IDENTIFICATION OF GANODERIC ACIDS AND DERIVATIVES FROM FRUITING BODIES OF *GANODERMA LUCIDUM*

A Project submitted to Central University of Punjab

For the award of

Master of Science

In

Life Sciences

(Department of Plant Sciences)

By

Agrah Pradhan

Supervisor

Dr Sanjeev Kumar

Department of Plant Sciences

School of Basic and Applied Sciences

Central University of Punjab, Bathinda

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Certificate

I declare that dissertation entitled “IDENTIFICATION OF GANODERIC ACIDS AND DERIVATIVES FROM POWDERED FRUITING BODIES OF *GANODERMA LUCIDUM*” has been prepared by me under guidance of Dr. Sanjeev Kumar, Associate Professor, Head of department, Department of Plant Sciences, School of Basic and applied sciences, Central university of Punjab. No part of this thesis has formed the basis for award of any degree or fellowship previously.

Agrah S Pradhan (Reg:16mslsps09)

Department of Plant Sciences, School of Basic and Applied Sciences,

Central University of Punjab, Bathinda (151001),

Punjab, India

Date: 30.05.2018

Certificate

I certify that AGRAH SUBASH PRADHAN has prepared his dissertation entitled "IDENTIFICATION OF GANODERIC ACIDS AND DERIVATIVES FROM POWDERED FRUITING BODIES OF *GANODERMA LUCIDUM*" for award of M.Sc degree of Central University of Punjab, under my guidance. He has carried out this work at Department of Plant Sciences and Department of Pharmaceutical Sciences, Central University of Punjab.

Dr. Sanjeev Kumar

Associate Professor,

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Date: 30.05.2018

Abstract

Ganoderic acids and other bioactive triterpene metabolites from crude extract of wood decaying fungus *Ganoderma lucidum* from two different hosts *Acacia* and *Azadirachta* were to be isolated as proposed. The method for extraction was simple and divided into three parts, 1. Solvent selection, 2. Dissolving metabolites into solvent for maximization of number of detected metabolites, 3. Extraction of metabolites from solvent. A variety of methods for maximizing dissolution in solvent based on pharmaceutical extraction protocols were employed. Sonication was chosen as the best method with detection of 5 distinct spots (metabolites) from TLC. Ethanol based sample extracts were packed inside silica gel column while using chloroform as the mobile phases in column chromatography. Some metabolites were isolated and confirmed by TLC. Due to time bound project work the further isolation was paused and the crude extracts were forwarded for GC-MS detection and analysis of constituent metabolites. After preparation of sample and analysis in gas chromatography – mass spectroscopy (GC-MS), 16 metabolites were found in chloroform extract of ganoderma having azadirachta as host and 24 metabolites were found from acacia host associated fungal crude extract. The detected metabolites were alkaloids, phenols, fatty acids (and precursors), esters, di-ene compounds and sterols (terpenes).Terpenes (C_{28}-Phytosterols) were found with molecular weight 396-398. 7,22-Ergostadienol with area 2.91% and 1.19% is found in Acacia and Azadirachta samples. Moreover another metabolite 7,22-Ergostadienone with area 0.61% is also found in Acacia (Host) sample this metabolite has various bio-active properties like anti-inflamatory and cytotoxic properties. These are derivatives of the ergosterols found in fungal body. These structures are products of lanosterol, (squalene derived products) they have many anthropocentric exploitive uses and they resemble the metabolite backbone (derivatives) we were initially looking for.

Keywords: Acacia, azadirachta, chloroform extract, sonication, TLC, GC-MS, column chromatography, terpenes.

Acknowledgement

With all due respect I express my version of sincere gratitude towards my supervisor Dr. Sanjeev Kumar, Associate Professor, HOD Plant Sciences and my co-supervisor Dr. Raj Kumar, Associate Professor, HOD Pharmaceutical Sciences; who have guided me through every step of my project with full interest and I am grateful to students from Plant Sciences Lab and Pharmaceutical Sciences Lab for providing me ample research environment and cooperating positively with my work and providing me with necessary help during the completion of the project with all the facilities given by the lab. The acknowledgement is empty without expressing gratitude towards Arindam Adhikary (Ph.D, Plant sciences lab) and Neha (M.Pharm, Pharmacutical sciences lab) for their valuable guidance and timely suggestions during the entire period of my project work. Lab Assistants of both labs were also very helpful for providing necessary requirements without which this work would not have been completed. Lastly I feel grateful to carry out research in the laboratories of university so I thank my university administration whose regulations made these efforts successful.

Agrah Pradhan

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List of abbreviations

Serial no	Name	Abbreviations.
1	Dimethylallyl diphosphate	DMAPP
2	Isopentenyl diphosphate	IPP
3	Geranyl pyrophosphate	GPP
4	(2E, 6E)-farnesyl pyrophosphate	(2E, 6E)-FPP/ FPP
5	Geranylgeranyl pyrophosphate	GGPP
6	Oxidosqualene/lanosterol cyclase	OSC
7	Squalene hopene cyclases	SHC
8	Lanosterol synthase	LSS
9	Cytochrome P_{450} superfamily	CYPs
10	Thin Layer Chromatography	TLC
11	Gas chromatography - Mass spectroscopy	GC-MS

CHAPTER 1

INTRODUCTION TO AREA OF RESEARCH

CHAPTER 1

INTRODUCTION

Fungi are the heroes in the race of evolution due to their unique environmental fitness. With diversity of about 2.2 million to 3.8 million species and a long history ranging about 900 million years, these organisms have efficiently adapted to almost every habitat present on earth. Survival ability of the fungus is result of actively metabolized natural product pathways. These bioactive properties have enabled fungi to successfully coexist in any ecological niche by inherent evolutionary ability to compete for nutrients, to put off predators, and interactions with organisms in the ecosystem. Discovery of natural product pathways therefore holds great promise for the bio-pharmaceutical industry. With 55,000 scaffolds terpenes are a major group of identified bioactive natural products (Chávez et al., 2016). These are incredibly structurally diverse class of natural products derived from 5C precursors DMAPP (dimethyl allyl diphosphate) and IPP (isopentenyl diphosphate) from mevalonate pathway (Kuzuyama et al., 2012).

Condensation of monomers (IPP and DMAPP) results in linear hydrocarbons of varying length C_{10}, C_{15} and C_{20} namely GPP (geranyl pyrophosphate), (2E,6E)-FPP ((2E, 6E)-farnesyl PP), and GGPP (geranylgeranyl PP) (Chang et al., 2010).

Terpenes synthesis occurs when linear hydrocarbons undergo a de-phosphorylation and cyclization cascade which is catalysed by enzymes known as terpene synthases. Depending on the length of the precursor molecule, fungal terpene synthases are known to produce sesquiterpenes (C_{15}), diterpenes (C_{20}) and triterpenes (C_{30}). Cytochrome P_{450} mono-oxygenases, oxido-reductases, and different group transferases modify the core terpene scaffold producing overabundance of terpenes (Janocha et al., 2015).

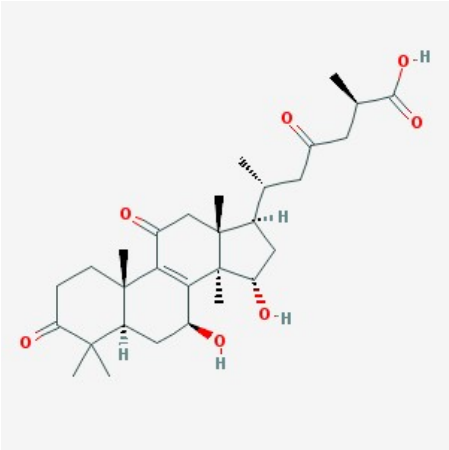


Figure 1.1: Ganoderic acid A ($C_{30}H_{44}O_7$) Boiling point 690.1 °C

Source: PubChem, Description: Data deposited in or computed by PubChem

Ganoderic acids are derivatives from lanosterols and they come in many forms with slight changes in core structure; GA-A/B/F/G/H/T/C_1/S/beta/etc (Cao et al., 2017). About 198 derivatives of GAs and similar metabolites have been isolated and characterized, among those GA-A and GA-B are the most well-known ones possessing several biological activities (Sabulal et al. 2015; Ulrike et al. 2015). These fungal species have become the model organisms for the extraction of terpenes and derivatives.

Hypothesis

The fungus is an active producer of triterpenes (terpenes in general) which have a great promise for the health industry. As it is reported a vast reservoir of terpenes is actively metabolized inside its fruiting body, though triterpenes are actively produces in primordial stage and in fruiting body they are present in low quantities. Collected samples were mature and dried fruiting bodies, it is probable that our samples may contain some amounts of triterpenes. Previously the fungal crude extract is known to have potential cytotoxic effects (Gill, B. S., Kumar, S. 2016), ganoderic acid being a major bioactive triterpene; the proposed hypothesis is that this crude extract may have different ganoderic acids or their derivatives based on its host.

CHAPTER 2
REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

Scientific classification:

Kingdom	Fungi
Division	Basidiomycota
Class	Agaricomycetes
Order	Polyporales
Family	Ganodermataceae
Genus	Ganoderma
Species	*G. Lucidum* ***(Curtis) P. Karst (1881)***

Table no. 1 - Scientific classification of *Ganoderma lucidum*

Ganoderma lucidum a macro-fungus synthesizes plethora of bioactive metabolites. It has also been popularised in the ancient medicinal texts of some regions of world. *G. lucidum*, also known as 'the mushroom of immortality' is one of the best-known medicinal macro fungi in the world. Modern pharmacological research has laid vision of potential therapeutic activities besides anti-inflammatory, anti-tumour, anti-hypertensive and immune-modulatory activities, they are also known to possess anti-thrombosis properties (Yi et. al., 2017).

The fungus is a storehouse of bioactive metabolites; making itself a ‘virtual cellular factory' for bio-pharma industry. Triterpenes and polysaccharides are found in excess in G. lucidum. It is known that at primordial stage the fungi contains maximum tri-terpenes; but in cultured mycelium and fruiting bodies low quantities are found. It also secretes proteins that can adequately disintegrate both cellulose and lignin. Such enzyme activities may act as valuable resource for biomass usage, fiber bleaching and organo-pollutant degredation (Chen et al., 2012). Our comprehension of G. lucidum science is restricted in spite of its part in conventional Chinese medicine and its amazing stockpile of bioactive metabolites.

The area of interest fungal triterpenes

The triterpenes are C_{30} prenyl chain derived metabolites that are found widely in nature as steroids and sterols. The biosynthetic pathway towards ergosterol, the major component of the plasma membrane in fungi, begins with the cyclization of squalene to lanosterol. Squalene-epoxidation occours forming 2,3-oxidosqualene (chair-boat-chair). Linear chain of polyisoprene is cyclized by oxidosqualene cyclase/lanosterol cyclase (OSC). Methyl migrations (two) and hydride shifts followed by deprotonation, results into lanosterol (Quin et al. 2014).

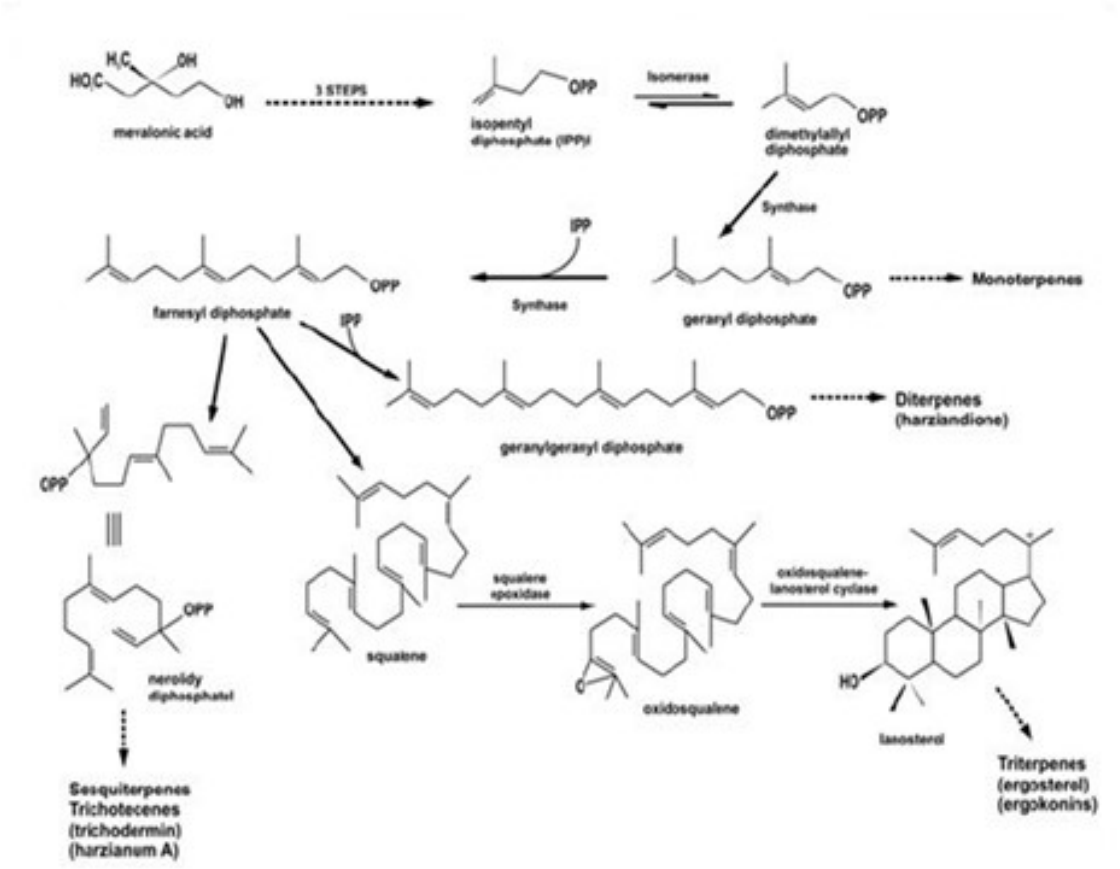


Fig. 2.1: Biosynthesis of different terpenes from Mevalonic acid (Rosa et al., 2005))

Prokaryotic and eukaryotic triterpene cyclases share a similar overall protein fold, with two α-helical and a membrane linking channel. Aromatic active site cavity extents up to two domains and the protonating group initiating cyclization are located at polar region. Triterpene cyclases contains conserved 16 amino

acid repeats of which Gln and Trp residues offer bonding network amid the α-helices (Quin et al. 2014) (stabilization during exergonic reaction). Bacterial SHC and eukaryotic OSC have diverged sequence and mechanism. Evolutionary divergence is observed both in SHC accepting squalene as a substrate and OSC accepting 2,3-oxidosqualene as substrate. Substrate activation is favoured by a protonating environment, aromatic residues which stabilizes the carbocation intermediates, forming lanosterol.

