

HERBAL FORMULATION COMPRISING EXTRACTS OF ADHATODA, HEDYCHIUM AND CURCUMA AS  
COUGH SYRUP

### Field of Invention

The present invention relates to an herbal formulation comprising *Adhatoda zeylanica*, *Hedychium spicatum* and *Curcuma longa*. Also provided are processes for the  
5 isolation, detection, and quantification of the active phytochemicals found in the herbs.

### Background of the Invention

Coughing is an important protective reflex and plays a critical role in the physiological defense mechanism that protects the airways from noxious irritants and augments the muco-ciliary clearance of airway secretions. It is also a prime mechanism  
10 for clearing secretions from the lungs when the natural ciliary function is compromised by an acute or chronic infection.

A cough may be produced by inflammatory, mechanical, chemical and/or thermal stimulation of the cough receptors. An inflammatory cough response is often initiated by edema and hyperemia of the respiratory mucous membranes or by irritation from the  
15 exudative process. Such stimuli may arise either in the airways (as in laryngitis, tracheitis, bronchitis and bronchiolitis) or in the alveoli (as in pneumonitis and lung abscess).

Further, coughing is an important defense mechanism, in the form of an explosive expiration, for clearing secretions and inhaled extraneous substances from the tracheo-bronchial tree. It is associated with other protective processes such as broncho-  
20 constriction and mucus secretion. Coughing in itself is not a disease but is often the most visible symptom in a wide variety of diseased states ranging from mild upper respiratory tract infection to serious illnesses.

In summary, coughing has two primary functions: (1) to prevent foreign material from entering the lower respiratory tract and (2) to clear foreign material and excessive  
25 secretions from the lower respiratory tract. When coughing inadequately serves its protective roles, atelectasis, gas-exchange abnormalities, pneumonia and bronchiectasis may ensue.

The Ayurvedic system of Indian traditional medicine provides many formulations concerning cough related disorders. A majority of the plants have already been  
30 investigated for their beneficial medicinal properties.

In India, Ayurveda has carried out studies for many generations and recorded medicinal uses of plants for over 5000 years. In the ancient classical texts, a number of herbs have been advocated for cough treatment. For example, Vasaka (*Adhatoda zeylanica*); Tulsi (*Ocimum tenuiflorum*); Yashtimadhu (*Glycyrrhiza glabra*); Kapoorkachri  
 5 (*Hedychium spicatum*); Sunthi (*Zingiber officinale*); Pippali (*Piper longum*); Kantakari (*Solanum xanthocarpum*); Malayvacha (*Alpinia galanga*); Haridra (*Curcuma longa*); Karpoor (*Cinnamomum camphora*); Guggulu (*Commiphora wightii*); Draksha (*Vitis vinifera*); Jufa (*Hyssopus officinalis*); Guduchi (*Tinospora cordifolia*); Jatiphal (*Myristica fragrans*); Gojivha (*Onosma bracteatum*); Amalaki (*Emblica officinalis*); Haritaki  
 10 (*Terminalia chebula*); Vibhitak (*Terminalia bellirica*); Maricha (*Piper nigrum*); Vidanga (*Embelia ribes*); Twak (*Cinnamomum zeylanicum*); Brihati (*Solanum indicum*); Pushkarmool (*Inula racemosa*); Banafsha (*Viola odorata*); Ghrit-kumari (*Aloe vera*); Lavanga (*Syzygium aromaticum*); Talish (*Abies pindrow*); Karkatshringi (*Pistacia integerrima*); Kasamarda (*Cassia occidentalis*); Karchoor (*Curcuma zedoaria*); Bharangi  
 15 (*Clerodendrum serratum*); Dugdhika (*Euphorbia thymifolia*); Soma (*Ephedra gerardiana*); Tailaparna (*Eucalyptus globulus*); Sarala (*Pinus roxburghii*) etc.

While studying some of these medicinal plants the present inventors have come across three particular plants that have useful medicinal properties. These plants are *Adhatoda zeylanica*, *Hedychium spicatum* and *Curcuma longa* and have been indicated for  
 20 the treatment of cough related disorders.

### 1. *Adhatoda zeylanica*: Syn: *Adhatoda vasica*

This plant is found in the western and central regions of the sub-tropical Himalayas at an altitude of 1500-2000 m and is abundant in Punjab and Uttaranchal. It is a well-known plant drug of the Indian system of medicine and is used for the treatment of various  
 25 diseases, particularly of the respiratory tract (Claeson et al., J. Ethnopharmacology 72; 1-20, 2000). The plant is bitter, astringent, expectorant, febrifuge, styptic and tonic. Its leaves are especially used for irritable cough and in hemoptysis. The leaf-juice along with the roots is used in phthisis, cough, hemoptysis and asthma. It is used mainly as a bronchodilator and as an expectorant. In the classical texts of Ayurveda viz. Charak,  
 30 Sushruta, Vagbhatta, Bhava Prakash, it is mentioned as a remedy for treating cold, cough, whooping cough, chronic bronchitis, asthma, epistaxis, tuberculosis, spasms and as an anthelmintic. It is an official drug of the Ayurvedic Pharmacopoeia of India. It is

employed in different forms, such as fresh juice, decoction, infusion, powder, alcoholic extract, liquid extract and syrup.

The leaves contain a very small amount of an essential oil. The alkaloid content in the leaves is 0.2 - 0.4% and in the bark it is about 0.35% (The Wealth of India, Vol.1, 31-32 (1948)). The major quinazoline alkaloids are vasicine, vasicinine, vasicinol, vasicol, vasicoline, adhatodine, vasicolinone and anisotine.

It has been shown that the alcoholic extract of *A. vasica* displays a marked dose dependent anti-tussive activity when given orally to guinea pigs in which the cough was induced by irritant aerosol. The activity was found to be comparable with codeine, suggesting it to be a potent anti-tussive agent (Dhuley et al., J. Ethnopharmacology 67: 361-65, 1999). The crude alcoholic extract of *Vasaka*, when administered orally to rats, exhibited bronchodilatory and respiratory stimulant activity. The extract was found to be more potent than its pure alkaloids viz. vasicine and vasicinone in respiratory ailments (Rao et al., Indian Veterinary Journal 58; 107-11, 1981).

*Adhatoda* has been shown to contain the alkaloids vasicinone, vasicine and vasicinol. Chemical modification of vasicine resulted in 6,7,8,9,10,12-hexahydroazepino-{2,1-b}-quinaxoline-12-one which exhibited significant bronchodilatory activity similar to sodium cromoglycate and aminophylline in rats under systemic anaphylaxis. Semi-synthetic derivatives of vasicine including bromhexine and ambroxol are used as mucolytic agents because of their indirect effect on enhancement of lysozyme levels in bronchial secretions.

*Adhatoda* has also been said to possess anti-inflammatory activity. Of the four fractions tested for anti-inflammatory activity via methanol extract, non-alkaloidal fraction, saponins and alkaloids, the alkaloidal fraction showed the most potent activity, which was found to be comparable to hydrocortisone.

The alcoholic extract of *Adhatoda* was found to be non-lethal in rats even when given in doses of 4 gm/kg. The intra-peritoneal LD<sub>50</sub> of the extract was found to be 581 mg/kg.

## 2. *Hedychium spicatum* Ham ex Smith:

This plant is found throughout the year in the plains and sub-Himalayan tracts, ascending up to 1200 m.

The main active constituents of the *Hedychium spicatum* are Furanoid diterpene – Hedychenone, 7-hydroxy hedychenone which were isolated from rhizomes. Distilled from roots of *Hedychium Spicatum* it contains mainly p-methoxy cinnamic acid. The dried rhizomes are subjected to steam-distillation to yield its principal constituent, the ethyl ester of *p*-methoxy cinnamic acid. Besides the ester, the essential oil contains ethyl cinnamate, d-sabinene, cineole, sesquiterpene, sesquiterpene alcohols, some pentadecane and traces of cinnamaldehyde. Besides the essential oil, the rhizomes contain starch (52%), organic acids including resinic acid, a glycoside, and ash (4.6%).

In a clinical study fifteen patients having tropical pulmonary eosinophilia were given dried powdered rhizomes of *H. spicatum* at a dose of 12 grams twice daily for four weeks. This resulted in a 60.54% reduction in total absolute eosinophil count, which was found to statistically significant. Also 86.6% of the patients showed signs of improvement when assessed for symptoms like dyspnoea, cough with expectoration, dry cough, palpitation, headache and fever. The results were found to be better when compared to the other group of patients treated with Rasamanikya and Kanakasava (Shaw B.P.; Nagarjun, 214-16, 1980).

The ethanolic extract did not produce any signs of toxicity or mortality during 72 hour of observation period when given orally to mice in graded doses of 2.5 to 10 gm/kg (Tandan et al., Indian J Pharmaceutical Sciences. 148-50, 1997). The LD<sub>50</sub> of the hexane soluble and benzene soluble fractions of the ethanolic extract was found to be more than 1 gm/kg with no signs of mortality (Srimal et al., Indian J Pharmacology 143-47, 1984).

### 3. *Curcuma longa* Linn

This plant is a native of Southern Asia, but it is cultivated throughout all the States of India.

The major chemical constituents are curcuminoids (~6%), the yellow coloring principles of which curcumin constitutes 50-60%; essential oil 2-7% with a high content of bisabolane derivatives. Sesquiterpenes including curzerenone, curculone, curcumenol, pyrocurzerenone, turmerones are present in the rhizome in addition to desmethoxycurcumin, bidesmethoxycurcumin, dihydrocurcumin, phytosterols, fatty acids, phenolics and a noncyclic peptide turmerin, polysaccharides, viz., ukonan A, B, C and D

composed of arabinose, xylose galactose, glucose rhamnose, mannose and galacturonic acid.

