Determination of Phytocompounds from methanol extracts of *Ganoderma lucidum* by LC-MS

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Abstract: Ganoderma is an herb that has been in use for more than 2000 years to alleviate a great numbers of health complaints. Thus the present study focused on the qualitative determination of phytochemicals, extraction and purification of bioactive compounds from *Ganoderma lucidum*. The preliminary phytochemical screening showed positive reactions for alkaloid, flavanoid, terpenoid, steroid, reducing sugars and carbohydrates tests except saponins. The LC-MS/MS study reported that totally 13 compounds were screened based on negative ionization $[M-H]^-$ and also 12 peaks were identified from FTIR spectrum with their functional groups. The peak showed (M-H)- ion at *m/z* 169 indicate the Gallic acid. The compound Theanine, Caffeic acid and Caffeine were assigned for peak as the MS pattern depicted a parent ion at *m/z* 174.2, 183.1 and 194.1respectively. The FT-IR spectrum was used to identify the functional groups of the active components present in extract based on the peaks values in the region of IR radiation. IR spectrum of the isolated compound was registered a broad band cm^{-1} and stretching band at 3277.49 to 410 cm^{-1}. Some bands in the FTIR spectrum of methanol extracts of *Ganoderma lucidum* showed different bands at range of 3277.49, 2923.05, 1636.19, 1373.48, 1033.1, 656.57, 589.71, 553.03, 520.68, 485.73, 433.75 and 410 cm^{-1}. A strong peak at 3277.49 cm^{-1} in *Ganoderma lucidum* corresponds to O-H/C-H stretching of the carboxylic acid group.

Key words: Phytochemical, *Ganoderma lucidum*, FT-IR, Methanol extracts and Ionization.

INTRODUCTION

Traditional medicines are used not only for primary health care of the poor in developing countries, but also in countries where conventional medicine is predominant in the national health care system . The herbal medicines serve the health needs of about 80% of the world s population, especially for millions of people in the vast rural areas of developing countries; more than 65% of the global population uses medicinal plants as a primary health care modality (Kamaraj *et al.*, 2012). Plants have been a major focus of investigations for novel biologically active compounds. However, filamentous fungi have been the producers of some of the most powerful secondary metabolites which have been developed into therapeutic agents (Roberts, 2004).

Mushrooms are the group of fungi which are widely used as food and medicine in different parts of the world since long time. Mushroom contain a vast amount of compound among which are polysaccharide and triterpenes. *Ganoderma* species belong to the division Basidiomycota, class Homobasidiomycetes, and order Aphyllophorales, family Polyporaceae (Alexopolus *et al.,* 1996). They are numbered among several species of wood degrading fungi (Jonathan *et al.,* 2008). Sheena *et al.,* (2003) reported that the major secondary metabolites of *Ganoderma lucidum* are ganodermic acid, triterpenes and carcinostatic polysaccharides. Medicinal mushrooms are widely used as traditional medicinal ingredients for the treatment of various diseases and related health problems. *Ganoderma* species like any other fungi grow wild on living or dead or dying wood log of hardwood and some times on dead roots (Prasad *et al,* 2008, Quereshi *et al.,* 2010).

Phytochemicals are non-nutritive plant chemicals having protective or disease preventive properties. The phytochemical screening of plants reveals the presence of primary and secondary metabolites that suggest the plant might be of medicinal or industrial importance. Phenolic acids are a group of secondary metabolites widely distributed in plants and several studies have reported their inhibition effect on the growth of pathogens and cancer cells (Rocha-Guzman *et al.,* 2009). For instance, a total of 15 phenolic acids detected in a Mexican plant *Quercus resinosa* were shown to be responsible for the growth inhibition of cervical cancer cell. A HPLC-MS technique is often used for separation, identification and quantitation of flavonoids and phenolic acids in plants. A total of 9 phenolic acids was separated from the leaves of Chinese sweet potato (*Ipomea batatas*) within 60 min by employing a gradient mobile phase of water/acetonitrile/glacial acetic acid (980/20/5, v/v/v, pH 2.68) and acetonitrile/glacial acetic acid (1000/5, v/v) with flow rate at 3 mL/min and detection at 325 nm (Zheng and Clifford, 2008). Herchi *et al.*(2011) developed a gradient solvent system of 0.5% acetic acid (A) and acetonitrile (B) to separate 5 phenolic acids in flax seed oil within 35 min with flow rate at 0.8 mL/min and detection by electrospray ionization (ESI)-time of flight (TOF)-mass spectrometry (MS). However, the number of phenolic acids separated is limited. However, the solvent system is quite complex and resolution remains inadequate as co-elution of peaks occurred. The present study was undertaken to extraction and purification of bioactive compounds from *Ganoderma lucidum* by using HPLC-MS-MS.

