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Original Article

Physicochemical, Phytochemical and HPTLC Analysis of a Novel Combined Herbal Formulation

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ABSTRACT

Background: *Cinnamomum zeylanicum* (Darchini), *Alpinia galanga* (Khulanjan), and *Withania somnifera* (Asgand) have been used in joint diseases by Unani scholars since long. The combination of these ingredients may be more effective than they are given alone as these drugs are having potent anti-inflammatory, antioxidant, analgesic and rejuvenating properties. As per the WHO guideline, standardization should be done before undertaking any experiment which claims safety and efficacy. Therefore, this study is aimed to establish the physicochemical and phytochemical quality standards for this novel combination of herbal formulation for future references. **Methods:** In present study, extractive values such as methanol extract, aqueous extract, chloroform extract and aqueous ethanol extract was obtained. Further, loss on drying, pH, ash values, fluorescence analysis, qualitative phytochemical analysis and HPTLC fingerprint profile were done. **Results:** Mean values of methanolic, water, chloroform, and aqueous ethanolic extracts were 10.4±0.77%, 13.0±1.3%, 2.3±0.26% and 15.28±1.18% respectively. Further, the total ash, acid insoluble ash, and water-soluble ash were 3.3±0.32%, 1.13±0.09%, and 1.72±0.2% respectively. pH of 1% extract was 5.57±0.16 and 5.37±0.32 for 10% extract. It was observed that the percentage of moisture content was 4.67±0.42%. Qualitative phytochemical analysis revealed that the extract contains carbohydrates, glycosides, phenolic compounds, flavonoids, proteins and amino-acids, alkaloids, and saponins. Further, HPTLC fingerprint revealed the presence of many plant metabolites and active ingredients. **Conclusion:** The data generated in this study have been reported for the first time for this novel drug combination, which could be used as a source for future studies.

Key words: Anti-inflammatory, Unani medicine, *Cinnamomum zeylanicum, Alpinia galanga, Withania somnifera.*

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INTRODUCTION

Inflammation is a defense mechanism of the immune system of the human to protect from the toxins, irradiations, irritants, and pathogens such as bacteria, viruses, etc. by eliminating or neutralizing injurious stimuli and promoting healing.[1,2]

Clinical features like pain, redness, swelling, warmth, and loss of function of the affected part are resulting from body's response against the infection or injury.[3] This occurs at the tissue level as a reaction to injurious stimuli.[4] This acute inflammation becomes chronic when it is not controlled or managed properly and producing vicious cycle.[5] Alterations in the inflammatory response from acute to chronic will have an influence on immune tolerance. Affected organs and tissues will lose their normal physiological functions and this ultimately leads to various non-communicable diseases in the individuals of any age group.[6] Autoimmune diseases, allergies, arthritis, cancers are manifestations of chronic inflammation or persistent inflammation.[7]

Numerous numbers of single and compound drugs, having anti-inflammatory action are mentioned in Unani system of medicine which are useful in acute and chronic inflammations. Though, many compound medicines which are having anti-inflammatory actions are advocated for the treatment of non-communicable diseases, all of them are not available due to many reasons such as some of their ingredients are not available at present or very expensive or difficulty in getting the genuine drugs. It is a timely need to discover new compound preparations with easily available raw material which are cheaper and effective. Therefore, after thorough literature survey in the Unani classical texts and available research data which justifies the action and uses related to joint diseases, a combination of three Unani single drugs, namely Darchini (*Cinnamomum zeylanicum*)[8-11] Khulanjan (*Alpinia galanga*)[12-15] and Asgand (*Withania somnifera*)[16-19] was selected for this study.

Inadequate standardized herbal products are the main weakness in the field of herbal medicines. Quality of the herbal medicines should be established before any experiments as to be conducted for what they are claimed for.[20] World Health Organization (WHO) has also emphasized this and drawn many guidelines such as WHO Quality control methods for medicinal plant materials, General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine (2000), etc.[21,22]

Though, standardization of herbal products is a challenging task as they contain many herbs having unknown active components and to ensure the production of batch-to-batch reproducibility, it is a must to maintain the consistency in the claimed efficacy of the herbal product at least ensuring the minimum quality standards. This study is aimed to establish the quality standards for the combination of Unani herbs, Darchini,

Khulanjan and Asgand which is essential for the experimental studies *in vitro* and *in vivo*. Hence, given protocol was implemented to establish quality standards for this novel combined herbal formulation.