The active agent in *Curcuma longa* has been identified as curcumin. In an in-vitro model using a hamster tracheal surface epithelial cell culture, curcumin was found to  
5 increase mucin release by directly acting on airway mucin secreting cells. This suggests a possible use as a mild expectorant (Lee et al., Planta Med 69; 523-26, 2003).

Curcumin has been found to inhibit cyclooxygenase-2 and nitric oxide synthase enzymes that mediate the inflammatory process. It was also found to strongly suppress tumour promotion. Curcumin has shown beneficial anti-oxidant properties. Powder of  
10 *Curcuma longa*, when administered to patients with various respiratory diseases, was found to offer relief of symptoms like dyspnoea, cough and sputum.

There are many cough syrups of both natural and synthetic origin available in the market. Acute cough and chronic cough are symptoms of conditions that may require medical attention. Generally, the cough syrups of synthetic origin produce good results but  
15 are always accompanied by one or the other side effects like drowsiness, sedation, dryness of mouth and respiratory tract and at times can be habit forming.

The drugs, which are used in the symptomatic treatment of the cough, are pharyngeal demulcents, expectorants, anti-tussives and bronchodilators. An ideal cough formulation should: (1) liquify the thick mucous and at the same time should help in  
20 expectorating it out, (2) sooth the irritated respiratory mucosa, (3) stop the irritating dry cough, (4) protect the respiratory tract from allergens and (5) improve the immunity of the respiratory tract.

#### Summary of the Invention

In one general aspect there is provided a herbal pharmaceutical formulation that includes  
25 extracts of Adhatoda, Hedychium and Curcuma, or a mixture of active ingredients that have been extracted from these herbs, and one or more pharmaceutically acceptable carriers and/or diluents.

Embodiments of the pharmaceutical formulation may include one or more of the following features. For example, the vasicine may make up from about  $17.44 \times 10^{-3}$  to about  $15.06 \times 10^{-3}$  % w/v, the ethyl p-methoxycinnamate may make up from about  $10.20 \times 10^{-3}$  to  
30 about  $9.02 \times 10^{-3}$  % w/v, and the curcuminoids may make up from about  $10.1 \times 10^{-3}$  to about  $8.47 \times 10^{-3}$  % w/v. In the formulation, the Adhatoda extract may make up from about 1.0 % w/v

to about 5.0 % w/v, the Hydechium extract may make up from about 0.1 % w/v to about 0.6 % w/v, and the Curcuma extract may make up from about 0.1 % w/v to about 0.7 % w/v.

The pharmaceutical formulation may be intended for oral use as a cough syrup.

In another general aspect, the pharmaceutical formulation may be prepared by:

- 5           a.       mixing a therapeutically effective amount of Adhatoda, Hedychium and Curcuma to get a combined extract;
- b.       adding one or more pharmaceutically acceptable carriers and/or diluents to the combined extract to form a final mixture; and
- c.       clarifying the final mixture.

10           Embodiments of the process of making the pharmaceutical formulation may further include detecting and quantifying the curcumin in the pharmaceutical formulation. The detecting and quantifying may include:

- a.       preparing a reference standard stock solution in an organic solvent;
- b.       preparing standard curves by using the solution of step a) and a blank organic  
15    solvent;
- c.       measuring the absorptions of the standard stock solution and a blank organic solvent at particular wavelengths after step b); and
- d.       calculating the percentage curcuminoids in the formulation after step c).

The wavelength for measuring absorption may be  $\lambda_{\max}$  418 nm.

20           The preparation of the pharmaceutical formulation may still further include detecting and quantifying vasicine and ethyl p-methoxycinnamate in the pharmaceutical formulation. The detecting and quantifying may include:

- a.       extracting the formulation with one or more organic solvents or mixtures thereof optionally containing water or base;
- 25           b.       concentrating the extracts of step a) and reconstituting the residue in second organic solvent;
- c.       spotting the solution obtained in step b) against a standard phytochemical marker on a HPTLC plate;

- d. developing the HPTLC plate of step c) in a mobile phase;
- e. optionally spraying or applying the detection reagent on the HPTLC plate of step d);
- f. scanning the plate of step e) at particular wavelengths; and
- 5 g. detecting and quantifying vasicine and ethyl p-methoxycinnamate after step f).

In another general aspect there is provided a process for the isolation of the phytochemical marker compound vasicine from *Adhatoda zeylanica*. The process of isolation include:

- a. extracting a selected powdered part of the plant with an organic solvent and then concentrating the extract to form a concentrate;
- 10 b. acidifying the concentrate and extracting the concentrate with a second organic solvent;
- c. basifying the concentrate and extracting the concentrate with the second organic solvent;
- d. concentrating the organic extracts; and
- 15 e. isolating vasicine.

Embodiments of the process may include one or more of the following features. For example, the isolation may be by crystallization. The organic solvent may be water soluble lower alkanols. The water soluble alkanols may be one or more of methanol, ethanol, n-propanol, isopropanol and mixtures thereof. The acidification of the concentrate may be performed with an aqueous organic or inorganic acid. The acidification may be performed with one or more of

20 sulphuric acid, hydrochloric acid, acetic acid, formic acid, phosphoric acid, nitric acid and mixtures thereof. The second organic solvent may be one or more of non-polar aliphatic, cycloalkyl solvents, ethers, esters, chlorinated hydrocarbons, ketones and mixtures thereof. The basification of the concentrate may be performed with one or more of ammonia, ammonium

25 hydroxide, triethylamine, dimethylamine, sodium hydroxide, potassium hydroxide, sodium carbonate, potassium carbonate, sodium bicarbonate, potassium bicarbonate, calcium carbonate, calcium hydroxide and mixtures thereof.

The process further include identifying vasicine in the extract. The identifying includes:

- a. after extracting the selected powdered part of the plant with an organic solvent, optionally filtering to form a test solution;
- b. applying a reference standard and test solution to a chromatoplate;
- c. developing the chromatoplate in a mobile phase;
- 5 e. drying the chromatoplate and spraying it with a spraying/developing agent; and
- f. identifying vasicine.

The process may still further include estimating the total alkaloid content in the *Adhatoda* extract by:

- a. after extracting the selected powdered part of the plant with an organic solvent,
- 10 acidifying the extract with an acid solution, refluxing, and filtering to form a filtrate;
- b. extracting the filtrate with an organic solvent to remove the organic compounds from the aqueous extract;
- c. basifying the aqueous extract with a base;
- d. extracting the solution with the organic solvent;
- 15 e. concentrating/evaporating the solution to form a residue;
- f. drying the residue; and
- g. estimating the total alkaloid content.

In another general aspect there is provided a process for the isolation of the phytochemical marker compounds ethyl p-methoxycinnamate and curcumin from the plants *Hedychium* spicatum and *Curcuma longa*, respectively. The process of isolation includes:

- a. extracting the selected powdered part of the plant with an organic solvent optionally containing water;
- b. concentrating the extracts;
- c. extracting the concentrated extracts with a second organic solvent and, optionally,
- 20 filtering the solution to remove the non-polar impurities;
- d. purifying the resultant organic extracts by column chromatography; and
- e. detecting/isolating the phytochemical markers.

Embodiments of the process of isolating may include one or more of the following features. For example, the isolation of the phytochemical markers may be by crystallization. The organic solvent may be water soluble lower alkanols. The second organic solvent may be one or more of non-polar aliphatic, cycloalkyl solvents, ethers, esters, chlorinated hydrocarbons, ketones and mixtures thereof.

The process may further include identifying the ethyl p-methoxycinnamate or curcumin in the extract. The identifying includes:

- a. after extracting the selected powdered part of the plant with an organic solvent, optionally filtering to form a test solution;
- 10 b. applying a reference standard and the test solution to a chromatoplate;
- c. developing the chromatoplate in a mobile phase;
- d. drying the chromatoplate and spraying it with a spraying/developing agent; and
- e. identifying p-methoxycinnamate or curcumin.

The process may further include estimating the curcuminoid content in the Curcuma extract. The estimating includes:

- a. after extracting the selected powdered part of the plant with an organic solvent, mixing the extract with an organic solvent;
- b. filtering the solution of step a);
- c. measuring the absorbance of the solution of step b) against an organic solvent as a
- 20 blank and then calculating the curcuminoids content.

The process of estimating may include one or more of the following features. For example, the organic solvent may be one or more of non-polar aliphatic, cycloalkyl solvents, ethers, esters, chlorinated hydrocarbons, ketones, lower alkanols and mixtures thereof. The absorbance may be measured at a wave length of 418 nm.

25 The details of one or more embodiments of the inventions are set forth in the description below. Other features, objects and advantages of the inventions will be apparent from the description and claims.

### Description of the Drawings

**Fig. I** shows TLC photograph of standard Vasicine against five different experimental batches of cough syrup.

**Fig. II** shows TLC photograph of standard ethyl p-methoxycinnamate against  
5 seven different experimental batches of cough syrup.

**Fig. III** shows TLC photograph of standard Curcumin against seven different experimental batches of cough syrup.

**Fig. IV** shows HPTLC chromatogram of Vasicine extracted from the syrup against standard Vasicine.

10 **Fig. V** shows HPTLC chromatogram of ethyl p-methoxycinnamate extracted from the syrup against Standard ethyl p-methoxycinnamate.

