

Design, Development and Phytochemical Evaluation of a Poly Herbal Formulation Linkus Syrup

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Abstract

The present work has been carried out on polyherbal formulation named as Linkus Syrup. The herbal formulation consists of *Glycyrrhiza glabra*, *Hyssopus officinalis*, *Piper longum* and *Alpinia galanga*. The Linkus syrup physico-chemical evaluation such as pH, density, identification of polysaccharide, tanning agents, ascorbic acid and shelf life was complied. The TLC and quantitative determination of alkaloid were quantified. Determination of biomarker has been validated for the analysis of vasicine. The study result revealed that Linkus syrup formulation was well standardized at different levels such as physic-chemical consistency and assay of bio marker compound.

Keywords

Syrup, Crude Herb, Anti-Tussive, Anti-Bronchitis, Anti-Pharyngitis, Sore Throat, Tracheitis, HPLC

1. Introduction

Cough is one of the most common symptoms for which patients seek medical attention from primary care physicians and pulmonologists [1]. These studies were carried out for the design, development and phytochemical evaluation of a poly herbal formulation named as Linkus syrup. The syrup consists of leaves of *Adhatoda vasica* used in the treatment of coughs, bronchitis, asthma, common cold, expectorant and antispasmodic (bronchodilator). The active components are vasicine (aka peganine) and vasicinone [2]. The root and rhizome of *Glycyrrhiza glabra* contain glycyrrhizin that inhibit growth and cytopathology of cytomegalovirus and are utilized in

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cough, catarrhal infections, asthma, bronchitis, coughs, arthritis and allergic complaints [3]-[5]. *Hyssopus officinalis* contains pinocampnone, camphone, pinocampheol, cineole used as an expectorant, bronchitis, respiratory infections, upper respiratory tract infections, coughs [3] [6]-[8]. The fruits and roots of *Piper longum* are attributed for diseases of respiratory tract, such as cough, bronchitis, asthma [9] and contain piperine, piperlongumine, piperlonguminine, methyl 3,4,5-trimethoxycinnamate [9]. *Alpinia galanga* has a bronchodilator and anti-inflammatory effect on bronchioles [10] [11] and the main components isolated from its rhizomes were galangoisoflavonoid, methyleugenol, galangin, acetoxychavicol acetate (ACA) and hydroxychavicol acetate In the standard German text, *Hager's Handbuch der Pharmazeutischen Praxis*, *Zingiber officinale* has been listed as being used against nervousness, chronic inflammation of the intestine, coughing, conditions of the urinary tract and lower abdomen, rheumatism, and a sore throat [12] and the chemical constituents are zingerone, shogaols and gingerols, zingiberene, bisbolone and cineol. The leaves and flower of *Althea officinalis* have demulcent, expectorant, diuretic, and emollient properties and generally used to treat ailments of the lungs and the urinary system [13]. The active constituents are Pectin, disaccharide sacharose, mucilage, Hypolaetin-8-glucoside, isoquercitrin, kaempferol, caffeic, *p*-coumaric acid, scopoletin, asparagines and many amino acids.

Cordia latifolia [14] is used to treat gastrointestinal, respiratory and dermatological disorders and the constituents cited were cordinoic acid, cordicilin, cordinol and cordicinol [15]. *Zizyphus jujuba* used for colds and coughs; they also have antifungal, antibacterial, antiulcer, anti-inflammatory, sedative and antiseptic properties [16] [17] and it possesses mauritine-A; amphibine-H, jubanine-A, jubanine-B; mucronine-D, and nummularine-B, sativanine-E, franguloline, ziziphines jujubosides [17]. *Onosma bracteatum* leaves and stems are used to treat bronchitis and asthma [18] and main phytochemicals present are naphthazarins, naphthoquinones, alkannins and Shikonins [19].

