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TLC-Bioautography Profiling of Pippali Extract for Antidiabetic and Antioxidant Effect

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Abstract:

Pippali, also known as Piper Longum, has potential therapeutic uses, including antidiabetic and free-radical scavenging. The constituents responsible for Pippali's antidiabetic and antioxidantproperties were investigated. The in vitro inhibition of α-amylase and α-glucosidase enzymes and evaluated antioxidant activity through DPPH scavenging activity were assessed. TLC-bioautographic-MS analysis identified specific compounds accountable for Pippali's antidiabetic and antioxidant activities. The study found a significant inhibitory effect on α-amylase and α-glucosidase enzymes, indicating its potential in regulating carbohydrate absorption and digestion. Piperine, a compound present in Pippali, exhibited prominent antioxidant properties and α-amylase inhibition, suggesting its crucial role in Pippali's antidiabetic and antioxidant effects. These findings offer valuable insights into Pippali's potential therapeutic applications in managing diabetes and associated complications.

Keywords:TLC-Bioautography, Pippali, α-Amylase, α-Glucosidase, Antioxidant

1.1 Introduction

Diabetes is a chronic non-communicable disease characterized by high blood sugar levels, and it is considered a significant global health concern. It falls into three main categories: type 1, type 2, and gestational diabetes. Type 1 diabetes, which used to be referred to as adolescent diabetes or insulin-dependent mellitus, primarily affects younger individuals. However, our main focus is on Type 2 diabetes, which isa common form, accounting for at least 90% of all diabetes cases. It is also known as

adult-onset diabetes or non-insulin-dependent diabetes. (1)Type 2 diabetes relies on digestive enzymes like α-amylase and α-glucosidase, which break down complex carbohydrates. Targeting α-glucosidase inhibition is a potential therapeutic approach for controlling postprandial hyperglycemia and managing Type 2 diabetes. Natural products with medicinal properties are recommended for this approach.(2)
The analysis of herbal medicine has a rich history, focusing on evaluating the quality and safety of medicinal plants. Various chromatographic and spectroscopic methods have been developed to identify medicinal plants' chemical constituents and bioactive compounds. Spectroscopic methods, such as infrared, UV-Vis, and NMR spectroscopy, provide information about structural characteristics and functional groups. Hyphenated techniques, which combine chromatographic and spectroscopic methods, enable precise identification and structural elucidation of individual compounds in complex plant extracts. These methods help standardize, quality control, and develop evidence-based formulations.Advancements in technology have streamlined the process of analyzing medicinal plants and assessing their biological activities, enabling the identification of lead compounds, elucidation of mechanisms of action, and the development of new drug candidates. Bioinformatics and computational modeling techniques help predict potential biological activities and optimize structures for enhanced therapeutic efficacy. By combining traditional knowledge with modern scientific approaches, researchers can contribute to the development of safe and effective herbal medicines.(3)
TLC is a cost-effective as well as sophisticated separation technique for separating compounds in samples. It can be combined with visualizable enzyme reactions to perform activity screening, known as the TLC-bioautography assay. This method offers rapid access to evidence on bioactive compounds' localization and biological activities in complex plant matrices.Combining TLC-bioautography with mass spectrometry enhances the technique's capabilities, enabling rapid, target-directed isolation and high-throughputof active molecules based on enzymatic potential over the Tlc-Plate. This efficient approach provides valuable insights into chemical structures and biological activities. This approach streamlines the process of identifying and characterizing bioactive compounds in herbal medicine samples, enabling rapid screening and isolation of active molecules for further investigation for therapeutic potential.(4)
Piper longum Linn. is world's significant as well as ancient spices, which is also known as 'Long pepper' and 'Pippali'. The fruits haveactivities including CNS depressants(5), antipyretic, analgesic, hepatoprotective(6), bioavailability enhancers, antioxidants (7), antidiabetic(8), and anti-inflammatory. The phytoconstituents of P.longum include volatile oil, and other minor alkaloids such as piplartin, piperlogumine, piperidine, starch, resin, and pungent alkaloid piperine, and many more. Pippali clinical studies show mechanisms reducing fasting plasma glucose, improved postprandial glucose, and hemoglobin A1c, improving glucose control indicators, confirming its clinical relevance and statistical significance.
To date, numerous preclinical and clinical investigations have been carried out on Pippali to explore its potential as an antidiabetic treatment. However, there remains a dearth of research data elucidating the specific bioactive compounds responsible for its therapeutic effects. Thus, the objective of the current study is to identify the bioactive compounds in Pippali that exhibit antidiabetic and antioxidant properties by employing a combination of thin-layer chromatography (TLC) coupled with bioautographic techniques and mass spectrometry (MS). The aim is to determine the compounds that demonstrate inhibitory activity against α-amylase and α-glucosidase, as well as possess free radicle scavenging abilities.