### Detailed Description of the Invention

The present inventors have a reasoned belief that three indigenous herbs, Adhatoda, Curcuma and Hydechium, are time-proven for their role in cough and related  
15 disorders and are known to enhance the immunity as well. In combination, they will provide a better management of cough. All the herbs are believed to possess the property of 'Kasahara' (relieve cough) and also are believed to be useful in 'Shwaasa' (asthma). *A. vasica* is said to promote good voice and is especially used as '*Kapha-nihsaaraka*' (expectorant) and as a '*Kaphaghna*' (mucolytic) agent. It also has a specific property of  
20 '*Chedana*' (scraps and liquefies the thick viscid cough) along with being a good '*Kapha-nihsaaraka*' (expectorant) drug. *C. longa* is '*Deepana*' i.e. aids in the digestion and is also '*Vishaghna*' (detoxifying), '*Shothahara*' (anti-inflammatory); '*Raktashodhaka*' (blood purifying) and '*Vrana-shodhana*' (antiseptic and disinfectant). *H. spicatum* is '*Kaphaghna*' (mucolytic), '*Vedanaasthapana*' (analgesic) and '*Jwaraghna*' (anti-pyretic).

25 These herbs have been shown to possess significant effects in etiology and symptoms of cough and, keeping in mind the desirable effects and synergism of herbs, the formulation has been conceptualized to serve as an ideal cough formulation. It has been conceived not only for providing symptomatic relief from cough but also to enhance the host-defense mechanism in order to strengthen the immune system. The formulation can

be used in at least the following conditions: (1) allergic cough, (2) dry irritating cough, (3) productive cough and (4) smoker's cough

The cough syrup of the present invention contains select herbs, along with throat soothers, which provide quick relief from coughs and prevent reoccurrence. Also the  
5 formulation is alcohol free, resulting in no drowsiness.

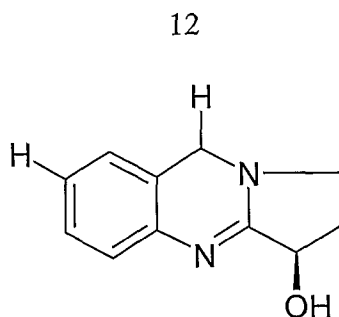
Ayurvedic medicines or herbal formulations are often criticized by the Western world and by regulatory agencies for the fact that there is no standardization or quantification of active principles in the dosage form. The present inventors have also provided a method of standardizing the formulation by adopting certain strict criterion for  
10 selecting raw materials, processing of the extraction of the herbs and manufacturing of the formulation. In this context, the present inventions provide a method of quantification of the active phytochemicals present in the formulation.

The present invention relates to a new herbal formulation which has been found to be effective in treatment and management of cough. The formulation of present invention  
15 ensures not only symptomatic relief from cough but also to enhance the host-defense mechanism in order to strengthen the immune system and at the same time is devoid of toxic side effects. Also provided is a process for the preparation and quantitative analysis of the individual phytochemicals used in the herbal formulation.

The combination of herbs has been selected on the basis of classical Ayurvedic  
20 literature survey and research studies conducted till date to work synergistically against coughs of varied etiologies.

The herbal pharmaceutical formulation includes extracts of *Adhatoda*, *Hedychium* and *Curcuma* or a mixture of their respective active ingredients that have been extracted along with one or more pharmaceutically acceptable excipients.

25 It has now been found that the active ingredients of the herbs can be isolated and quantified. The process for the isolation of the phytochemical marker compound-Vasicine, as denoted by Formula I,

**FORMULA I**

from *Adhatoda zeylanica*, includes:

- a) extracting the selected powdered part of the plant with a suitable organic solvent,  
optionally containing water, and then concentrating the extract;
- b) acidifying the concentrate of step a) and extracting it with second organic solvent;
- c) basifying the concentrate obtained in step b) and extracting it with second organic  
solvent;
- d) concentrating the organic extracts obtained in step b) and c); and
- e) isolating the phytochemical marker, which may optionally be completed by  
crystallization.

Vasicine is present in leaves of *Adhatoda zeylanica*. The leaves of *Adhatoda zeylanica* should be crushed and powdered so that the particle size of the powder is such that not less than (NLT) 95% of the particles pass through a size #30 mesh screen. This powder is extracted three times with an organic solvent.

Suitable organic solvents include one or more of water soluble lower alkanols, such as methanol, ethanol, n-propanol and isopropanol and mixtures thereof at a temperature between 35°C to 80°C. The powder is extracted at the reflux temperature of the solvent used. The combined extract is then concentrated under reduced pressure. The concentrated extract is then suspended in an acid solution, which may be optionally aqueous in nature. Suitable acid solutions may be organic or inorganic and include one or more of sulphuric acid, hydrochloric acid, acetic acid, formic acid, phosphoric acid, nitric acid and mixtures thereof.

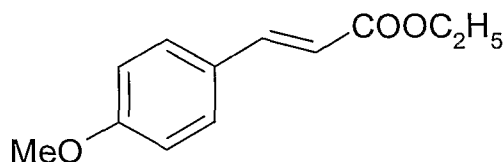
The solution is then repeatedly partitioned with a second organic solvent. Suitable second organic solvents include one or more of non-polar aliphatic or cycloalkyl solvents, such as hexane, heptane, petroleum ether, cyclohexane; ethers such as diethyl ether, diisopropyl ether; esters, such as ethyl acetate, methyl acetate, ethyl formate, methyl

formate, isobutyl acetate, n-butyl acetate; chlorinated hydrocarbons, such as methylene chloride, ethylene chloride, chloroform and carbon tetrachloride; ketones, such as acetone, ethyl methyl ketone, diisobutyl ketone, methyl isobutyl ketone; and mixtures thereof.

The organic phase is separately collected and the aqueous phase is basified with the base. Suitable bases include one or more of ammonia, ammonium hydroxide, triethylamine, dimethylamine, sodium hydroxide, potassium hydroxide, sodium carbonate, potassium carbonate, sodium bicarbonate, potassium bicarbonate, calcium carbonate, calcium hydroxide and mixtures thereof.

The basified aqueous phase is repeatedly partitioned with the second organic solvents described above and the collected organic phases are combined. The combined organic layer obtained is then freed from traces of water by passing it through an anhydrous sodium sulphate. The organic solvent is recovered under reduced pressure to get a solid light brown residue, which is crystallized from the organic solvent discussed above.

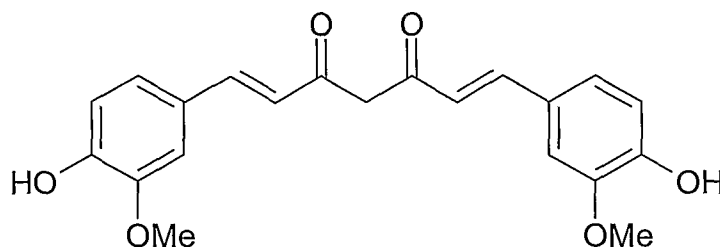
The process for the isolation of the phytochemical marker compounds ethyl p-methoxycinnamate of Formula II



**Formula II**

20

or curcumin of Formula III



**Formula III**

from the plants *Hedychium spicatum* and *Curcuma longa* respectively, includes:

- a) extracting the selected powdered part of the plant with a suitable organic solvent, which may optionally contain water;
- b) concentrating the extracts obtained in step a);
- c) extracting the concentrated extracts of step b) with a second organic solvent, and if  
5 required, filtering the solution to remove the non-polar impurities;
- d) purifying the resultant organic extracts of step c) by column chromatography; and
- e) detecting and isolating the phytochemicals markers, optionally by crystallization, after step d).

Ethyl p-methoxycinnamate is present in the rhizomes of *Hedychium spicatum*.

- 10 The rhizomes of *Hedychium spicatum* should be powdered to a particle size wherein not less than 95% material should pass through size #30 mesh. This is extracted three times with an organic solvent. Suitable organic solvents include one or more of water soluble lower alkanols, optionally containing water, such as methanol, ethanol, n-propanol and isopropanol or mixtures thereof at a temperature between 35 to 80°C. The powder is  
15 extracted at the reflux temperature of the solvent used.

- The combined extracts are then concentrated under reduced pressure. The concentrated extracts are then mixed with water and the aqueous layer is washed with second organic solvents. Suitable second organic solvents include one or more of non-polar aliphatic or cycloalkyl solvents, such as hexane, heptane, petroleum ether,
- 20 cyclohexane; ethers, such as diethyl ether, diisopropyl ether; esters, such as ethyl acetate, methyl acetate, ethyl formate, methyl formate, isobutyl acetate, n-butyl acetate; chlorinated hydrocarbons, such as methylene chloride, ethylene chloride, chloroform and carbon tetrachloride; ketones, such as acetone, ethyl methyl ketone, diisobutyl ketone, methyl isobutyl ketone; and mixtures thereof.

- 25 The organic washings are pooled together, dried over a suitable drying agent and filtered. The organic extracts are further concentrated under reduced pressure and subjected to column chromatographic purification through a silica gel column and eluted with the second organic solvents in the increasing order of polarity. The fractions are collected separately. The fractions are scanned for ethyl p-methoxycinnamate presence by  
30 Thin Layer Chromatography (hereinafter referred to as TLC) using chloroform: methanol :: 9:1 mobile phase and Anisaldehyde in sulphuric acid as a developing spray. The fractions

having ethyl p-methoxycinnamate as observed by its TLC pattern are combined and concentrated under vacuum. The crude ethyl p-methoxycinnamate is then crystallized from the second organic solvent. The identity of this phytochemical was further confirmed through spectroscopic data generated.

