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Chromatographic Studies on Extracts of *Ganoderma lucidum*

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ABSTRACT

Ganoderma lucidum is considered to be a natural medicine that promotes longevity and maintains the vitality of human beings. Its beneficial clinical effects in patients with hepatitis, hyperglycemia, chronic bronchitis, cancer, muscular dystrophy, arteriosclerosis, hypertension, hypercholesterolemia, and leukopenia have been confirmed in pharmacologic studies in recent years. The fruiting bodies, mycelia, and spores have recently received more and more attention not only as home remedies but also as new drug sources. Being an important medicinal mushroom, it was thought necessary to scan the mushroom for its chemical constituents and to work out it's chromatographic profile via thin layer chromatographic and high-performance thin layer chromatographic approaches. Present work sheds light on the qualitative chemical examination of Ganoderma extracts and their further chromatographic analysis. Standard marker viz., beta-sitosterol was used in the process. Some major phytoconstitents found to be present were triterpenoids and polysaccharides which have been held responsible for most of the pharmacological activities of the mushroom.

Keywords: *Ganoderma lucidum*; TLC; HPTLC; Phytochemistry; Polysaccharides; Triterpenoids.

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INTRODUCTION

Ganoderma lucidum (Curtis) P. Karst.

Ganoderma lucidum, an oriental fungus, has been used in China, Korea, and Japan for centuries. It was considered to preserve the human vitality and to promote longevity. In addition, *Ganoderma lucidum* has been used to treat various human diseases such as allergy, arthritis, bronchitis, gastric ulcer, hyperglycemia, hypertension, chronic hepatitis, hepatopathy, insomnia, nephritis, neurasthenia, scleroderma, inflammation, and cancer. Different compounds with various biological activities were extracted from mycelia, the fruiting bodies or spores of *Ganoderma lucidum*, and some of them are linked to possible therapeutic effects.

Source: Icons of Medicinal Fungi, Science Press, Beijing, China

Reishi mushroom

Ganoderma lucidum

Family

Ganodermataceae

Common names

United States	:	Reishi mushroom (*Herbs of Commerce*), ganoderma
China	:	Ling zhi, ling zhi cao, ling chih, hong ling zhi, chi zhi
Japan	:	Reishi, mannentake; rokkaku reishi (antler form)
Korea	:	Young ji
Vietnam	:	Ling chi

Taxonomical classification

Kingdom	:	Fungi
Phylum	:	Basidiomycota
Class	:	Agaricomycetes
Order	:	Polyporales
Family	:	Ganodermataceae
Genus	:	Ganoderma
Species	:	*G. lucidum*

History

Numerous legends surrounding reishi mushroom provide an historical record which spans 2000 years. Traditionally, it was used in China by Taoist monks to promote a centered calmness, improve meditative practices, and attain a long and healthy life. Reishi mushroom has also been revered in Japanese culture where it is considered to be the most important of all the Japanese medicinal polypores [sarunokoshikake].[1] The characters making up the Chinese name for reishi mushroom [ling zhi] originally depicted a "shaman crying for rain", represent the magical or divine properties which were associated with ling zhi. Reishi mushroom has also been commonly referred to as the "mushroom of immortality", "ten-thousand-year mushroom", "mushroom of spiritual

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potency", and "spirit plant".[2,3] Reishi mushroom was listed among the superior tonics [Shang pin] in the most famous of all Chinese materia medicas, the *Shen Nung Ben Cao Jing* (206 BC-AD 8).[1,4] Superior herbs were among the most highly regarded of all medicines since they were considered to prolong life, prevent aging, boost qi, make the body light and limber, and corresponded to heaven. Reishi mushroom was listed as the most respected out of the 120 superior tonics cited. Specifically, in this text, red reishi was reported to treat binding in the chest, tonify the heart, nourish the center, sharpen the wit, and improve memory. In addition to its physical properties, reishi was said to "cultivate virtue".[5]

Ganoderma possesses the following pharmacological properties according to an overview of the American Herbal Pharmacopoeia (2006).

Analgesic; anti-inflammatory; antitumor; antiviral; hepatoprotective; hypoglycemic; hypocho-lesterolemic; hypotensive (ACE inhibitor); immune-modulating: increases IL-1-b, IL-2, and IL-6, increases cytotoxicity of T lymphocytes, increases TNF-a in macrophage cultures; inhibits platelet aggregation.[6]

Chemical constituents

Triterpenes: More than 100 different highly oxygenated lanostanoid triterpenes have been identified in reishi mushroom. These include multiple pairs of C-3 stereoisomers and C-3/C-15 positional isomers.[7] The predominant triterpenes are ganoderic acids A-Z. Other triterpenes include ganoderal A and B,[8,9] ganoderol A and B,[8] epoxyganoderiol A-C,[9] ganoderenic acid A-D,[10] ganodermic acids,[11] ganoderiol A-I,[12,13] ganodermanontriol, ganodermatriol,[13] ganolucidic acids A-E, lucidone A-C, lucidenic acids A-M,[14-17] lucidadiol, and lucidal.[18]

Polysaccharides: Numerous bioactive polyglycans (polysaccharides) are contained in all parts of the fruiting body. Among those present in reishi mushroom are neutral polysaccharides (b-1-3, b-1- 6 homo D-glucan), acidic glucan and polyglycan, proteinbound heteroglucan, arabinoxyoglucan (a highly branched heteroglucan), a heteroglycan with a b-1-4 core, and peptidoglycans (ganoderan A, B, and C). The basic structure of the major glucans b-1-3 and b-1-6 homo D-glucan is b-1-3 D-glucopyronan with 1-15 units of b- 1-6 monogluosyl side chains.[19]

Amino acids (mol %): Serine (15.2), alanine (14.8), glycine (12.7), threonine (12.4), aspartic acid (9.9), glutamic acid (8.1), proline (6.9), valine (5.3), and other minor amino acids.[20]

Other constituents: Steryl esters (ergosterol), adenosine (5'deoxy-5'-methylsulphinyladenosine),[7] fungal lysozyme, fatty acids, and a protease.[21-23]

Ganoderma lucidum is a very well researched mushroom. Being a popular medicinal mushroom and being used in Traditional Chinese Medicine for a long time, researchers from all over the world have got increased interest in these mushroom and lot of research has been carried out on the phytochemical and pharmacological profile of *Ganoderma lucidum* in past 20-30 years.