MATERIALS AND METHODS

Source of the Drug

Ingredients of the novel herbal formulation were procured from authentic Unani raw material suppliers from Delhi. Each individual herb was authenticated by the scientist (Dr. Sunita Garg – Emeritus Scientist) from National Institute of Science Communication and Information Resources (NISCAIR), New Delhi. Voucher Specimens NISCAIR/RHMD/Consult/2018/3306-07-1, NISCAIR/RHMD/Consult/2018/3306-07-2, and NISCAIR/RHMD/ Consult/-2018/3306-07-3 for Khulanjan, Darchini, and Asgand respectively were deposited at the herbarium of NISCAIR, New Delhi.

Chemicals and Instruments

Standard analytical grade chemicals, solvents and reagents were procured and used in the experiments.

Experiment Procedures

All the experiments were carried out according to the standard methods in triplicate to get the mean values.

Extractive Values and Ash Values

Extractive values were determined with the different solvents such as chloroform, methanol, aqueous-ethanol (50%) and distilled water by utilizing Soxhlet apparatus.[23] As per the WHO guidelines, total ash, acid insoluble ash and water-soluble ash were determined.[24]

Preliminary Phytochemical Screening

Fehling's test, Molisch test, and Benedict's test were done to screen the carbohydrates. Glycosides was detected by the Borntrager's test. Ferric Chloride test was carried out to screen the phenolic compounds and Ammonia test for flavonoids. Ninhydrin test, Millon's test, Xanthoprotein test, and Biuret test were used to identify proteins and amino acids. Alkaloids, Saponin, Steroid, and Tannins were screened by Wagner's test, foam test, Liebermann - Burchard's test, and Breamer's test respectively.[25-27]

Moisture Content and Determination of pH

Moisture content was determined by loss on dry method; and pH of 1% and 10 % solution of the hydroethanolic extracts were determined using standard glass electrode.[24]

Exposure to Different Chemical Reagents and Fluorescence Analysis

The extract was allowed to react with hydrochloric acid, sodium hydroxide, nitric acid, ferric chloride, and picric acid and the color of the resultant was recorded.[28] Fluorescence analysis was carried out for the extract exposed to UV light (254 nm and 366 nm) and day light.[23]

HPTLC Analysis

Fresh extract of 50% aqueous-ethanol solvent was used to develop HPTLC fingerprint analysis. Stationary phase was pre-coated silica gel 60 F_{254} Aluminum plates (Merck, KgaA, Germany) and HPTLC grade solvent system, Butanol, Acetic acid, Water was used in the ratio of 4:0.2:5.8. Plate was scanned using CAMAG HPTLC Densitometer. UV 366 nm and anisaldehyde sulphuric acid detection systems were used and the detection was done using deuterium and tungsten lamp.[29]

RESULTS

Extractives values of different solvents give the idea regarding the amount of chemical constituents found in the respective extract. Foreign matters such as sand and soil contamination are determined by evaluating the ash values. The results of the extractive values and the ash values, such as total ash, acid-insoluble ash, water-soluble ash, moisture content and pH values are summarized in Table 1.

All the tests were repeated three times and the mean values of the readings with their standard deviation were given.

Extract was allowed to react with chemicals such as con. HNO_3, 10% Aq. NaOH, con. H_2SO_4, con. HCl, Iodine and brown, dark brown, black, dark brown, and brownish yellow colour reactions were observed respectively. Fluorescence analysis showed brown, dark brown and brown fluorescence when the extract was exposed to UV 254 nm, UV 366 nm and normal ordinary light respectively. Qualitative tests for glycosides, alkaloids, flavonoids, phenolic compounds, carbohydrates, proteins and amino acids were done to identify the active plant metabolites. All the results are depicted in the Table 2.

Table 1: Results of physico-chemical analysis.