1.2 Material and Method:

1.2.1Procurement of Pippali: Pippali Sample 1 (Fresh Pippali) and Sample 2 (Stored Pippali) were procured from ManakarnikaAushadhalaya with Authentication Certificate (Batch no-

SHPL/01/22, Exp. Date -05/24)

1.2.2Extraction of Pippali:

1.2.2.1Sample 1: Initially the sample was converted to fine powder. The 25g of fine powder was taken and Soxhlet with 250ml of Ethanol. The sample was allowed to Soxhlet at 40°C for 8hr. The liquid extracts so obtained were subjected to rotary evaporator to concentrate the extract. The final product sample was re-suspended in respective solvents, dried and filtered with Whatsman filter paper.
1.2.2.2Sample 2: Initially the sample was converted to fine powder. The 25g of fine powder was taken and Soxhlet with 250 ml of Ethanol. The sample was allowed to Soxhlet at 40°C for 8hr. The liquid extract so obtained were subjected to a rotary evaporator to concentrate the extract. The final product sample was re-suspended in respective solvents, dried and filtered with Whatman filter paper. (9)

1.2.3Invitro Assay:

Invitro assays have been performed before TLC-Bioautography in order to determine the optimum activity when compared against the standard for their respective activity.

1.2.3.1 DPPH free radicle scavenging activity:

Preparation of Solutions:

Standard stock preparation: 1mg of ascorbic acid was dissolved in 1ml of DMSO. (1000ug/ml)
Sample stock preparation: 1mg of extract was dissolved in 1ml of DMSO. (1000ug/ml) subsequent dilution of standard and sample of 100ug/ml to 500 ug/ml were made in DMSO
DPPH solution(0.135mM): 13.31mg of DPPH was transferred to 25ml volumetric flask, 10ml methanol was added to the flask to dissolve it and final volume was made up to the mark with methanol. 10ml of the stock solution was pipette out and further diluted to 100ml to get 0.135mM solution.
Procedure of assay:

In this experiment, 20 µL of each diluted sample was mixed with 180 µLDPPH solution in methanol (0.135mM) in a separatewells of a 96-well plate. Plate was then incubated for 30 min at normal temperature in a dark location. After incubation, the absorbance of each mixture was notetedat 517 nm using a spectrophotometer. Ascorbic acid was used as acontrol to evaluate effectiveness of Pippali, and its efficacy was compared to that of control. The percentage of antioxidant activity was calculated and plotted against the concentration of the Pippali extract to determine its antioxidant potential. The equation used was:

$$\%\ scavenging\ activity = \frac{1 - Asample}{Acontrol} \times 100$$

1.2.3.2 α-amylase Activity:

Preparation of Solution:

Standard preparation: 13 mg of acarbose tablet powder was dissolved in 1 ml of DMSO (di methyl sulphoxide) i.e; 5000ug/ml. Ultrasonicate for 15 min.
Sample preparation: 5mg of extract was dissolved in 1ml of DMSO (5000ug/ml)
The standard and the sample were dilutions of 1000 ug/ml to 2500ug/ml was prepared in DMSO.
Sodium Phosphate Buffer: (0.02M, 6.8Ph)
NaH_2PO_4: Initially NaH_2PO_4 of 0.02 M was prepared 276mg in 100ml of dis water

Na_2HPO_4: Na_2HPO_4 of 0.02M was prepared by dissolving 284mg in 100 ml of dis water.
0.006M NaCl: 3.52mg of NaCl was dissolved in 10 ml of dis water.
The solvent with lower Ph was added to the solvent with higher Ph.
0.1% Starch: 0.1gm of starch in 100ml dis water.
α-amylase stock: 8mg of α-amylase was added to 10 ml of sodium phosphate buffer i.e; 4units/ml
1M HCl: 0.83ml of HCl was dissolved in 10ml of dis water.
Iodide solution: Iodine solution (5mM) was added to Potassium iodide solution (5mM)
5mM Iodine solution: 12.69mg of Iodine crystal were dissolved in 10ml dis water. Ultrasonicate for 10min.
5mM Potassium iodide solution: 8.3mg KI in 10ml of dis water.