MATERIALS AND METHODS

Phytochemical Analysis

Ganoderma lucidum powder (authenticated sample obtained from Gano Excel Industries Sdn Bhd, Malaysia) was packed in soxhlet extractors and extracted with petroleum ether (60–80°C) till complete extraction. The solvent from the petroleum ether extract was eliminated under reduced pressure (yield- 0.51 % *w/w*). The defatted marc was extracted with ethanol (95 % *v/v*) to obtain the ethanolic extract (yield- 3.05 % w/w). The marc left after the ethanolic extraction was macerated with distilled water for 24 hr and the aqueous extract was finally obtained by vacuum drying (yield- 0.62 %*w/w*). The extracts were studied for their phytochemical profiling as follows:

Physical Examination of the Extracts

The dried extracts of *Ganoderma lucidum* were evaluated for physical parameters such as consistency, color, odor and taste. The results are shown in Table 1.

Characterization of Extracts by Qualitative Chemical Tests

The petroleum ether, ethanolic and aqueous extracts were subjected to following chemical tests:

Test for Carbohydrates

The test solution was prepared by dissolving test extract in water or respective solvent, hydrolysed with 2N HCl and subjected to following tests:

Fehling's Test (for reducing sugars)

Test solution is treated with few drops of Fehling's reagent [Dissolve 34.66g of Copper sulphate in distilled water and make upto 500 ml (solution A). Dissolve 173 g of potassium sodium tartarate and 50 g of sodium hydroxide in distilled water and make volume upto 500 ml (solution B). Mix two solutions in equal volume prior to use for detection of reducing sugars.] It gives brick red color on warming.[24]

Molisch's Test

Test solution is treated with few drops of Molisch's reagent (10 g α-naphthol in 100 ml of 95% ethanol). Then 2 ml of conc. Sulphuric acid is added slowly from sides of the test tube. It shows purple ring at the junction of two layers.[24]

Test for Proteins

The extract is dissolved in water or 95% ethanol for preparing the test solution.

Millons Test

Test solution is treated with Millon's reagent [Dissolve 1 g of mercury in 9 ml of fuming nitric acid, after cooling, add equal volume of distilled water]. Protein is stained red on warming.[24]

Xanthoproteic Test

Test solution treated with conc. nitric acid and boiled gives yellow precipitate.

Biuret Test

Test solution treated with 10% sodium chloride and 1% copper sulphate (1 drop). Solution gives violet / purple color. On addition of alkali, it becomes dark violet.

Table 1: Physical characteristics of *Ganoderma lucidum* extracts.

Consistency	Color	Odor	Taste	Extractive value (% *w/w*)
Petroleum ether extract				
Semisolid	Pale Yellow	Characteristic	Characteristic	0.51 %
Ethanolic extract				
Semisolid	Brownish black	Characteristic	Characteristic	3.05 %
Aqueous extract				
Solid	Black	None	Characteristic	0.62 %

Test for Alkaloids

5 ml of the extract was evaporated to dryness. The residues were taken in 5 ml of hydrochloric acid, saturated with sodium chloride and filtered. The filtrates were separately tested with following reagents:

Dragendorff's Test

The acidic solution treated with Dragendorff's reagent (potassium bismuth iodide) gives orange precipitate.

Mayer's Test

Test solution with Mayer's reagent (potassium mercuric iodide) gives cream colored precipitate.

Hager's Test

The acidic solution treated with Hager's reagent (saturated picric acid solution) gives yellow precipitate.

Wagner's Test

Test solution treated with Wagner's reagent (iodine- potassium iodide solution) gives reddish brown precipitate.[24]

Test for Steroids

The extracts were dissolved in chloroform.

Libermann Burchard Test

To the test solution few drops of acetic anhydride were added and conc. sulphuric acid added from sides of test tube, shaken and allowed to stand. Lower layer turns bluish green indicating the presence of sterols.

Salkowski Test

Test extract solution was treated with few drops of conc. sulphuric acid, shaken and allowed to stand. Lower layer turns red indicating the presence of sterols.

Test for Glycosides

The test solution was prepared by dissolving extract in 95% ethanol or aqueous alcoholic solution.

Keller Killiani Test (for digitoxose)

Test solution is treated with few drops of Ferric chloride solution and mixed, and then sulphuric acid containing ferric chloride solution is added, it forms two layers. Lower layer shows reddish brown color while upper layer turns bluish green.

Baljet Test

Test solution treated with sodium picrate gives yellow to orange colour.

Legal Test

Test solution treated with a drop of 2% sodium nitroprusside and a drop of sodium hydroxide is then added. Production of a deep red color constitutes a positive test.

Test for Saponins

The test solution was prepared by dissolving extract in water.

Foam Test

Test solution on shaking shows foam formation, which is stable for at least 15-20 min.

Hemolysis Test

The test extract solution was subjected to hemolytic test. 1ml of blood was diluted with 10 ml of sodium citrate (36.5g/l) and separately 10 mg of extract was dissolved in phosphate buffer pH 7.4 and made up to 100 ml. Drop of blood was taken over slide and observed under microscope (40x) for the presence of intact RBC, then 1-2 drops of saponin solution was put over the blood and observed under microscope for hemolysis.[25]

Test for Tannins

The extract is dissolved in 95% ethanol or respective solvent.

Gelatin- Lead Acetate Test

Test solution treated with lead acetate solution. This solution gives a white precipitate, when a 1% solution of gelatin containing 10% sodium chloride is added.

Test for Phenolics

The extract is dissolved in 95% ethanol or respective solvent.

Ferric Chloride Test

The solution treated with few drops of ferric chloride solution gives dark color.

Test for Pentose Sugar

Heat the test solution in a test tube with an equal volume of hydrochloric acid containing a little phloroglucinol. Formation of a red color indicates pentoses.

Test for Flavonoids

The extract is dissolved in 95% ethanol or respective solvent.

Shinoda Test

Test solution taken in a test tube. Few magnesium ribbons are dipped and conc. HCl is added over them. Magenta color develops indicating presence of flavonoids.

Test for Triterpenoids

The test solution was prepared by dissolving extracts in chloroform.

Libermann Burchard Test

To the test solution few drops of acetic anhydride were added and mixed well. Then few drops of conc. sulphuric acid added from sides of test tube. Red color is produced in the lower layer indicates the presence of triterpenes.

Salkowski Test

Test extract solution was treated with few drops of conc. sulphuric acid, shaken and allowed to stand. Lower layer turns golden yellow indicating the presence of triterpenoids. Results have been shown in Table 2.