2. Material and Method

Herbs were bought through Supply Chain Department of the Herbion Pakistan (Pvt.) Limited, Karachi from the local market. All samples were stored in dark at 23°C. The herbs utilized in Linkus syrup were morphologically compared with the authentic specimen available at QC department of Herbion Pakistan Pvt. Ltd., they details are as follows, *Adhatoda vasica* reference No. B18, *Glycyrrhiza glabra* reference No.M4, *Piper longum* reference No. F1, *Viola odorata* reference No. B2, *Hyssopus officinalis* reference No.Z1, *Alpinia galangal* reference No. A2, *Cordia latifolia* reference No. S9, *Althea officinalis* reference No. K3, *Zizyphus vulgaris* reference No.U3, *Onosma bracteatum* reference No. B1. These herbs have also been authenticated and verified by Prof. Dr. Iqbal Azhar, Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi. and chemically for biomarker of some herbs. All the other chemicals and reagents were of pure analytical grade and obtained from local supplier.

2.1. Development of Linkus Syrup

With extensive literature review and traditional knowledge research and development department of Herbion Pakistan Pvt. Limited was successfully developed the formulation for cough and cold which named as Linkus Syrup.

2.1.1. Selection and Evaluation of Herbs

Table 1 represents the quantity and pharmacological action of herbs used in this preparation.

All herbs were tested for their prescribed part, macro and microscopic descriptions, identification, chemical assays, foreign matter, total ash, Acid insoluble ash, alcohol and water soluble part of extract. These properties with specifications were listed in **Table 2**.

2.1.2. Preparation of Extracts

Each herb was extracted individually in vacuum jacketed extractor with water five times to the amount of herb taken. Stir for 10 minutes and then start heating. Heat to boil. The temperature should be from 100°C - 110°C. Then slow down the temperature to 80°C - 90°C. Amount of the herb, extraction time and percent yield are mentioned in the **Table 3**.

2.1.3. Preparation of Linkus Syrup

625 liter deionized water was took in manufacturing tank. Heated and boil for 40 to 45 minutes. When boiling

Table 1. The herbs used, quantity and pharmacological actions.

S.No	Active ingredients	Common name	Quantity*	Pharmacological Action
01	<i>Adhatoda vasica</i> (dry leaves Extract)	Arusa	600 mg	Bronchodilator and expectorant, use in breathlessness
02	<i>Glycyrrhiza glabra</i> (Dry root extract)	Mulaithi	75 mg	Anti-inflammatory and it reduces inflammation of the respiratory tract, relieve a spasmodic cough
03	<i>Piper longum</i> (Dry fruit extract)	Piplamol	100 mg	Prevents recurrent attacks of bronchial asthma, immune-modulator activity, anti-allergic
04	<i>Viola odorata</i> (Dry fruit extract)	Banafsha	25 mg	Expectorant
05	<i>Hyssopus officinalis</i> (Dry leaves extract)	Zufa	50 mg	antiseptic, cough reliever, and expectorant, soothing and cough suppressant properties. antibacterial activity against <i>S. aureus</i>
06	<i>Alpinia galanga</i> (Dry root extract)	galangal	50 mg	Cough and respiratory troubles, cold, flu, lung diseases, phlegm
07	<i>Cordia latifolia</i> (Dry fruit extract)	Lasorda	100 mg	against <i>H. influenzae</i>
08	<i>Althea officinalis</i> (Dry extract of flower)	Khatmi	100 mg	cough suppression, bronchodilator activity
09	<i>Zizyphus jujuba</i> (Dry fruit extract)		100 mg	colds and coughs, antibacterial, antiseptic properties, produced sound sleep
10	<i>Onosma bracteatum</i> (Dry leaves Extract)		100 mg	bronchitis and asthma, restlessness antibacterial activity against <i>S. aureus</i>

*amount of herb used in the preparation of 10 ml syrup.

Table 2. Specification and analytical method of raw material used in Linkus syrup by quality operation.