	Parameter	Mean with standard deviation
1.	Extractive Values (%)	
	Methanol extract	10.4 ± 0.77 %
	Aqueous extract	13.0 ± 1.3 %
	Chloroform extract	2.3 ± 0.26 %
	Aqueous Ethanol extract	15.28 ± 1.18%
2.	Ash values	
	Total ash	3.3 ± 0.32 %
	Acid insoluble ash	1.13 ± 0.09 %
	Water soluble ash	1.72 ± 0.2 %
3.	Loss on drying (%)	4.67 ± 0.42 %
4.	pH of the drug	
	pH of 1% of the drug	5.57 ± 0.16
	pH of 10% of the drug	5.37 ± 0.32

Table 2: Results of Phytochemical analysis of the aqueous ethanol extract.

Phytochemical	Test	Results
Carbohydrates	Molisch test,	+
	Fehling's test	+
	Benedict's test	+
Glycosides	Borntrager's test.	+
Phenolic compounds	Ferric Chloride test	+
Flavonoids	Shinoda Test	+
Proteins and Amino acids	Ammonia test	+
	Millon's test	+
	Biuret test	+
	Xanthoprotein test	+
	Ninhydrin test	+
Alkaloids	Dragendroff's Test	+
	Mayer's test	+
	Wagner's test	+
	Hager's	+
Saponins	Foam test	+

All the tests were repeated three times

The chromatograms were developed and scanned at 366 nm and after derivatization with anisaldehyde sulphuric acid at 580 nm. The HPTLC fingerprints of this combined herbal formulation are depicted in Figures 1, 2 and the results of HPTLC fingerprints are given in Tables 3 and 4.

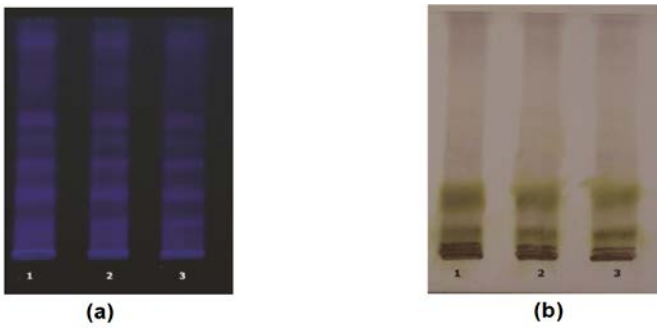

Figure 1: HPTLC fingerprint of hydroethanolic extract of the combined herbal formulation (a) at UV 366nm. (b) after derivatization with anisaldehyde sulphuric acid at 580nm.

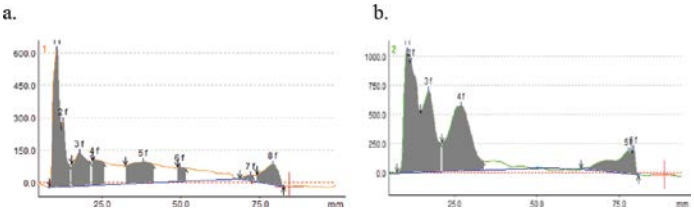

Figure 2: HPTLC Densitogram of hydroalcoholic extract of herbal formulation. (a) at UV 366nm (b) upon derivatized with Anisaldehyde Sulphuric acid at 580nm.

Table 3: Peak list of aqueous-alcoholic extract of the combined herbal medicine at UV 366nm.

Peak no	Y-Pos	Area	Area %	Height	R_f value
1	10.8	1413.19	31.23	629.73	0.01
2	12.7	481.99	10.65	302.68	0.04
3	17.9	718.96	15.89	152.51	0.11
4	22.7	397.14	8.78	117.34	0.18
5	38.2	790.33	17.47	98.03	0.40
6	49.6	159.29	3.52	65.89	0.57
7	72.1	63.57	1.41	25.68	0.89
8	79.2	499.95	11.05	93.57	0.99

Table 4: Peak list of aqueous-alcoholic extract of the combined herbal formulation upon derivatized with anisaldehyde sulphuric acid at 580nm.

Peak no	Y-Pos	Area	Area %	Height	R_f value
1	10.5	1949.98	14.61	1034.52	0.01
2	11.7	2429.33	18.20	943.16	0.02
3	17.1	3024.42	22.66	693.94	0.10
4	27.0	4359.32	32.66	555.66	0.24
5	77.8	1364.07	10.22	190.88	0.97
6	79.4	219.85	1.65	211.85	0.99

DISCUSSION

Traditional systems of medicines have been practiced since very long and it became scientific even before Christ. Considering the popularity and wide practice of traditional medicines all over the world, the WHO advocates, supports, and promotes these traditional medical systems in primary and secondary health care. However, at present, most of the traditional practitioners are treating the patients with natural remedies without giving due consideration for the quality and standards of the herbal drugs. This is due to improper identification, adulteration, contamination, substitution, compromised in manufacturing procedures, adopting wrong procedures, etc. which leads to batch to batch and manufacturer to manufacturer variation for a same drug. Hence, desired outcome is not attained in the present era, as such, to build up the confident of the patients towards traditional systems of medicines, practice with standard herbal drugs is paramount important.