MATERIALS AND METHODS

Collection of Sample

Ganoderma lucidum was randomly collected from nearby Orathanadu, Thanjavur district, Tamil Nadu, India and it was authenticated from the Rapinat Herbarium and centre for molecular systematic St. Joseph's college, Tiruchirappalli, Tamil Nadu, India. Collected samples were brought to laboratory in polythene bags and cleaned thoroughly with fresh water to remove adhering debris and associated biota. And it was cleaned using brush for the removal of the epiphytes with distilled water. After cleaning the fungus was dried in shade at room temperature for a week.

Extraction of bioactive compounds

The whole *Ganoderma lucidum* was initially rinsed thrice in distilled water and dried on paper towelling, and samples (25g) cut into fine pieces and boiled with 100ml of sterile distilled water for 5 minutes. The crude extract was passed through Whatman no.1 filter paper and the filtrates stored at 4°C for further use to perform various assays for determination of bioactivity.

Preliminary Phytochemical Screening

The different Qualitative chemical tests can be performed for establishing a profile of given extract for its chemical composition. The extract was then subjected to qualitative chemical tests for various phytoconstituents (Raaman, 2006 and Shamaki *et al.*, 2012).

Test for alkaloids: The extract was warmed with 2% H_2SO_4 for two minutes. It was filtered and few drops of below reagent were added and indicated the presence of alkaloids. Picric acid (1%) a formation of yellow precipitation indicated positive.

Test for flavonoids: A small quantity of the extract was heated with 10mL of ethyl acetate in boiling water for three minutes. The mixture was filtered differently and the filtrate used for the following test.

Test for terpenoids: The extract was mixed with 2ml of chloroform and conc.H2SO4(3ml) was carefully added to form a layer. A reddish brown coloration of the interface was formed to show positive result of the presence of terpenoids.

Test for Phytosterols: To the extract, 3 ml of acetic anhydride was added and mixed. To this one or two drops of concentrated H2SO4 were added slowly along the sides of the test tube. An array of colour change showed the presence of phytosterols.

Test for steroids: 2ml of acetic anhydride was added to extract along with 2ml of H2SO4.The colour changed from violet to blue or green in samples which indicated the presence of steroids.

Test for saponins: A small quantity of extract was diluted with 4 ml of distilled water. The mixture was shaken vigorously and then observed on standing for stable brake.

Test for reducing sugar: A small fraction of the extracts were added vigorously with 5 ml of distilled water and filtered to the filtrates while equal volumes of Fehling`s solution A and B added and were shaken vigorously. A brick red precipitation indicated positive.

Test for carbohydrates: The extract was shaken vigorously with water and then filtered. To the aqueous filtrate was added few drops of Molisch reagent, followed by vigorous shaking again, concentrated H2SO4 1 ml was carefully added to form a layer below the aqueous solution. A brown ring at the interface indicated the positive test

Test for Glycosides: To the extract, few drops of 10% NaOH were added to make it alkaline. Then freshly prepared sodium nitroprusside was added to the solution. Presence of blue colouration indicated the presence of glycosides in the extract.

Test for Proteins: An aliquot of 2 ml of extract was heated with 1 drop of 2 % CuSO4 solution. To this 1 ml of ethanol (95%) was added, followed by excess of KOH Pellets. Pink colour in the ethanolic layers indicated the presence of proteins.

Test for Phenolic Compounds: To the extract, few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of phenolic compounds.