5 Curcumin is found in the rhizomes of *Curcuma longa*. The rhizomes are powdered so the particle size of the powder is such that not less than 95% material should pass through a size #30 mesh. This powder is extracted in the similar manner as described in the case of ethyl p-methoxycinnamate. The concentrated extract is then subjected to column chromatographic purification through silica gel 100-200 mesh column and eluted  
10 with second organic solvents in the increasing order of polarity. The fractions are collected separately. The fractions are scanned for the presence of curcumin by TLC using chloroform:methanol::95:5 mobile phase and detection is done under a UV / Fluorescence detector. The fractions having curcumin as observed by TLC pattern are combined and concentrated under vacuum. The crude curcumin is then crystallized from the solvent  
15 selected as the second organic solvent.

Also provided is a method of identification of the active phytochemicals vasicine, ethyl p-methoxycinnamate and curcumin from their respective plant extracts. The method includes:

- a. applying the reference standard and test solution to the chromatoplate;
- 20 b. developing the chromatoplate of step (a) in a suitable mobile phase;
- c. drying the chromatoplate of step (b) and if required, spraying it with suitable spraying/developing reagent; and
- d. identifying vasicine, ethyl p-methoxycinnamate or curcumin after step (c).

The reference standard solution is prepared by dissolving vasicine, ethyl p-methoxycinnamate or curcumin separately in an organic solvent and optionally filtering the  
25 solution.

The test solution is prepared by dissolving the extract of Adhatoda, Hedychium or curcuma separately in an organic solvent and optionally filtering the solution.

Suitable organic solvents include one or more of lower alkanols, such as methanol,  
30 ethanol, n-propanol, isopropanol and mixtures thereof.

Suitable solvents used for the mobile phase include one or more of methanol, ethanol, n-propanol, isopropanol, hexane, heptane, petroleum ether, cyclohexane, diethyl ether, diisopropyl ether, ethyl acetate, methyl acetate, ethyl formate, methyl formate, isobutyl acetate, n-butyl acetate, methylene chloride, ethylene chloride, chloroform, carbon tetrachloride, acetone, ethyl methyl ketone, diisobutyl ketone, methyl isobutyl ketone, 1,4-dioxane, toluene, ammonia solution, glacial acetic acid and mixtures thereof.

Suitable spraying/developing reagents include one or more of anisaldehyde-sulphuric acid reagent, glacial acetic acid, sulphuric acid, dragendorff's reagent and mixtures thereof.

The method of identification of vasicine, ethyl p-methoxycinnamate and curcumin in their respective plant extracts is performed through thin layer chromatography using a silica gel 60 F<sub>254</sub> pre-coated Aluminium foil plate with layer thickness 0.2 mm (1.05554, Merck). Dragendorff's and Anisaldehyde in sulphuric acid are used as spraying or detecting reagent for Vasicine and ethyl p-methoxycinnamate respectively. In case of curcumin, evaluation of chromatographic plate is done under visible/fluorescent light. For this purpose a standard solution of marker compounds is used against which the extracts are spotted on TLC plate. The mobile phase is prepared using 1,4-dioxane:toluene: methanol:ammonia in a ration of 25:01:01:0.5 for Vasicine and hexane:acetone in a ratio of 80:20 for ethyl p-methoxycinnamate. Chloroform:ethyl acetate:glacial acetic acid in a ratio of 95:5:1 is used as the mobile phase for curcumin. The photograph of the developed TLC after scanning is taken using Camag's photodocumentation unit in visible/fluorescent light.

Also provided is a method for the estimation of total alkaloids content in Adhatoda extracts. The method includes:

- a. acidifying the plant extract with an acid solution, optionally containing water, refluxing and filtering it;
- b. extracting repeatedly the filtrate of step a) with an organic solvent to remove the organic components from the aqueous extract;
- c. basifying the combined aqueous extract of step b) with a base;
- d. extracting the solution of step c) with an organic solvent; and
- e. concentrating/evaporating and then drying the product of step d) for estimating the total alkaloid content.

The acid solution used may optionally be aqueous in nature. Suitable acid solutions may be organic or inorganic including one or more of sulphuric acid, hydrochloric acid, acetic acid, formic acid, phosphoric acid, nitric acid and mixtures thereof.

5            Suitable organic solvents include one or more of non-polar aliphatic and cycloalkyl solvents, such as hexane, heptane, petroleum ether, cyclohexane; ethers, such as diethyl ether, diisopropyl ether; esters, such as ethyl acetate, methyl acetate, ethyl formate, methyl formate, isobutyl acetate, n-butyl acetate; chlorinated hydrocarbons, such as methylene chloride, ethylene chloride, chloroform and carbon tetrachloride; ketones, such as acetone,  
10    ethyl methyl ketone, diisobutyl ketone, methyl isobutyl ketone; and mixtures thereof.

Suitable bases include one or more of ammonia, ammonium hydroxide, sodium hydroxide, calcium hydroxide, calcium carbonate, potassium hydroxide, potassium carbonate and mixtures thereof.

For example, the Adhatoda extract may be mixed with hydrochloric acid and  
15    refluxed on a water bath for a half an hour and then diluted with water. The resulting mass is then filtered through a Whatman No. 41 filter paper. The clear filtrate is extracted with chloroform for a minimum of four times and the chloroform extract is discarded. The aqueous layer is then basified with a dilute ammonia solution (up to pH 9-10) and extracted with chloroform. The chloroform extracts are then dried and evaporated in a pre-weighed  
20    beaker to dryness on a water bath. The extracts are dried in an oven for 1 hour at a temperature of 105°C and then cooled in a desiccator and weighed.

The percentage Alkaloid content can be calculated by using the following formula:

$$\begin{array}{rcccl} & \text{Wt. of residue} & 100 & & \\ & \text{\% Alkaloids} = & \text{-----} & \times & \text{-----} \times 100 \\ & & \text{Wt. of sample} & (100- \text{LOD}) & \end{array}$$

LOD equals the loss on drying.

Also provided is a method for the estimation of the curcuminoid content in a Curcuma extract. The method includes:

- a. mixing the extract with an organic solvent;
- 30    b. filtering the solution of step a); and

- c. measuring the absorbance of the solution of step b) against the organic solvent as a blank and then calculating the curcuminoid content.

Suitable organic solvents includes one or more of non-polar aliphatic or cycloalkyl solvents, such as hexane, heptane, petroleum ether, cyclohexane; ethers, such as diethyl ether, diisopropyl ether; esters, such as ethyl acetate, methyl acetate, ethyl formate, methyl formate, isobutyl acetate, n-butyl acetate; chlorinated hydrocarbons, such as methylene chloride, ethylene chloride, chloroform and carbon tetrachloride; ketones, such as acetone, ethyl methyl ketone, diisobutyl ketone, methyl isobutyl ketone; lower alkanols, such as methanol, ethanol, n-propanol, isopropanol and mixtures thereof.

- 10 The organic solvent used as a blank for measuring the absorbance in step c) represents the organic solvent alone (e.g. without plant extract).

To the curcuma extract, methanol is added and the solution is sonicated in an ultrasonic water bath. The solution is filtered through a Whatman No. 41 filter paper and the absorbance of the solution is measured at 425 nm using methanol as blank.

- 15 The total curcuminoids are calculated by using Formula given below:

$$\% \text{ Curcuminoids content} = \frac{\text{ASPL} \times 1250}{1560 \times \text{wt. of sample}}$$

ASPL equals the absorbance of sample at 425 nm.

- 20 Also provided is a process for the preparation of a herbal pharmaceutical formulation which comprises extracts of *Adhatoda*, *Hedychium* and *Curcuma* or a mixture of active ingredients that have been extracted from these herbs with pharmaceutically acceptable carriers and/or diluents. The process includes:

- a. mixing the individual extract of *Adhatoda*, *Hedychium* and *Curcuma* in a  
25 therapeutically effective amount to get a combined extract;  
b. adding one or more pharmaceutically acceptable carriers and/or excipients; and  
c. clarifying the final mixture by a suitable means if required.

The extracts may be mixed at a concentration range of: *Adhatoda* extract containing vasicine in a quantity of about 10 mg/100 ml to about 5000 mg / 100 ml;

- 30 *Hedychium* extract containing ethyl p-methoxycinnamate in a quantity of about

10 mg/100 ml to about 5000 mg/100 ml; and Curcuma extract containing curcumin in a quantity of about 10 mg/100 ml to about 5000 mg/100 ml. The extracts are mixed together and one or more pharmaceutically acceptable carriers are added.

Suitable pharmaceutically acceptable carriers include one or more of water  
5 miscible pharmaceutically acceptable co-solvents comprising propylene glycol, sorbitol, glycerol, polyethylene glycol and mixtures thereof. Purified water may also be added to the extract. The resultant mixture is then optionally filtered through a Sparkler Filter/centrifuge to remove any insoluble foreign particulate matter. To the clear solution obtained pharmaceutically acceptable preservatives may be added.

10 Suitable pharmaceutically acceptable preservatives include one or more of propyl paraben, methyl paraben, and quaternary ammonium salts, such as cetrimide and mixtures thereof along. Optionally, one or more suitable flavoring agents and sequestering agents may be added.

The formulation can be made palatable by adding to it one or more sweetening  
15 agents. Suitable sweetening agents include one or more of invert sugar syrup, xylitol, mannitol, sugar syrup, jaggery, honey and maltose syrup. Non-sugar based sweetening agents may also be used.

The syrup obtained is then finally filtered through a size #100 mesh nylon cloth to  
20 remove the foreign particulate matter. The oral syrup can be then packed in a suitable container and stored in a cool, dry place away from direct sunlight.

