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Phytochemical evaluation and Biomarker isolation from a Poly-herbal formulation

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ABSTRACT

Plant derived medicines are popularly used nowadays considering their natural origin and low side effects. Phytochemical compounds are responsible for their various biological activities. Qualitative evaluation of such formulations is a challenging task due to high variability of their chemical components. This work aimed at preparing and evaluating a polyherbal anti-stress medicine, formulated using four potent Ayurvedic drugs namely *Withania somnifera, Valeriana wallichii, Bacopa monnieri* and *Piper longum* and also isolating a bioactive marker compound from the same.The formulation was prepared as per classical methods of Ayurveda and analysed for physicochemical characters like loss on drying, total ash content, acid insoluble ash, water soluble extractive value and alcohol soluble extractive value by standard methods. Phytochemical analysis was performed for detecting presence of carbohydrates, phenols, flavonoids, tannins, alkaloids, steroids, Terpenoids, glycosides and saponins and for estimating active groups like total phenolic content, flavonoids, tannins and saponins. High Performance Thin Layer Chromatography fingerprint profiling was performed to confirm the presence of various phytocompounds. The biomarker compound was isolated by column chromatography method and characterized by UV-Visible, H^1-NMR, C^{13}-NMR and Mass spectroscopy methods. Physicochemical analysis showed presence of about 18% water soluble components and about 14% alcohol soluble components. Preliminary phytochemical analysis confirmed presence of carbohydrate, phenols, flavonoids, tannins, alkaloids, steroids, glycosides, phytosterols and saponins. Quantitative analysis showed presence of about 7.6% phenols, 1.3% flavonoids, 1.1% tannins and 3.5% saponins. Spectroscopic study indicated isolated compound is a phenolic compound 3, 4, 5-tri hydroxybenzoic acid.

Keywords: Antioxidants, bioactive compound, phenolic compound, flavonoid, memory, anti-stress, *Withania somnifera, Valeriana wallichii, Bacopa monnieri, Piper longum*

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INTRODUCTION

Plants have been used as medicine for treatment in different health conditions since time immemorial. Plant derived medicines are popularly used nowadays considering their natural origin and low side effects[1-2]. Stress, insomnia and memory related complaints are now a days increasing as result of our changing life styles and as a part of symptoms of age related diseases. Many remedies are available in allopathic, traditional and herbal medicines. As far as herbal medicines are considered quality assessment and quality control is a challenging task as these are prone to natural variations. Secondary metabolites present in these medicines are considered as the compounds responsible for their therapeutic efficacy. It is believed to be based on synergistic activity of a group of active phytoconstituents, present in the medicine.Isolation of bioactive compound from a plant based formulation is of growing concern as it may be used as a tool for standardization of herbal drugs.

A polyherbal formulation prepared from four potent neuroprotective ayurvedic drugs namely *Withania somnifera, Valeriana wallichii, Piper longum* and *Baccopa monnieri* is taken for the present study. Withania somnifera generally referred as Indian ginseng or winter cherry is a very potent ayurvedic drug having wide range of pharmacological activities such as anti-inflammatory, antioxidant, anti-stress, effective in conditions of insomnia, memory loss and is a known neuroprotective[3-6] drug. The main active compounds in the root of this plant are alkaloids, steroidal lactones, withanolides and withaferins. Valeriana wallichii, Indian valerian, an ingredient in a number of Ayurvedic Medicine such as Dhanwantharam kashayam, Vilwadi gulika, Jatiphaladi curna, Mahanaryana thailam, Dhanwantharam thailam, etc., is reported to be used effectively in conditions like anxiety, emotional stress, insomnia and nervous conditions[7-9.]

The principal active compound reported is valepotrates and valeric acid. Piper longum is another important medhyarasayana drug in Ayurveda which is reported as a remedy for sleeping problem, arthritic conditions and it is a bioavailability enhancer[10-12].The most popular neurotonic herb, Baccopa monnieri, commonly known as Brahmi, is a cognitive enhancer and used in various neurological conditions[13-15.]Literatures are available for the traditional use of Brahmi for enhancing memory and alleviating anxiety neurosis. Saponins are responsible for its neuroactivity.