Chromatographic Studies of Extracts

Thin Layer Chromatography

Thin layer Chromatography (TLC) is a method of analysis in which the stationary phase, a finely divided solid, is spread as a thin layer on a rigid supporting plate; and the mobile phase, a liquid is allowed to migrate across the surface of the plate. Although separation efficiencies equivalent to those obtained with gas or high-pressure liquid chromatography cannot be obtained by this method, it has the advantages of speed, versatility and simplicity.[26]

Preparation of sample

Approximately 10 mg of each extract dissolved in respective solvent.

Preparation of plates

Coating of glass plates with adsorbent layer can be achieved by spreading, pouring, spraying or dipping. Most uniform layers are achieved by the spreading technique. For this purpose, slurry of silica gel is prepared by mixing it in distilled water and triturating uniformly in a pestle-mortar. A uniform layer of the adsorbent material is applied to a clean glass plate with the help of a glass rod. The plates are allowed to dry in air at room temperature and then activated at 120°C in an oven for 30 min.

The thickness of the layer of stationary phase is important to the success of the chromatography as excessively thick layers allow the solutes to

Table 2: Qualitative chemical examination of *Ganoderma lucidum* extracts.

Tested for	Petroleum ether extract	Ethanolic extract	Aqueous extract
Carbohydrates			
Fehling's Test	-	-	+
Molisch Test	-	-	+
Hasch Test	-	-	+
Proteins			
Millon's Test	-	+	+
Xanthoproteic Test	-	+	+
Biuret Test	-	+	+
Alkaloids			
Dragendorff Test	-	-	-
Wagner's Test	-	-	-
Mayer's Test	-	-	-
Hager's Test	-	-	-
Glycosides			
Keller Kiliani Test	-	-	-
Baljet Test	-	-	-
Legal Test	-	-	-
Saponins			
Foam Test	-	-	-
Hemolytic Test	-	-	-
Phenolics			
$FeCl_3$ Test	-	+	+
Flavonoids			
Shinoda Test	-	+	+
Tannins			
Lead Acetate Test	-	+	+
Steroids			
Libermann Burchard	+	-	-
Salkowski Test	+	-	-
Triterpenoids			
Libermann Burchard Test	-	+	-
Salkowski Test	-	+	-

+ indicates presence and – indicates absence of the test compound

diffuse literally, and band broadening results. Layers from 0.1 to 2.0 mm in depth are used most often, with thinner ones (250μm) being most suitable for precise separations and thicker coatings for preparative work, due to their greater solute capacity.[26]

Sample application and development

After the plates have been dried and conditioned, the samples are spotted usually with a capillary tube or a microlitre syringe. Samples may be applied as spots or as thin streaks, but it is essential that all of the solvent is evaporated between repeated applications and the area of sample application be kept as small as possible, because the bands will broaden as they travel up the plate.

For ascending development of the thin layer chromatogram, the plate is placed in a rectangular glass jar that contains developing solvent to a depth of about 0.5 cm. The atmosphere of the jar should be saturated completely with the mobile phase before development, a process usually performed by lining the jar with a piece of filter paper that has been wet with mobile phase, which acts as a wick and thus saturates the jar with solvent vapors. Chromatography in saturated atmosphere has the following advantages-

(1) It yields straight solvent fronts.

(2) Developing time is reduced to one-third.

(3) R_f values are much less than in unsaturated jars.

The solvent is allowed to move up the plate until it has travelled a distance of about 15 cm from the point of application of the sample, on a 20 cm plate. The plate is then removed from the tank, the mobile phase front is marked by scratching the surface, and the solvent is evaporated in an oven or, if the sample is heat labile, in the air.

Detection methods

Once the chromatogram has been developed, the solute spots must be made visible in order to determine their R_f values.

The most routinely used method of detection is examination of the plate under an ultraviolet (UV) light to detect fluorescence, using light sources that have their maximum emission lines at 254nm or 365nm.

The more specific methods of detection involve spraying the plates with reagents designed to react with specific functional groups to produce visible derivatives.

Chromatographic procedure

The solvent system was poured to a depth of 0.5 cm in a rectangular chromatographic glass chamber. The chamber was lined with a piece of filter paper to ensure proper saturation. The spots of extract were applied on a silica gel-G plate with the help of capillary tube. The distance between two spots was kept approximately 2.0 cm. The applied spots were dried at room temperature and the plate was gently placed inside the glass chamber. The angle of the plate with the vertical was kept approximately 15°. The chromatogram was developed till the solvent front migrated to about 10.0 cm. The plate was taken out and the solvent front was marked. The plate was dried at room temperature and inspected either under UV light or sprayed with the specific detecting reagent. The colored spots were marked and the R_f value of each separated component was calculated.[26]

Detecting reagents used

(1) UV at 254 nm.

(2) Anisaldehyde- Sulphuric acid reagent.

Chromatographic Profiling of *Ganoderma lucidum* Extracts

Petroleum ether extract

Following solvent systems were tried for achieving the best separation in case of petroleum ether extract of *G. lucidum* (Table 3):

Toluene: Ethyl Acetate (9:1)

Toluene: Ethyl Acetate (8:2)

Toluene: Ethyl Acetate (7:3)

Toluene: Ethyl Acetate (6:4)

Toluene: Ethyl Acetate (5:5)

Hexane: Ethyl Acetate (9:1)

Hexane: Ethyl Acetate (8:2)

Hexane: Ethyl Acetate (7:3)

Hexane: Ethyl Acetate (6:4)

Hexane: Ethyl Acetate (5:5)

Table 3: Thin layer chromatographic profile of *Ganoderma lucidum* petroleum ether extract

Sl. No.	Solvent System	Number of Spots	Resolution
1	Toluene: Ethyl Acetate (9:1)	2	Poor
2	Toluene: Ethyl Acetate (8:2)	**3**	**Excellent**
3	Toluene: Ethyl Acetate (7:3)	3	Very Good
4	Toluene: Ethyl Acetate (6:4)	2	Fair
5	Toluene: Ethyl Acetate (5:5)	2	Poor
6	Hexane: Ethyl Acetate (9:1)	2	Good
7	Hexane: Ethyl Acetate (8:2)	3	Good
8	Hexane: Ethyl Acetate (7:3)	2	Fair
9	Hexane: Ethyl Acetate (6:4)	2	Fair
10	Hexane: Ethyl Acetate (5:5)	2	Poor

Table 4: TLC profile of petroleum ether extract of *Ganoderma lucidum* in Toluene: Ethyl Acetate (8:2).