The invention also provides a method for the detection and quantification of  
vasicine and ethyl p-methoxycinnamate in a herbal pharmaceutical formulation, which  
comprises extracts of *Adhatoda*, *Hedychium* and *Curcuma* or a mixture of active  
ingredients that have been extracted from these herbs with pharmaceutically acceptable  
25 carriers and/or diluents. The method includes:

- a. extracting the formulation with an organic solvent, optionally containing water or base;
- b. concentrating the extracts of step a) and reconstituting the residue in a second organic solvent;
- 30 c. spotting the solution obtained in step b) against a standard phytochemical marker on a HPTLC plate;

- d. developing the HPTLC plate of step c) in a mobile phase;
- e. optionally spraying or applying the detection reagent on the HPTLC plate of step d);
- f. scanning the plate of step e) at particular wavelengths; and
- g. detecting and quantifying Vasicine and ethyl p-methoxycinnamate, after step f).

5           The oral syrup prepared above is further diluted with distilled water and extracted with an organic solvent. The extracts in organic solvent are dried over a suitable drying agent and then concentrated under a vacuum to remove the organic solvent. The residue obtained is reconstituted in an aliquote of the second organic solvent. The test solution is spotted against standard phytochemical marker solutions on HPTLC plates and the plates  
10       are allowed to develop in a saturated chamber containing a mobile phase. After complete development, the plates are optionally sprayed with a solution of spray reagents and dried in oven at 105°C for about 5 minutes. The plates are then scanned at various wavelengths to detect and quantify the phytochemical markers present in the formulation against standard phytochemical markers.

15           Suitable organic solvents include one or more of alkanols, such as methanol, ethanol, n-propanol, isopropanol, n-butanol, isobutanol, tert-butanol and n-octanol; ketones selected from acetone, ethyl methyl ketone, methyl isobutyl ketone and diisobutyl ketone; esters selected from methyl formate, ethyl formate, methyl acetate, ethyl acetate, n-propyl acetate, isopropyl acetate, n-butyl acetate, isobutyl acetate and tert-butyl acetate;  
20       chlorinated hydrocarbons selected from methylene chloride, chloroform, carbon tetrachloride and ethylene chloride; aromatic hydrocarbons selected from xylene, toluene, benzene, substituted benzene, substituted toluene and substituted benzyl; aliphatic hydrocarbons, such as hexane, heptane and petroleum ether; cycloalkyl hydrocarbons, such as cyclopentane, cyclohexane, cycloheptane, substituted cyclohexane and substituted  
25       cycloheptane; ether selected from tetrahydrofuran, 1,4-dioxane, diethyl ether, diisopropyl ether, methyl tert-butyl ether, acetonitrile and mixtures thereof.

          A suitable second organic solvent includes one or more of methanol, ethanol, isopropanol, n-propanol, acetone, acetonitrile, diethyl ether, N,N-dimethylformamide, N,N-dimethylacetamide, dimethylsulphoxide, tetrahydrofuran and mixtures thereof.

30           The standard phytochemical marker solutions are prepared by dissolving a known quantity of each of vasicine and ethyl p-methoxycinnamate separately in a known quantity

of the second organic solvent mentioned above. The solution may be sonicated and then made up to a desired fixed volume using the same solvent. These standard solutions are used as reference standard solutions.

Suitable spraying reagents include one or both of Dragendorff's reagent and  
 5 anisaldehyde sulphuric acid reagent. For example, the spraying reagent may be Dragendorff's reagent for the detection of Vasicine and Anisaldehyde sulphuric acid reagent for the detection of ethyl p-methoxycinnamate.

HPTLC was performed using a silica gel 60 F<sub>254</sub> pre-coated Aluminium foil plate with a layer thickness of 0.2 mm (1.05554, Merck). The reference and test solutions are  
 10 applied on the plates in a fixed volume using a hypodermic syringe. The distance between two spots is kept at about 10 mm having a band length of about 8 mm. The mobile phase used can be selected from a mixture of solvents described as first organic solvent. For example, the mobile phase may be 1,4- dioxane: toluene:methanol: ammonia present at a ratio of 50:2:2:1 for the detection of vasicine or chloroform:acetone present at a ratio of 80  
 15 : 20 for the detection of ethyl p-methoxycinnamate.

For the purpose of developing HPTLC plates, the mobile phase is placed in a glass chamber having dimensions of 20 cm x 10 cm. After saturating the glass chamber with the mobile phase for about 60 minutes, HPTLC plates are placed into the chamber and the development distance of 8 cm from point of application is selected. After complete  
 20 development, the plate was removed from the chamber and dried using hot air blower.

The plate is scanned at two wavelengths; 292 nm for the determination of vasicine and 310 nm for determination of ethyl p-methoxycinnamate using Camag's High Performance Thin Layer Chromatographic system equipped with Linomat V and Photo documentation unit.

25 The content of vasicine is calculated by using Formula I provided below:

$$\% \text{ Content of Vasicine} = \frac{A_{\text{SPL}}}{A_{\text{STD}}} \times \frac{D_{\text{STD}}}{D_{\text{SPL}}} \times 100$$

The content of ethyl p-methoxycinnamate is calculated by using Formula II provided below:

$$\begin{array}{l} \text{\% Content of Ethyl p-methoxycinnamate} \\ 5 \end{array} = \frac{A_{\text{SPL}}}{A_{\text{STD}}} \times \frac{D_{\text{STD}}}{D_{\text{SPL}}} \times 100$$

Wherein,

$A_{\text{SPL}}$  = Area of sample corresponding to vasicine or ethyl p-methoxycinnamate

$A_{\text{STD}}$  = Area of Standard solution of vasicine or ethyl p-methoxycinnamate

$D_{\text{STD}}$  = Dilution of reference standard solution

10  $D_{\text{SPL}}$  = Dilution of test sample

The TLC plate, after spraying with spray reagent and drying, can be photographed using the Camag's photodocumentation unit. The TLC is shown here as Figure I and Figure II in the accompanied drawing.

15 The HPTLC chromatograms of two different phytochemical markers are shown as Figure IV (Vasicine) and Figure V (Ethyl p-methoxycinnamate).

Also provided is a method for the detection and quantification of curcumin in a herbal pharmaceutical formulation, which comprises extracts of *Adhatoda*, *Hedychium* and *Curcuma* or a mixture of active ingredients that have been extracted from these herbs with  
20 pharmaceutically acceptable carriers and/or diluents. The method includes:

- a. preparing a reference standard stock solution in an organic solvent;
- b. preparing standard curves by using the solution of step a) and a blank organic solvent;
- c. measuring the absorptions of standard stock solution and a blank organic solvent at  
25 particular wavelengths after step b); and
- d. calculating the % age curcuminoids in a formulation after step c).

The reference standard stock solution is prepared by dissolving curcuminoids in an organic solvent.

Suitable organic solvents include one or more of non-polar aliphatic or cycloalkyl  
30 solvents, such as hexane, heptane, petroleum ether, cyclohexane; ethers, such as diethyl

ether, diisopropyl ether; esters, such as ethyl acetate, methyl acetate, ethyl formate, methyl formate, isobutyl acetate, n-butyl acetate; chlorinated hydrocarbons, such as methylene chloride, ethylene chloride, chloroform and carbon tetrachloride; ketones, such as acetone, ethyl methyl ketone, diisobutyl ketone, methyl isobutyl ketone; lower alkanols, such as  
 5 methanol, ethanol, n-propanol, isopropanol, n-butanol; 1,4-dioxane, toluene, ammonia solution, ammonium hydroxide and mixtures thereof.

The blank organic solvent that is used for measuring the absorptions in step c) represents the organic solvent alone or without plant extract.

The content of curcumin is calculated by using Formula III provided below:

$$10 \quad \% \text{ Content of Curcumin} = \frac{A_{\text{SPL}}}{W_{\text{SPL}}} \times 100$$

Wherein

$A_{\text{SPL}}$  = Concentration of Test sample against working curve

15  $W_{\text{SPL}}$  = Qty. of Sample taken in ml.

The results of three different batches of cough syrup are shown below in Table 1:

**Table 1: Content of Phytochemical markers in the oral syrup**

| Expt. No. | % w/v of Vasicine      | % w/v of Ethyl p-methoxycinnamate | % w/v of Curcumin      |
|-----------|------------------------|-----------------------------------|------------------------|
| I         | $17.44 \times 10^{-3}$ | $9.17 \times 10^{-3}$             | $10.10 \times 10^{-3}$ |
| II        | $15.17 \times 10^{-3}$ | $9.02 \times 10^{-3}$             | $8.47 \times 10^{-3}$  |
| III       | $15.06 \times 10^{-3}$ | $10.20 \times 10^{-3}$            | $9.46 \times 10^{-3}$  |

Also provided is a method of treating cough of varied etiologies in a patient in  
 20 need thereof, the method includes administering a pharmaceutical composition which comprises extracts of *Adhatoda zeylanica*, *Hedychium spicatum* and *Curcuma longa* and one or more pharmaceutically acceptable carriers/excipients.

The following examples are intended to illustrate the invention and not to be construed as limiting the scope of the invention in any way.

**EXAMPLE 1****Isolation of Vasicine from *Adhatoda Zeylanica* Leaves**

Powdered leaves of *A. zeylanica* (1.0 Kg) were extracted three times with methanol (3 x 3.5 Ltrs) at 65° C for 4 hours each. The combined methanolic extract was  
5 concentrated to 1/5<sup>th</sup> of its original volume under reduced pressure. The concentrated extract was suspended in 2% sulphuric acid (1 Ltr) and partitioned three times with chloroform (3 x 500 ml). The chloroform washings were discarded and the aqueous phase was basified with ammonia solution. The basified aqueous phase was partitioned three times with chloroform (3 x 500 ml). The combined chloroform extract obtained was freed  
10 from traces of water by passing through anhydrous sodium sulphate and concentrated under reduced pressure to get solid light brown residue. The residue was crystallized from ethanol to yield a crystalline material having melting point 198° C

**EXAMPLE 2****Isolation of Ethyl p-Methoxycinnamate from *Hedychium Spicatum* Rhizomes**

15 Powdered hedychium spicatum rhizomes (1.0 Kg) were extracted with 50% alcohol (3 x 3.5 liter) at 60° C for 4 hours. The extract was concentrated to 1/5<sup>th</sup> of its original volume under reduced pressure.