Tri-terpenes biosynthesis

More than 150 triterpenes have been isolated until now and are being investigated keenly for wide range of health benefits. Upstream of cyclization in mevalonic acid pathway constitutes 13 genes encoding 11 enzymes (Diner et al. 2018). Two genes encode for Acetyl-CoA, C-acetyltransferases and farnesyl diphosphate synthases; remaining are encoded by single-copy genes. Lanosterol synthase (LSS) synthesizes a cyclic intermediate of triterpenes and ergosterols; Lanosterol from where pathways diverge. The steps following cyclization include a series of oxidation, reduction and acylation reactions. Oxidations are catalysed by cytochrome P_{450} superfamily proteins (CYPs) hence modification of the lanosterol skeleton (Chen et al. 2012). Ergosterols and ganoderic acids are the major product class of tri-terpenes produced from the lanosterol skeleton. Ganoderic acid A (among many GAs) suppresses proliferation and invasion and induces apoptosis in human osteosarcoma cells (Shao et al. 2015), inhibits proliferation, invasion, and promotes apoptosis in human hepatocellular carcinoma cells (Wang et al. 2017).

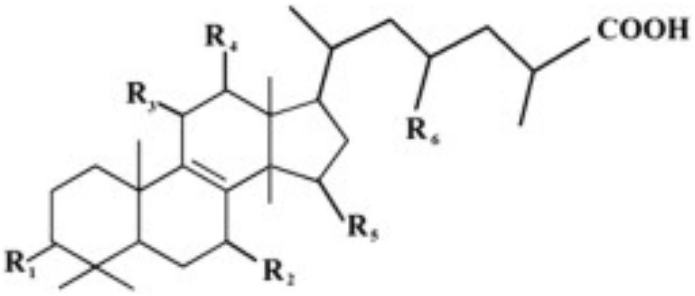


Fig.2.2: Core molecular structure of GAs, different R-groups (R1–R6) define GA-subtype and biochemical properties
(Figure adapted from (Liu et al. 2012))

Objectives of the study

• Identification of Ganoderic acids and associated triterpene derivatives from powered fruiting body of fungal sample.

• Gas chromatography and Mass Spectroscopic analysis of fungal crude extract from two different hosts, *Acacia* and *Azadirachta*.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

SAMPLE COLLECTION

Powdered fungal sample were collected from Plant Sciences lab, which were previously collected and deposited by former PhD student, Balraj Singh Gill. Samples were collected from two different plant hosts namely *Acacia* and *Azadirachta*.

CHOOSING THE PROPER SOLVENT

The fungal sample were weighed in equal parts each of 0.5 grams and dissolved in 10 ml of ethanol, chloroform, petroleum-ether respectively. The sample were left overnight and Thin Layer Chromatography (TLC) checks were done to predict the total number of metabolites dissolved in the solvent and further extracted as prominent spots in the TLC plate. Maximum number of metabolites was identified by ethanol extracts; therefore it was selected as the proper solvent.

CHOOSING THE PROPER MOBILE PHASE

TLC running was performed in silica gel plates as stationary phase and chloroform: methanol (10:1) as mobile phase. The mobile phase must be the solvent whose polarity should be such that the flow rate of metabolites would be uniform and slow. The movement of metabolites in ethanol is fast and no movement was shown in ethyl acetate so chloroform was checked and found out to be better. The movement of metabolites in chloroform was slow and it could be aided with ethanol (chloroform: ethanol; 10:1) for desirable speed and fine separation.

TLC PLATES VISUALIZATION

Plates were visualized after drying and dipping TLC plates in iodine crystals, resulting in iodine binding with metabolites and resulting in a dense dark line with interspaced dots identified as metabolites.

ISOLATION OF BIOACTIVE COMPONENTS INTO SOLVENT

• **Maceration**

In this method sample (0.5g) were taken in a round bottom flask (50ml) and refluxed for 24 hours. Extracts were taken in two round bottom flasks respectively one containing 10 ml of methanol and containing 10 ml chloroform. After 24 hours TLC was checked and it is found that in the methanol extract 2-3 major spots are visible and similarly in the chloroform extract 2-3 major spots are present.

• **Sonication**

0.5 grams of sample was mixed with 10 ml of ethanol in a beaker and kept for sonication for about one hour in the sonicator setting the temperature at 55 degrees. The sample was filtered and collected in an round bottom flask (50ml) and concentrated by using the rotor-evaporator apparatus and TLC check was performed whereby resulting in 4-5 spots in the TLC plate unevenly interspaced (maximum number of metabolites visible and separated). The sonicator temperature and duration of placing sample in sonicator can be tangled with as the metabolite we need to identify is ganoderic acid and derivatives who have boiling point near to 690.1^{o}C (GA-A).

• **Maceration + Sonication**

0.5 grams of sample was mixed in 10 ml of ethanol in a beaker and macerated and sonicated afterwards as described above (purpose is disruption of cellular membranes so as to extract maximum number of metabolites from the sample and dissolve them in solvent). 3 spots observed in TLC.

• **Sonication + Refluxing**

0.5 grams of sample with 10 ml ethanol in beaker were sonicated for an hour with temperature set to 55 degrees and refluxed afterwards in the apparatus for 24 hours. Resulting extract was checked in TLC plate and 3 prominent spots were found.

• **Column Chromatography**

Column for chromatography was collected from pharmaceutical sciences lab and it was packed with semi aqueous silica gel and sample extract with solvent was stacked on the top. Chloroform was the selected mobile phase for separation of metabolites, it was poured from the top of column and the solvent passes through the silica gel packed material carrying with it the metabolites stacked at the top. Metabolites move according to their relative sizes and molecular weights and are influences by the mobile phase flowing down the column and accordingly the metabolites flow out of the silica gel packed column wherein the metabolite with low molecular weight and small size are driven out first and accordingly metabolites separate in ascending order of molecular masses and sizes. Test tubes and holders from Plant Sciences Lab were employed for the chromatographic experiment. The column was not let to dry out and was always packed with mobile phase (the gauge was closed when column was not running and at the top of column a cotton plug is inserted to avoid impurities being mixed with our sample), hence metabolites were separated slowly with uniform flow rate and drying was prevented. Metabolites were isolated and estimation of total amount of mobile phase needed for isolation of metabolites was done. And the extraction was put on hold as the flow rate was disturbed and separation was hampered.

1. Identification

NMR (to be carried out in future)

Isolating pure crystals from crude extract

The powdered fruiting body (0.5 g) is mixed with 10 ml ethanol and heated in the heating mantle for about 25-20 minutes and shaken at intervals; metabolites present in the powder could have been made soluble in the solvent (ethanol). The extract is filtered; the solvent is covered in parafilm and left overnight in low temperature conditions. The sample is observed the next day for formation of crystals of pure metabolites, which could thereby sent for NMR analysis. It was hypothesised that the metabolites would cluster together in

ethanol and form crystals which could be seen by naked eyes, after 24 hours crystals formed were analysed and found impure and minute in sizes.

Converting Ganoderic acids into salts and detection

As we were searching for ganoderic acids in the sample we would look for acids, metabolites of similar molecular wt., sizes, boiling points, etc., accordingly was the process. First of all 10 grams of crude sample from acacia host was sonicated with 10 ml of ethanol and products were filtered out. Pet ether (10 ml) was added to the sample to dissolve all the impurities of low polarities. Base (5-6 pellets of NaOH) was then added to the sample and all the acids present in the sample have been converted to salts, and the salts reside in the middle layer between the two layer of solvents, wherein Pet ether (top layer) and ethanol (basal layer). The results (salts) were analysed in GC-MS apparatus by dissolving them with chloroform in vials.

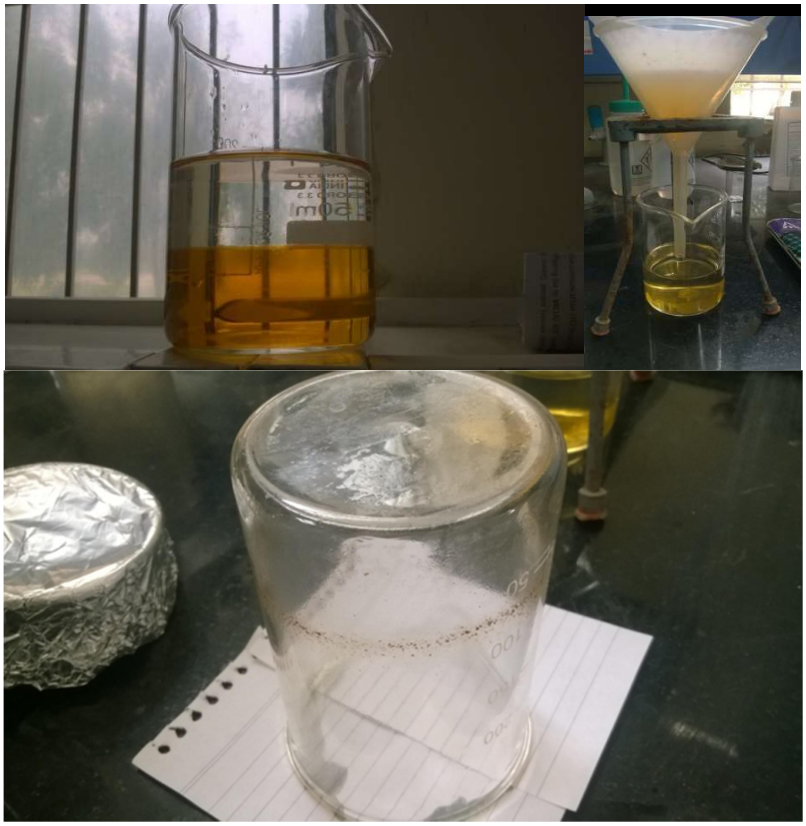


Figure 3.1: Conversion of acidic metabolites into salts; - Two layers of solvents with their constituent metabolites are well separated, upper one being pet ether and lower

one ethanol. In next figure the solvents were separated and layer of salt retained in the beaker was extracted and remaining salts were filtered out.

GC-MS Analysis

Fungal samples from two different hosts Azadiracta and Acacia were analyzed in search for metabolites present in both of them by gas chromatography and mass spectroscopy.

1. Sample Collection

Samples were collected from different hosts of *Ganoderma* namely *Acacia* (host) and *Azadirachta* (host) by former Ph.D student Balraj Singh Gill from different locations in Bathinda (Chetak Park, Bir Talab etc.). The mycelium of the fungal samples were converted into powdered form and stored for analysis as the chemical constituents do not change in converting the samples into powder form.

2. GC-MS sample preparation

Fungal samples 10 grams each was mixed with 10 ml of ethanol (high polarity) and sonicated for about one hour, temperature of the sonicator was set to 55^0C. Following sonication the samples were filtered with filter paper and concentrated in rotavaporator. Vials of adequate sizes were borrowed from Central Instrumentation Lab (Central University of Punjab) and sample were loaded in them and marked accordingly (different samples from different hosts). Chloroform was added to samples in the vials and samples were sent for GC-MS analysis. Detection would be maximized when sample is diluted properly otherwise, sample may pose harm to the gas column used for detection.

GC-MS ANALYSIS PARAMETERS

Column Oven Temp. :60.0 °C
Injection Temp. :250.00 °C
Injection Mode :Splitless
Sampling Time :1.00 min
Flow Control Mode :Pressure (Pressure :150.0 kPa)
Total Flow :18.0 mL/min

Column Flow :2.51 mL/min

Linear Velocity :57.7 cm/sec

Purge Flow :3.0 mL/min

Split Ratio :5.0

Oven Temperature

Rate	Temperature (^{O}C)	Hold time (minute)
--	60.0	1.00
30.00	270.0	0.00
10.00	310.0	5.00

Table no. 2 – Oven temperature setup for GC oven

Equilibrium Time: 3.0 min

IonSourceTemp: 200.00 °C

Interface Temp. : 280.00 °C

Solvent Cut Time: 2.50 min

Detector Gain Mode: Relative

Detector Gain: 1.84 kV +0.00 kV

Threshold: 0

MS Table

Start time	**3.00 min**
End Time	**17.00 min**
ACQ mode	**Scan**
Event time	**0.50 sec**
Scan speed	**1666**
Start m/z	**20.00**
End m/z	**800.00**

Table no. 3: Mass Spectroscopy parameters

CHAPTER 4

RESULTS

CHAPTER 4

RESULTS

Choosing the proper solvent and mobile phase

Proper solvent (ethanol) and proper mobile phase (chloroform) were selected out of many solvents available in the laboratory according to their metabolite dissolution properties (solvent) and metabolite mobility, preferably slow (mobile phase).