Spot No.	R_f Value	Color in UV (254 nm)[a]	Color in Sunlight[b]
1.	0.14	Light grey	Dark Blue
2.	0.42	Light grey	Violet
3.	0.95	Dark grey	Light blue

Detecting Reagents:
a. UV at 254 nm
b. Anisaldehyde-Sulphuric acid and heating at 110°C for 5 min

On the basis of above observations, the solvent system Toluene: Ethyl acetate (8:2) was found to exhibit best resolution with 3 distinct spots (Table 4).

Ethanolic extract

Following solvent systems were tried for achieving the best separation in case of ethanolic extract of *Ganoderma lucidum* (Table 5 and Table 6):

Dichloromethane: Methanol (9:1)
Chloroform: Methanol (9:1)
Chloroform: Methanol (8:2)
Chloroform: Methanol (7:3)
Chloroform: Methanol (6:4)
Chloroform: Methanol: Toluene (8:1:1)
Chloroform: Methanol: Toluene (8:3:1)
Chloroform: Methanol: Toluene (8:4:2)
Chloroform: Methanol: Toluene (8:4:4)
Chloroform: Methanol: Toluene (8:2:1)
Chloroform: Methanol: Toluene (7:2:1)
Chloroform: Methanol: Toluene (5:5:0.5)
Ethyl acetate: Methanol: Water (11:7:3)
Chloroform: Methanol: Water (6:2:2)
Chloroform: Methanol: Water (7:3:1)
Chloroform: Glacial Acetic Acid: Methanol: Water (16:8:3:2)

Aqueous Extract

Following solvent systems were tried for achieving the best separation in case of aqueous extract of *Ganoderma lucidum* (Table 7):

Butanol: Acetic acid: Water (4:1:2)

Table 5: Thin layer chromatographic profile of *Ganoderma lucidum* ethanolic extract.

Sl. No.	Solvent System	Number of Spots	Resolution
1	Dichloromethane: Methanol (9:1)	5	Good
2	Chloroform: Methanol (9:1)	4	Poor
3	Chloroform: Methanol (8:2)	4	Poor
4	Chloroform: Methanol (7:3)	4	Poor
5	Chloroform: Methanol (6:4)	4	Poor
6	Chloroform: Methanol: Toluene (8:1:1)	5	Good
7	Chloroform: Methanol: Toluene (8:3:1)	5	Fair
8	Chloroform: Methanol: Toluene (8:4:2)	5	Fair
9	Chloroform: Methanol: Toluene (8:4:4)	5	Fair
10	Chloroform: Methanol: Toluene (8:2:1)	6	Excellent
11	Chloroform: Methanol: Toluene (5:5:0.5)	4	Poor
12	Ethyl acetate: Methanol: Water (11:7:3)	3	Poor
13	Chloroform: Methanol: Water (6:2:2)	4	Poor
14	Chloroform: Methanol: Water (7:3:1)	4	Poor
15	Chloroform: Glacial Acetic Acid: Methanol: Water (16:8:3:2)	3	Poor

Table 6: TLC profile of ethanolic extract of *Ganoderma lucidum* in Chloroform: Methanol: Toluene (8:2:1).

Spot No.	R_f Value	Color in UV (254 nm)[a]	Color in Sunlight[b]
1.	0.04	Black	Dark grey
2.	0.06	Black	Dark grey
3.	0.64	Black	Light blue
4.	0.71	Black	Dark blue
5.	0.74	Black	Light purple
6.	0.93	Black	Violet

Detecting Reagents:
a. UV at 254 nm
b. Anisaldehyde-Sulphuric acid and heating at 110°C for 5 min

Table 7: Thin layer chromatographic profile of *Ganoderma lucidum* aqueous extract.

Sl. No.	Solvent System	Number of Spots	Resolution
1	Butanol: Acetic acid: Water (4:1:2)	1	Poor
2	Butanol: Acetic acid: Water (4:1:3)	1	Poor
3	Butanol: Acetic acid: Water (4:1:5)	1	Poor

Detecting Reagents:
a. UV at 254 nm
b. Anisaldehyde-Sulphuric acid and heating at 110°C for 5 min

Butanol: Acetic acid: Water (4:1:3)
Butanol: Acetic acid: Water (4:1:5)

Co-chromatography of the Extracts with Standard Marker

The extracts were subjected to co-chromatography on TLC plates using the best solvent systems with standard marker viz., β-sitosterol

to characterize the extracts for the presence of these phytochemical marker. It was found that β-sitosterol is present in the petroleum ether and ethanolic extracts of *Ganoderma lucidum.* Further confirmation of the presence of these markers in the extracts was done using high performance thin layer chromatography (HPTLC).

High Performance Thin Layer Chromatography

HPTLC is a versatile separation technique, which is official in most of the Pharmacopoeia intended for determining the content uniformity, purity, assay value, dissolution, drug-drug interaction and bioavailability of the drugs. It is precisely for these reasons that almost every research laboratory today is equipped with HPTLC system.[27,28] It simultaneously handles a number of samples even of divergent nature and composition supporting several analysis at a given time. It can be considered a time machine that can speed the work usually impractical with other analytical techniques.[29]

Crude drug extract standardization is the first step towards standardization of herbal medicines. Various tests for the overall standardization are well described e.g. in the Indian Herbal Pharmacopoeia. These standardization tests are limited to HPTLC characterization, for the purpose of routine quality control.

In the eastern hemisphere, the purpose of analysis and its quantification is "fingerprint" the total extract. The western countries on the other hand, believe in the "active principle" or "marker" concept i.e. the medicinal properties are attributed to a compounds solely. Since these terms can also lead to controversy "known fractions" may be used as a more accurate description of the purpose of analysis.

The "total extract" can only be described by "fingerprint" technique, in which a large amount of chromatographic data of "standard" is compared with the data from the samples. This characterization data is obtained from multiwavelength scanning in UV, fluorescence scan, *in-situ* UV spectra, image comparison and where applicable, data after post chromatographic derivatisation.

The "standard" extract would be a certified plant material, extracted in a well-documented way and whose biological activity has been proved for the intended use.

