum, C. asiatica,* and *W. somnifera* and punched tablet was dissolved in 90% alcohol and extract was used for the assay.

Acetylcholinesterase inhibition assay

Cholinesterase inhibition assay was performed in flat-bottom 96-well microplates using colorimetric method by Ellman *et al*. and was adapted by Okello *et al.* The run volume consisted of 5µL of bovine AChE solution, 200 µL of 0.1 M phosphate buffer (pH 8), 5 µL of DTNB in 0.1M phosphate buffer (pH 7) and 5µL of test extract. All the reactants were mixed and preincubated for about 15 min at 30°C. The reaction was started by adding 5 µL of ATChI at a final concentration of 0.5 mM. As a control, the inhibitor solution was replaced with buffer. The control was assayed three times. To check any nonenzymatic hydrolysis in the final reaction mixture, two blanks for each run were prepared in triplicate. One blank consisted of buffer replacing enzyme and a second blank had buffer replacing substrate. Change in absorbance at 405 nm was measured on a TECAN Sunrise Microplate 96-well plate reader for a period of 6 min at 30°C.[11,12]

RESULTS AND DISCUSSION

Piperine

Piperine is one of the important therapeutic phytoconstituents, and in this study the quantification of *Piperine* in the formulation and extract was determined by rapid HPTLC method. The solvent system used to develop chromatogram was toluene:ethyl acetate (9:1) and R_f value 0.24 and the details are given in Table 2. The developed plates were scanned at 254, 366, and in visible light [Figure 1]. Three-dimensional (3D) chromatogram of *Piperine* is shown in Figure 2.

Asiaticoside

C. asiatica is also popularly known as brahmi and is one of the traditional herbs used in Ayurveda for memory-related disorders. The chief important constituent is *Asiaticoside*. Solvent system used for quantification of *Asiaticoside* was ethylacetate:methanol:water (10:2.5:1) and the R_f value was 0.71. The details are given in Table 3. The developed plates were scanned in visible light only [Figure 3]. 3D chromatogram of *Asiaticoside* is shown in Figure 4.

Table 2: Details of quantification of *Piperine* in the formulation and *Piper longum* extract

Sample	R_f	Area	Percentage content (w/w)
Piperine (standard)	0.24	30649.4	97
Pepper extract	0.24	38694.4	2.44
SF	0.24	31146.4	1.97

SF: Sample formulation

Table 3: Details of quantification of *Asiaticoside* in the formulation and *Centella asiatica* extract

Sample	R_f	Area	Percentage content (w/w)
Asiaticoside (standard)	0.71	26512.2	95
CE	0.71	18621.4	1.33
SF	0.72	9990.8	0.71

CE: *Centella* extract; SF: Sample formulation

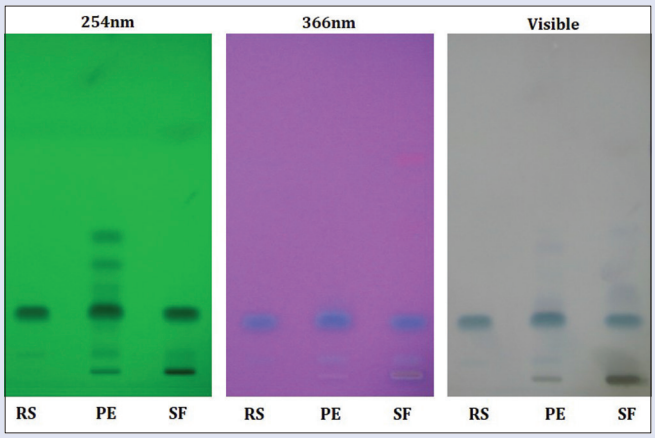

Figure 1: Thin-layer chromatography of *Piperine* at 254 nm, 366 nm, and in visible light