Name of the Herbs	Drug Parts	Description	Identification	Assay	Foreign Matter	Total ASH	Acid Insoluble Ash	Alcohol Soluble Extraction	Water Soluble Extraction
<i>Adhatoda vasica</i>	dried leaves	Macroscopic and Microscopic	By TLC Spot observed	About 1.3% of alkaloids as vascine by HPLC	NMT 2.0%	NMT 17.0%	NMT 1.0%	NLT 6.0%	NLT 27.0%
<i>Glycyrrhiza glabra</i>	Rhizome dark brown to black brown extract	Soluble in Water	Through taste: Sweetish bitter (Organoleptic)	Glycyrrhizin content present in Glycyrrhiza glabra by HPLC	---	---	---	---	---
<i>Piper longum</i>	Fruiting spikes	Macroscopic	Spot observed by TLC	NLT 5% of Piperine by HPTLC	NMT 2.0%	NMT 8.0%	NMT 1.0%	NLT 9.0%	NLT 35.0%
<i>Viola odorata</i>	Dried leaves	Viola has characteristic odor	Macroscopic (As per Flora of Pakistan page # 4)	---	NMT 3.0%	NMT 15.0%	---	---	---
<i>Hyssopus officinalis</i>	Flowering tops	Macroscopic. Microscopic	Spot observed by TLC	---	NMT 2%	NMT 10%	NMT 2.5%	water soluble extractive: NLT 10%	---
<i>Alpinia galanga</i>	Dried rhizome	Macroscopic and Microscopic	---	---	NMT 2.0%	NMT 2.0%	---	NLT 6.0%	NLT 13.0%
<i>Cordia latifolia</i>	Dried fruit	Macroscopic	---	---	NMT 2.0%	NMT 3.0%	NMT 1.0%	---	---
<i>Althea officinalis</i>	Dried seeds	Macroscopic and Microscopic	---	---	NMT 2.0%	NMT 8.0%	NMT 1.5%	NLT 10.0%	NLT 18.0%
<i>Zizyphus jujuba</i>	Dried mature fruit of	Macroscopic and Microscopic	---	---	NMT 1.0%	NMT 4.5%	NMT 0.2%	NLT 25.0%	NLT 45.0%
<i>Onosma bracteatum</i>	Dried leaves and stem	Macroscopic and Microscopic	---	---	NMT 2.0%	NMT 26.0%	NMT 4.0%	NLT 1.0%	---

Table 3. Extraction of Linkus syrup herbs.

Herb	Herb Quantity	Quantity of Thick Extract	Extraction Time	Percent Yield
<i>Adhatoda vasica</i> Nees.	18.0 kg	3.60 kg	1 hour	20.0%
<i>Piper longum</i> L.	3.0 kg	0.75 kg	20 minutes	25.00%
<i>Cordia latifolia</i> L.	3.0 kg	0.75 kg	20 minutes	25.00%
<i>Althaea officinalis</i> L.	3.0 kg	0.75 kg	20 minutes	25.00%
<i>Zizyphus vulgaris</i> Lam.	3.0 kg	0.75 kg	20 minutes	25.00%
<i>Onosma bracteatum</i> Wall	3.0 kg	0.75 kg	20 minutes	25.00%
<i>Glycyrrhiza glabra</i> L.	2.3 kg	0.56 kg	15 minutes	24.30%
<i>Alpinia galanga</i> (L.) Wild.	1.5 kg	0.37 kg	10 minutes	24.60%
<i>Hyssopus officinalis</i> L.	1.5 kg	0.37 kg	10 minutes	24.60%
<i>Viola odorata</i> L.	0.8 kg	0.19 kg	10 minutes	23.70%

was started, add 1750 kg sugar slowly with constant stirring. Continue heating with constant stirring till it became homogenize syrup. Separately dissolved Methyl Paraben, Propyl Paraben, Citric acid and Glycerin in 83 liter deionized Water, mixed, heat and boiled thoroughly. Mix this solution to the homogenized syrup and stirred for 20 minutes. Took 416 Liters of Purified water in jacketed kettle and add to it 73.75 kg combine extract. Heat till it was fully dissolved and then filter the solution. Add the filtered solution to homogenized syrup prepared previously, stirred and mix for 20 min. After mixing, allow the syrup to cool down by chilled water circulation. Circulation was continued until the temperature of the syrup reached to “room temperature”. Mixed the flavors like Peppermint Oil, Clove Oil and Propylene glycol, then made up the volume of syrup to 1250 liters with deionized water. Afterward allow final mixing by stirring for half an hour. This process represented here in **Table 4**.