HPTLC fingerprint data comparison of such a "standard" with that of a sample can be accepted as the rapid, reliable and modern procedure for routine quality control as shown in the literature with various examples.[30,31]

The fingerprint method of analysis requires the complete standard extract and not just one fraction. These standard and sample extracts are HPTL chromatographed side by side. Then the TLC scanner is used to multiwave scan the plate, record UV absorbance spectra of all fractions, fluorescence if any, image documented by a digital camera in UV 254 and UV 366 nm. Occasionally, post chromatography derivatisation may be carried out for sensitivity reasons.

HPTLC fingerprint of a crude drug extract comprises of the following steps.

Development of Separation Method by HPTLC

A best resoluting system (mobile phase) is a primary requirement to obtain efficient results by HPTLC. This can be achieved by selecting the best mobile phase in repeated conventional TLC practices.

Multiwavelength Scanning

Since there are several fractions, a multiwavelength scan is almost always required. The procedure to arrive at the multiple wavelengths is as follows.

The UV absorbance spectra of all fractions are first recorded. The λ_{max} of each major or important fraction is then chosen as one of the wavelengths. Peaks at origin and at solvent front, if any are not taken into account for finger print. Sometimes, a compromise wavelength for two or more fractions may be found, as UV-spectra are not very distinct. A chromatogram containing e.g. 6 peaks, when scanned at 5 wavelengths gives up 5×6 peaks i.e. 30 peaks for comparison, without repeating chromatography. This is the purpose of multiwavelength scan.

These data can also be used for quantification, as one of the multiwavelength will be the λ_{max} of known fraction. The relative area percentage of the corresponding peaks must be within tolerance limits, set out by the analytical method. The tolerance limits can be set to a small value (large peaks) or large value (smaller peaks), for example.

A large number of batches must be analyzed first, to arrive at suitable tolerance values. This parameter ensures that peaks at the same R_f in standard and sample have similar quantity, even though of known origin.

Fluorescence Scan

Fluorescence scan (incident wavelength 366 nm, emitted > 400 nm) gives us unique information and is important data. A fluorescence scan and UV absorbance scan of the same chromatographed samples usually differ substantially.

UV Absorbance Spectra

UV absorbance spectra of major fractions provide essential data for three purposes. First, to make the selection of multiwavelength for scanning. Secondly to characterize a peak for comparison, without what it is. Characterisation is done by R_f as well as $\lambda_{max.}$ Thirdly, a modern HPTLC instrumentation allows a library of spectra to be created. This library can be used to search the matching spectra at that R*f*. Once a spectrum library is created, it may be possible to identify a crude extract using the library.

Image of Chromatographic Plate

Image of the chromatographic plate under UV 254 nm as well as 366 nm is taken for visual documentation. This visual aspect is unique to HPTLC.

In UV 254 nm, the background i.e. plate appears green or blue, due to the fluorescence indicator incorporated in the adsorbent while the fractions appears as dark spots due to fluorescence quenching. This image is nonspecific as only black spots are seen on a bright fluorescence background.

In UV 366 nm, fractions, which have native fluorescence, appear colored while the background is dark. This allows even faint fractions to be seen. More important, the fractions may have different colors, which can make this image quite specific. White light must be completely excluded when observing/photodocumenting fluorescence otherwise it interferes in the visibility fractions.

Post Chromatography Derivatisation

Optionally or additionally, post chromatography derivatisation can be done where a class of compounds present is to be quantified.

As per the guidelines of FIP for standardization of herbal medicinal products (HMPs), chemical markers whose therapeutic potential has not been investigated properly, but they present in a considerable amount in the drugs, can also be used to standardize the extracts and HMPs.

Countries like India, which cater both to domestic market and export, need to develop methods of analysis that can satisfy the needs of both types of analysis i.e. "finger print" and "quantification of known fraction". HPTLC has several advantages in herbal analysis as it is very fast (15-20 samples/hour), minimum little cleanup is required and most important, a huge amount of data is obtained from a chromatogram.

Selection of HPTLC Plates

The precoated and preactivated TLC plates (E. Merk No. 5548) of silica gel 60 $F_{254+366}$ with the support of aluminium sheets having thickness of 0.1 mm and size 20 × 20 cm were used.

Sample preparation: Accurately 2 gm of drug extract was weighed and dissolved with 20 ml of respective solvent. It was then extracted for 30 min by refluxing on water bath at 60°C to 70°C. The extract was cooled, filtered and finally volume made upto 20 ml with solvent.

Application of sample: The extract sample was applied in the form of a band using CAMAG LINOMAT V, an automatic sample application device, maintaining a band width 13.3 mm, space 9mm, 15 µl sec/. The quantity of sample applied was 5 µl to 10 µl.

HPTLC development: Following mobile phases were selected experimentally.

1. Toluene: Ethyl Acetate (8:2) for petroleum ether extract
2. Chloroform: Methanol: Toluene (8:2:1) for ethanolic extract

The plates were developed by placing in presaturated or precoated tank (12 cm height) with mobile phase for 2 hrs. The plates were dried by evaporating the solvent either at room temperature or by spraying hot air by air dryer.

Establishment of assay conditions: Standard and sample solutions were applied as 6 mm wide bands by means of Linomat applicator.

Linomat 5 Application Parameters

Spray gas	:	Inert gas
Sample solvent type	:	Methanol
Dosage speed	:	150 nl/s
Predosage volume	:	0.2 µl
Sequence		
Syringe size	:	100 µl
Number of tracks	:	6
Application position Y	:	8.0 mm
Band length	:	6.0 mm
Distance between tracks	:	13.3 mm
Position of first track X	:	10.0 mm
Scanner settings		
Plate size (with x-height)	:	10×10 cm
Application position Y	:	10.00 mm
Position of solvent front	:	95.00 mm
Scan start position Y	:	12.4 mm
Scan end position Y	:	95.0 mm
Scan start position X	:	10 mm
Distance between tracks X	:	13.3 mm
Number of tracks	:	7
Lamp	:	D2 & W
Monochromator bandwidth	:	20 mm
Slit dimension	:	6.0 × 0.45 mm
Data resolution	:	100 µm/ step
Display scaling	:	1000 AU
Measurement type	:	Remission
Measurement mode	:	Absorption
Scanning speed	:	20 mm/s
Optical filter	:	Second order
Zeroing mode	:	Automatic
Quick scan from	:	10.0 mm to 94.0 mm, all tracks
Sensitivity	:	Automatic
PM High voltage	:	277 V
Integration Parameters		
Data Filtering	:	Savitsky-Golay 7
Baseline correction	:	Lowest slope
Peak threshold min. slope	:	5
Peak threshold min. height	:	10 AU
Peak threshold min. area	:	50
Peak threshold max. height	:	990 AU
Track start position	:	15.6 mm
Track end position	:	94.0 mm
Calibration Parameter		
Calibration method	:	Single level
Internal standard	:	No
Statistics	:	CV
Results calculated for	:	Area only
Spot check mode	:	Automatic
Peak window size (auto)	:	3000 µm

Detection/scanning

CAMAG densitometric evaluation system with Camag software version 4.06 was used for scanning of thin layer chromatogram objects in reflectance or transmission mode by absorbance at 254 nm (Figures 1-6).