After partitioning the extract above in petroleum ether (3 x 500ml), all petroleum ether extracts were combined and passed through sodium sulphate. The petroleum ether  
20 extract was evaporated under reduced pressure to get the residue. The ether extract was chromatographed through a silica gel column eluting first with hexane and then with ethyl acetate: hexane mixture in increasing order of polarity.

The fractions obtained in hexane: ethyl acetate (95: 5) were combined because of identical TLC (Chloroform: methanol 9: 1) pattern. Upon evaporation, a residue was formed which  
25 after crystallization from ethyl acetate provided crystals having a melting point 50° C.

**EXAMPLE 3****Isolation of Curcumin from *Curcuma Longa* Rhizomes**

Powdered *curcuma longa* rhizomes (1 Kg) were extracted with 3 x 3.5 liters of methanol at 65°C for 3 hours followed by filtration of the mixture. All the extracts were  
30 combined and concentrated under reduced pressure to 1/5<sup>th</sup> of its original volume. An

equal amount of water was added, the methanol was distilled off and the aqueous layer was cooled to room temperature. The aqueous layer was partitioned with an equal volume of chloroform three times. All the chloroform extracts were combined and passed through sodium sulphate for drying. Next the extracts were concentrated under reduced pressure.

- 5 The resulting material was chromatographed through a silica gel 100-200 mesh column and eluted first with petroleum ether and then with an increasing volume of ethyl acetate in petroleum ether. The different fractions were collected and each fraction was eluted by TLC using mobile phase chloroform: methanol at a ratio of 95:5. The developed plate was then observed under UV. All the fractions having curcumin were combined as
- 10 observed by their TLC pattern. The pooled fractions were concentrated under reduced pressure and crystallized curcumin was formed from the acetone and petroleum ether mixture (m.p. 183<sup>0</sup>C).

#### EXAMPLE 4

##### **Method of Detection of Vasicine, Ethyl p-Methoxycinnamate and Curcumin from**

15 **Extracts of Adhatoda, Hedychium and Curcuma**

Thin Layer Chromatography was carried out using a Silica gel 60 F<sub>254</sub> pre-coated aluminum foil plate with a layer thickness 0.2 mm (1.05554, Merck).

##### **a) Preparation of the spraying reagents**

###### **1. Preparation of Solution A**

- 20 Preparation of Dragendorff's reagent:

Basic bismuth nitrate (0.85 grams) was dissolved in glacial acetic acid (10 mL) and water (40 mL) under heating. If necessary the solution is filtered.

###### **2. Preparation of Solution B**

Potassium iodide (8 grams) was dissolved in water (30 mL).

- 25 3. A stock solution was prepared by mixing Solution A and Solution B in 1:1 ratio. Stock solution (1 mL) was mixed with glacial acetic acid (2 mL) and water (10 mL).

###### **4. Preparation of anisaldehyde-sulphuric acid reagent:**

Anisaldehyde (0.5 mL) was mixed with glacial acetic acid (10 mL), methanol (85 mL) and concentrated sulphuric acid (5 mL) in that order. The reagent has only limited stability and was discarded when the color has turned to red-violet.

#### **b) Preparation of mobile phase**

##### 5 1. Mobile phase for vasicine

1,4 dioxane (AR Grade) (25 mL), toluene (AR Grade) (0.1 mL), 1 ml methanol (AR Grade) and ammonia solution (0.5 mL) were measured in a 50 mL stoppered conical flask. About 20 mL of the mobile phase was shaken/sonicated and transferred into a 10x10 cm TLC chamber and allowed to equilibrate for 60 minutes.

##### 10 2. Mobile phase for ethyl p-methoxycinnamate

Hexane (AR Grade) (80 mL) and acetone (AR Grade) (20 mL) were measured individually using graduated measuring cylinders and transferred separately to a 100 mL volumetric flask. The mobile phase was shaken/sonicated and then about 20 mL of the mobile phase was transferred into a 10x10 cm TLC chamber and allowed to equilibrate for 30 minutes.

##### 15 3. Mobile phase for curcumin

Chloroform (AR Grade) (95 mL), ethyl acetate (AR Grade) (5 mL) and glacial acetic acid (1 mL) were measured individually using graduated cylinders/pipettes and transferred separately to a 100 mL volumetric flask. The mobile phase was shaken/sonicated and about 20 mL of the mobile phase was then transferred into a 10x10 cm TLC chamber and  
20 allowed to equilibrate for 60 minutes.

#### **c) Preparation of reference standard solution**

##### 1. Preparation of vasicine reference standard:

A vasicine reference standard (2 mg) was weighed accurately and transferred to a 5 mL volumetric flask. Then methanol (AR Grade) (3.0 mL) was added and sonicated in an  
25 ultrasonic water bath until it dissolved. The volume was made up with methanol. Aliquotes of the resulting solution were used as reference standard solutions for vasicine.

##### 2. Preparation of ethyl p-methoxycinnamate reference standard:

A ethyl p-methoxycinnamate reference standard (2 mg) was weighed out and transferred to a 5 mL volumetric flask. Methanol (AR Grade) (3.0 mL) was added and sonicated in an

ultrasonic water bath until it dissolved. The volume was made up with methanol. The aliquotes of the resulting solution were used as reference standard solution for ethyl p-methoxycinnamate.

### 3. Preparation of curcumin reference standard:

- 5 Curcumin (2 mg) was weighed out as a reference standard and transferred to a 5 mL volumetric flask. Methanol (3.0 mL) was added (AR Grade) and sonicated in an ultrasonic water bath until it dissolved. The volume was then made up with methanol. The aliquotes of resulting solution were used as reference standard solution for curcumin.

### d) Preparation of test solution

- 10 1. Preparation of adhatoda extract test solution

Adhatoda extract (500 mg) was weighed out and transferred to a 50 mL conical flask. Methanol (10 mL) was added to and sonicated in an ultrasonic water bath for 10 minutes. After sonication, the conical flask was heated to a boil for a few seconds. The contents of conical flask were filtered through a Whatman No.41 filter paper. The resulting solution  
15 was used as a test solution.

2. Preparation of hedychium extract test solution

Hydechium extract (500 mg) was weighted and transferred to a 50 mL conical flask. Methanol (10.0 mL) was added to and sonicated in an ultrasonic water bath for 10 minutes. After sonication, the conical flask was heated to a boil for few seconds. The contents of  
20 the conical flask was then filtered through a Whatman No.41 filter paper. The resulting solution was used as test solution.

3. Preparation of curcuma extract test solution

Curcuma extract (500 mg) was weighed out and transferred to a 50 mL conical flask. Methanol (10.0 mL) was added to and sonicated in an ultrasonic water bath for 10 minutes.  
25 After sonication, the conical flask was heated to a boil for few seconds. The contents of the conical flask was then filtered through a Whatman No.41 filter paper. The resulting solution was then used as a test solution.

### e) Procedure

1. Procedure for detection of vasicine

10 and 20  $\mu$ l each of reference standard and test solutions respectively were applied individually to the chromatoplate in duplicate. The chromatoplate in above mobile phase was developed about 7.0 cm from the point of application. The plate from the TLC chamber was removed and dried with hot air blower. The chromatoplate was sprayed with dragendorff's reagent (10-15 mL) and the plate in visible light.

## 2. Procedure for detection of ethyl p-methoxycinnamate

10 and 20  $\mu$ l each of reference standard and test solutions respectively were applied individually to the chromatoplate in duplicate. The chromatoplate in above mobile phase was developed about 7.0 cm from the point of application. The plate from the TLC chamber was removed and dried with hot air blower. The chromatoplate was sprayed with anisaldehyde-sulphuric acid reagent (10-15 mL) and dried in an oven for 5-10 minutes under observation.

## 3. Procedure for detection of curcumin

10 and 20  $\mu$ l each of reference standard and test solutions respectively were applied individually to the chromatoplate in duplicate and chromatoplate was developed in the above mobile phase about 7.0 cm from the point of application. The plate was removed from the TLC chamber and dried with a hot air blower. The plate was then evaluated in visible/ fluorescent light.

### **f) Inference / TLC fingerprint pattern**

#### 20 1. Vasicine

A band corresponding to vasicine was observed in test sample at the same  $R_f$  (approx. 0.44) and having the same color. A photograph was taken with the help of Camag's photodocumentation unit in visible/fluorescent light. A characteristic fingerprint pattern was obtained.

#### 25 2. Ethyl p-methoxycinnamate

A band corresponding to ethyl p-methoxycinnamate was observed in test sample at the same  $R_f$  (approx. 0.40) and having same color. Took photograph with the help of Camag's photodocumentation unit in visible light. A characteristic fingerprint pattern was obtained.

## 3. Curcumin

A band corresponding to curcumin was observed in test sample at the same R<sub>f</sub> (approx. 0.31) and having same color. Took photograph with the help of Camag's photodocumentation unit in visible/fluorescent light. A characteristic fingerprint pattern was obtained.