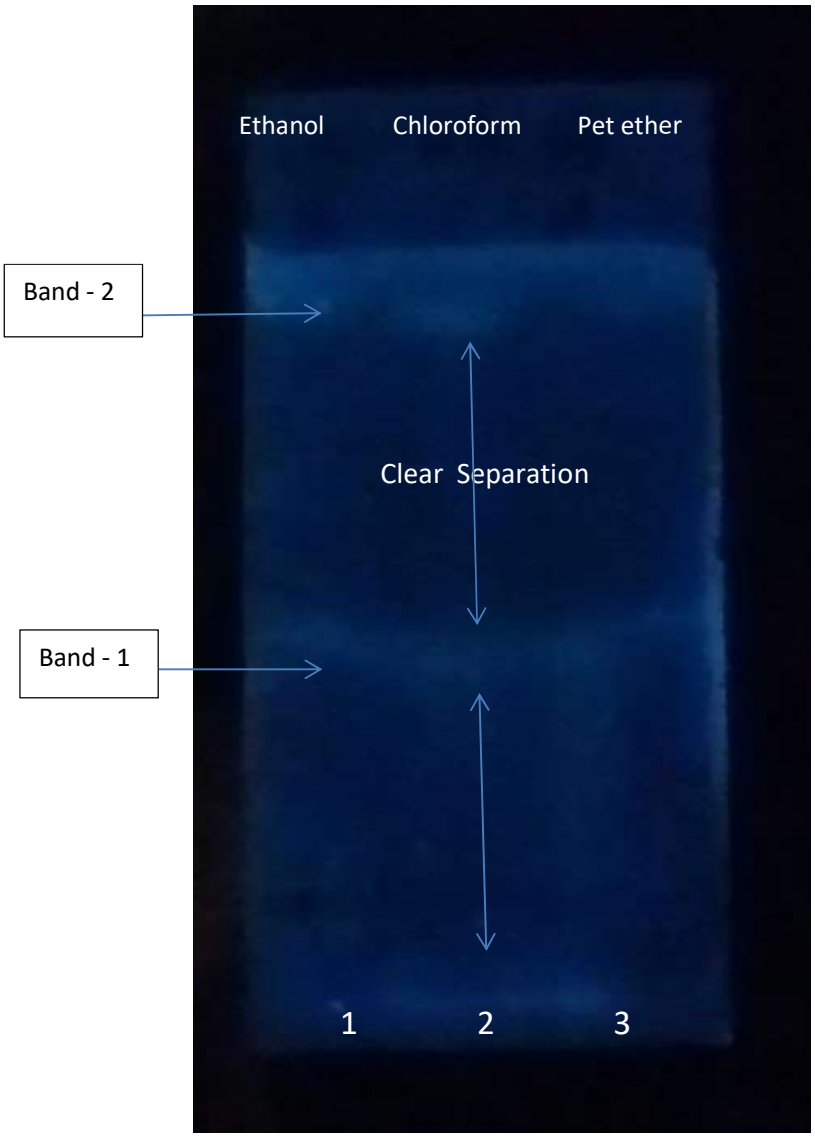


Figure 4.1: TLC check for choosing proper mobile phase

Isolation of bioactive components into solvent

Following methods were followed for dissolving the compounds present in fungal powder into selected solvent (ethanol). TLC checks were performed and number of compounds (spots in TLC plate) detected were noted.

S. no.	Methods followed	TLC spots	Compounds	Selection
1	Maceration	2-3	3	no
2	Sonication	4-5	5	yes
3	Maceration + Sonication	3	3	no
4	Sonication + Refluxing	3	3	no

Table no.4: Methods applied for dissolving compounds in solvent

Column Chromatography

After mobile phase and solvent were selected, the crude extracts were dissolved in solvent and sonicated and loaded on top of silica gel column, thereafter mobile phase chloroform were loaded and column chromatography was performed.

Conformations about the separation of metabolites by column chromatography is established by repeated TLC checking of the contents of test tubes from respective apparatus (as that was the only tool for separation of metabolites), further within the test tubes mobile phase and assisting metabolites are present; those can be seen in TLC plates. This simply means that the metabolites present in the column stacked above the silica gel are interacting with the mobile phase and are relatively dispersed according to their sizes, the small one coming out first and the bulky ones latter.

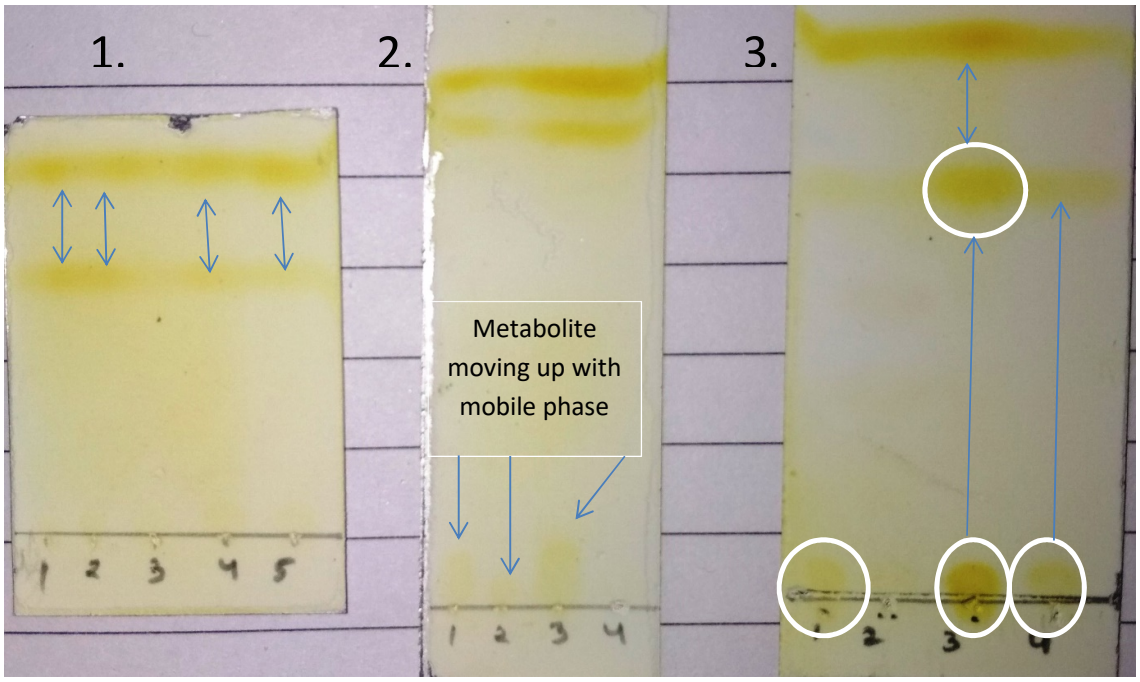


Figure 4.2: TLC plates showing progress in column chromatography

The above shown three TLC plates depict a picture of isolation through column chromatography. The 3 plates listed as 1, 2 and 3 are placed in the order in which the TLC checks were performed after starting column chromatography. The first plate shows no isolation rather two bands are seen forming distinctly and the separation between the two bands is clearly visible thus they represent two different compounds. The second TLC plate contains 4 samples and out of those 3 contains metabolites of interest which moved with mobile phase (indicated by arrows). The third plate contains 4 samples and here sample 3 and 4 showed distinct bands well separated from one another and the metabolite of interest eluting slowly.

The TLC plates show metabolites present in the sample indicated below and the movement of the metabolite under influence of mobile phase and if the metabolites present are a mixture bands would be visible and the separation of bands is directly related to the effective separation by mobile phase.

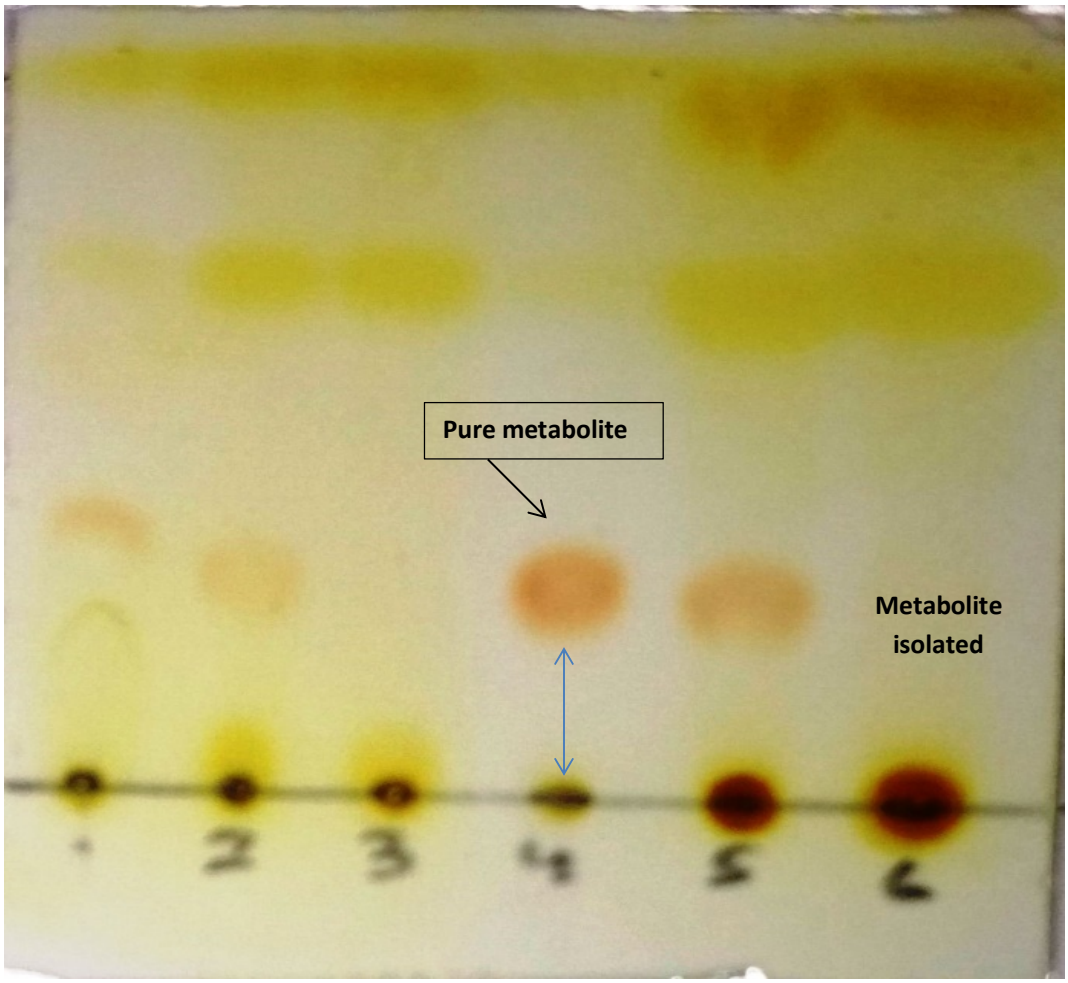


Figure 4.3: TLC plate showing single metabolite at 4th sample

The TLC plate above was run in chloroform: methanol (10:1), the metabolites present in each insertion by capillary needles. The selections of these 6 test tubes were also a matter of fine precision. Conditioned under uniform flow rate the test tubes in the stand were loaded with mobile phase flowing out of column, when all test tubes were full 6 was selected out of 40. From each column of test tubes one or two are selected accordingly and the selection should be dispersive for ensuring proper detection of separated metabolites. This TLC plate shows that there was a metabolite which was finely separated and retained a definite position (band) in the TLC plate. The metabolite can be seen slightly visible in samples 1, 2; moreover in sample 4, 5 the bands are clearly visible, herein the 4^{th} sample shows pure metabolite with much less impurities and 5^{th} one shows metabolite with impurities but the 6^{th} sample does

not contain any amount of the metabolite as shown in TLC plate. Therefore it can be assumed that the shown metabolite is finally isolated from the cluster of metabolites contained in sonicated sample.

The initial metabolites with low molecular weights have been isolated and it is the starting step of separation of the 5 detected metabolites in the sonicated sample. This means other remaining metabolites are left intact in the silica gel column. As GA has higher molecular weight so it will elute slowly, accordingly we can concidear that maybe the last or the second last metabolite could be ganoderic acid if flow rate is kept constant (516.675 g/mol).

The objective isolation is put on hold due to time constraints which limit the dissertation completion by May 2018 so didn't proceed for column chromatography. The process is kept on hold for future and the project is forwarded in the direction of detection of metabolites from crude samples by GC-MS method.

GC-MS data analysis

Literature was searched and protocols for detection of triterpene metabolites were analysed. The parameters of oven temperature, pressure, injection time etc. for gas chromatography and conditions for mass analysis were investigated and the crude samples were analysed. Samples were dissolved in chloroform and the precaution is taken to make the sample less concentrated for better detection and less harm to column in the apparatus.

Ganoderic acid salts MS analysis

The formations of salts of ganoderic acid as mentioned in the methodology section were detected by GC-MS analysis. Here out of the 15 peaks representing metabolites by gas chromatography neither one correlates with ganoderic acid or its derivatives but when MS MS Library analysis was done the structure was anticipated.

Name: Androstane-3,2'-thiazolidine with formula - $C_{21}H_{35}NS$ (although not a C_{30} metabolite but still it is a squalene derived structure).

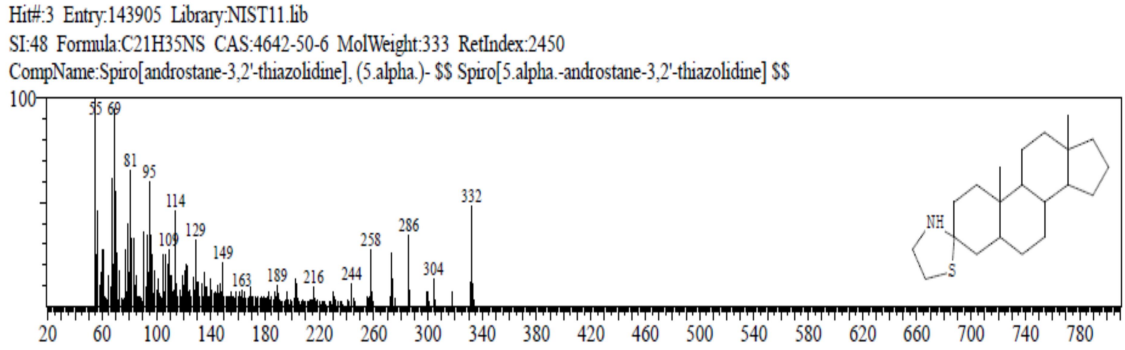


Figure 4.4: Structural similarities in MS library search.