Procedure of Assay:

The α-amylase inhibitory activity of pippali was determined using a 96-well plate. In each defined well, 40 µL amylase solution (5 units/ml in sodium phosphate buffer, pH 6.8). The mixture was then incubated at 37ºC for 30min. After the incubation period, 40 µL of starch solution (0.1%) was added to the mixture. Following a 10 min interval, 20 µL of HCl (1M) was added. To measure the absorbance, 100 µL of iodide solution (5mM iodide + 5mM potassium iodide in dis water) was added, and the absorbance was recorded at 580nm. Acarbose served as standard in this assay, providing a reference for comparison.

$$Percentage\ inhibition\ of\ \alpha - \text{amylase} = \frac{1-Asample}{Acontrol} \text{x} 100$$

1.2.3.3 α-Glucosidase Activity:

Preparation of Solvents:

Standard preparation: 13 mg of acarbose tablet powder was dissolved in 1 ml of DMSO (dimethylsulphoxide) i.e; 5000ug/ml. Vortex for 15 min. Further, 0.2ml was taken from stock and dissolved to 1ml of DMSO (1000ug/ml).
Sample preparation: 5mg of extract was dissolved in 1 ml of DMSO (5000ug/ml). Further 0.2ml was diluted to 1ml(1000ug/ml).
Dilutions of concentration 250 to 450 ug/ml were prepared for both standard and sample.
Potassium Phosphate buffer: (0.1M, Ph 6.8)
Potassium phosphate dibasic: 1.742g was dissolved in 100ml of dis. water.
Potassium Dihydrogen Orthophosphate: 1.366g in 100ml dis water
The solvent with lower Ph was added to the solvent with higher Ph.
α glucosidase solution: 0.1 mg of α-glucosidase was added to 10ml of potassium phosphate buffer
Para-nitrophenyl-α-D-glucopyranoside(5mM): 3.021mg of para-nitrophenyl-α-D-glucopyranoside was dissolved in 2ml of dis water.
0.2M sodium carbonate: 210mg of sodium carbonate was dissolved in 10ml of dis water.

The procedure of assay:

In this experiment, 120 µL of sample mixed with 20 µL of α-glucosidase solution (1 U/mL in 0.1 M potassium phosphate buffer, pH 6.8) in separate wells of a 96-well plate. Thenit was incubated for 20 min. Then further 20 µL of para-nitrophenyl-α-D-glucopyranoside (5mM) was added, and the mixture was incubated for 20min. The reaction was stopped by adding 80 µL of 0.2 M sodium carbonate. The absorbance of the mixtures was measured at 405 nm. Acarbose served as the standard in this assay, providing a reference for comparison.

$$Percentage\ inhibition\ of\ \alpha - \text{glucosidase} = \frac{1-Asample}{Acontrol} \text{x} 100$$

1.2.4 TLC-Bioautography

1.2.4.1 Antioxidant activity:

Procedure:A DPPH stock solution (5mM) and Pippali extract (1000ug/ml) were prepared in methanol, and applied to a TLC plate. The plate was developed usingToulene: Ethyl acetate: formic acid: methanol (6:6:1.8:0.25v/v/v/v) as a solvent system.Plate was then sprayed with methanolic DPPH solution. Yellowish bands appeared against a purple background, representing antioxidant properties. Compounds in the separated bands reacted with the DPPH radical, causing a change in color.

1.2.4.2 α-Amylase Activity:

α-Amylase solution was prepared by dissolving 10 mg in 20 ml of sodium acetate buffer and stored at 4°C. The Tlc plate was developed and dipped in enzyme solution.Plate was then incubated in a humid desiccator for 1.5 hours, then dipped in a 1% starch solution and iodine solution. The enzyme activity was observed, with brown spots.