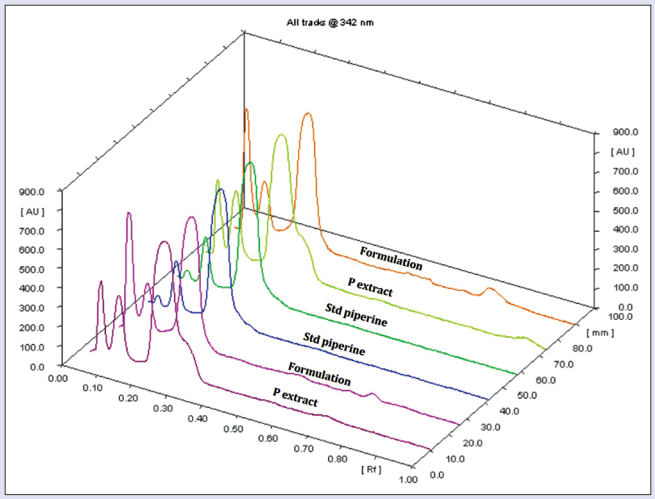

Figure 2: Three-dimensional chromatogram of *Piperine*

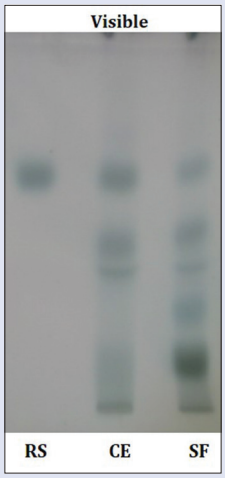

Figure 3: Thin-layer chromatography of *Asiaticoside* in visible light

Withanolide-A

Ashwagandha is one of the oldest medicinal herbs widely used in the Ayurvedic system of medicine for various health complications. It contains various constituents and *Withanolide-A* is one among having wide therapeutic actions on memory-related deficits. Solvent system used for quantification of *Withanolide-A* was toluene:ethylacetate:formic acid (5:5:1) and the R_f value was 0.77; the details are given in Table 4. The developed plates were scanned at 254 and in visible light [Figure 5]. 3D chromatogram of *Withanolide*-A is shown in Figure 6.

Acetylcholinesterase inhibition assay

The assessment of cholinesterase inhibition was done with hydroalcoholic extracts of *P. longum, C. asiatica,* and *W. somnifera* and punched tablet formulation. The results of the assay are more promising by inhibiting the enzyme acetylcholinesterase that is one of the hallmarks in controlling the disease symptom. Table 5 and Figure 7 show the results of acetylcholinesterase inhibition assay.

SUMMARY AND CONCLUSION

The proposed HPTLC method for standardizing and validating the formulation as per the ICH guidelines and was very rapid and accurate for quantitative estimation of *Piperine, Asiaticoside,* and *Withanolide-A* in the extracts and tablet formulation. The method is very reliable and suitable for estimation of marker compound present in the formulation which is having most effective therapeutic efficacy and found to be very helpful in detecting the quality of the extracts and formulation made out of it. The formulation was also evaluated for its efficacy by *in vitro* acetylcholinesterase inhibition and was found to be more promising in controlling symptoms of memory-related deficits.