HPLC Conditions

UHPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with two Shimadzu UHPLC: Nexera UHPLC system Column: Shim-pack XR-ODS III (100 x 2 mm, 2.2 µm particle size) Column temp.40ºC. The mobile phase consisted of (A) 0.1% formic acid in water and (B) Acetonitrile. Both mobile phases were filtered through a cellulose nitrate filter, diameter 47 mm, pore size 0.45 µm (Sartorius, Goettingen, Germany). After the gradient separation, the column was equilibrated for 5 min using the initial solvent composition. The flow rate was set to 1 mL/min, The samples were kept in amber vials at 4 °C in the autosampler, and the injected volume was 5 µL. The separation was performed at 25.0 ± 0.1 °C.

MS/MS Conditions : LC-MS/MS System (Make: Shimadzu Corporation, Kyoto, Japan, Model: LCMS 8040, Triple Quadrupole) Ionization: ESI (Positive / Negative) ,Ion spray voltage: +4.5 kV / −3.5 kV , Dwell time 5 msec. / Pause time 1 msec Ambient CDL Temperature : 250º C Block Temperature : 400º C Detector voltage : 1.3kv Nebulizer Gas flow: 1.5 l/min Drying gas : 10 L/min Detection. The mobile phase was filtered through a 0.22 µ membrane and degassed using ultrasonicator.

Extraction and Purification

A method based on Kao *et al.* (2008) was modified and used for extraction of phytocompounds from *Ganoderma lucidum*. A 0.25 g of *Ganoderma lucidum* powder sample was mixed with 15 mL of methanol (100%). The mixture was then shaken at 60 °C for 3 h, centrifuged at 10,000 rpm for 30 min and the supernatant was evaporated to dryness under vacuum using a rotary evaporator. The residue was dissolved in 10 mL of deionized water. Next, the crude extract was subjected to purification in a SPE cartridge based on a method described by Inbaraj *et al.*, (2010). Initially, 5 mL of crude extract was poured into a vial and adjusted to pH 7 with 2% sodium hydroxide. Then 1 mL was collected and poured into a C18 cartridge (500 mg/3 mL, 55 µm, 70 A°), which was previously activated sequentially with 10 mL each of methanol and deionized water. The phytocompounds were eluted with 15 mL of deionized water. The volume of eluents was optimized for complete elution by evaluating 2, 3, 4 and 5 mL of methanol (100%). Each eluate was then evaporated to dryness and dissolved in 1 mL of deionized water.

FTIR Spectroscopic Analysis

Fourier transform infrared spectrophotometer is perhaps the most powerful tools for identifying the types of chemical bonds (functional groups) present in compounds. Dried powders of different solvent extracts of each plant material were used for FTIR analysis. 10mg of the dried extract powder was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample disc. The powdered sample of each plant specimen was loaded in FTIR Spectroscope (Shimadzu, IR Affinity1, Japan), with a scan range from 400 to 4000 cm-1 with a resolution of 4cm-1.

Results and Discussion

Qualitative phytochemical screening

The preliminary phytochemical screening of *Ganoderma lucidum* revealed that the extracts contain carbohydrates, glycosides, triterpenoids and phenolic compounds. The preliminary phytochemical screening showed positive reactions for alkaloid, flavanoid, terpenoid, steroid, reducing sugars and carbohydrates tests except saponins (Table 1). Mushrooms contain a wide variety of bioactive molecules, such as terpenoids, steroids, phenols, nucleotides and their derivatives, glycoproteins, and polysaccharides. The chemical constituents of *G. lucidum* include polysaccharides, proteins, nucleosides, fatty acids, sterols, and triterpens. Polysaccharides, peptidoglycans, and triterpenes are three major physiologically active constituents in *G. lucidum* (Boh *et al*. 2007; Zhou et al. 2007). This result was in accordance to the previously reported literature (Shamaki *et al*., 2012). Phytochemical constitutes of plants serves as defense mechanism against by many microorganisms. Thus the preliminary screening test may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and improvement.

