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Detection of Squalene compound in *Ganoderma lucidum* through thin-layer Chromatography (TLC)

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ABSTRACT: Thin-layer chromatography is a simple, *inexpensive,* easy, and reliable method widely used for detection of various toxins present in food and plant materials in Plant Pathology. Thin layer chromatography can also be used to identify the nature of different plant compounds: anti-oxidative, antibacterial, or antifungal. In this study, we have used this method to detect the antiviral compounds in the *Ganoderma lucidum. Ganoderma lucidum,* one of the medicinal mushrooms, has antiviral properties against the groundnut bud necrosis virus infecting tomatoes. The antiviral compound squalene has already been reported. Hence, the presence of squalene in different extracts of *G. lucidum, viz*., secondary metabolites, mycelial extracts, and basidiocarp extract, was analysed by the thin layer chromatography (TLC) method. The results of red brick colour formation and refractive index in the different extracts of *G. lucidum* have confirmed the presence of squalene. Analysing the costs of the various techniques, TLC emerged as the most affordable method for sample analysis. TLC is the most convenient and economical method for finding compounds in various microorganisms.

Key words: *Ganoderma lucidum*, Squalene, Thin-layer chromatography (TLC)

INTRODUCTION

Ganoderma lucidum (Fr. Karst) it belongs to the Class: Agaricomycetes, phylum: Basidiomycota, order: Polyporales, family: Ganodermataceae and commonly known as Reishi or Lingzhi. It is globally used for medicinal purpose especially herbal medicine. It prolonging human life because it has a several bioactive compounds and metabolites especially triterpenoids, meroterpenoids, sterols, polysaccharides, lectins, proteins, peptidoglycans (Cor *et al*., 2018). The extracts of *Ganoderma viz*., basidiocarp, mycelium and culture filtrates have antibacterial, antiviral, and antifungal properties and cures several diseases in humans (Liu and Zhang 2005; Teng *et al*., 2011). Different parts of *G. lucidum* are commercially available, including mycelia, spores, and fruit body, and are sold as many different products, including powders, dietary supplements, and herbal tea (Wachtel-Galor and Sissi 2011). Because of its importance and demand in wild grown *Ganoderma*. It was commercially cultivated in China and Japan (Chang and Buswell 2008). According to a research of *G. lucidum* non-volatile ingredients, the mushroom includes 1.8% ash, 26-28% carbohydrate, 3-5% crude fat, 59% crude fiber, and 7-8% crude protein (Mau, *et al*., 2001).These comprise extracts and separated components in a variety of formulations that are sold all over the world as pills, lotions, syrups, and hair tonics.

G. lucidum is an enormous, dark mushroom that stands out for having a glossy surface (including a kidney-shaped cap that is red-varnished) and a woody texture. Fresh mushrooms are flat, squishy, cork-like, lack gills on the underside, and exude spores through tiny pores. Depending on the mushroom's age, the pores on its underside may be white or brown (Arora, 1986). It is widely grown in Europe, Asia, North and South America, especially in temperate rather than subtropical regions. Chen (1999) described the nature of *G. lucidum* growth on the bases and stumps of a wide variety of deciduous trees, including oak, maple, elm, willow, sweetgum, magnolia, and locust. Less frequently found on coniferous trees (such as larix, ptea, and pinus).

Apart from this, metabolites, and culture filtrates of *Ganoderma* used to manage the diseases in plantsdue its antimicrobial properties. Also, the metabolites of *Ganoderma lucidum* and *Ganoderma applanatum* were used to manage the viral disease like against tobacco mosaic virus (Kovalenko *et al*., 2008). After a decade, Sangeetha *et al*., 2020 reported the antiviral activity of *G. lucidum* against bud blight disease in tomato caused groundnut bud necrosis virus. Also found the squalene is the triterpenoid compound responsible for the antiviral activity by GC-MS analysis. This study was formulated to confirmed the presence of squalene in *G. lucidum* through thin layer chromatography.

Thin layer chromatography is one of the most widely used separation techniques in aflatoxins analysis. It is made up of a stationary phase made of silica, alumina, or cellulose immobilized on an inert material called the matrix, which is made of glass or plastic. The mobile phase is a methanol, acetonitrile, and water mixture that transports the sample through the solid stationary phase. The distribution of aflatoxins between the mobile and stationary phases in TLC is primarily determined by differences in analyte solubility between the two phases. Depending on their molecular structures and interactions with the stationary and mobile phases, different analytes either adhere to the stationary phase more or remain in the mobile phase, allowing for rapid and effective separation (Betina, 1985).