This paper discusses evaluation of a polyherbal formulation by physicochemical and phytochemical methods and the isolation of biomarkers from it.

MATERIALS AND METHODS:

All the chemicals used were of AR grade purchased from Merck and Nice Chemicals. Authenticated herbal samples were collected from the factory, The Arya Vaidya Pharmacy (Coimbatore) Limited.

Preparation of Formulation

The authenticated samples of *Withania somnifera* and *Valeriana wallichii* roots were collected, preprocessed and dried. These dried raw materials were finely pulverized and mixed with fine powder of piper longum fruits. This mixture was then taken for Bhavana process as detailed in classical texts of Ayurveda. Fresh juice of Brahmi was used as the bhavana dravya or liquid medium for the process[16-21]. The process of bhavana was repeated for seven times[22]. After bhavana the powder was dried.

Qualitative Analysis

Physicochemical analysis: Physicochemical analysis such as loss on drying, Total Ash, Acid insoluble ash, water soluble extractive value and Alcohol soluble extractive value of the formulation was done as per the standard methods[23-24].

Extraction: 100 g of the formulation in the dried form was taken in thimble and extracted with 70% methanol using Soxhlet apparatus for 6 hours. The solvent free extract was used for the following tests.

Phytochemical analysis: Qualitative phytochemical tests for the identification of phenols, tannins, flavonoids, alkaloids, Terpenoids, steroids, glycosides, phytosterols and saponins were carried out for the formulation by standard methods[25-27].

Quantitative Analysis

According to the preliminary phytochemical results some of the active phytogroups were estimated[28-30].

Determination of total phenolic content:

The total phenolic content was determined using Folin-ciocalteu reagent. Appropriately diluted standard and samples were made up to 3.5 ml with distilled water in a series of test tubes. These tubes were then treated with 0.5 ml 2 N Folin-ciocalteu's reagent and incubated for 3 minutes at room temperature. The reaction was then neutralized by the addition of 1 ml 20% sodium carbonate. The reaction mixture was then incubated at room temperature for 90 minutes after which the absorbance was read at 760nm using UV/Visible spectrophotometer (Shimadzu Model 1800) and the percentage phenolic content is calculated from its graph

Determination Total flavonoids:

Aluminum chloride colorimetric method was used for flavonoids determination. Each sample were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm using UV/Visible spectrophotometer and the calibration curve was determined by preparing quercetin solutions at concentrations 12.5 to 100 g in methanol.

Quantitative determination of tannins:

The tannin content in the formulation was estimated by Folin-Denis Method. Tannin like compounds reduces phosphotungstomolybdic acid in alkaline solution to produce a highly coloured solution, the intensity of which is proportional to the amount of tannins present. The intensity is measured using UV/Vis spectrophotometer at 700 nm. The standard tannic acid is prepared by dissolving 100 mg of tannic acid in 100 ml of distilled water. To 100 µl of appropriately diluted methanol extract, 0.5 ml Folin-Dennis reagent 1 ml sodium carbonate solution are added and diluted to 1 ml. It was incubated for 30 minutes. The absorbance is read using UV/Vis spectrophotometer. The percentage of tannins is calculated

Quantitative determination of saponins:

20 g of the powder was extracted with distilled water three times and evaporated to get the residue. To 1 g of the water extract taken in a round bottom flask 10 ml of 10% Hydrochloric acid in Methanol was added. It was then refluxed on a water bath for three hours and then neutralized with sodium carbonate. To this was added 20 ml distilled water by extraction with ethyl acetate. The ethyl acetate washings were combined and evaporated to dryness under vacuum. The weight of the saponins was noted and the percentage was calculated.

Heavy metal analysis:

Heavy metal estimations were carried out by ICPMS. Lead, Arsenic, Cadmium and Mercury were estimated.