For identification of bioactive compounds in *Ganoderma lucidum* without standard in single mass spectrometer with ESI (Electrospray ionization) mode and triple quadrupole tandem mass spectrometer (MS/MS) (Table.2). The peak showed (M-H)- ion at *m/z* 169 indicate the Gallic acid. The compound Theanine, Caffeic acid and Caffeine were assigned for peak as the MS pattern depicted a parent ion at *m/z* 174.2, 183.1 and 194.1respectively. Similarly, Peak 29 was identified to be Caffeoyl-hexose-deoxyhexoside based on the parent ion at *m/z* 487 and the fragment ion obtained at *m/z* 308 by expulsion of Caffeic acid moiety and at *m/z* 179 due to loss of Deoxyhexose plus hexose moieties (Rivera-Pastrana *et al.,* 2010). The peak 5 and 6 were characterized as Ferulic acid and Theacrine based on the parent ion at *m/z* 194.1 and 224.2. MS profiling of peak 7 and 8 yielded a parent ion at *m/z* 290.2 and 302.2 corresponding to Catechin and Quercetin. The Epigallo Catachin was assigned for peak 9 based on the [M-H]- value at *m/z* 306.2. Peaks 10 and 11 with parent ions at *m/z* 442 and 458.3 were characterized as a Catechin gallate and Epicatachin gallate. The compounds Quercetin hexoside and Rutin were corresponding on the parent ions at *m/z* 463 and 610.5 (Table.2). In earlier study eported that the peak 19 with a parent ion at *m/z* 473 was characterized to be a derivative of Chicoric acid (dicaffeoyltartaric acid), as daughter ions were generated at *m/z* 311 and 293 owing to the loss of Caffeic acid moiety and Caffeic acid plus H_2O molecule, respectively (Schutz *et al.,* 2005).

The FT-IR spectrum was used to identify the functional groups of the active components present in extract based on the peaks values in the region of IR radiation. When the extract was passed into the FT-IR, the functional groups of the components were separated based on its peaks ratio. IR spectrum of the isolated compound was registered a broad band cm^{-1} and stretching band at 3277.49 to 410 cm^{-1}. Some bands in the FTIR spectrum of methanol extracts of *Ganoderma lucidum* showed different bands at range of 3277.49, 2923.05, 1636.19, 1373.48, 1033.1, 656.57, 589.71, 553.03, 520.68, 485.73, 433.75 and 410 cm^{-1}(Fig.1). A strong peak at 3277.49 cm^{-1} in *Ganoderma lucidum* corresponds to O-H/C-H stretching of the carboxylic acid group. The alkyl (C-H) and carbonyl (C=O) groups were seen at 2923.05 and 1636.19 cm^{-1} respectively. The peak at 1033.1 cm^{-1} was due to the C-N stretching of the aromatic amines. FTIR spectroscopy is proved to be a reliable and sensitive method for detection of bio molecular composition. In the previous study reported that the FTIR spectrum shows a very high intense band at 1744 cm-1 associated with the existence of the ester carbonyl functional group (O-C=O) and very weak shoulder peak of cis double-bond (C=C) stretching was noticed at ~1655 cm^{-1} (Veeraprakash *et al.,* 2018).

CONCLUSION

Mushrooms are the group of fungi which are widely used as food and medicine in different parts of the world since long time. In this present study revealed that the rich composition of phytochemicals were showed in the methanol extracts of *Ganoderma lucidum*. The LC-MS/MS study reported that totally 13 compounds were screened based on negative ionization $[M-H]^-$ and also 12 peaks were identified from FTIR spectrum with their functional groups. LCMS and FTIR parameters, which could serve as important and has commercial interest in both research institutes and pharmaceuticals companies for the manufacturing of the innovative drugs. In the present study concluded that the potential bioactive compounds are presented in the *Ganoderma lucidum* and it's conformed by LCMS and FTIR parameters. Further research is needed to investigate the discovery of bioactive compounds and their structure and efficacy in clinical trials.