1.2.4.3 α-Glucosidase Activity:

α-glucosidase stock solution was prepared by dissolving 100 U in sodium acetate buffer and stored at 4°C. A TLC plate was developed and dipped in the enzyme solution. The plate was then incubated for 1-2 hours in adesiccator. The plate was then dipped in 2-naphthyl-α-D-glucopyranoside and Fast-Blue B salt solution in ratio 1 :1, and incubated for an additional 2 hours. The enzyme activity was visible with white spots on a purple/violet background.(10)

1.2.5 Mass Study:

Sample Preparation: The TLC-Bioautograms showed zones of DPPH, amylase, and glucosidase inhibition. Targeted spots were scrapped off the TLC plate and dissolved in HPLC-grade methanol. The mixture was centrifuged at 4000 RPM for 10 minutes to separate silica gel particles from compounds. Supernatants were collected and filtered using a 0.2 µm polytetrafluoroethylene membrane filter. The samples were prepared for analysis, enabling identification and characterization of compounds in targeted spots.

Mass spectral Analysis: The 3 samples from TLC-Bioautography were analysed at Savitribai Phule Pune University-Central Instrumentation Facility. HRMS has been performed using source type ESI.(9)

1.3 RESULTS:

1.3.1 Invitro Assays:

1.3.1.1 DPPH Free-Radicle Scavenging Activity: Pippali contains a wide range of phytochemicals that contribute to its antioxidant activity. The ethanolic extract of Pippali has exhibited significant free radicle scavenging activity against DPPH radicals, with an average inhibition of 77% at a concentration of 1000 ug/ml andat same concentration Ascorbic acid, demonstrated an average inhibition of 79%. These results indicate that Pippali possesses notable antioxidant properties, comparable to the positive control.

Pippali, a plant with rich alkaloid content, has antioxidant properties that enhance the body's antioxidant enzyme system. These enzymes combat reactive oxygen species, neutralize trace elements, and prevent cellular damage. Maintaining normal glutathione levels is crucial for individuals with diabetes, as low levels can cause diabetic nephropathy. Pippali's naturally occurring antioxidants may provide therapeutic benefits for various conditions, including type 2 diabetes.

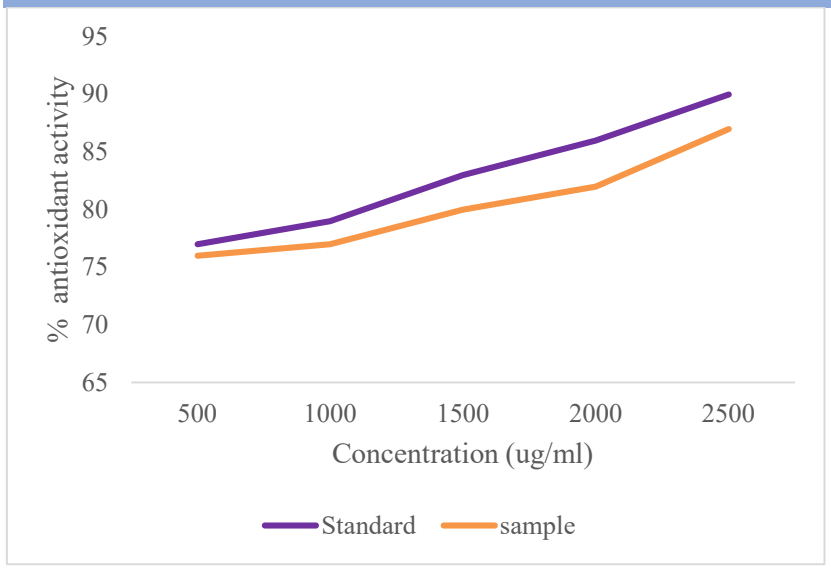


Fig:1.1 Graph of concentration vs %inhibition of DPPH

1.3.1.2 α-Amylase Activity:

α-amylase, a calcium-containing enzyme, is essential for breaking down polysaccharides into monosaccharides in the oral cavity. Inadequate carbohydrate and lipid metabolism can lead to elevated blood glucose levels, causing Type 2 diabetes mellitus (T2DM). Pippali, a natural alternative, has been investigated for its potential as an α-amylase inhibitor. Results show significant inhibition at a concentration of 2500 µg/ml, with Pippali showing 76% of inhibition and acarbose showing 86% inhibition at the same concentration. These findings suggest Pippali's potential as a natural alternative to drugs for inhibiting α-amylase activity.