Isolation of Bioactive Compound:

The compounds present in the methanol extract of the formulation were separated by Column chromatography method. Silica gel was used as stationary phase and the extract was eluted through the wet filled column using Toluene: Chloroform: Methanol (4:3:3) solvent system. 5ml of fractions were collected and pooled according to Thin Layer Chromatography results. The third fraction which answered positive to phenolic tests was concentrated. The crystals obtained after keeping for three weeks were recrystallized from methanol.

Characterization: The crystals obtained were characterized by usual techniques like UV-Visible spectroscopy, H^1 and C^{13} NMR spectroscopy, and mass spectroscopy. The results were compared with results from the standard data library and other literatures.

HPTLC finger printing: HPTLC finger printing was carried out for methanol extract of the formulation with isolated Gallic acid. Silica gel 60/F254 readymade plates were used for spotting. Solvent system used was Toluene: Chloroform: Methanol (4:3:3). The plate was visualized at 254 nm.

RESULTS AND DISCUSSION:

Results of physicochemical analysis are detailed in table 1. The loss on drying indicates the volatile matter and moisture content of the formulation. Ash percentage value indicated that the presence of inorganic compounds. Acid insoluble ash content is low. The alcohol soluble and water soluble extractive values give the percentage of secondary metabolites in it. The pH is on the acidic side. Results of preliminary phytochemical analysis conducted on the methanol extract of the formulation are shown in Table 2. The presence of these compounds may contribute to the activity of the formulation. The active metabolites phenols, flavonoids, tannins and saponins are quantified as they may contribute largely to the biological activity of the formulation and the results are tabulated in Table 3. The results obtained for heavy metal analysis are given in Table 4. The four metals present are found to be within the permissible limits that may enhance metal-protein complex formation.

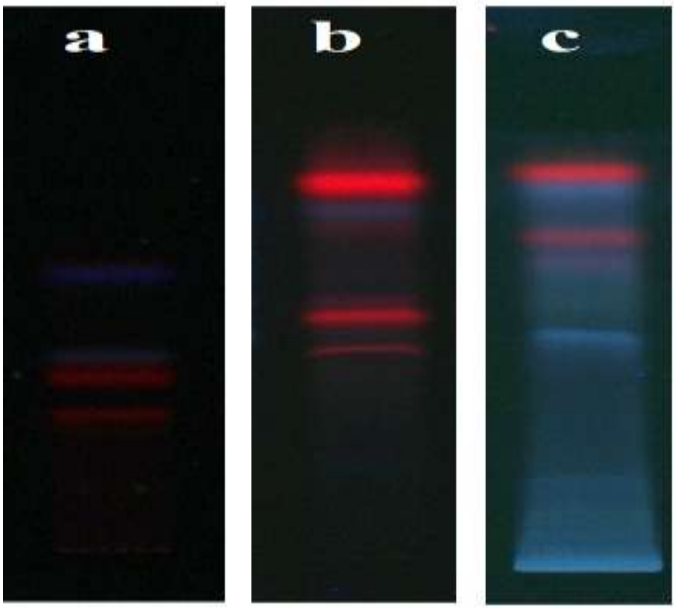

Figure 1: Showing HPTLC Finger Printing (a) hexane extract, (b) ethyl acetate extract, (c) methanol extract

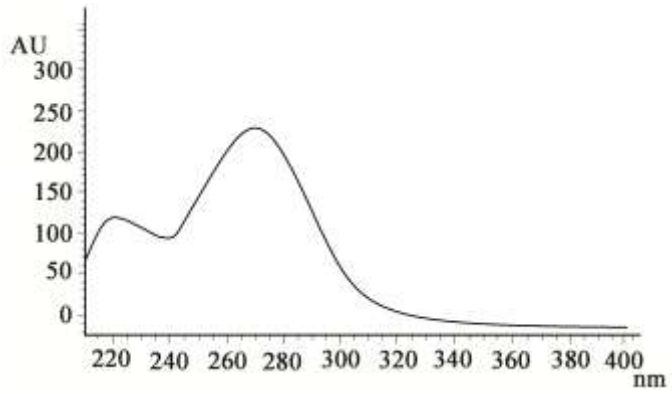

Figure 2: Showing UV-Vis Spectrum of Isolated Compound

Table 1 Showing Results of Physicochemical Analysis

Sl. No.	Tests	Results (%)
1	Loss on drying	6.73
2	pH	4.97
3	Ash	6.75
4	Acid Insoluble Ash	1.11
5	Water soluble Extractive	18.08
6	Alcohol soluble extractive	13.96
7	Bulk density	0.48