Table. 1 Qualitative phytochemical analysis of methanol extracts of *Ganoderma lucidum*

S.No	Phytochemical analysis	Observation
1	Alkaloids	Presence
2	Flavonoids	Presence
3	Terpenoids	Presence
4	Phytosterol	Presence
5	Steroids	Presence
6	Saponins	Absence
7	Reducing sugar	Presence
8	Carbohydrate	Presence
9	Glycosides	Presence
10	Proteins	Presence
11	Phenol	Presence

Table.2 Phytocompounds screened by LC-MS/MS in methanol extracts of *Ganoderma lucidum*

S.No	Compound	Parent Ion (m/z)	[M-H] Negative Ionization	
			Absolute intensity	Relative intensity
1	Gallic acid	169	150285	100
2	Theanine	174.2	24145	16.0
3	Caffeic acid	183.16	4170	2.7
4	Caffeine	194.19	15785	10.5
5	Ferulic acid	194.18	33838	22.5
6	Theacrine	224.2	69207	46.0
7	Catechin	290.2	91338	60.7
8	Quercetin	302.2	28308	18.8
9	EpiGallo catachin	306.2	23335	15.5
10	Catachin gallate	442	15504	10.3
11	Epicatachin gallate	458.3	34260	22.8
12	Quercetin hexoside	463	11908	7.9
13	Rutin	610.5	150285	100

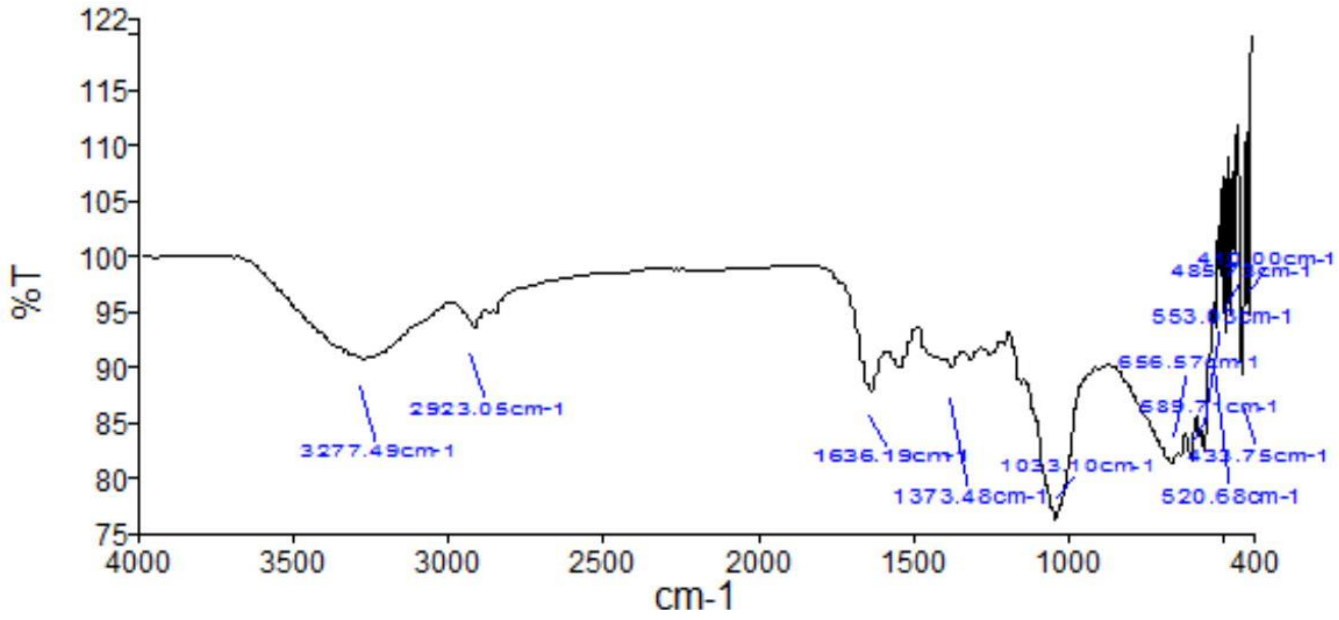

Fig.1 FT-IR spectrum in methanol extracts of *Ganoderma lucidum*

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