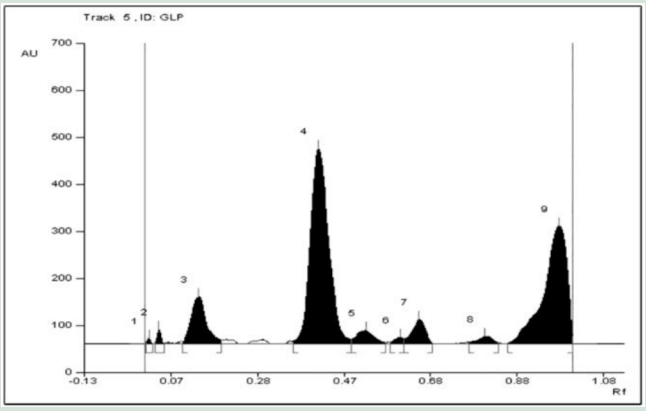

Figure 1: HPTLC Densitogram of petroleum ether extract of *Ganoderma lucidum.*

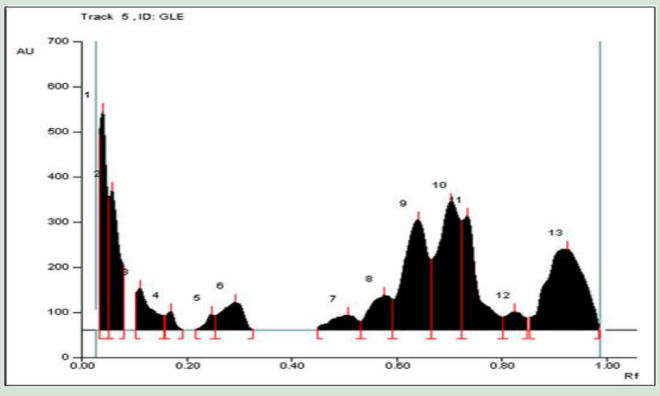

Figure 2: Densitogram of ethanolic extract of *Ganoderma lucidum.*

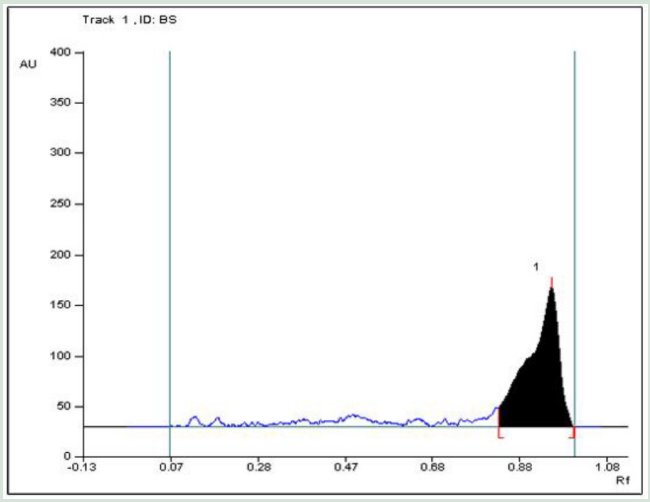

Figure 3: Densitogram of standard β-sitosterol in Toluene: Ethyl Acetate (8:2).

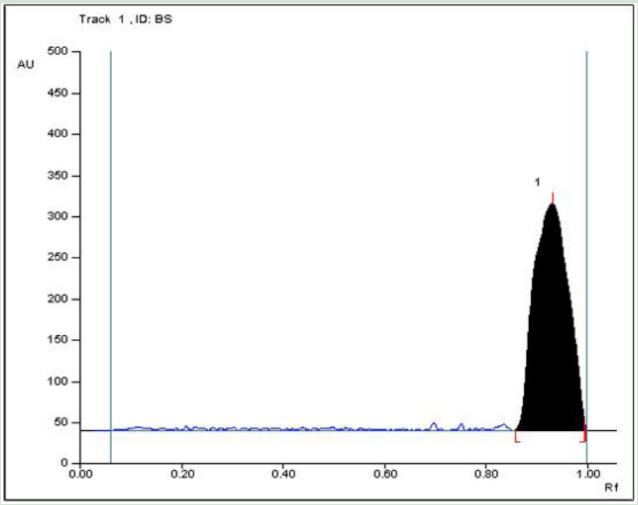

Figure 4: Densitogram of standard β-sitosterol in Chloroform: Methanol:Toluene (8:2).