Thin-layer chromatography (TLC) analysis was processed based on the principle of compound and movement of metabolites using the mobile phase in the silica-coated TLC plate and compound nature was identified by Rf value. This technique is used largely for metabolomics studies. Favre and Ryder (1996) detected squalene epoxidase in human pathogenic fungus *viz.*, *Trichophyton rubrum* by thin layer chromatography analysis.

The thin-layer chromatography method was used by Kowalska and Sajewicz (2022) to identify different compounds in botanicals. Additionally, it is used to check for antibiotics in food. According to Ciela *et al.* (2012), TLC can be used to differentiate between various drug metabolites. In the quest for diverse physiological qualities of botanical material, TLC-based screening techniques are frequently employed to identify the antibacterial, enzyme-inhibiting, and free radical scavenging activities of medicinal plants (Kagan *et al.*, 2014).

Silica gel TLC plates were frequently used for the qualitative and semi-quantitative determination of triterpenes isolated from different species of *Ganoderma*. Different solvents, such as chloroform-methanol-water (30:4:1) and chloroform-diethyl ether-methanol (9:1:1), were used for TLC analysis by Kohda *et al*. (1985). Furthermore, Casalicchio *et al*. (1975) used a TLC chromatographic method to assess the content of fatty acids in *G. lucidium*. In order to distinguish adenine, adenosine, uracil, and uridine from the fruiting bodies of *G. lucidum*, Huie and Di (2004) utilized TLC chromatography. Singh *et al.* (2012) reported the triterpenoid variability in different *Ganoderma* species, especially *G. applantum*, *G. lucidum* and *G. tsuga* collected in different parts of India

Dhara *et al*. (2010) used the TLC method to characterize and quantify phytosterol and other minor components present in three Indian minor seed oils: mahua (*Madhuca latifolia*), sal (*Shorearobusta)*, and mango kernel (*Mangifera indica*). Sasikala and Sundaraganapathy (2017) detected the presence of triterpenoids in the hydroalcoholic extract of *Ipomoea aquatica* using TLC analysis.

Holzl and Dormann (2021) separated the complex lipid mixtures from plant tissues by thin-layer chromatography (TLC). The glass plates coated with silica gel are used as the stationary phase and an organic solvent as the mobile phase, using different solvent systems to separate polar membrane lipids or nonpolar lipids by TLC. Depending on the complexity of the lipid mixture, lipids are separated using one- or two-dimensional TLC systems.

Kaya *et al*. (2011) identified the presence of high amounts of lipids, squalene compounds in the *Aurantiochytrium sp.* using TLC with time- of-flight mass spectrometry. Nakazawa *et al.*, 2014 investigated on the squalene production from 176 strains of *Aurantiochytrium* sp. (Labyrinthulomycetes) using TLC and identified the squalene-rich strain which contained approximately 1 g/L of culture volume. As a result, we used TLC to determine the presence of squalene in various *G. lucidum* extracts.

MATERIALS AND METHODS

A. Sample preparation

The *Ganoderma lucidum* mycelial culture were sub cultured in a Mushroom complete medium (MCM) (Lee *et al.*, 2009) and this medium were sterilized in autoclave 121°C for 20 min and used for further studies. Single disc fungal cultures were inoculated into MCM broth and incubated for 20 days at 25 °C on an orbital shaker. After 20 days the cultures were filtered through Whatman No.4 filter paper and centrifuged at 7000 rpm at 4 °C for 10 min to separate cell mass from the mushroom complete broth. Also, the mycelial mat was separately taken for extraction. After that, the supernatant was transferred into conical flasks and added with an equal volume of ethyl acetate and kept in a shaker for 24 h. After that, solvents were separated using separating funnel. Separated solvents were concentrated in vacuum flash evaporator at 40°C, 150 rpm. Then the solvents were evaporated overnight. The metabolites were collected from evaporator by gently scrapping with scarpee and dissolved in HPLC- grade methanol and filtered using a 0.2 µm syringe filter and obtained 2 mL of metabolite for TLC analysis**.** As the same above procedure was followed to extract metabolites from the Mycelial mat and Basidiocarp (Fig. 1).