3. Phytochemical Evaluations

Brown color syrup was prepared with characteristic odor, having pH in between 3.0 - 6.0, and density 1.25 - 1.35 g/cm³. Identification of polysaccharides, tanning agent, and ascorbic acid were performed by test tubes method. While the identification of vasicine was done by TLC.

3.1. Physical Evaluation

3.1.1. Appearance

Brown color viscosity syrup with characteristic odor, Determination was conducted visually.

3.1.2. pH Determination

Dissolved 10 ml of syrup in 100 ml of water. Filtered and determine pH potentiometrically Standard: 3.0 - 6.0

3.1.3. Density

Determination was conducted by SP XII method 3 and it was according to Standard: 1.25 to 1.35 g/cm³.

3.1.4. Qualitative Identification Reactions

Placed 5 ml of the preparation to a flat-bottomed 50 ml flask. Added 20 ml of 96% spirit and mixed; precipitated suspension was formed (**polysaccharides**). Left the obtained solution for 1 hour for layer separation and filter carefully through the folded paper filter (Solution A).

Added 3 drops of Ferric chloride solution to 3 ml of the solution A. After shaking greenish-yellow coloring appears (**tanning agents**). To 3 ml of A solution added 3 drops 0.1% spirit solution of sodium 2,6-dichlorofenolindofenolite solution had a reddish-violet coloring at mixing (**ascorbic acid**).

Table 4. Manufacturing of Linkus syrup.

Ingredients for 1 lot	Quantity	Unit
	Active	
Thick combined Extract	73.75	Kg
	Excipient	
Sucrose	1750	Kg
Anhydrous citric acid	5	Kg
Methyl Paraben	2.5	Kg
Propyl Paraben	0.5	Kg
Glycerin	25	Lit
Propylene glycol	2.5	Lit
Peppermint oil	0.9375	Lit
Clove oil	0.3125	Lit
Purified water	Up to 1250	Lit

Preparation of 0.1% Alcohol Solution of Sodium 2, 6-Dichlorphenolindophenolat

Placed 0.1 g of sodium 2, 6-dichlorphenolindophenolyat in 100 ml of measuring retort and dissolve it in 30 ml of 96% ethyl alcohol and then made up the volume with 96% ethyl alcohol. Shelf life of solution was 1 month

3.1.5. Qualitative Identification by TLC

Applied 0.05 ml of solution I to chromatographic plate “Silica gel 60 F₂₅₄” (size 10 × 20 cm manufactured by Merck) in the form of spot. Dry the plate in hot air for 5 minutes and placed it in the chamber preliminary saturated for 1 hour with a mixture of the following solvent: 96% alcohol : chloroform : hydrous ammonia (30:19:1).

When the front of solvents was reached the plate edge, took it out from the chamber and dried in a drying oven for 3 minutes at 100°C. Then apply Dragendorff reagent to the plate. 1 reddish pink color spot (vasicine) was shown on the plate in UV light at wave length 254 nm R_f(0.4).

The physicochemical and organoleptic parameters so generated for standardization were further determined by HPLC and UV spectrophotometers as mentioned by using colorimeter.

3.2. Quantitative Determination of Total Alkaloids

About 12 g (exact weight, with accuracy to 0.0001 g) (about 10 ml) of preparation was placed in a 100 ml dividing funnel. Added 1 ml of hydrous ammonia, mixed then adds 20 ml of water and mixed again. Added 25 ml of chloroform to the obtained solution in the funnel. Shake carefully for 3 minutes. After full division of layers. Filtered lower chloroform layer through the paper filter with anhydrous sodium sulfate (about 10 g) in 500 ml round-bottomed flask.

Repeated extraction procedure 4 times more using 25 ml portions (5 times in total). Collected chloroform layers in 500 ml round bottom flasks... Combined chloroform extraction steamed to dryness on a water bath under vacuum. The dry residue was dissolved in 3 ml of 96% alcohol (Solution 1).

Transferred 1 ml of solution 1 to 50 ml volumetric flask. Added 1 ml of hydrous ammonia and made up the mixture volume with 96% alcohol to the mark. To 2 ml of the obtained solution add 10 ml of 96% alcohol and mixed.