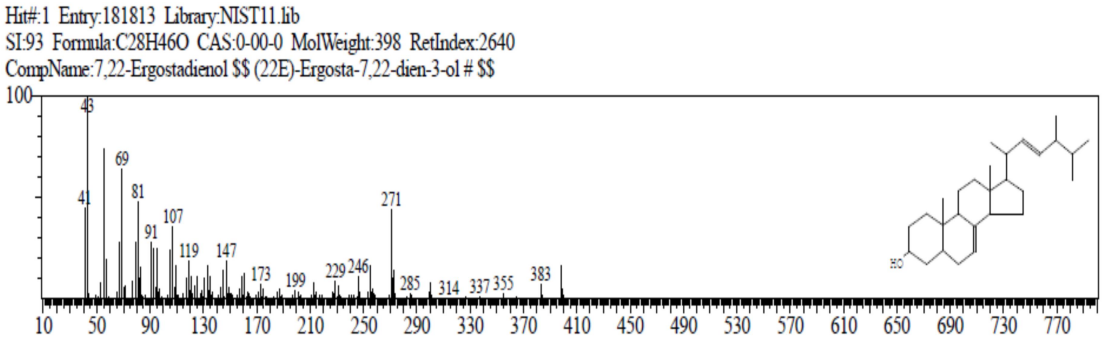
GC-MS analysis for crude extract of two host species

The analysis results in conclusions that 7,22-Ergostadienol found in crude fungal extracts of Acacia and Azadirachta hosts are the derived products of the terpene pathway and the type of sterols (ergosterols) identified are derivative of the same pathway having lanosterol as precursor and triterpenes as products. 7,22-Ergostadienone with area 0.61% was found in fungal sample from acacia host. Thus overall with area 2.91% the concentration of desired metabolites is in higher amount in *Acacia* sample over *Azadirachta*.

Metabolites detected in fungal samples from *Acacia* (host)

- **7,22-Ergostadienol (Area 2.91% and Height 1.98%)**

MS Library analysis



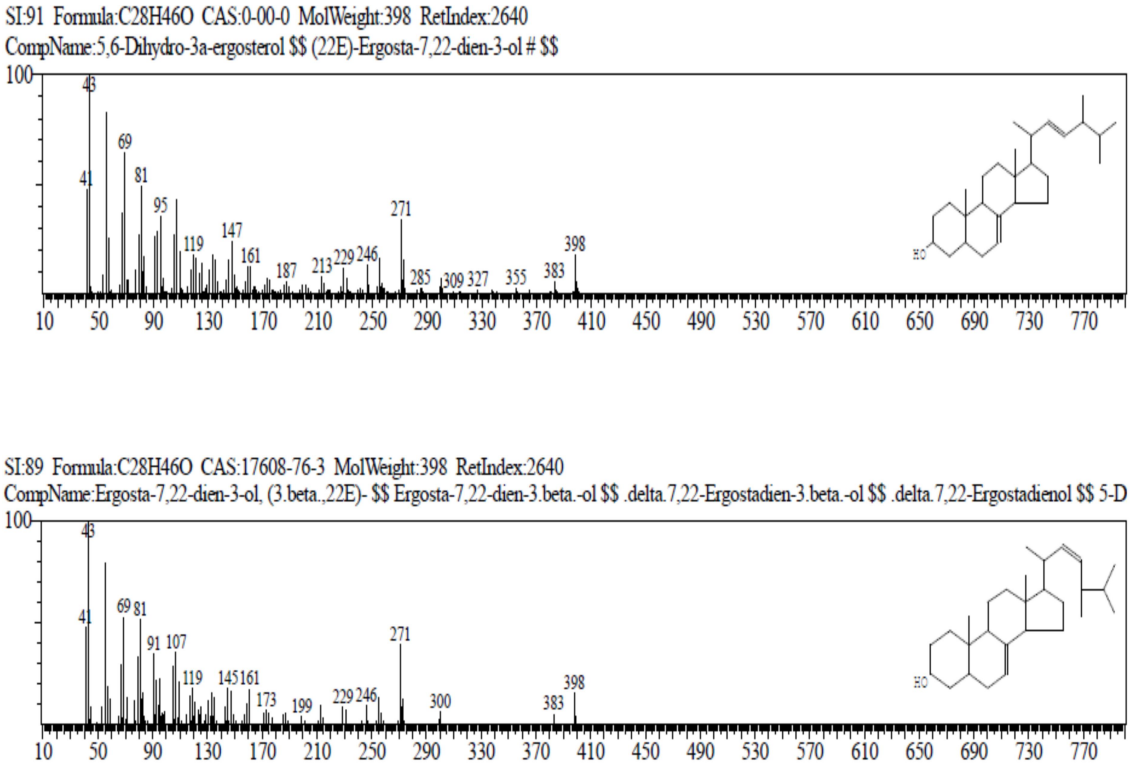


Figure 4.5: MS library search for structural similarities with 7,22-Ergostadienol in *Acacia* sample.

- **7,22-Ergostadienone (area 0.61% and height 0.43%)**

MS Library analysis

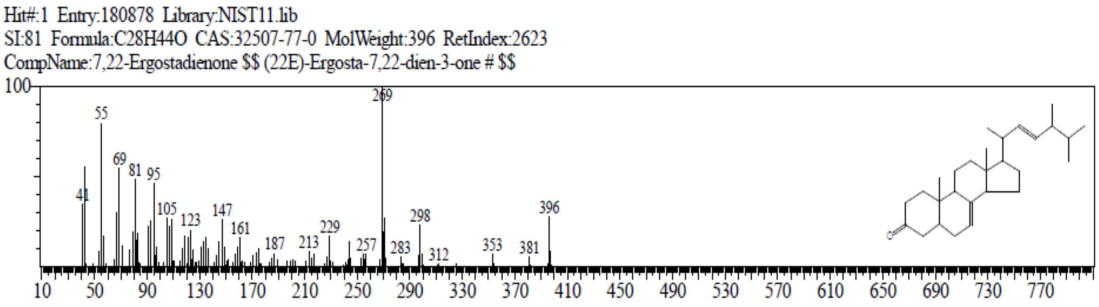


Figure 4.6: MS library search for structural similarities with 7,22-Ergostadienone

Metabolites detected from fungal samples of *Azadirachta* (host)

- **7,22-Ergostadienol (area 1.19%) (height 0.68%)**

MS Library analysis

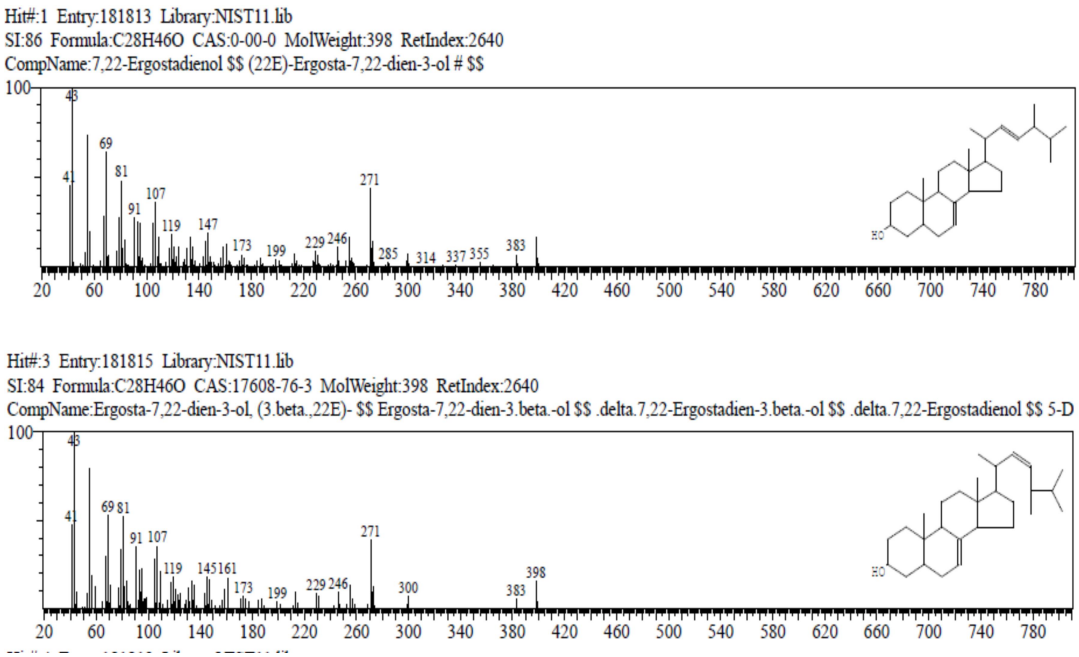


Figure 4.7: MS library search for structural similarities with 7,22-Ergostadienol in *Azadirachta* sample.

CHAPTER 5

DISCUSSION

CHAPTER 5

Discussion

Identification of individual components from the mixture can be done by separating them from one another and several methods are there for separation all listed under a common title, Chromatography. Chromatography being the standard method of separation of metabolites due to their affinity with mobile phase or solvent is employed for separation of metabolites from the mixture. Thus silica gel column chromatography was employed for separation and TLC was used for visualization of separation and separated individual metabolites. The solvent system and mobile phase were standardized. Proper solvent is described as the one in which maximum number of compounds from the fungal powder could be dissolved in, hence ethanol was selected. Similarly the mobile phase used was chloroform : methanol (10:1) because the metabolites were not moving upwards in TLC plates by any other solvent used (e.g. pet ether or ethyl acetate), the movement was still limited in chloroform but in ethanol the movement was fast because of its high polarity. Thus 10:1 ratio of chloroform and methanol were established to provide proper movement for the metabolites. For isolation of metabolites we need to be clear about the pharmaceutical approaches of isolating metabolites from mixture. Proper solvent was selected (ethanol) and metabolites were dissolved in it from fungal extract by sonicating the sample in a beaker. Column chromatography was put on hold; due for future because of the dissertation deadline time constraints due to which GC-MS detection of crude extract became the secondary objective.

The GC-MS data was analysed and plethora of compounds were found ranging from alkaloids, alkaloids, phenols, esters, fatty acids (precursors and derivatives), glutamate pathway derivatives (Azadirachta host) and sterols (terpenes). From a total of 24 compounds found in fungal crude extract (acacia) 14.36% area of fatty acids and 3.52% area of sterols were found; moreover 16 compounds found in fungal crude extract (azadirachta) 4.38 % area of fatty acids were found and 1.19% area of sterols were found. Looking for terpenes (tri-terpenes) was our query and it was found out that 7,22-Ergostadienol with

area 1.19% and height 0.68% is found in Acacia and Azadirachta samples. Moreover another metabolite 7,22-Ergostadienone with area 0.61% and height 0.43% is also found in Acacia (Host) sample. GC-MS detected ergosterols which primarily are steroidal metabolites sterols (C_{28}). Terpenes are thus detected and structures are identified.

Sterols (structural stability and signalling purposes (Tiwari et al. 2007)) are synthesized by enzymes located in the ER membrane and are enriched in the plasma membrane where they increase the permeability barrier of the membrane and thus are important to maintain the membrane potential (Haines, 2001). Ergosterols are synthesized from lanosterol; ganoderic acids and clavaric acids are also synthesized from lanosterol. Backbone is the same in derivatives of lanosterol; are interconverted by oxidation, reductions and acetylations (Tiwari et al. 2007). Under interaction with proteins of cytochrome P450 superfamily lanosterol skeleton shows significant modifications. Modification of lanosterol skeleton provides scopes for ergosterol production; Ergosterol is a sterol found in fungal cell membranes that serves the same function as cholesterol in animal cells (Lv et al. 2012). Crude fungal extract of acacia sample contains 7,22-Ergostadienone (C28) which has anti-inflammatory property (Horng et al. 2008),(Vinci et al. 2008). The precursor squalene alone exhibits chemopreventive activity. Although it is a weak inhibitor of tumor cell proliferation, it contributes either directly or indirectly to the treatment of cancer due to its potentiation effect (Harivardhan et al. 2009).

SUMMARY

SUMMARY

Though initial plan was separation of ganoderic acid (dissolved in solvent) and its derivatives from the fungal crude extract, according to their molecular weight and size in the chromatographic apparatus, about 2 metabolites were isolated from the mixture before the flow rate was disturbed. The two metabolites detected were of low molecular weight and sizes which could be explained why they eluted faster in chromatographic apparatus. This experiment can be repeated in future by providing undisturbed flow rate and NMR analysis of speculated isolated samples.

From the host dependent crude extract of *G. lucidum* GC-MS analysis, we conclude that the levels of active bio-constituents in crude extract of *G. lucidum* growing on *Acacia*, were found to be higher as compared to *Azadirachta* this finding was supported by identification of about 24 metabolites in fungal sample having *Acacia* host and about 16 metabolites were detected in *Azadirachta*. These molecules range from phenols, fatty acids, esters, alkaloids etc with different concentrations. The molecules we were searching were ganoderic acid and derivatives. 7,22-Ergostadienol with area 2.91% and 1.19% is found in Acacia and Azadirachta samples. Moreover another metabolite 7,22-Ergostadienone with area 0.61% is found in Acacia associated fungal sample thus giving insights about the pathway where lanostrol bio-synthesizes into ergostrols, ganoderic acids and derivatives with modifications on core structure. Metabolites present in *Acacia* were more in number. 2.91% area with 7,22-Ergostadienol and 0.61% area with 7,22-Ergostadienone presents us 3.52% total area covered by terpene derivatives in *Acacia* sample which is quantitatively much higher than that of *Azadirachta* sample with area 1.19% of total terpene metabolites. As both of them were mature and dried samples when collected thus the content of metabolites says something about chemovariance.