Traditional medicines are required to be proved as safe and effective via modern scientific methods, in order to be accepted by the present world. Further, when the medicines are experimented for their safety and efficacy, it is essential to standardize the medicines to ensure certain standards of the drugs. Keeping in view the importance of quality control, the drugs such as Darchini, Asgand and Khulanjan mentioned in the Unani system of medicine for treating arthritis related conditions are combined to form a novel herbal formulation which was standardized according to the principles and guidelines to standardize the herbal drugs drawn by WHO.

Identity and ensure the purity of raw materials are one of the main objectives in quality standard of herbal drugs. Genuineness of raw materials was established scientifically identified and authenticated by an expert scientist at NISCIR, New Delhi. Ash value is an essential parameter to detect adulteration and contaminations with sands and other foreign materials and helps to assess the gross value of inorganic materials like phosphates, carbonates, oxalates, and silicates present in the drug. The total ash, acid insoluble ash, and water-soluble ash are 3.3 ± 0.32 %, 1.13 ± 0.09 %, and 1.72 ± 0.2 % respectively which could be used as a standard reference for this compound preparation. Moisture content of these drugs were evaluated by loss on drying method, and it was 4.67 ± 0.42 %.

Extractive index is a vital parameter in the standardization procedures which gives the idea of how much active ingredients are found in a particular extract. The aqueous-ethanolic extract dissolves almost all the phytochemicals as it is a most potent solvent system.[23] This was revealed by the results that aqueous ethanolic extractive index is higher than other extractive indexes such as alcoholic extract, water extract, petroleum ether extract and chloroform extract (Table 1). As more active ingredients are found in aqueous ethanolic extract of *darchini, Asgand* and *Khulanjan*, which could be an effective drug for inflammation. Further, pH of the 1% and 10% solutions of aqueous ethanolic extract were 5.57 ± 0.16 and 5.37 ± 0.32 which showed that the extract of the research drug is slightly acidic. Specially, weak acids are present as non-ionic form and diffuse easily through cell membranes. Therefore, this combined herbal medicine will have a higher absorption in the stomach which has highly acidic environment.[30]

Phenolic, flavonoid compounds and other plant metabolites are important in maintaining the health. These metabolites are mainly responsible for anti-inflammatory and antioxidant activities which protect the plant from various diseases and injurious stimuli.[31] Qualitative phytochemical analysis revealed the presence of glycosides, alkaloids, flavonoids, phenolic compounds, carbohydrates, proteins and amino acids in this compound preparation. HPTLC method has been an outstanding technique to identify the plant as it produces the fingerprint profile which can be used as a marker to compare the later batches to maintain the consistency of the

safety and efficacy. It is also used to identify and separate the active constituents both qualitatively and quantitatively. HPTLC chromatogram of this combined herbal medicine, showed seven major spots under UV 366nm at R_f values 0.14, 0.28, 0.40, 0.50, 0.57, 0.78, 0.92 and under visible region after derivatizing with Anisaldehyde Sulphuric acid and heating at 105°C shows two spots at R_f values 0.11, 0.41.

CONCLUSION

Traditional medical systems such as Ayurveda, Unani, Siddha, etc. are based on the unique theories and concepts. The drugs prescribed in these systems are based on their concepts whereas modern medicines are based on evidence-based medicine using the data from clinical trials, experimental studies, cell line studies and toxicity studies. Major setback of the traditional medicines is unavailability of pharmacopeial quality standards on raw material / formulations which may result in mild to serious adverse effects. Thus, world health organization emphasizes to have a standardization of herbal drug for the assessment of safety, efficacy, and quality to prepare a drug with reproducible therapeutic effect. The data generated in this study have been reported for the first time for this novel drug combination, which could be used as a source for future studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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