Absorbance was measured on 302 nm by using 96% spirit as blank solution.

Total alkaloids were calculated as vasicine in percentage (X) through below formula:

$$X = \frac{10 \times 12 \times 50 \times 3 \times D \times 100}{2 \times 1 \times m \times 1000 \times 430} = \frac{900 \times D}{430 \times m}$$

where:

D = Optical density of the tested solution;
 m = weight of preparation in grams;
 430 = Specific parameter of vasicine absorption.

3.3. Determination of Vasicine by HPLC

3.3.1. Sample Preparation

About 12 g (exact weight) of preparation placed in a 100 ml dividing funnel. Added directly 20 ml of water, and 3 ml of hydrous ammonia, then carefully mixed the contents. The organics were extracted with 50 ml of chloroform (3 times in total). Combined chloroform extraction evaporated to dryness under vacuum. Dissolved the dry residue in 20 ml of mobile phase and transferred quantitatively into a 25 ml volumetric flask. The solution made up to the mark with mobile phase. Filtered the obtained solution through a filter with pore size 0.45 μm and used for chromatography.

3.3.2. Vasicine Standard Solution Preparation

Placed about 50 mg (exact weight) of RSO vasicine standard solution in a 50 ml volumetric flask and dissolved in mobile phase.

The volume of the solution was brought up to the mark with mobile phase. Placed 1 ml of the obtained solution in a 25 ml volumetric flask and diluted with mobile phase to the mark. Filtered the obtained solution if necessary. Used fresh solution.

Chemical analysis

HPLC was performed using Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA). Chromatographic separation and detection of samples were performed.

Chromatography conditions—liquid chromatograph with UV detector.

Column—chromatography column μ BondapakTM C18 10 μm 125°A 3.9 \times 300 mm or equivalent.

Mobile phase—degassed mixture consisting of acetonitrile: 0.1 M phosphate buffer: glacial acetic acid (15: 85: 1 v/v).

Flow rate—0.7 ml/min.

Wave length—300 nm.

Column temperature—room temperature.

Vasicine content in Linkus Syrup should not be less than 0.3 mg per 10 ml was calculated by the following formula:

$$X = \frac{A_{smp} \times W_{std} \times f \times Smp - \text{dilution} \times P \times D \times 10}{A_{std} \times W_{smp} \times Std - \text{dilution} \times 100}$$

f—0.838 conversion factor of vasicine Hydrochloride to vasicine

A_{SMP}—Mean value of peak area of tested solution samples

A_{STD}—Mean value of peak area of standard solution samples

W_{SMP}—Preparation weight, mg

W_{STD}—Standard weight, mg

P—Percent purity of standard sample, %

D—Density of syrup

3.3.3. Preparation of Phosphate Buffer

5.68 gm of sodium di-hydrogen phosphate and 3.63 gm potassium di-hydrogen phosphate transferred to 1000 ml volumetric flask, added 100 ml of water and mixed. Brought the solution volume to the mark with water [20]. Other parameters and norms of developed Linkus syrup were summarized in **Table 5**.

After evaluating the above parameters the preparation of Linkus syrup was established.

4. Results and Discussion

Standardization of biologically active material in the poly herbal formulation and quantitative analysis cover identification of chemical components where as quantitative assay measure the identification and level of bio-

Table 5. Different physico-chemical parameters.

Parameters	Method/Norms
Appearance	Brown color viscosity syrup with characteristic odor. Determination was conducted visually.
PH	3.0 - 6.0
Density	1.25 to 1.35 g/cm ³
<u>Originality</u>	
Polysaccharides	Qualitative reactions with: With of 96% spirit Should be precipitate suspension was formed
Tanning agents	With of Ferric chloride solution 3% Should be appears of greenish-yellow coloring
Ascorbic acid	With of spirit solution of sodium 2,6-dichlorofenolindofenolite Appeared reddish-violet coloring
Alkaloid vasicine determination	TLC, 1 reddish pink color spot (vasicine) was shown on the plate after treatment with Dragendorff reagent in UV light at wave length 254 nm
Methylparahydroxybenzoat Propylparahydroxybenzoat	HPLC, Periods of 2 main peaks retention should match to peaks retention periods of on standard solutions
<u>Quantitative determination</u>	
Total alkaloids	Spectrophotometric determination , Total alkaloids calculated as vasicine should not be less than 0.035% and not more than 0.07%
Methylparahydroxybenzoat Propylparahydroxybenzoat	HPLC, Methylparahydroxybenzoate 9.0 - 11.0 mg/10 ml of preparation Propylparahydroxybenzoate 1.8 - 2.2 mg/10 ml of preparation