**EXAMPLE 5****Estimation of Total Alkaloids in Adhatoda Extracts**

Adhatoda extract (5.0 gm) was placed in a round bottom flask and 0.1 N hydrochloric acid (50 mL) was added and refluxed on a water bath for 30 minutes. The volume was then made up to 100 mL and was filtered through a Whatman No. 41 filter paper. The filtrate was transferred to a separating funnel and extracted four times with each of 100 mL of chloroform. The chloroform layer was discarded. The aqueous layer was basified with a dilute ammonia solution (up to pH 9-10) and was extracted four times with each of the 100 mL of chloroform. The chloroform layers were collected and passed over an anhydrous sodium sulphate. The chloroform layer was evaporated in a pre-weighed beaker to dryness on a water bath. The beaker was dried in an oven for 1 hour at temperature 105°C. The beaker was cooled in a desiccator and the weight was noted.

The % Alkaloid content was calculated using the Formula:

$$\% \text{ Alkaloids} = \frac{\text{Wt. of residue}}{\text{Wt. of sample}} \times \frac{100}{(100 - \text{LOD})} \times 100$$

LOD equals Loss on drying.

The total alkaloid content should be from about 0.005% to about 25%.

**EXAMPLE 6****Estimation of Total Curcuminoids in Curcuma Extracts**

Curcuma extract (100 mg) was weighed and placed into a 100 mL volumetric flask. Methanol (AR Grade) (40 mL) was added to and sonicated in an ultrasonic water bath for 15 minutes. The volume was made up with methanol and filtered through a Whatman No. 41 filter paper. 2 mL of filtrate was pipetted into a 25 mL volumetric flask and the

volume was made up with methanol. The absorbance of the solution at 425 nm was measured using methanol as blank.

The % curcuminoids content was calculated using the Formula:

$$5 \quad \% \text{ curcuminoids content} = \frac{A_{\text{SPL}} \times 1250}{1560 \times \text{wt. of sample}}$$

$A_{\text{SPL}}$  equals the Absorbance of sample at 425 nm. The total curcuminoid content should be from about 0.01% to about 35%.

### EXAMPLE 7

#### 10 Manufacturing Procedure of a Pediatric Formulation

##### 1. Manufacturing Formula

| S. No. | Ingredients          | Quantity          |
|--------|----------------------|-------------------|
| 1      | Adhatoda Extract     | 1.0 – 5.0 %w/v    |
| 2      | Hydechium Extract    | 0.1 - 0.6 %w/v    |
| 3      | Curcuma Extract      | 0.1 – 0.7 %w/v    |
| 4      | Propylene Glycol USP | 1 – 4 % w/v       |
| 5      | Methyl Paraben IP    | 0.18 – 0.22 %w/v  |
| 6      | Propyl Paraben IP    | 0.02 – 0.024 %w/v |
| 7      | Sugar Syrup IH       | 40 – 100 %w/v     |
| 8      | Flavors              | 0.15 – 1.7 % v/v  |
| 9      | Purified Water IP    | q.s.              |

## 2. Preparation of Extract solution

To the extracts of Adhatoda, Hydechium and Curcuma were added a quantity of propylene glycol and purified water and stirred well for 15-20 minutes. This extract solution was filtered through sparkler filter press using filter pad and filter aid.

## 5 3. Preparation of preservative solution:

To methyl paraben and propyl paraben were added a quantity of propylene glycol and dissolved by stirring for 15-20 minutes.

## 4. Mixing

10 Invert sugar syrup was transferred to a steam jacketted kettle and warmed to 45-50°C. To this, extract solution was added and mixed well for 15-20 minutes. A preservative solution was added to the syrup and mixed well for 10-15 minutes. The pH of the syrup was checked. Flavor was added to the syrup and mixed well for 15-20 minutes and final volume was made up with purified water and again mixed well. The syrup is filtered through a size #100 mesh nylon cloth and transferred to a storage tank.

15

## EXAMPLE 8

### Detection and Quantification Of Vasicine From The Syrup

Instrument: Camag's High Performance Thin Layer Chromatographic system equipped with Linomat V and photo documentation unit.

Solvent system: 1,4 Dioxane : Toluene : Methanol: Ammonia solution

20

25

01

01

0.5

Stationary phase: Merck 1.05548, HPTLC Silica gel GF<sub>254</sub> Aluminium coated plate

Development distance: 8 cm from the point of application

Detector: Camag's TLC Scanner-3

Integrator: Camag's WinCats Software

25

Band Length: 8 mm

Distance between two tracks: 10 mm

Wavelength for recording the chromatogram: 292 nm

Application volume: 5 and 10  $\mu\text{L}$  of reference standard and 10 and 20  $\mu\text{L}$  of test sample

### **Preparation of Reference Standard Solution**

Approximately vasicine (25 mg) was dissolved in methanol (25 mL) by sonication and heating. The resulting solution was used as a reference standard solution for vasicine.

### **5 Preparation of test solution**

About 50 mL of the sample was transferred to a separating funnel. Distilled water (50 mL) and ammonia solution (2.5 mL) were added and mixed well. This was extracted five times with each 120mL mixture of chloroform : n-butanol : methanol (25:03:01). The organic layer was separated and passed over anhydrous sodium sulphate. The organic layer was evaporated to dryness on a boiling water bath. The residue was then reconstituted in methanol (10 mL). The resulting solution was transferred to a 25 mL volumetric flask, rinsed twice with each 5mL of methanol and transferred to the same volumetric flask. The volume of the volumetric flask was made up with methanol. The resulting solution was used as test sample.

### **15 Preparation of Solvent Solution**

1,4- dioxane AR (25 mL), toluene AR (0.1 mL) and methanol and ammonia solution (0.5 mL) were measured out and transferred to a 100 mL stoppered conical flask. This solution was sonicated for 2-3 minutes and 20 mL was then transferrerd to a 10x10 cm HPTLC chamber. The HPTLC chamber was saturated for 30 minutes.

### **20 Procedure**

5  $\mu\text{L}$  and 10  $\mu\text{L}$  of the reference standard and 10  $\mu\text{L}$  and 20 $\mu\text{L}$  of test solutions werer applied to a 10x10 cm HPTLC plate separately. The plate was developed in the above solvent system over a distance of 8 cm from the point of application. The plate was dried with the help of hot air-blower.

### **25 Quantification/calculation**

The plate was scanned at  $\lambda_{\text{max}}$  292 nm using deuterium lamp and the content of vasicine was calculated in the product.

The % content of vasicine in the finished product

$$= \frac{A_{\text{SPL}}}{A_{\text{STD}}} \times \frac{D_{\text{STD}}}{D_{\text{SPL}}} \times 100$$

5

Wherein,

$A_{\text{SPL}}$  = Area of sample corresponding to Vasicine

$A_{\text{STD}}$  = Area of Vasicine

$D_{\text{STD}}$  = Dilution of reference standard solution

10

$D_{\text{SPL}}$  = Dilution of test sample

### Results

| Expt. No. | % w/v                  |
|-----------|------------------------|
| I         | $17.44 \times 10^{-3}$ |
| II        | $15.17 \times 10^{-3}$ |
| III       | $15.06 \times 10^{-3}$ |

### EXAMPLE 9

#### Detection and Quantification Of Ethyl P-Methoxycinnamate from the Syrup

15 Instrument: Camag's High Performance Thin Layer Chromatographic system equipped with Linomat V and photo documentation unit.

Solvent system: n-Hexane : Acetone

80 20

Stationary phase: Merck 1.05548, HPTLC Silica gel GF<sub>254</sub> Aluminium coated plate

20

Development Distance: 8 cm from the point of application

Detector: Camag's TLC Scanner-3

25 Integrator: Camag WinCats Software

Band Length: 8 mm

Distance between two tracks: 10 mm

30

Wavelength for recording the chromatogram: 310 nm

Application volume: 5 and 10  $\mu\text{L}$  of reference standard and 10 and 20  $\mu\text{L}$  of test sample

### Preparation of Reference Standard Solution

Ethyl p-methoxycinnamate (25 mg) was dissolved in methanol (25 mL). The resulting  
5 solution was used as a reference standard solution for ethyl p-methoxycinnamate.

### Preparation of test solution

50 mL of the sample was transferred to a separating funnel. 50 mL of distilled water  
added and mixed well. The solution was extracted five times with each 120 mL mixture  
of chloroform : n-butanol : methanol (25:03:01). The organic layer was separated and  
10 passed over an anhydrous sodium sulphate. The organic layer was evaporated to dryness  
on a boiling water bath. Next, the residue was reconstituted in methanol (10 mL). The  
resulting solution was then transferred to a 25 mL volumetric flask. The vessel was rinsed  
twice with each 5 mL of methanol and then transferred the rinsing to the same volumetric  
flask. The volume of volumetric flask was made up with methanol. The resulting solution  
15 was used as a test sample.

### Preparation of Solvent Solution

N-hexane AR (80 mL) and Acetone AR (20 mL) were measured using the same  
measuring cylinder and transferred to a 100 mL stoppered conical flask. The flask was  
sonicated for 2-3 minutes for proper mixing and approximately 20 mL was transferred to a  
20 10x10 cm HPTLC chamber. The HPTLC chamber was saturated for 30 minutes.

### Procedure

5  $\mu\text{L}$  and 10  $\mu\text{L}$  of the reference standard solution and 10 and 20  $\mu\text{L}$  of test solutions were  
applied to a 10 x 10 cm HPTLC plate separately. The plate in the above solvent system  
was then developed over a distance of 8 cm from the point of application. The plate was  
25 dried with the help of hit air-blower.

### Quantification/calculation

The plate was scanned at  $\lambda_{\text{max}}$  310 nm using a deuterium lamp. The content of ethyl p-methoxycinnamate was calculated in the product.

The % content of ethyl p-methoxycinnamate is calculated by the formula below:

$$= \frac{A_{\text{SPL}}}{A_{\text{STD}}} \times \frac{D_{\text{STD}}}{D_{\text{SPL}}} \times 100$$

5   Wherein,

$A_{\text{SPL}}$  = Area of sample corresponding to ethyl p-methoxycinnamate.