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SUPPLEMENTARY DATA

Sample-1: Crude extract of *Ganoderma lucidum* from Acacia (host)

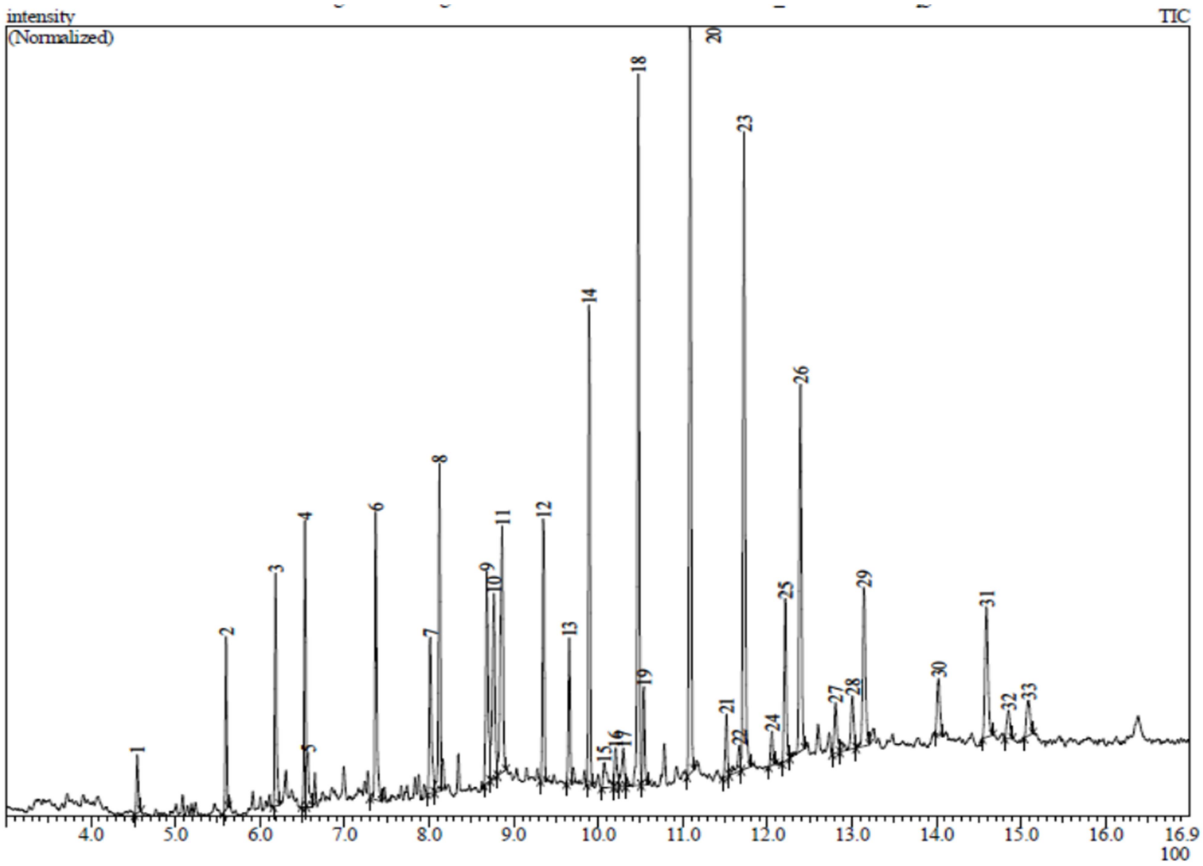

Figure: GC graph of crude sample of *Ganoderma lucidum* from Acacia host.

Serial no.	Compound	Retention time	Area %	Height %
1	(Z) 3-Tridecene **(Alkaloid)**	4.550	0.76	0.88
2	(Z) 3-Tridecene **(Alkaloid)**	5.599	1.78	2.63
3	2,4-bis(1,1-dimethylethyl)- phenol **(Phenol)**	6.184	2.83	3.55
4	(E) 3-Tetradecene	6.533	3.12	4.36
5	2-methyl-tridecane	6.567	0.81	0.83
6	Cetene **(Alkaloid)**	7.369	3.86	4.38
7	n-Hexadecanoic acid **(Anti-inflamatory property)**	8.013	2.58	2.38
8	Cetene **(Alkaloid)**	8.124	4.37	5.00

9	Oleic Acid **(Fatty acid)**	8.683	3.56	3.23
10	(E)-9-Octadecenoic acid ethyl ester **(Ester)**	8.766	3.50	2.81
11	Cycloeicosane **(Precursor of fatty acid)**	8.864	4.73	3.75
13	Cyclotetracosane **(Precursor of fatty acid)**	9.658	1.93	2.22
15	Eicosylester-2-ethylbutyricacid **(Fatty acid)**	10.067	0.76	0.37
16	2-hydroxy-1-(hydroxymethyl hexadecanoic acid) **(Fatty acid)**	10.207	0.72	0.59
17	Diisooctyl phthalate	10.298	0.77	0.58
18	Hexadecamethyl-heptasiloxane	10.474	9.78	10.84
19	Heptadecyl heptafluorobutyrate	10.538	1.47	1.49
21	Heptadecyl heptafluorobutyrate	11.516	0.94	0.94
22	1,1'-hexadecylidenebis-cyclopentane	11.667	0.71	0.38
24	2-methyloctacosane	12.055	0.54	0.52
25	2-methoxy-methyl ester tricosanoic acid **(Fatty acid)**	12.213	2.66	2.47
27	1-Hexacosene **(Di-ene compound)**	12.807	1.01	0.77
28	2,4,6-triphenyl-pyridine **(Alkaloid)**	13.006	1.31	0.81
31	7,22-Ergostadienol **(C28-lanosterol (ergosterol) backbone)**	14.592	2.91	1.98
32	7,22-Ergostadienone **(C28-lanosterol (ergosterol) backbone)**	14.852	0.61	0.43
	Total		100.00	100.00

Table: Metabolites from Ganoderma crude extract in Acacia sample

Sample-2 Crude extract of *Ganoderma lucidum* from Azadirachta (host)

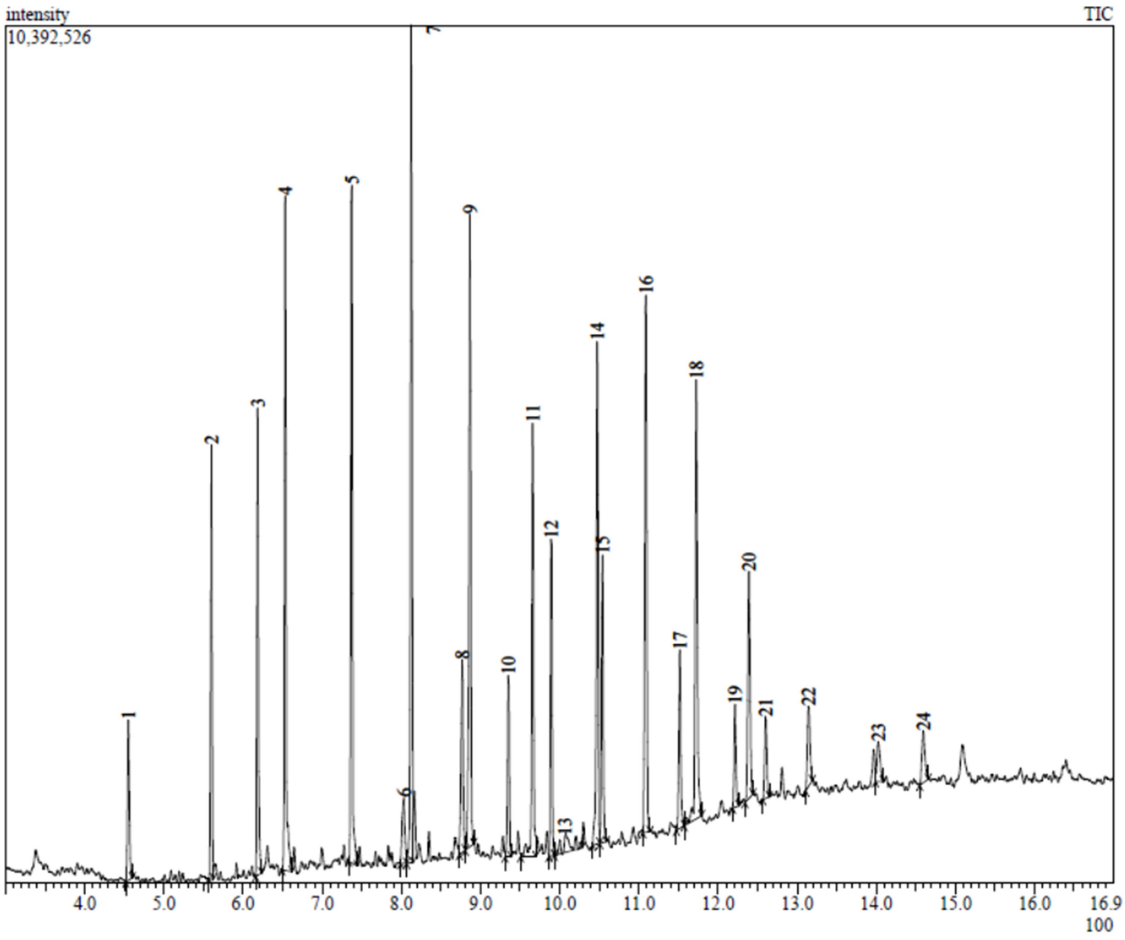

Figure: GC graph of crude sample of *Ganoderma lucidum* from Azadirachta host.

Serial no.	Compound	Retention time	Area %	Height %
1	(Z) 2-Tridecene **(Alkaloid)**	4.551	2.09	2.11
2	(Z) 3-Tridecene **(Alkaloid)**	5.599	4.37	5.70
3	2,4-bis(1,1-dimethylethyl)-phenol (**Phenol)**	6.184	5.09	6.13
4	Cetene **(Alkaloid)**	6.534	7.53	8.86

5	Cetene **(Alkaloid)**	7.371	8.34	8.89
6	Butyl undecyl ester phthalic acid **(Ether)**	8.030	1.32	0.86
7	Cetene **(Alkaloid)**	8.126	9.87	11.00
8	(Z)-6-Octadecenoic acid **(Fatty Acid)**	8.769	2.95	2.53
9	Heptadecyl heptafluorobutyrate **(Ester)**	8.867	8.20	8.30
10	Heptadecyl heptafluorobutyrate **(Ester)**	9.659	5.68	5.69
11	Isohexyl 3-phenylprop-2-glutaric acid **(Glutamate pathway deriative)**	10.067	1.03	0.20
12	Heptadecyl heptafluorobutyrate **(Ester)**	10.541	3.64	3.78
13	Octadecyl trifluoroacetate **(Ester)**	11.518	2.61	2.34
14	2-methoxy-methylester tricosanoic acid **(Fatty acid)**	12.215	1.43	1.35
15	Octadecyl trifluoroacetate **(Ester)**	12.603	1.27	1.07
16	7,22-Ergostadienol **(C_{28}-lanosterol (ergosterol) backbone)**	14.595	1.19	0.68
	Total		100%	100%

Table: Metabolites from Ganoderma crude extract in Azadirachta sample

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Urkund Analysis Result

Analysed Document:	plag chek doc.docx (D39472522)
Submitted:	5/30/2018 4:28:00 AM
Submitted By:	agrah23@gmail.com
Significance:	0 %

Sources included in the report:

Instances where selected sources appear:

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IDENTIFICATION OF GANODERIC ACIDS AND DERIVATIVES FROM POWDERED FRUITING BODIES OF Ganoderma lucidum INTRODUCTION Fungi are the heroes in the race of evolution due to their unique environmental fitness. With diversity of about 2.2 million to 3.8 million species and a long history ranging about 900 million years, these organisms have efficiently adapted to almost every habitat present on earth. This ability to survive is because of the actively metabolized natural product pathways of the organism and these products have antimicrobial, antifungal, immunosuppressive or cytotoxic effects. These bioactive properties have enabled fungi to successfully coexist in any ecological niche by inherent evolutionary ability to compete for nutrients, to put off predators, and communications with organisms in the ecosystem. Discovery of natural product pathways and fungi engineering therefore holds great promise for the bio-pharmaceutical industry. With 55,000 scaffolds terpenes are a major group of identified bioactive natural products. These are incredibly structurally diverse class of natural products derived from 5C precursors DMAPP (dimethylallyl diphosphate) and IPP (isopentenyl diphosphate) from mevalonate pathway. Condensation of monomers (IPP and DMAPP) results in linear hydrocarbons of varying length C10, C15 and C20 namely GPP (geranyl pyrophosphate), (2E,6E)-FPP ((2E, 6E)-farnesyl PP), and GGPP (geranylgeranyl PP). Terpenes synthesis occurs when linear hydrocarbons undergo a de-phosphorylation and cyclization cascade which is catalysed by enzymes known as terpene synthases. Depending on the length of the precursor molecule, fungal terpene synthases are known to produce sesquiterpenes (C15), diterpenes (C20) and triterpenes (C30). Cytochrome P450 mono-oxygenases, oxido-reductases, and different group transferases modify the core terpene scaffold producing overabundance of terpenes.