Table 2 Showing Results of Phytochemical Analysis

Sl. No.	Phytochemicals	Test	Results
1	Carbohydrates	Molisch's Test	Present
		Fehling's Test	
2	Phenols	Phosphomolybdic acid Test	Present
		Ferric chloride Test	
3	Flavonoids	Schinoda Test	Present
4	Tannins	Braemer's Test	Present
5	Steroids	Liebermann-Burchard's Test	Present
6	Terpenoids	Liebermann-Burchard's Test	Absent
7	Alkaloids	Dragendroff's Test	
		Mayer's Test	
		Wagner's Test	Present
		Hager's Test	
8	Glycosides	Legal's Test	Present
9	Phytosterols	Salkowski's Test	Present
		Liebermann-Burchard's Test	
10	Saponins	Foam Test	Present

Table 3 Showing Results of Quantitative Analysis

Sl. No.	Phytochemical	Result (%)
1	Total Phenol content	7.64
2	Flavonoids	1.28
3	Tannin	1.06
4	Saponins	3.54

It shows similar spots at Rf value 0.65 of standard Gallic acid. HPTLC profiles of different extracts of the formulation are shown in figure 1. Figure 2 shows the UV/Visible spectrum of the isolated compound. Two peaks obtained at 220 and 271 nm which are similar to that of standard Gallic acid spectrum[31-32]. The H^1-NMR spectrum is given in figure 3. And it gives δ values at 1.3, 4.02, and 7.02. C^{13} spectrum is shown in figure 4 which shows δ values at 78.2(C7), 114.6(C3, C4, C5) and quadrat at 136.5(C2, C6). Figure 5 shows the mass spectrum of the formulation. The molecular weight is 170 and the $[M^+ +H]$ peak is obtained at m/z value 171 and first fragmentation was obtained at 153 ($[M^+ +H]$- H2O) and base peak at 126 ($[M^+ +H]$-COOH) [33-35]. From the spectral data, the isolated compound is found to be 3, 4, 5-tri hydroxyl benzoic acid (Gallic acid)[36] and shown in figure 6. Figure 7 represents the HPTLC finger printing of formulation with isolated Gallic acid.

Table 4 Showing Results of Heavy Metal Analysis

Sl. No.	Heavy metal	Result (ppm)	Limit (ppm)
1	Lead	4.47	<10.0
2	Arsenic	0.27	<3.0
3	Cadmium	0.23	<0.30
4	Mercury	Not detected	<1.0