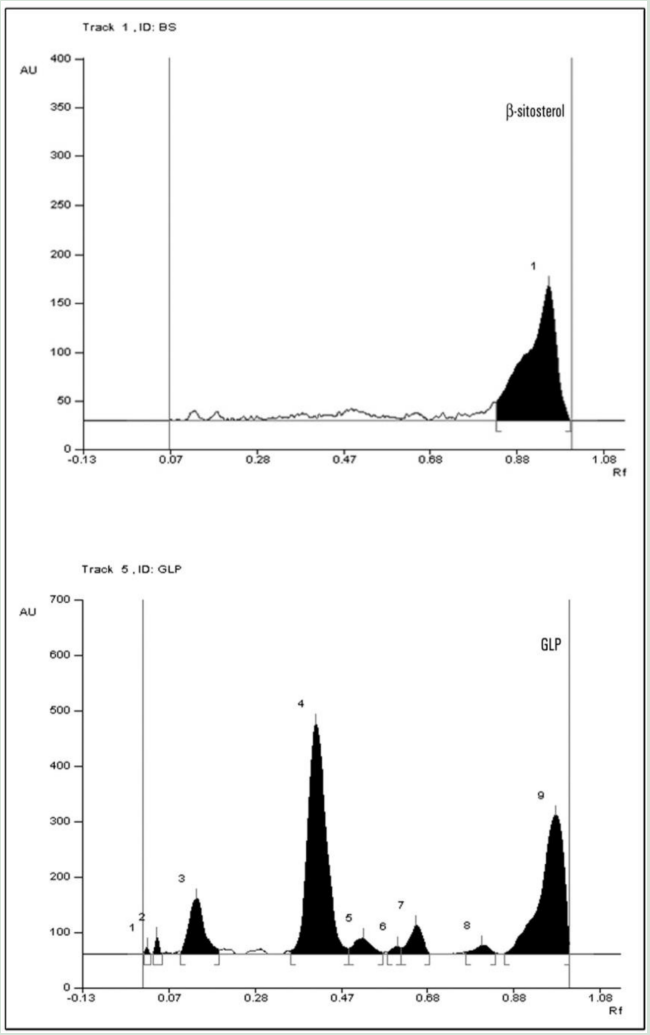

Figure 5: HPTLC densitometric profile of *Ganoderma lucidum* petroleum ether extract and standard β-sitosterol in Toluene:Ethyl Acetate (8:2) at 254 nm.

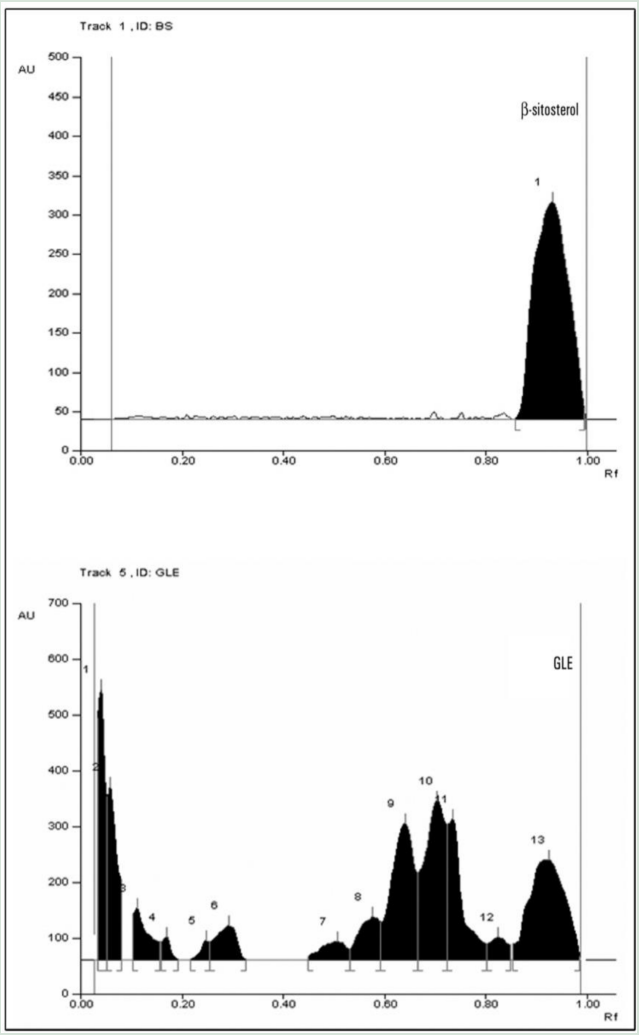

Figure 6: HPTLC densitometric profile of standard β-sitosterol and *Ganoderma lucidum* ethanolic extract in Chloroform: Methanol: Toluene (8:2:1) at 254 nm.

UV Overlain Spectral Comparisons of Marker Compounds Utilizing HPTLC for Ascertaining the Presence of Standard Marker in *Ganoderma lucidum* Extract

The UV spectrum of the detected compound in the extract is a unique identification marker for ascertaining its presence in the extract which can be analyzed using the spectral comparison function on the HPTLC instrument. The overlain spectra of the standard compound and the compound present in the extract gives a clear picture of the nature of the compound and rules out the possibilities of the presence of any other similar compound in the extract. This fact was given due consideration and the presence of β-sitosterol in the petroleum ether extract was confirmed using this technique. The overlain spectra are shown in Figure 7. Rf values, λmax and %area for petroleum ether extract and ethanolic extract as compared to standard β-sitosterol have been shown in Tables 8 and 9.

RESULTS AND DISCUSSION

Petroleum ether, ethanolic and aqueous extracts of *Ganoderma lucidum* was screened for their phytochemical profiles. Physical characterization of the extracts was followed by qualitative chemical examination using specific chemical tests for different categories of phytoconstituents to ascertain their presence in the tested extracts.

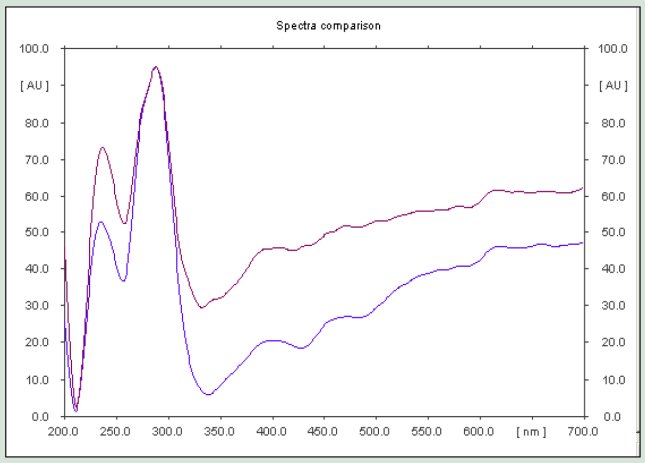

Figure 7: Overlain UV spectra of standard β-sitosterol and β-sitosterol present in the petroleum ether extract of *Ganoderma lucidum*.

Table 8: HPTLC profile of petroleum ether extract of *Ganoderma lucidum* and β- sitosterol in Toluene: Ethyl Acetate (8:2)

Ganoderma lucidum			β- sitosterol		
R_f value	λ_{max}	%Area	R_f value	λ_{max}	%Area
0.14	254	7.64	-	-	-
0.21	254	0.83	-	-	-
0.28	254	1.51	-	-	-
0.42	254	47.92	-	-	-
0.53	254	4.68	-	-	-
0.60	254	1.27	-	-	-
0.65	254	5.76	-	-	-
0.80	254	2.09	-	-	-
-	-	-	-	-	-
0.95	254	28.30	0.95	254	100

Table 9: HPTLC profile of ethanolic extract of *Ganoderma lucidum* and β- sitosterol in Chloroform: Methanol: Toluene (8:2:1).