Ganoderic acid A (C30H44O7) Source: PubChem Ganoderic acids are derivatives from lanosterols and they come in many forms with slight changes in core structure; GA-A/B/F/G/H/T/C1/S/beta/etc. About 198 derivatives of GAs and similar metabolites have been isolated and characterized, among those GA-A and GA-B are the most well-known ones possessing several biological activities. The metabolites present in fungal body are a matter of evolutionary wonder but for us humans we strive to derive anthropocentric bias in every entity we come across, and terpenes being the promising candidate in pharmaceutical and health science research, humans exploit nature in search of better and everlasting sources of terpenes now a days cannabis plants are looked into for terpenes. These fungal species have become the model organisms for the process and are cultured and harvested for product based market which is involved in extraction of triterpenes from the fungal body; they employ a plethora of processes for extraction of metabolites. Hypothesis The fungus is an active producer of triterpenes (terpenes in general) which have a great promise for the health industry. As it is reported a vast reservoir of terpenes is actively metabolized inside its fruiting body, though triterpenes are actively produces in primordial stage and in fruiting body they are present in low quantities. Collected samples were mature and dried fruiting bodied, it is probable that our samples contain some amounts of triterpenes. Previously the crude extract is known to have potential cytotoxic effects, ganoderic acid being a major bioactive triterpene; the proposed hypothesis is that this crude extract contains ganoderic acid or its derivatives. The pathway starting from mevalonic acid to squalene to lanostrol and further into ergostrol where several ganoderic acids and derivatives are synthesized is well known; thereby any part

of the pathway can be detected to state the presence of this model of metabolite interchanges. REVIEW OF LITERATURE Scientific classification:

Kingdom Fungi Division Basidiomycota Class Agaricomycetes Order Polyporales Family Ganodermataceae Genus Ganoderma Species G. Lucidum (Curtis) P. Karst (1881) Scientific classification of Ganoderma lucidum G. lucidum a macro-fungus synthesizes plethora of bioactive metabolites. It has also been popularised in the ancient medicinal texts of some regions of world. Ganoderma lucidum, also known as 'the mushroom of immortality' is one of the best-known medicinal macro fungi in the world. Modern pharmacological research has laid vision of potential therapeutic activities besides anti-inflammatory, anti-tumour, anti-hypertensive and immune-modulatory activities, they are also known to possess anti-thrombosis properties (Yi et. al. 2017). The fungus is a storehouse of bioactive metabolites; making itself a ‘virtual cellular factory' for bio-pharma industry. Triterpenoids and polysaccharides are found in excess in G. lucidum. It is known that at primordial stage the fungi contains maximum tri-terpenoids; but in cultured mycelium and fruiting bodies low quantities are found. It also secretes proteins that can adequately disintegrate both cellulose and lignin. Such enzyme activities may act as valuable resource for biomass usage, fiber bleaching and organo-pollutant degredation. Our comprehension of G. lucidum science is restricted in spite of its part in conventional Chinese medicine and its amazing stockpile of bioactive metabolites. The area of interest fungal triterpenoids

The triterpenoids are C30 prenyl chain derived metabolites that are found widely in nature as steroids and sterols. The biosynthetic pathway towards ergosterol, the major component of the plasma membrane in fungi, begins with the cyclization of squalene to lanosterol. Squalene-epoxidation occours forming 2,3-oxidosqualene (chair-boat-chair). Linear chain of polyisoprene is cyclized by oxidosqualene cyclase/lanosterol cyclase (OSC). Methyl migrations (two) and hydride shifts followed by deprotonation, results into lanosterol (Quin et al. 2014).

Biosynthesis of different terpenes from Mevalonic acid (Rosa et al., 2005)) Prokaryotic and eukaryotic triterpene cyclases share a similar overall protein fold, with two α-helical and a membrane linking channel. Aromatic active site cavity extents up to two domains and the protonating group initiating cyclization are located at polar region. Triterpene cyclases contains conserved 16 amino acid repeats of which Gln and Trp residues offer bonding network amid the α-helices (Quin et al. 2014) (stabilization during exergonic reaction). Bacterial SHC and eukaryotic OSC have diverged sequence and mechanism. Evolutionary divergence is observed both in SHC accepting squalene as a substrate and OSC accepting 2,3-oxidosqualene as substrate. Substrate activation is favoured by a protonating environment, and aromatic residues which stabilizes the carbocation intermediates, forming lanosterol.

Tri-terpenes biosynthesis

More than 150 triterpenoids have been isolated until now and are being investigated keenly for wide range of health benefits. Upstream of cyclization in mevalonic acid pathway constitutes 13 genes encoding 11 enzymes. Two genes encode for Acetyl-CoA, C-acetyltransferases and farnesyl diphosphate synthases; remaining are encoded by single-copy genes. Lanosterol synthase (LSS) synthesizes a cyclic intermediate of triterpenoids and

ergosterols; Lanosterol from where pathways diverge. The steps following cyclization include a series of oxidation, reduction and acylation reactions. Oxidations are catalysed by cytochrome P450 superfamily proteins (CYPs) hence modification of the lanosterol skeleton. Ergosterols and ganoderic acids are the major product class of tri-terpenoids produced from the lanosterol skeleton. Ganoderic acid A (among many GAs) suppresses proliferation and invasion and induces apoptosis in human osteosarcoma cells (Shao et al. 2015), inhibits proliferation, invasion, and promotes apoptosis in human hepatocellular carcinoma cells (Wang et al. 2017).

Core molecular structure of GAs, different R-groups (R1–R6) define GA-subtype and biochemical properties (figure adapted from (Liu et al. 2012)) Objectives of the study • Identification of Ganoderic acids and associated triterpenoid derivatives from powered fruiting body of fungal sample. • Gas chromatography and Mass Spectroscopic analysis of fungal crude extract from two diff hosts, Acacia and Azadirachta. MATERIALS AND METHODS SAMPLE COLLECTION Powdered fungal sample were collected from Plant Sciences lab, which were previously collected and deposited by former PhD student, Balraj Singh Gill. Samples were collected from two different plant hosts namely Acacia and Azadirachta. CHOOSING THE PROPER SOLVENT The fungal sample were weighed in equal parts each of 0.5 grams and dissolved in 10 ml of ethanol, chloroform, pet-ether respectively. The sample were left overnight and Thin Layer Chromatography (TLC) checks were done to predict the total number of metabolites dissolved in the solvent and further extracted as prominent spots in the TLC paper. Maximum number of metabolites was identified by ethanol extracts; therefore it was selected as the proper solvent. CHOOSING THE PROPER MOBILE PHASE TLC running was performed in silica gel plates as stationary phase and chloroform: methanol (10:1) as mobile phase. The mobile phase must be the solvent whose polarity should be such that the flow rate of metabolites would be uniform and slow. The movement of metabolites in ethanol is fast and no movement was shown in ethyl acetate so chloroform was checked and found out to be better. The movement of metabolites in chloroform was slow and it could be aided with ethanol (chloroform: ethanol; 10:1) for desirable speed and fine separation. TLC PLATES VISUALIZATION Plates were visualized after drying and dipping TLC plates in iodine crystals, resulting in iodine binding with metabolites and resulting in a dense dark line with interspaced dots identified as metabolites. ISOLATION OF BIOACTIVE COMPONENTS INTO SOLVENT • Maceration In this method sample (0.5g) were taken in an RB and refluxed for 24 hours. Extracts were taken in two RBs respectively one containing 10 ml of methanol and containing 10 ml chloroform. After 24 hours TLC was checked and it is found that in the methanol extract 2-3 major spots are visible and similarly in the chloroform extract 2-3 major spots are present. • Sonication 0.5 grams of sample was mixed with 10 ml of ethanol in a beaker and kept for sonication for about one hour in the sonicator setting the temperature at 55 degrees. The sample was filtered and collected in an RV and concentrated by using the rotavaporator apparatus and TLC check was performed whereby resulting in 4-5 spots in the TLC plate unevenly interspaced (maximum number of metabolites visible and separated). The sonicator temperature and duration of placing sample in sonicator can be tangled with as the metabolite we need to identify is Ganoderic acid and derivatives who have boiling point near to 690.1oC (GA-A). • Maceration + Sonication 0.5 grams of sample was mixed in 10 ml of ethanol in a beaker and macerated and sonicated afterwards as described above (purpose is

disruption of cellular membranes so as to extract maximum number of metabolites from the sample and dissolve them in solvent). 3 spots observed in TLC. • Sonication + Refluxing 0.5 grams of sample with 10 ml ethanol in beaker were sonicated for an hour with temperature set to 55 degrees and refluxed afterwards in the apparatus for 24 hours. Resulting extract was checked in TLC plate and 3 prominent spots were found. • Column Chromatography Column for chromatography was collected from pharmaceutical sciences lab and it was packed with semi aqueous silica gel and sample extract with solvent was stacked on the top. Chloroform was the selected mobile phase for separation of metabolites, it was poured from the top of column and the solvent passes through the silica gel packed material carrying with it the metabolites stacked at the top. Metabolites move according to their relative sizes and molecular weights and are influences by the mobile phase flowing down the column and accordingly the metabolites flow out of the silica gel packed column wherein the metabolite with low molecular weight and small size are driven out first and accordingly metabolites separate in ascending order of molecular masses and sizes. Test tubes and holders from Plant Sciences Lab were employed for the chromatographic experiment. The column was not let to dry out and was always packed with mobile phase (the gauge was closed when column was not running and at the top of column a cotton plug is inserted to avoid impurities being mixed with our sample), hence metabolites were separated slowly with uniform flow rate and drying was prevented. Metabolites were isolated and estimation of total amount of mobile phase needed for isolation of metabolites was done. And the extraction was put on hold as the flow rate was disturbed and separation was hampered. 1. Identification NMR (to be carried out in future) Isolating pure crystals from crude extract The powdered fruiting body (0.5 g) is mixed with 10 ml ethanol and heated in the heating mantle for about 25-20 minutes and shaken at intervals; metabolites present in the powder could have been made soluble in the solvent (ethanol). The extract is filtered; the solvent is covered in parafilm and left overnight in low temperature conditions. The sample is observed the next day for formation of crystals of pure metabolites, which could thereby sent for NMR analysis. It was hypothesised that the metabolites would cluster together in ethanol and form crystals which could be seen by naked eyes, after 24 hours crystals formed were analysed and found impure and minute in sizes. Converting Ganoderic acids into salts and detection As we were searching for ganoderic acids in the sample we would look for acids, metabolites of similar molecular wt., sizes, boiling points, etc., accordingly was the process. First of all 10 grams of crude sample from acacia host was sonicated with 10 ml of ethanol and products were filtered out. Pet ether (10 ml) was added to the sample to dissolve all the impurities of low polarities. Base (5-6 pellets of NaOH) was then added to the sample and all the acids present in the sample are now converted to salts, and the salts reside in the middle layer between the two layer of solvents, wherein Pet ether (top layer) and ethanol (basal layer). The results (salts) were analysed in GC-MS apparatus by dissolving them with chloroform in vials.

Conversion of acidic metabolites into salts; Explanation - Two layers of solvents with their constituent metabolites are well separated, upper one being pet ether and lower one ethanol. In next figure the solvents were separated and layer of salt retained in the beaker was extracted and remaining salts were filtered out.

GC-MS Analysis Fungal samples from two different hosts Azadiracta and Acacia were analyzed in search for metabolites present in both of them by gas chromatography and mass spectroscopy. 1. Sample Collection Samples were collected from different hosts of Ganoderma namely Acacia (host) and Azadirachta (host) by former Ph.D student Balraj Singh Gill from different locations in Bathinda (Chetak Park, Bir Talab etc.). The mycelium of the fungal samples were converted into powdered form and stored for analysis as the chemical constituents do not change in converting the samples into powder form. 2. GC-MS sample preparation Fungal samples 10 grams each was mixed with 10 ml of ethanol (high polarity) and sonicated for about one hour, temperature of the sonicator was set to 550C. Following sonication the samples were filtered with filter paper and concentrated in rotavaporator. Vials of adequate sizes were borrowed from Central Instrumentation Lab (Central University of Punjab) and sample were loaded in them and marked accordingly (different samples from different hosts). Hplc grade chloroform was added to samples in the vials and samples were sent for GC-MS analysis. Detection would be maximized when sample is diluted properly otherwise, our sample may pose harm to the gas column used for detection. GC-MS ANALYSIS PARAMETERS Column Oven Temp. :60.0 °C Injection Temp. :250.00 °C Injection Mode :Splitless Sampling Time :1.00 min Flow Control Mode :Pressure (Pressure :150.0 kPa) Total Flow :18.0 mL/min Column Flow :2.51 mL/min Linear Velocity :57.7 cm/sec Purge Flow :3.0 mL/min Split Ratio :5.0 Oven Temperature Rate Temperature (OC) Hold time (minute) -- 60.0 1.00 30.00 270.0 0.00 10.00 310.0 5.00 Oven temperature setup for GC oven

Equilibrium Time: 3.0 min IonSourceTemp: 200.00 °C Interface Temp. : 280.00 °C Solvent Cut Time: 2.50 min Detector Gain Mode: Relative Detector Gain: 1.84 kV +0.00 kV Threshold: 0

MS Table Start time 3.00 min End Time 17.00 min ACQ mode Scan Event time 0.50 sec Scan speed 1666 Start m/z 20.00 End m/z 800.00 Mass Spectroscopy parameters

RESULTS Proper solvent (ethanol) and proper mobile phase (chloroform) were selected out of many solvents available in the laboratory according to their metabolite dissolution properties (solvent) and metabolite mobility, preferably slow (mobile phase).

Band - 2

Band - 1

Clear Separation

1 2 3

Ethanol Chloroform Pet ether

TLC check for choosing proper mobile phase After selected mobile phase and solvent were put to use, the crude extract were dissolved in solvent and sonicated and loaded on top of silica gel column, thereafter mobile phase chloroform were loaded and column chromatography was performed.

Conformations about the separation of metabolites by column chromatography is established by repeated TLC checking of the contents of test tubes from respective apparatus (as that was the only tool for separation of metabolites), further within the test tubes mobile phase and assisting metabolites are present; those can be seen in TLC plates. This simply means that the metabolites present in the column stacked above the silica gel are interacting with the mobile phase and are relatively dispersed according to their sizes, the small one coming out first and the bulky ones latter.

Metabolite moving up with mobile phase

1. 2. 3.

TLC plates showing progress in column chromatography The above shown three TLC plates depict a picture of isolation through column chromatography. The 3 plates listed as 1, 2 and 3 are placed in the order in which the TLC checks were performed after starting column chromatography. The first plate shows no isolation rather we see two bands forming distinctly and the separation between the two bands is clearly visible thus they represent two different compounds. The second TLC plate contains 4 samples and out of those 3 contains metabolites of interest which moved with mobile phase (indicated by arrows). The third plate contains 4 samples and here sample 3 and 4 showed distinct bands well separated from one another and the metabolite of interest eluting slowly. The TLC plates show metabolites present in the sample indicated below and the movement of the metabolite under influence of mobile phase and if the metabolite present is a mixture bands would be visible and the separation of bands is directly related to the effective separation by mobile phase.