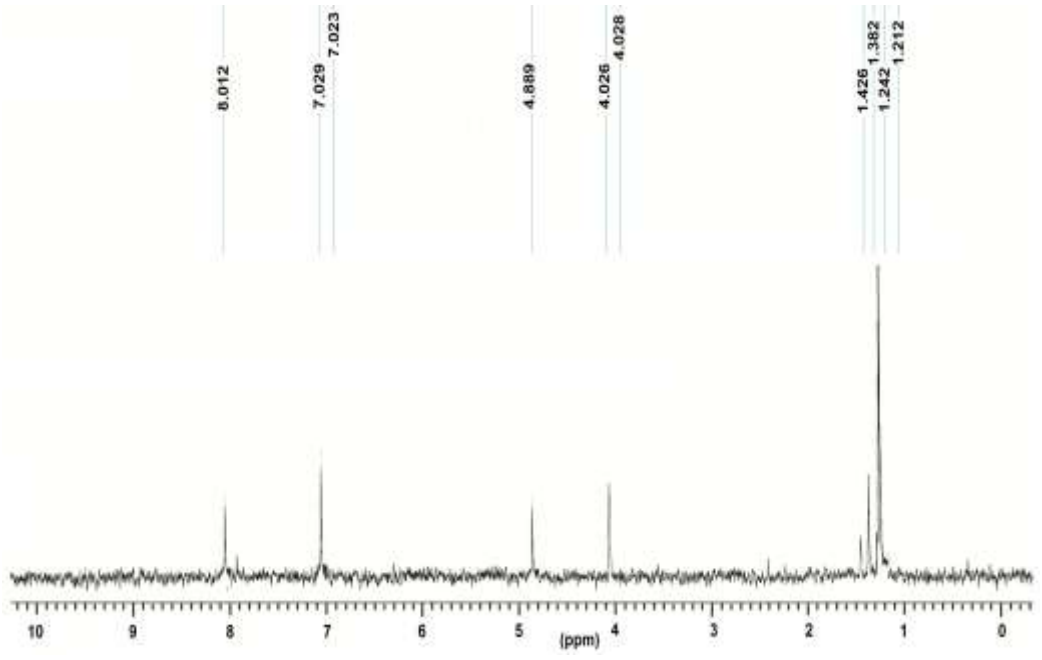

Figure 3: Showing H^1 NMR

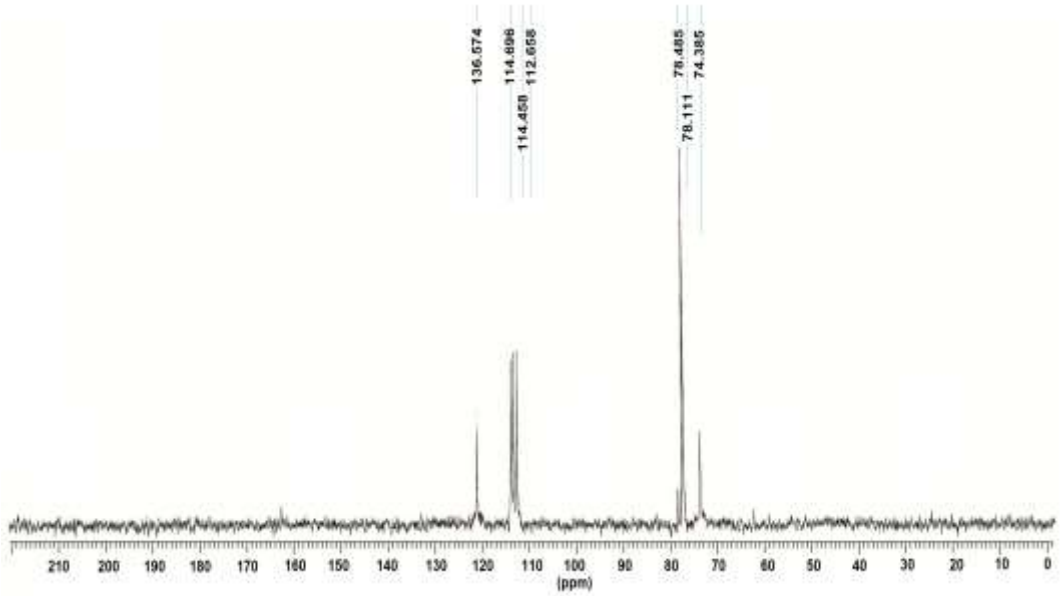

Figure 4: Showing C^{13} NMR

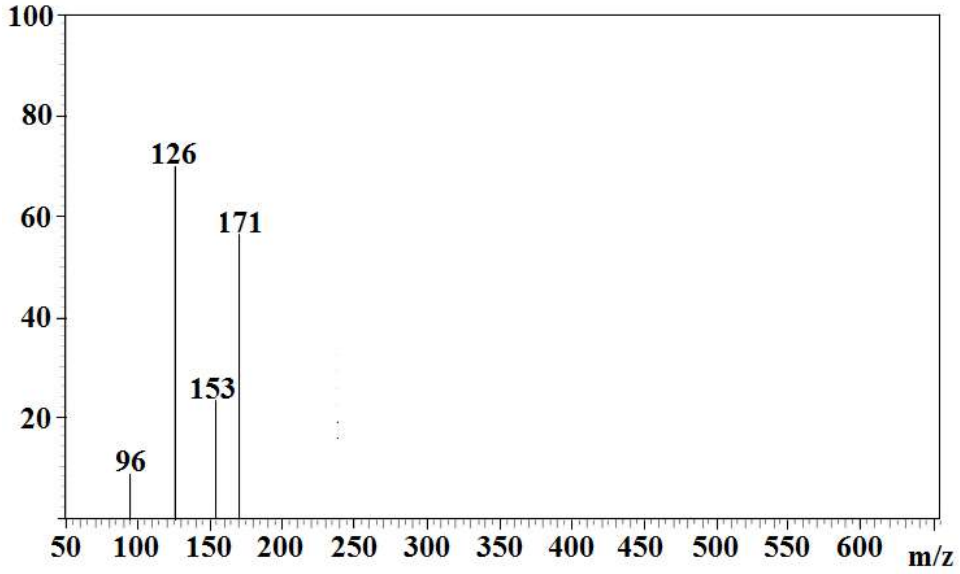

Figure 5: Showing Mass Spectrum

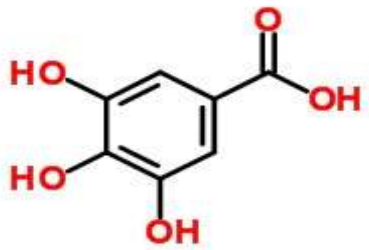

Figure 6: Showing Structure of Gallic Acid

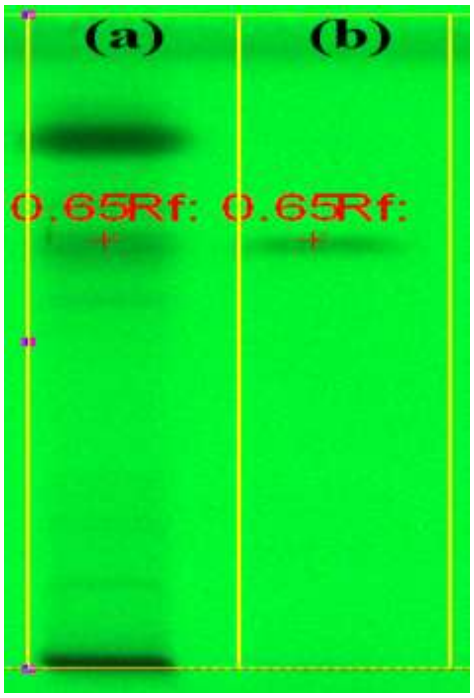

Figure 7: HPTLC of formulation with marker compound

CONCLUSION:

The physicochemical analysis conducted on the formulation gives an overview on the qualitative aspects of the herbal combination. The presence of secondary metabolites, which are the biologically active principles in herbal medicines, are confirmed by preliminary phytochemical analysis. These secondary metabolic products of plant have protective or disease preventing properties, strong antioxidant, protects cells from oxidative damage, hormonal actions, and stimulation of enzymes, anticancer and antimicrobial activities[38]. Isolation of bioactive compounds from herbal medicine is growing concern nowadays as it plays crucial role in development and modernisation of herbal medicines. The isolated Gallic acid is a known phenolic acid which is present in almost all plant species. It is known to have anti-inflammatory, anti-mutagenic, anti-cancer activity[38-41] and it shows cytotoxicity against cancer cells without harming healthy cells. Gallic acid is a potent antioxidant. It is very effective in prevention and treatment of stress related disorders as it preserves antioxidant levels by inhibition of lipid peroxidation [42-43]. Studies supports its neuroprotective activity in oxidative stress induced rat models[44-46]. It is a polar molecule and the neuroprotective effects of gallic acid and its derivatives seem to depend more on their molar polarities rather than antioxidant activities in human SH-SY5Y cell lines[47]. Some of the pharmacological effects of the formulation might be due to the presence of Gallic acid. The scope of this study can be extended to find the contributory role of Gallic acid to the formulation.

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