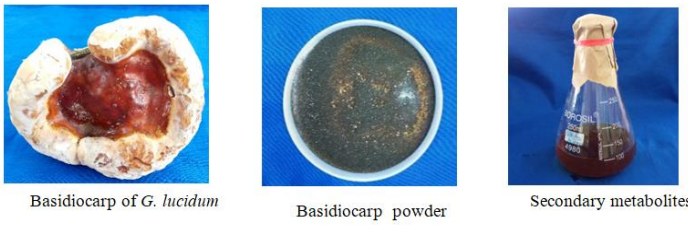

Fig. 1. Extraction of metabolites from basidiocarp of *G.lucidum.*

Thin Layer Chromatography (TLC). Squalene standard purchased from the sigma was used in this study to identify the triterpenoid compound (squalene) present in the *G. lucidum* metabolites. The secondary metabolites 20 µL were dotted in the TLC plate. Thin-layer chromatography plate coated with silica gel of 20× 20 cm (TLC Silica gel 60 F254, CAT No. # HX880096). Mobile phase hexane: chloroform (9:1) was used in the TLC tank. Plates were kept in the mobile phase for separation of the secondary metabolites and standard in the TLC tank for 25 min. As negative control methanol was used. After that 10 per cent of sulphuric acid (H_2SO_4) was sprayed like mist into the TLC plate. Then the plates were heated at 70° C to visualize the triterpenoids in the samples and standard. Pink to brick red colour was formed based on the concentration and calculated by colour variation. The squalene spots were classified into following concentration; ++ (extreme); + (high); ± (medium); – (low), trace or no squalene. This procedure is given by Nakazawa *et al. (*2014), was followed.

Retention or retardation factor *(R_f)* value is calculated by following formula

$$= \frac{\text{distance travelled by the component}}{\text{distance travelled by the solvent}}$$

RESULT AND DISCUSSION

The presence of squalene in *G. lucidum* was further confirmed by Thin Layer Chromatography (TLC). The methanolic fractions of basidiocarp, mycelial mat and secondary metabolites (broth culture) of *G. lucidum* were subjected to TLC. The samples of about 20 µl were spotted in silica coated aluminium TLC plate. The plates were observed with pink to brick red colour spots when sprayed with 10 per cent sulphuric acid. Strong red brick colour spots (+) were obtained for standard squalene with R_f of value 0.93 (Fig. 2). Other spots were also obtained in the same distance with R_f of value 0.93. Among all the samples analysed, secondary metabolites containing sample was found with dense spots of squalene (+ high), followed by light pink colour (±) with the sample of mycelial mat representing the medium size spots and mild pink colour (-) for the basidiocarp sample, while no colour (--) was noticed with the methanol control (Table 1). This result showed that, dense squalene spots were found in the secondary metabolites sample when compared to others. From this result the presence of squalene in *G. lucidum* was confirmed. Similarly, Su *et al*. (2001) determined that the ganoderic acids B and C are specific components of *Ganoderma* by TLC. The presence of these substances was confirmed by compounds exhibiting characteristic bright red spots on a TLC plate after spraying with 10% H_2SO_4 and followed by heating. TLC has also been used to distinguish between various *Ganoderma* species.

Similarly, Yang *et al*. (2012) detected the triterpenes and polysaccharides by using high-performance thin-layer chromatography (HPTLC) method. This study stood evidence for TLC is the effective method to detect the triterpenoids in *G. lucidum*. Strong evidence to our study, Thakur *et al*. (2014) estimated the triterpenoids from fruit body extracts of *G. lucidum* through silica gel thin layer chromatography with an eluent of chloroform: methanol (10:1) for the first extract and dichloromethane for the second extract.