Metabolite isolated

Pure metabolite

TLC plate showing single metabolite at 4th sample The TLC plate above clearly depicts such a picture; the TLC plate was run in chloroform: methanol (10:1), the metabolites present in each insertion by capillary needles is justified starting from 1 to 6 (depends on how many samples one wants to check at a time). The selections of these 6 test tubes were also a matter of fine precision. Conditioned under uniform flow rate the test tubes in the stand were loaded with mobile phase flowing out of column, when all test tubes were full 6 was selected out of 40. From each column of test tubes one or two are selected accordingly and the selection should be dispersive for ensuring proper detection of separated metabolites. This TLC plate shows that there was a metabolite which was finely separated and retained a definite position (band) in the TLC plate. The metabolite can be seen slightly visible in samples 1, 2; moreover in sample 4, 5 the bands are clearly visible, herein the 4th sample shows pure metabolite with much less impurities and 5th one shows metabolite with impurities but the 6th sample does not contain any amount of the metabolite as shown in TLC plate. Therefore can be assumed that the sample metabolite is finally isolated from the cluster of metabolites as known from sonicated sample analysis. The initial metabolites with low molecular weights have been isolated and it is the starting step of separation of the 5 detected metabolites in the sonicated sample. This means other remaining metabolites are left intact in the silica gel column. As GA

has higher molecular weight so it will elute slowly, accordingly we can concidear that maybe the last or the second last metabolite could be ganoderic acid if flow rate is kept constant (516.675 g/mol). The column chromatographic isolation is put on hold due to mobile phase unavailability and lack of pre-planning of quantitative volume of mobile phase for running the whole of column chromatographic apparatus for crude fungal samples from two different host plants. The process is kept on hold for future and the dissertation is forwarded in the direction of detection of metabolites from crude samples by GC-MS method.

GC-MS data analysis Literature was searched and protocols for detection of triterpenoid metabolites were analysed. The parameters of oven temperature, pressure, injection time etc. for gas chromatography and conditions for mass analysis were investigated and the crude samples were analysed. Samples were dissolved in chloroform and the precaution is taken to make the sample less concentrated for better detection and less harm to column in the apparatus. Ganoderic acid salts MS analysis The formations of salts of ganoderic acid as mentioned in the methodology section were detected by GC-MS analysis. Here out of the 15 peaks representing metabolites by gas chromatography neither one correlates with ganoderic acid or its derivatives but when MS MS Library analysis was done the structure was anticipated. Name: Androstane-3,2′-thiazolidine with formula - C21H35NS (although not a C30 metabolite but still it is a squalene derived structure).

Structural similarities in MS library search.

GC-MS analysis for crude extract of two host species The analysis results in conclusions that 7,22-Ergostadienol found in crude fungal extracts of Acacia and Azadirachta hosts are the derived products of the terpenoid pathway and the type of sterols (ergosterols) identified are derivative of the same pathway having lanosterol as precursor and triterpenes as products. 7,22-Ergostadienone with area 0.61% was found in fungal sample from acacia host. Thus overall with area 2.91% the concentration of desired metabolites is in higher amount in Acacia sample over Azadirachta. Metabolites detected in fungal samples from Acacia (host) • 7,22-Ergostadienol (Area 2.91% and Height 1.98%)

MS Library analysis

MS library search for structural similarities with 7,22-Ergostadienol in Acacia sample. • 7,22-Ergostadienone (area 0.61% and height 0.43%) MS Library analysis

MS library search for structural similarities with 7,22-Ergostadienone Metabolites detected from fungal samples of Azadirachta (host) • 7,22-Ergostadienol (area 1.19%) (height 0.68%) MS Library analysis

MS library search for structural similarities with 7,22-Ergostadienol in Azadirachta sample. Discussions Identification of individual components from the mixture can be done by separating them from one another and several methods are there for separation all listed under a common title, Chromatography. Chromatography being the standard method of separation of metabolites due to their affinity with mobile phase or solvent is employed for separation of metabolites from the mixture. Thus silica gel column chromatography was

employed for separation and TLC was used for visualization of separation and separated individual metabolites. The solvent system and mobile phase were standardized by experimentations. Where the proper solvent is describable as the one in which maximum number of components from the fungal powder could be dissolved in, hence ethanol was taken into consideration. Similarly the mobile phase used was chloroform : methanol (10:1) because the metabolites were not moving upwards in TLC plates by any other solvent used (e.g. pet ether or ethyl acetate), the movement was still limited in chloroform but in ethanol the movement was fast because of its high polarity. Thus 10:1 ratio of chloroform and methanol were established to provide proper movement for the metabolites. For isolation of metabolites we need to be clear about the pharmaceutical approaches of isolating metabolites from mixture. Proper solvent was selected (ethanol) and metabolites were dissolved in it from fungal extract by sonicating the sample in a beaker One of the metabolites from the 5 detected metabolites in TLC spots in Sonicated sample was isolated from the mixture. Thus the sample now contains 4 other metabolites which should contain ganoderic acid and is left for future study. This states that we could not isolate ganoderic acid from the sample rather came across related C-28 terpenes of the pathway which could have further converted into a tri-terpene. The GC-MS data is analysed and 7,22-Ergostadienol with area 1.19% and height 0.68% is found in Acacia and Azadirachta samples. Moreover another metabolite 7,22-Ergostadienone with area 0.61% and height 0.43% is also found in Acacia (Host) sample. Thus it is clear that GC-MS detection was done for ergosterols which primarily are steroidal metabolites sterols (C28). Terpenes are detected and structures are identified. SUMMARY Statements like, ganoderic acids having therapeutic properties are easy to say but hard to prove, with over 150 derivatives, GAs constitute the most diverse family of triterpenoid metabolites; these are not limitations rather maybe more derivatives could be seen in future. To establish therapeutic potential of GAs careful studies of activity of specific derivatives and the coordinated action of derivatives must be revisited. It may seem unlikely but therapeutic potential of GAs could be established as a cluster of interacting molecules coordinating for benefits. Anticancer potential of various ganoderic acids can be explored through in-vivo studies. Though initial plan was separation of ganoderic acid (dissolved in solvent) and derivatives from the fungal crude extract, according to their molecular weight and size in the chromatographic apparatus, the process was simple and I had isolated about 2 metabolites from the mixture before the flow rate was disturbed. This experiment can be repeated in future by providing undisturbed flow rate and NMR analysis of speculated isolated samples. From the host dependent crude extract of G. lucidum GC-MS analysis, we conclude that the levels of active bio-constituents in crude extract of G. lucidum growing on Acacia, were found to be higher as compared to Azadirachta this finding was supported by identification of about 33 metabolites in fungal sample having Acacia host and about 24 metabolites were detected in Azadirachta. 7,22-Ergostadienol with area 2.91% and 1.19% is found in Acacia and Azadirachta samples. Moreover another metabolite 7,22-Ergostadienone with area 0.61% is found in Acacia associated fungal sample thus giving insights about the pathway starting from mevalonic acid to squalene to lanostrol and further synthesizing ergostrols, ganoderic acids and derivatives with modifications on core structure. Thus we cannot definitely conclude to what were the parameters which affected the metabolite production in Azadirachta but we can however say that metabolites present in Acacia were more in number. 2.91% area with

7,22-Ergostadienol and 0.61% area with 7,22-Ergostadienone presents us 3.52% total area covered by terpenoid derivatives in Acacia sample which is quantitatively much higher than that of Azadirachta sample with area 1.19% of total terpenoid metabolites. As both of them were mature and dried samples when collected thus the content of metabolites says something about chemovariance.

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IDENTIFICATION OF GANODERIC ACIDS AND DERIVATIVES FROM POWDERED FRUITING BODIES OF Ganoderma lucidum INTRODUCTION Fungi are the heroes in the race of evolution due to their unique environmental fitness. With diversity of about 2.2 million to 3.8 million species and a long history ranging about 900 million years, these organisms have efficiently adapted to almost every habitat present on earth. This ability to survive is because of the actively metabolized natural product pathways of the organism and these products have antimicrobial, antifungal, immunosuppressive or cytotoxic effects. These bioactive properties have enabled fungi to successfully coexist in any ecological niche by inherent evolutionary ability to compete for nutrients, to put off predators, and communications with organisms in the ecosystem. Discovery of natural product pathways and fungi engineering therefore holds great promise for the bio-pharmaceutical industry. With 55,000 scaffolds terpenes are a major group of identified bioactive natural products. These are incredibly structurally diverse class of natural products derived from 5C precursors DMAPP (dimethylallyl diphosphate) and IPP (isopentenyl diphosphate) from mevalonate pathway. Condensation of monomers (IPP and DMAPP) results in linear hydrocarbons of varying length C10, C15 and C20 namely GPP (geranyl pyrophosphate), (2E,6E)-FPP ((2E, 6E)-farnesyl PP), and GGPP (geranylgeranyl PP). Terpenes synthesis occurs when linear hydrocarbons undergo a de-phosphorylation and cyclization cascade which is catalysed by enzymes known as terpene synthases. Depending on the length of the precursor molecule, fungal terpene synthases are known to produce sesquiterpenes (C15), diterpenes (C20) and triterpenes (C30). Cytochrome P450 mono-oxygenases, oxido-reductases, and different group transferases modify the core terpene scaffold producing overabundance of terpenes.

Ganoderic acid A (C30H44O7) Source: PubChem Ganoderic acids are derivatives from lanosterols and they come in many forms with slight changes in core structure; GA-A/B/F/G/H/T/C1/S/beta/etc. About 198 derivatives of GAs and similar metabolites have been isolated and characterized, among those GA-A and GA-B are the most well-known ones possessing several biological activities. The metabolites present in fungal body are a matter of evolutionary wonder but for us humans we strive to derive anthropocentric bias in every entity we come across, and terpenes being the promising candidate in pharmaceutical and health science research, humans exploit nature in search of better and everlasting sources of terpenes now a days cannabis plants are looked into for terpenes. These fungal species have become the model organisms for the process and are cultured and harvested for product based market which is involved in extraction of triterpenes from the fungal body; they employ a plethora of processes for extraction of metabolites. Hypothesis The fungus is an active producer of triterpenes (terpenes in general) which have a great promise for the health industry. As it is reported a vast reservoir of terpenes is actively metabolized inside its fruiting body, though triterpenes are actively produces in primordial stage and in fruiting body they are present in low quantities. Collected samples were mature and dried fruiting bodied, it is probable that our samples contain some amounts of triterpenes. Previously the crude extract is known to have potential cytotoxic effects, ganoderic acid being a major bioactive triterpene; the proposed hypothesis is that this crude extract contains ganoderic acid or its derivatives. The pathway starting from mevalonic acid to squalene to lanostrol and further into ergostrol where several ganoderic acids and derivatives are synthesized is well known; thereby any part

of the pathway can be detected to state the presence of this model of metabolite interchanges. REVIEW OF LITERATURE Scientific classification:

Kingdom Fungi Division Basidiomycota Class Agaricomycetes Order Polyporales Family Ganodermataceae Genus Ganoderma Species G. Lucidum (Curtis) P. Karst (1881) Scientific classification of Ganoderma lucidum G. lucidum a macro-fungus synthesizes plethora of bioactive metabolites. It has also been popularised in the ancient medicinal texts of some regions of world. Ganoderma lucidum, also known as 'the mushroom of immortality' is one of the best-known medicinal macro fungi in the world. Modern pharmacological research has laid vision of potential therapeutic activities besides anti-inflammatory, anti-tumour, anti-hypertensive and immune-modulatory activities, they are also known to possess anti-thrombosis properties (Yi et. al. 2017). The fungus is a storehouse of bioactive metabolites; making itself a ‘virtual cellular factory' for bio-pharma industry. Triterpenoids and polysaccharides are found in excess in G. lucidum. It is known that at primordial stage the fungi contains maximum tri-terpenoids; but in cultured mycelium and fruiting bodies low quantities are found. It also secretes proteins that can adequately disintegrate both cellulose and lignin. Such enzyme activities may act as valuable resource for biomass usage, fiber bleaching and organo-pollutant degredation. Our comprehension of G. lucidum science is restricted in spite of its part in conventional Chinese medicine and its amazing stockpile of bioactive metabolites. The area of interest fungal triterpenoids

The triterpenoids are C30 prenyl chain derived metabolites that are found widely in nature as steroids and sterols. The biosynthetic pathway towards ergosterol, the major component of the plasma membrane in fungi, begins with the cyclization of squalene to lanosterol. Squalene-epoxidation occours forming 2,3-oxidosqualene (chair-boat-chair). Linear chain of polyisoprene is cyclized by oxidosqualene cyclase/lanosterol cyclase (OSC). Methyl migrations (two) and hydride shifts followed by deprotonation, results into lanosterol (Quin et al. 2014).

Biosynthesis of different terpenes from Mevalonic acid (Rosa et al., 2005)) Prokaryotic and eukaryotic triterpene cyclases share a similar overall protein fold, with two α-helical and a membrane linking channel. Aromatic active site cavity extents up to two domains and the protonating group initiating cyclization are located at polar region. Triterpene cyclases contains conserved 16 amino acid repeats of which Gln and Trp residues offer bonding network amid the α-helices (Quin et al. 2014) (stabilization during exergonic reaction). Bacterial SHC and eukaryotic OSC have diverged sequence and mechanism. Evolutionary divergence is observed both in SHC accepting squalene as a substrate and OSC accepting 2,3-oxidosqualene as substrate. Substrate activation is favoured by a protonating environment, and aromatic residues which stabilizes the carbocation intermediates, forming lanosterol.