marker in the extract establish the standard of that particular compound for validation. Linkus is a poly herbal formulation manufactured by Herbion Pakistan (Pvt.) Limited. In order for the standardization of the procedure of the finished product and there in the five different herbal drugs of different batches were analyzed for physico-chemical parameters. The drugs in the Linkus are *Glycyrrhiza glabra*, *Hyssopus officinalis*, *Piper longum* and *Alpinia galangal*. The formulation organoleptic characteristics such as identification by TLC, assay of alkaloid content, foreign matter, total ash, acid insoluble ash, alcohol; soluble extraction and water soluble extraction are adequately presented in **Table 2**. Thereafter the extraction of different herbal drugs is given in **Table 3**, giving details of quantity used, quantity of thick extract, extraction time taken and percentage yield of the extract. Preparation of manufacturing of the syrup is depicted in **Table 3**. Physico-chemical evaluation such as appearance, pH determination plays a significant role in the quality assessment. The identification with quantitative reaction and preparation of 0.1% alcohol solution exhibit the indication polarity concern of plant materials. TLC was done to augment the determination of alkaloids components and there in the identification of vasicine was located. All these values show that the physico-chemical parameters adopted the values were within the limits and repeated experiments confirmed the verification. HPLC was performed to confirm the quantitative and qualitative presence of vasicine for validation. The present standardization under taken reveal compliance with all the physico-chemical and analytical procedures, therefore it is concluded that Linkus syrup is well standardized product at the base line parameters.

If these analytical profiles compared with the work of Modi *et al.* where Zeal cough syrup employing physico-chemical test and bioassay marker compounds agree with quality and potency [21]. In another study Desai and co worker validated the, manufacturing procedure of Vasu cough syrup with the poly herbal formulation for compliance for their reproducibility [22]. The work done by Khadair and associates validation analysis of hederacoside C, the marker of Ivy plant and percentage recovery was given as 99.6% [23]. In a patent of cough syrup the extract of *Adhatoda vasica* along with *Hedychium* and *Curcuma* cough syrup has been validated in greater details [24]. Therefore the Linkus syrup standardization fall under the specific guide lines of quality herbal medicine and in line with different as mentioned earlier follow the prerequisite for global harmonization.

5. Conclusion

In this study for medicinal plants component, *Glycyrrhiza glabra*, *Hyssopus officinalis*, *Piper longum* and *Alpinia galangal* were formulated for the indication of anti-tussive activity. The physico chemical and organoleptic

parameter data so generated were recorded for standardization. TLC was carried out on the aqueous extract and Rf value (0.4) of vasicine as raddish pink spot on exposure to Dragendroff and UV was realized. Quantitative and spectrophotometric estimation of alkaloid was found between the range of 0.035% to 0.07%. Determination of vasicine as biomarker by HPLC in linkus syrup determined as 0.3 mg per 10 mL. The analytical profile of linkus syrup and there in vasicine have been validated to proved that linkus syrup in different batches confirmed the verification. Further study on Linkus syrup is being carried out to determine the efficacy as anti-tussive agent and will be reported elsewhere. The following animal model of cough was used to investigate the anti-tussive potential of Linkus. Healthy Wistar albino rats were treated orally with the recommended doses of Linkus. After 15 minutes, they were individually placed in a closed Plexiglass chamber (20 × 10 × 10 cm) and exposed to citric acid inhalation (0.1 g/ml) for 7 minutes. The cough reflexes were counted for the last 5 minutes and compared with those of the control animals. The results obtained showed good responses.

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