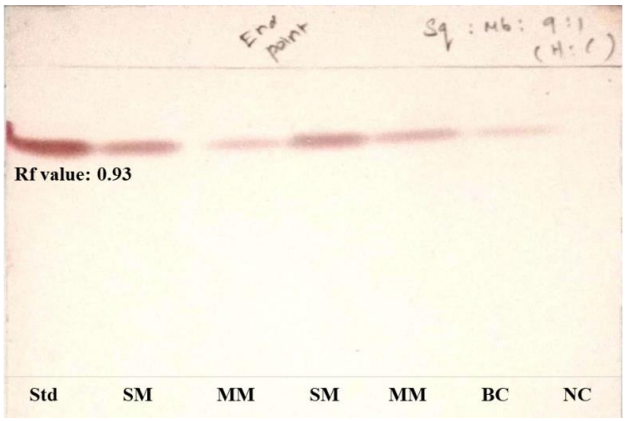

Fig. 2. Detection of squalene in the bio-extracts of *G. lucidum* using TLC. Std - Standard, SM - Secondary metabolite, MM- Mycelial mat, BC - Basidiocarp, NC - Negative control (Methanol).

Table 1: *Thin-layer chromatography* (TLC) analysis of presence of squalene in the metabolites of *G. lucidum*.

Samples	Sample Name	Colour of spots	Squalene
Std	Standard	Brick red colour	++
SM	Secondary metabolites extract	Pink colour	+
MM	Mycelial mat extract	Light pink colour	±
SM	Secondary metabolites extract	Pink colour	+
MM	Mycelial mat extract	Light pink colour	±
FB	Basidiocarp extract	Mild pink colour	-
NC	Negative control (Methanol)	No colour	--

++ Extreme; + High; ± Medium; - Low; -- Absent

The macro basidiomycetous fungi are natural sources of phenols, triterpenoids, terpenoids and sterols. The basidiomycetous fungus *Ganoderma lucidum* (Fr. Karst) is valued for its medicinal properties in treating assorted diseases and expanding life span. *G. lucidum* possesses bioactive metabolites, such as polysaccharides, lectins, proteins, peptidoglycans, triterpenoids, meroterpenoids, sterols and alkaloids (Cor *et al.*, 2018). These molecules broadly possess antiviral (El-Mekkawy *et al*., 1998), antifungal (Ferreira *et al*., 2015) and antibacterial (Stojkovic *et al*., 2014) activities. These novel molecules are extracted from basidiocarps, mycelium and culture filtrates. The presence of certain volatile compounds in *G. lucidum* was responsible for salicylic acid signaling pathway, inducing systemic resistance in plants (Cavalcanti *et al*., 2007). Hence, hypothetically assuming that, application of *G. lucidum* biomolecules on plants may activate various signaling processes, might result in the reduction of infection of GBNV in cowpea and tomato (Sangeetha *et al*., 2020).

TLC studies were taken up to further confirm the presence of squalene in the mycelial mat, fruiting body and secondary metabolites of *G. lucidum*. The highest amount of squalene was detected in secondary metabolites fraction extracted from the 20 days old broth culture of (Fig. 2). Similarly, Theresa *et al*. (2022) reported a high amount of phenolic and triterpenoid compounds in the methanolic extract of *Ganoderma lucidum* when compared to the water extracts. Like our results, pink spots were observed in TLC plates sprayed with anisaldehyde-sulfuric acid reagent, which indicate the presence of triterpenes. Similarly, Squalene epoxidase was detected in human pathogenic fungus *viz.*, *Trichophyton rubrum* by thin layer chromatography (Favre and Ryder 1996). Similar to the present results, Nakazawa *et al.* (2014) detected the squalene production in an algae *Aurantiochytrium* sp. using thin layer chromatography and quantified the squalene using HPLC analysis. Like our investigation, Razafindralambo *et al*. (1993) used thin layer chromatography (TLC) and first discovered the antifungal lipopeptides in the supernatant of *B. amyloliquefaciens* PG12. The presence of antimicrobial compounds in different plants was analysed using TLC-direct bioautography as a high throughput method (Choma *et al.*, 2015). Similarly, Hanani *et al.* (2017) detected the alkaloids, tannins, flavonoids, steroids, triterpenoids, saponins, phenols, glycosides, and carbohydrates in alcoholic, chloroform, and aqueous extracts of *Mirabilis jalapa*, a potential herb-drug product in Indonesia, using thin layer chromatography (TLC).

CONCLUSIONS

Various chromatographic methods were used for the analysis of secondary metabolites and compounds from the samples. Apart from all, thin-layer chromatography is an easy and cost-effective, and conventional method widely used to find the aflatoxins and various toxins from food products. The presence of an antiviral compound *viz.*, squalene from different extracts of *G. lucidum* was easily confirmed by this TLC method.

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Conflicts of Interest. None.

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