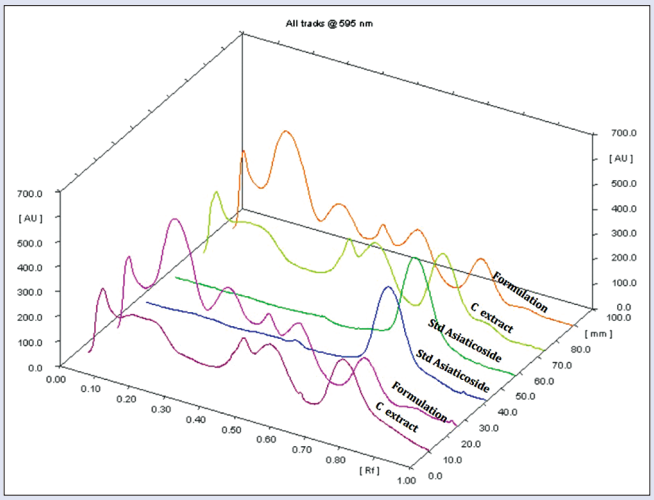

Figure 4: Three-dimensional chromatogram of *Asiaticoside*

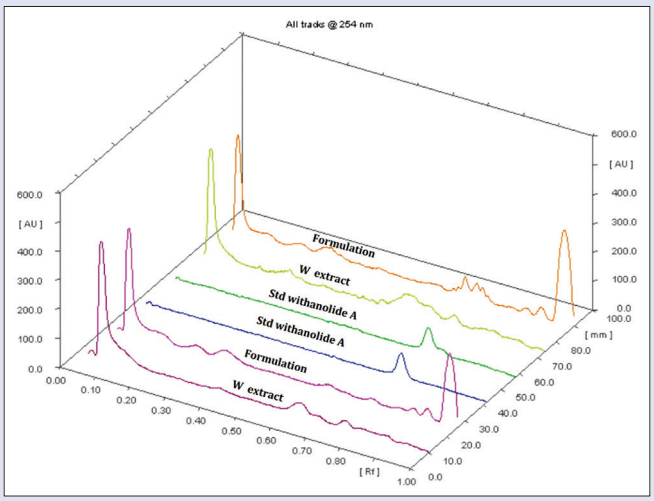

Figure 6: Three-dimensional chromatogram of *Withanolide*– A

Table 4: Details of quantification of *Withanolide-A* in the formulation and *Withania* extract

Sample	R_f	Area	Percentage content (w/w)
Withanolide -A (standard)	0.77	2276.9	95
WE	0.76	973.3	0.81
SF	0.76	2546.3	2.12

SF: Sample formulation; WE: *Withania* extract

Table 5: Results of acetylcholinesterase inhibition assay

Extracts	IC_{50} value (µg)
Piper longum extract	43.06
Centella asiatica extract	116.03
Withania somnifera extract	52.91
Tablet formulation extract	70.00
Rivastigmine standard	10.32

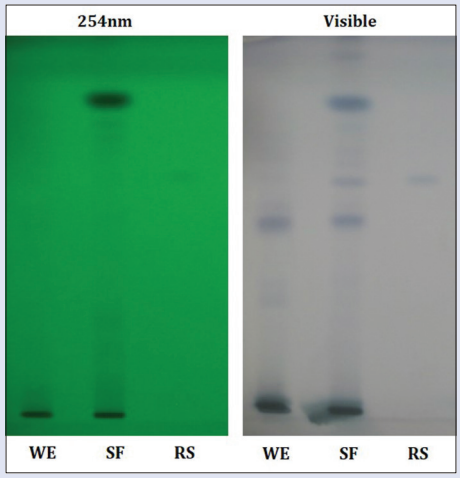

Figure 5: Thin-layer chromatography of *Withanolide-A* at 254 nm and in visible light

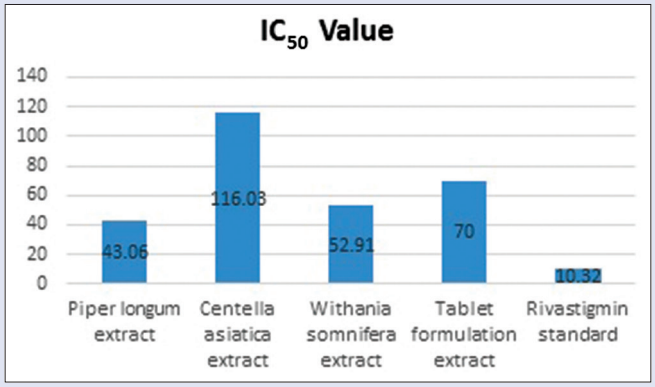

Figure 7: IC_{50} value of acetylcholinesterase inhibition assay

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Nil.

Conflicts of interest

There are no conflicts of interest.

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