$A_{\text{STD}}$  = Area of ethyl p-methoxycinnamate

$D_{\text{STD}}$  = Dilution of reference standard solution

$D_{\text{SPL}}$  = Dilution of test sample

10

### Results

| Expt. No. | % w/v                  |
|-----------|------------------------|
| Expt-I    | $9.17 \times 10^{-3}$  |
| Expt-II   | $9.02 \times 10^{-3}$  |
| Expt-III  | $10.20 \times 10^{-3}$ |

### EXAMPLE 10

15

#### Detection and Quantification of Curcumin from the Syrup

**Instrument:** Perkin Elmer's UV Spectrophotometer

#### Preparation of Reference Standard Stock Solution

Approximately 25 mg of curcuminoids were dissolved in methanol (50 mL) by sonication. The resulting solution was used as a stock solution for a reference standard.

20

#### Preparation of standard curve

4 mL, 5 mL, 6 mL, 7 mL and 8 mL of stock solution were taken as a reference standard solution and transfer to a 25 mL volumetric flasks. The volume of volumetric flasks was made up with methanol.

#### Procedure

25

The absorption was measured at a  $\lambda_{\text{max}}$  418 nm of each concentration-using methanol as blank and a standard linearity curve was plotted and the absorption of test sample was measured.

**Quantification/Calculation:**

The % content of curcuminoids in the finished product =  $\frac{A_{SPL}}{W_{SPL}} \times 100$

5

Wherein

$A_{SPL}$  = Concentration of test sample against working curve

$W_{SPL}$  = Qty. of sample taken in mL

10

**Results**

| Expt. No. | % w/v                  |
|-----------|------------------------|
| Expt-I    | $10.10 \times 10^{-3}$ |
| Expt-II   | $8.47 \times 10^{-3}$  |
| Expt-III  | $9.46 \times 10^{-3}$  |

While the present invention has been described in terms of its specific embodiments, certain modifications and equivalents will be apparent to those skilled in the art and are included within the scope of the present invention.

15

We Claim:

1. A herbal pharmaceutical formulation comprising extracts of *Adhatoda*, *Hedychium* and *Curcuma*, or a mixture of active ingredients that have been extracted from these herbs, and one or more pharmaceutically acceptable carriers and/or diluents.
2. The pharmaceutical formulation of claim 1, wherein the vasicine comprises from about  $17.44 \times 10^{-3}$  to about  $15.06 \times 10^{-3}$  % w/v, the ethyl p-methoxycinnamate comprises from about  $10.20 \times 10^{-3}$  to about  $9.02 \times 10^{-3}$  % w/v, and the curcuminoids comprises from about  $10.1 \times 10^{-3}$  to about  $8.47 \times 10^{-3}$  % w/v.
3. The pharmaceutical formulation of claim 1, wherein the Adhatoda Extract comprises from about 1.0 % w/v to about 5.0 % w/v, the Hydechium Extract comprises from about 0.1 % w/v to about 0.6 % w/v, and the Curcuma Extract comprises from about 0.1 % w/v to about 0.7 % w/v.
4. The pharmaceutical formulation of claim 1, wherein the formulation is intended for oral use as a cough syrup.
5. The pharmaceutical formulation of claim 1, wherein the pharmaceutical formulation is prepared by:
  - a. mixing a therapeutically effective amount of *Adhatoda*, *Hedychium* and *Curcuma* to get a combined extract;
  - b. adding one or more pharmaceutically acceptable carriers and/or diluents to the combined extract to form a final mixture; and
  - c. clarifying the final mixture.
6. The pharmaceutical formulation of claim 5, wherein the preparation of the pharmaceutical formulation further comprises detecting and quantifying the curcumin in the pharmaceutical formulation, the detecting and quantifying comprising:
  - a. preparing a reference standard stock solution in an organic solvent;
  - b. preparing standard curves by using the solution of step a) and a blank organic solvent;
  - c. measuring the absorptions of the standard stock solution and a blank organic solvent at particular wavelengths after step b); and
  - d. calculating the percentage curcuminoids in the formulation after step c).

7. The pharmaceutical formulation of claim 6, wherein the wavelength for measuring absorption is  $\lambda_{\max}$  418 nm.
8. The pharmaceutical formulation of claim 5, wherein the preparation of the pharmaceutical formulation further comprises detecting and quantifying vasicine and ethyl p-methoxycinnamate in the pharmaceutical formulation, the detecting and quantifying comprising:
  - a. extracting the formulation with one or more organic solvents or mixtures thereof optionally containing water or base;
  - b. concentrating the extracts of step a) and reconstituting the residue in second organic solvent;
  - c. spotting the solution obtained in step b) against a standard phytochemical marker on a HPTLC plate;
  - d. developing the HPTLC plate of step c) in a mobile phase;
  - e. optionally spraying or applying the detection reagent on the HPTLC plate of step d);
  - f. scanning the plate of step e) at particular wavelengths; and
  - g. detecting and quantifying vasicine and ethyl p-methoxycinnamate after step f).
9. A process for the isolation of the phytochemical marker compound vasicine from *Adhatoda zeylanica*, the process comprising:
  - a. extracting a selected powdered part of the plant with an organic solvent and then concentrating the extract to form a concentrate;
  - b. acidifying the concentrate and extracting the concentrate with a second organic solvent;
  - c. basifying the concentrate and extracting the concentrate with the second organic solvent;
  - d. concentrating the organic extracts; and
  - e. isolating vasicine.
10. The process of claim 9, wherein the isolation comprises crystallization.

11. The process of claim 9, wherein the organic solvent comprises water soluble lower alkanols.
12. The process of claim 11, wherein the water soluble alkanols comprise one or more of methanol, ethanol, n-propanol, isopropanol and mixtures thereof.
13. The process of claim 9, wherein the acidification of the concentrate is performed with an aqueous organic or inorganic acid.
14. The process of claim 13, wherein the acidification is performed with one or more of sulphuric acid, hydrochloric acid, acetic acid, formic acid, phosphoric acid, nitric acid and mixtures thereof.
15. The process of claim 9, wherein the second organic solvent comprises one or more of non-polar aliphatic, cycloalkyl solvents, ethers, esters, chlorinated hydrocarbons, ketones and mixtures thereof.
16. The process of claim 9, wherein the basification of the concentrate is performed with one or more of ammonia, ammonium hydroxide, triethylamine, dimethylamine, sodium hydroxide, potassium hydroxide, sodium carbonate, potassium carbonate, sodium bicarbonate, potassium bicarbonate, calcium carbonate, calcium hydroxide and mixtures thereof.
17. The process of claim 9, further comprising identifying vasicine in the extract, wherein the identifying comprises:
  - a. after extracting the selected powdered part of the plant with an organic solvent, optionally filtering to form a test solution;
  - b. applying a reference standard and test solution to a chromatoplate;
  - c. developing the chromatoplate in a mobile phase;
  - e. drying the chromatoplate and spraying it with a spraying/developing agent; and
  - f. identifying vasicine.
18. The process of claim 9, further comprising estimating the total alkaloid content in the Adhatoda extract by:
  - a. after extracting the selected powdered part of the plant with an organic solvent, acidifying the extract with an acid solution, refluxing, and filtering to form a filtrate;

- b. extracting the filtrate with an organic solvent to remove the organic compounds from the aqueous extract;
  - c. basifying the aqueous extract with a base;
  - d. extracting the solution with the organic solvent;
  - e. concentrating/evaporating the solution to form a residue;
  - f. drying the residue; and
  - g. estimating the total alkaloid content.
19. A process for the isolation of the phytochemical marker compounds ethyl p-methoxycinnamate and curcumin from the plants *Hedychium spicatum* and *Curcuma longa*, respectively, the process comprising:
- a. extracting the selected powdered part of the plant with an organic solvent optionally containing water;
  - b. concentrating the extracts;
  - c. extracting the concentrated extracts with a second organic solvent and, optionally, filtering the solution to remove the non-polar impurities;
  - d. purifying the resultant organic extracts by column chromatography; and
  - e. detecting/isolating the phytochemical markers.
20. The process of claim 19, wherein the isolation of the phytochemical markers comprises crystallization.
21. The process of claim 19 wherein the organic solvent comprises water soluble lower alkanols.
22. The process of claim 19, wherein the second organic solvent comprises one or more of non-polar aliphatic, cycloalkyl solvents, ethers, esters, chlorinated hydrocarbons, ketones and mixtures thereof.
23. The process of claim 19, further comprising identifying the ethyl p-methoxycinnamate or curcumin in the extract, wherein the identifying comprises:
- a. after extracting the selected powdered part of the plant with an organic solvent, optionally filtering to form a test solution;
  - b. applying a reference standard and the test solution to a chromatoplate;
  - c. developing the chromatoplate in a mobile phase;

- d. drying the chromatoplate and spraying it with a spraying/developing agent;  
and
  - e. identifying p-methoxycinnamate or curcumin.
24. The process of claim 19, further comprising estimating the curcuminoid content in the *Curcuma* extract, wherein the estimating comprises:
- a. after extracting the selected powdered part of the plant with an organic solvent, mixing the extract with an organic solvent;
  - b. filtering the solution of step a);
  - c. measuring the absorbance of the solution of step b) against an organic solvent as a blank and then calculating the curcuminoids content.
25. The process of claim 24, wherein the organic solvent comprises one or more of non-polar aliphatic, cycloalkyl solvents, ethers, esters, chlorinated hydrocarbons, ketones, lower alkanols and mixtures thereof.
26. The process of claim 24, wherein the absorbance is measured at a wave length of 418 nm.