Ganoderma lucidum			β- sitosterol		
R_f value	λ_{max}	% Area	R_f value	λ_{max}	% Area
0.04	254	10.23	-	-	-
0.06	254	8.58	-	-	-
0.11	254	3.85	-	-	-
0.17	254	0.97	-	-	-
0.25	254	0.83	-	-	-
0.29	254	3.63	-	-	-
0.51	254	2.31	-	-	-
0.58	254	4.60	-	-	-
0.64	254	15.78	-	-	-
0.71	254	17.70	-	-	-
0.74	254	10.67	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
0.82	254	2.03	-	-	-
0.93	254	18.82	0.93	254	100

Ganoderma lucidum petroleum ether extract was pale yellow colored and semisolid in consistency (yield- 0.51% w/w). Ethanolic extract was brownish black and semisolid with a yield of 3.05% w/w. Aqueous extract was solid and black colored (yield 0.62% w/w).

Ganoderma lucidum petroleum ether extract revealed the presence of steroids as major constituents. The ethanolic extract showed the presence of proteins, phenolics, flavonoids, tannins and triterpenoids. Aqueous extract was rich in carbohydrates, proteins, flavonoids, phenolics and tannins.

Thin layer chromatographic studies were carried out to find out the best solvent systems for the various extracts of *Ganoderma lucidum* and following were the best solvent systems.

Petroleum ether extract- Toluene: ethyl acetate (8:2)

Ethanolic extract- Chloroform: Methanol: Toluene (8:2:1)

The extracts were subjected to co-chromatography on TLC plates using the best solvent systems with standard marker viz., β-sitosterol to characterize the extracts. It was found that β-sitosterol is present in the petroleum ether and ethanolic extracts. Further confirmation of the presence of these markers in the extracts was done using high performance thin layer chromatography (HPTLC). HPTLC confirmed the presence of β-sitosterol in the petroleum ether and ethanolic extracts. The % area of β-sitosterol in the petroleum ether extract was 28.30 at a R_f value of 0.95 when visualized at 254 nm using toluene: ethyl acetate as the solvent system. Ethanolic extract showed a percentage area of 18.82%. Solvent system used was chloroform: methanol: toluene (8:2:1). Detection was done at 254 nm and R*f* noted for β-sitosterol was 0.93. All these analyses were performed using pure β-sitosterol as a standard.

Further the overlain spectral analysis function of the HPTLC instrument was exploited to confirm the presence of β-sitosterol in the extracts to rule out the possibilities of the presence of any other similar compounds. Overlain UV spectra are an accurate confirmation of the presence of the

compound when a reference standard has been used in the analysis. The presence of β-sitosterol was confirmed in the tested extracts (Figure 7).

CONCLUSION

Ganoderma lucidum is a wide spectrum multitalented mushroom which can help cure many human aliments if clinical trials are done with this mushroom on such diseases. Owing to its immunomodulatory and anticancer properties, it is gaining attention worldwide and attracting the attention of the west significantly. Being in the neutraceutical market for many years, *Ganoderma lucidum* is a major part of health supplements and beverages like coffee and tea.[32,33] Many countries have approved its use as medicine too. It is the need of the hour to understand the usefulness of medicinal mushrooms in better interest of human population all over the world. The phytochemical profile and chromatographic studies will be an aid to the quality control of formulations containing *Ganoderma lucidum* and its extracts.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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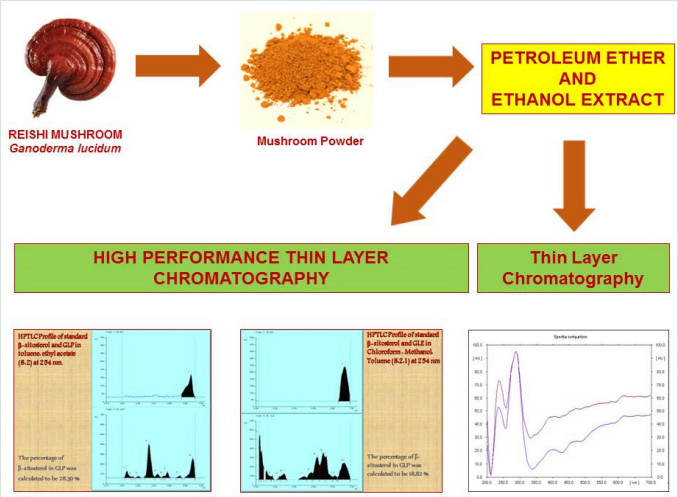
GRAPHICAL ABSTRACT



SUMMARY

- *Ganoderma lucidum* is considered to be a natural medicine that promotes longevity and maintains the vitality of human beings. Its beneficial clinical effects in patients with hepatitis, hyperglycemia, chronic bronchitis, cancer, muscular dystrophy, arteriosclerosis, hypertension, hypercholesterolemia, and leukopenia have been confirmed in pharmacologic studies in recent years. The fruiting bodies, mycelia, and spores have recently received more and more attention not only as home remedies but also as new drug sources. Being an important medicinal mushroom, it was thought necessary to scan the mushroom for its chemical constituents and to work out its chromatographic profile via thin layer chromatographic and high-performance thin layer chromatographic approaches.
- Owing to its immunomodulatory and anticancer properties, it is gaining attention worldwide and attracting the attention of the west significantly. Being in the neutraceutical market for many years, *Ganoderma lucidum* is a major part of health supplements and beverages like coffee and tea. Many countries have approved its use as medicine too. It is the need of the hour to understand the usefulness of medicinal mushrooms in better interest of human population all over the world. The phytochemical profile and chromatographic studies will be an aid to the quality control of formulations containing *Ganoderma lucidum* and its extracts.

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Prof. Dr. V.K. Dixit has served as Professor and head, Department of Pharmaceutical Sciences, Dr. Harisingh Gour Vishwavidyalaya, Sagar, India having a teaching experience of about 35 years at Undergraduate and 30 years at Postgraduate level. Now Prof. Dixit has retired for the department and being actively involved in research on natural products and plant biotechnology, he has many national and international research and review articles to his credit. He has authored many books which have become the landmarks in Pharmaceutical education. He has guided many Ph.D. and M.Pharm. students during his tenure. Now Dr Dixit is the Editor-in-Chief of Indian Journal of Natural Products. He is an active member of APTI and Society of Pharmacognosy.

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