Tri-terpenes biosynthesis

More than 150 triterpenoids have been isolated until now and are being investigated keenly for wide range of health benefits. Upstream of cyclization in mevalonic acid pathway constitutes 13 genes encoding 11 enzymes. Two genes encode for Acetyl-CoA, C-acetyltransferases and farnesyl diphosphate synthases; remaining are encoded by single-copy genes. Lanosterol synthase (LSS) synthesizes a cyclic intermediate of triterpenoids and

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disruption of cellular membranes so as to extract maximum number of metabolites from the sample and dissolve them in solvent). 3 spots observed in TLC. • Sonication + Refluxing 0.5 grams of sample with 10 ml ethanol in beaker were sonicated for an hour with temperature set to 55 degrees and refluxed afterwards in the apparatus for 24 hours. Resulting extract was checked in TLC plate and 3 prominent spots were found. • Column Chromatography Column for chromatography was collected from pharmaceutical sciences lab and it was packed with semi aqueous silica gel and sample extract with solvent was stacked on the top. Chloroform was the selected mobile phase for separation of metabolites, it was poured from the top of column and the solvent passes through the silica gel packed material carrying with it the metabolites stacked at the top. Metabolites move according to their relative sizes and molecular weights and are influences by the mobile phase flowing down the column and accordingly the metabolites flow out of the silica gel packed column wherein the metabolite with low molecular weight and small size are driven out first and accordingly metabolites separate in ascending order of molecular masses and sizes. Test tubes and holders from Plant Sciences Lab were employed for the chromatographic experiment. The column was not let to dry out and was always packed with mobile phase (the gauge was closed when column was not running and at the top of column a cotton plug is inserted to avoid impurities being mixed with our sample), hence metabolites were separated slowly with uniform flow rate and drying was prevented. Metabolites were isolated and estimation of total amount of mobile phase needed for isolation of metabolites was done. And the extraction was put on hold as the flow rate was disturbed and separation was hampered. 1. Identification NMR (to be carried out in future) Isolating pure crystals from crude extract The powdered fruiting body (0.5 g) is mixed with 10 ml ethanol and heated in the heating mantle for about 25-20 minutes and shaken at intervals; metabolites present in the powder could have been made soluble in the solvent (ethanol). The extract is filtered; the solvent is covered in parafilm and left overnight in low temperature conditions. The sample is observed the next day for formation of crystals of pure metabolites, which could thereby sent for NMR analysis. It was hypothesised that the metabolites would cluster together in ethanol and form crystals which could be seen by naked eyes, after 24 hours crystals formed were analysed and found impure and minute in sizes. Converting Ganoderic acids into salts and detection As we were searching for ganoderic acids in the sample we would look for acids, metabolites of similar molecular wt., sizes, boiling points, etc., accordingly was the process. First of all 10 grams of crude sample from acacia host was sonicated with 10 ml of ethanol and products were filtered out. Pet ether (10 ml) was added to the sample to dissolve all the impurities of low polarities. Base (5-6 pellets of NaOH) was then added to the sample and all the acids present in the sample are now converted to salts, and the salts reside in the middle layer between the two layer of solvents, wherein Pet ether (top layer) and ethanol (basal layer). The results (salts) were analysed in GC-MS apparatus by dissolving them with chloroform in vials.

Conversion of acidic metabolites into salts; Explanation - Two layers of solvents with their constituent metabolites are well separated, upper one being pet ether and lower one ethanol. In next figure the solvents were separated and layer of salt retained in the beaker was extracted and remaining salts were filtered out.

GC-MS Analysis Fungal samples from two different hosts Azadiracta and Acacia were analyzed in search for metabolites present in both of them by gas chromatography and mass spectroscopy. 1. Sample Collection Samples were collected from different hosts of Ganoderma namely Acacia (host) and Azadirachta (host) by former Ph.D student Balraj Singh Gill from different locations in Bathinda (Chetak Park, Bir Talab etc.). The mycelium of the fungal samples were converted into powdered form and stored for analysis as the chemical constituents do not change in converting the samples into powder form. 2. GC-MS sample preparation Fungal samples 10 grams each was mixed with 10 ml of ethanol (high polarity) and sonicated for about one hour, temperature of the sonicator was set to 550C. Following sonication the samples were filtered with filter paper and concentrated in rotavaporator. Vials of adequate sizes were borrowed from Central Instrumentation Lab (Central University of Punjab) and sample were loaded in them and marked accordingly (different samples from different hosts). Hplc grade chloroform was added to samples in the vials and samples were sent for GC-MS analysis. Detection would be maximized when sample is diluted properly otherwise, our sample may pose harm to the gas column used for detection. GC-MS ANALYSIS PARAMETERS Column Oven Temp. :60.0 °C Injection Temp. :250.00 °C Injection Mode :Splitless Sampling Time :1.00 min Flow Control Mode :Pressure (Pressure :150.0 kPa) Total Flow :18.0 mL/min Column Flow :2.51 mL/min Linear Velocity :57.7 cm/sec Purge Flow :3.0 mL/min Split Ratio :5.0 Oven Temperature Rate Temperature (OC) Hold time (minute) -- 60.0 1.00 30.00 270.0 0.00 10.00 310.0 5.00 Oven temperature setup for GC oven

Equilibrium Time: 3.0 min IonSourceTemp: 200.00 °C Interface Temp. : 280.00 °C Solvent Cut Time: 2.50 min Detector Gain Mode: Relative Detector Gain: 1.84 kV +0.00 kV Threshold: 0

MS Table Start time 3.00 min End Time 17.00 min ACQ mode Scan Event time 0.50 sec Scan speed 1666 Start m/z 20.00 End m/z 800.00 Mass Spectroscopy parameters

RESULTS Proper solvent (ethanol) and proper mobile phase (chloroform) were selected out of many solvents available in the laboratory according to their metabolite dissolution properties (solvent) and metabolite mobility, preferably slow (mobile phase).

Band - 2

Band - 1

Clear Separation

1 2 3

Ethanol Chloroform Pet ether

TLC check for choosing proper mobile phase After selected mobile phase and solvent were put to use, the crude extract were dissolved in solvent and sonicated and loaded on top of silica gel column, thereafter mobile phase chloroform were loaded and column chromatography was performed.

Conformations about the separation of metabolites by column chromatography is established by repeated TLC checking of the contents of test tubes from respective apparatus (as that was the only tool for separation of metabolites), further within the test tubes mobile phase and assisting metabolites are present; those can be seen in TLC plates. This simply means that the metabolites present in the column stacked above the silica gel are interacting with the mobile phase and are relatively dispersed according to their sizes, the small one coming out first and the bulky ones latter.

Metabolite moving up with mobile phase

1. 2. 3.

TLC plates showing progress in column chromatography The above shown three TLC plates depict a picture of isolation through column chromatography. The 3 plates listed as 1, 2 and 3 are placed in the order in which the TLC checks were performed after starting column chromatography. The first plate shows no isolation rather we see two bands forming distinctly and the separation between the two bands is clearly visible thus they represent two different compounds. The second TLC plate contains 4 samples and out of those 3 contains metabolites of interest which moved with mobile phase (indicated by arrows). The third plate contains 4 samples and here sample 3 and 4 showed distinct bands well separated from one another and the metabolite of interest eluting slowly. The TLC plates show metabolites present in the sample indicated below and the movement of the metabolite under influence of mobile phase and if the metabolite present is a mixture bands would be visible and the separation of bands is directly related to the effective separation by mobile phase.

Metabolite isolated

Pure metabolite

TLC plate showing single metabolite at 4th sample The TLC plate above clearly depicts such a picture; the TLC plate was run in chloroform: methanol (10:1), the metabolites present in each insertion by capillary needles is justified starting from 1 to 6 (depends on how many samples one wants to check at a time). The selections of these 6 test tubes were also a matter of fine precision. Conditioned under uniform flow rate the test tubes in the stand were loaded with mobile phase flowing out of column, when all test tubes were full 6 was selected out of 40. From each column of test tubes one or two are selected accordingly and the selection should be dispersive for ensuring proper detection of separated metabolites. This TLC plate shows that there was a metabolite which was finely separated and retained a definite position (band) in the TLC plate. The metabolite can be seen slightly visible in samples 1, 2; moreover in sample 4, 5 the bands are clearly visible, herein the 4th sample shows pure metabolite with much less impurities and 5th one shows metabolite with impurities but the 6th sample does not contain any amount of the metabolite as shown in TLC plate. Therefore can be assumed that the sample metabolite is finally isolated from the cluster of metabolites as known from sonicated sample analysis. The initial metabolites with low molecular weights have been isolated and it is the starting step of separation of the 5 detected metabolites in the sonicated sample. This means other remaining metabolites are left intact in the silica gel column. As GA

has higher molecular weight so it will elute slowly, accordingly we can concidear that maybe the last or the second last metabolite could be ganoderic acid if flow rate is kept constant (516.675 g/mol). The column chromatographic isolation is put on hold due to mobile phase unavailability and lack of pre-planning of quantitative volume of mobile phase for running the whole of column chromatographic apparatus for crude fungal samples from two different host plants. The process is kept on hold for future and the dissertation is forwarded in the direction of detection of metabolites from crude samples by GC-MS method.

GC-MS data analysis Literature was searched and protocols for detection of triterpenoid metabolites were analysed. The parameters of oven temperature, pressure, injection time etc. for gas chromatography and conditions for mass analysis were investigated and the crude samples were analysed. Samples were dissolved in chloroform and the precaution is taken to make the sample less concentrated for better detection and less harm to column in the apparatus. Ganoderic acid salts MS analysis The formations of salts of ganoderic acid as mentioned in the methodology section were detected by GC-MS analysis. Here out of the 15 peaks representing metabolites by gas chromatography neither one correlates with ganoderic acid or its derivatives but when MS MS Library analysis was done the structure was anticipated. Name: Androstane-3,2′-thiazolidine with formula - C21H35NS (although not a C30 metabolite but still it is a squalene derived structure).

Structural similarities in MS library search.

GC-MS analysis for crude extract of two host species The analysis results in conclusions that 7,22-Ergostadienol found in crude fungal extracts of Acacia and Azadirachta hosts are the derived products of the terpenoid pathway and the type of sterols (ergosterols) identified are derivative of the same pathway having lanosterol as precursor and triterpenes as products. 7,22-Ergostadienone with area 0.61% was found in fungal sample from acacia host. Thus overall with area 2.91% the concentration of desired metabolites is in higher amount in Acacia sample over Azadirachta. Metabolites detected in fungal samples from Acacia (host) • 7,22-Ergostadienol (Area 2.91% and Height 1.98%)

MS Library analysis

MS library search for structural similarities with 7,22-Ergostadienol in Acacia sample. • 7,22-Ergostadienone (area 0.61% and height 0.43%) MS Library analysis

MS library search for structural similarities with 7,22-Ergostadienone Metabolites detected from fungal samples of Azadirachta (host) • 7,22-Ergostadienol (area 1.19%) (height 0.68%) MS Library analysis

MS library search for structural similarities with 7,22-Ergostadienol in Azadirachta sample. Discussions Identification of individual components from the mixture can be done by separating them from one another and several methods are there for separation all listed under a common title, Chromatography. Chromatography being the standard method of separation of metabolites due to their affinity with mobile phase or solvent is employed for separation of metabolites from the mixture. Thus silica gel column chromatography was

employed for separation and TLC was used for visualization of separation and separated individual metabolites. The solvent system and mobile phase were standardized by experimentations. Where the proper solvent is describable as the one in which maximum number of components from the fungal powder could be dissolved in, hence ethanol was taken into consideration. Similarly the mobile phase used was chloroform : methanol (10:1) because the metabolites were not moving upwards in TLC plates by any other solvent used (e.g. pet ether or ethyl acetate), the movement was still limited in chloroform but in ethanol the movement was fast because of its high polarity. Thus 10:1 ratio of chloroform and methanol were established to provide proper movement for the metabolites. For isolation of metabolites we need to be clear about the pharmaceutical approaches of isolating metabolites from mixture. Proper solvent was selected (ethanol) and metabolites were dissolved in it from fungal extract by sonicating the sample in a beaker One of the metabolites from the 5 detected metabolites in TLC spots in Sonicated sample was isolated from the mixture. Thus the sample now contains 4 other metabolites which should contain ganoderic acid and is left for future study. This states that we could not isolate ganoderic acid from the sample rather came across related C-28 terpenes of the pathway which could have further converted into a tri-terpene. The GC-MS data is analysed and 7,22-Ergostadienol with area 1.19% and height 0.68% is found in Acacia and Azadirachta samples. Moreover another metabolite 7,22-Ergostadienone with area 0.61% and height 0.43% is also found in Acacia (Host) sample. Thus it is clear that GC-MS detection was done for ergosterols which primarily are steroidal metabolites sterols (C28). Terpenes are detected and structures are identified. SUMMARY Statements like, ganoderic acids having therapeutic properties are easy to say but hard to prove, with over 150 derivatives, GAs constitute the most diverse family of triterpenoid metabolites; these are not limitations rather maybe more derivatives could be seen in future. To establish therapeutic potential of GAs careful studies of activity of specific derivatives and the coordinated action of derivatives must be revisited. It may seem unlikely but therapeutic potential of GAs could be established as a cluster of interacting molecules coordinating for benefits. Anticancer potential of various ganoderic acids can be explored through in-vivo studies. Though initial plan was separation of ganoderic acid (dissolved in solvent) and derivatives from the fungal crude extract, according to their molecular weight and size in the chromatographic apparatus, the process was simple and I had isolated about 2 metabolites from the mixture before the flow rate was disturbed. This experiment can be repeated in future by providing undisturbed flow rate and NMR analysis of speculated isolated samples. From the host dependent crude extract of G. lucidum GC-MS analysis, we conclude that the levels of active bio-constituents in crude extract of G. lucidum growing on Acacia, were found to be higher as compared to Azadirachta this finding was supported by identification of about 33 metabolites in fungal sample having Acacia host and about 24 metabolites were detected in Azadirachta. 7,22-Ergostadienol with area 2.91% and 1.19% is found in Acacia and Azadirachta samples. Moreover another metabolite 7,22-Ergostadienone with area 0.61% is found in Acacia associated fungal sample thus giving insights about the pathway starting from mevalonic acid to squalene to lanostrol and further synthesizing ergostrols, ganoderic acids and derivatives with modifications on core structure. Thus we cannot definitely conclude to what were the parameters which affected the metabolite production in Azadirachta but we can however say that metabolites present in Acacia were more in number. 2.91% area with

7,22-Ergostadienol and 0.61% area with 7,22-Ergostadienone presents us 3.52% total area covered by terpenoid derivatives in Acacia sample which is quantitatively much higher than that of Azadirachta sample with area 1.19% of total terpenoid metabolites. As both of them were mature and dried samples when collected thus the content of metabolites says something about chemovariance.

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