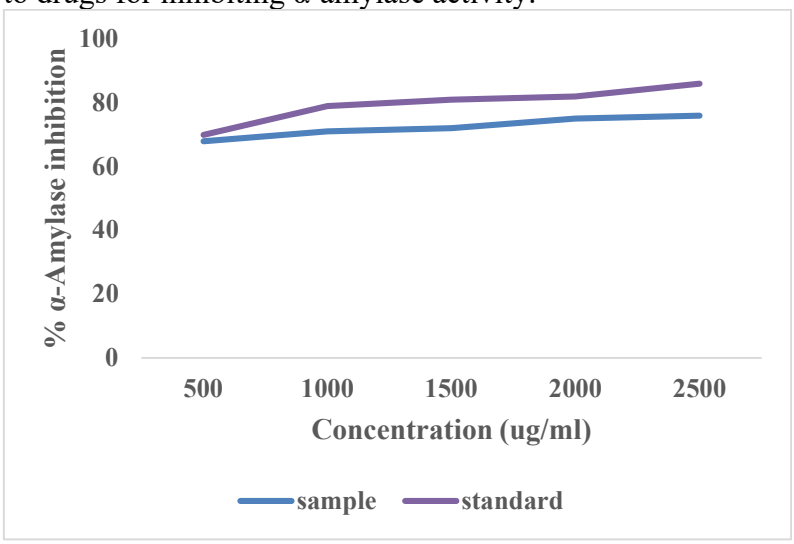


Fig:1.2 Graph of concentration vs %inhibition of α-Amylase

Studies have shown the effectiveness of α-amylase inhibitors like acarbose in treating diabetes. Acarbose, a pseudotetrasaccharide, regulates postprandial glucose levels (PPG) and hemoglobin A1c in hyperglycemia by blocking or slowing down starch absorption in the gastrointestinal tract. This is

achieved by inhibiting the hydrolysis of 1,4-glycosidic linkages, preventing starch breakdown into simpler sugars like maltose. Oxidative stress, a common complication of diabetes, is caused by prolonged high blood glucose levels. To mitigate this, compounds rich in phenols, flavonoids, and alkaloids are recommended due to their antioxidant properties.

In summary, α-amylase inhibitors like acarbose helps in managing diabetes by regulating PPG and HbA1c. Additionally, phenolic, flavonoid, and alkaloid compounds are recommended due to their antioxidant properties, which can help alleviate complications associated with diabetes-induced oxidative stress.

1.3.1.3 α-Glucosidase Activity:

Pippali, a natural therapy, has shown significant potential against α-glucosidase activity, with an average inhibition of 81% at a 2500 µg/ml concentration. This is significantly higher than acarbose's 92% inhibition, suggesting that Pippali possesses a more efficient and substantial α-glucosidase inhibition potential compared to acarbose. This suggests that Pippali may be a promising option for regulating postprandial blood glucose levels in Type 2 diabetes patients.A study found that natural α-glucosidase inhibitors, like acarbose, have therapeutic efficacy not solely due to delayed digestion of complex carbohydrates but also due to colonic starch fermentation. This suggests that α-glucosidase inhibitors could be effective treatment options for Type 2 diabetes, targeting postprandial hyperglycemia. Their safety profile ensures they do not pose a risk of hypoglycemic events or life-threatening complications, making them a suitable and acceptable therapeutic approach for managing Type 2 diabetes.

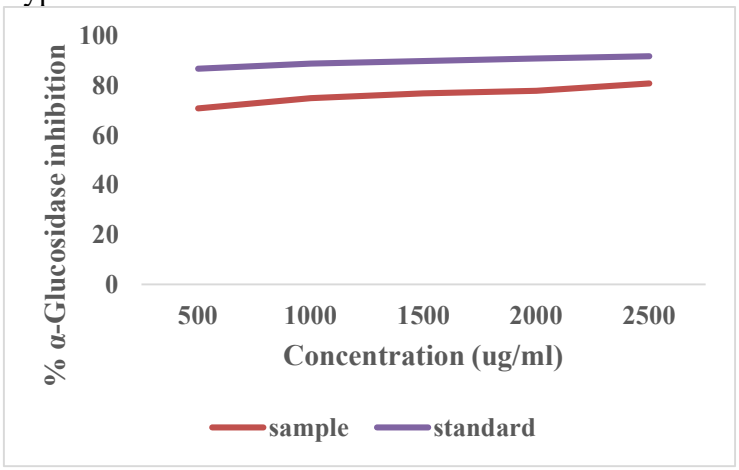
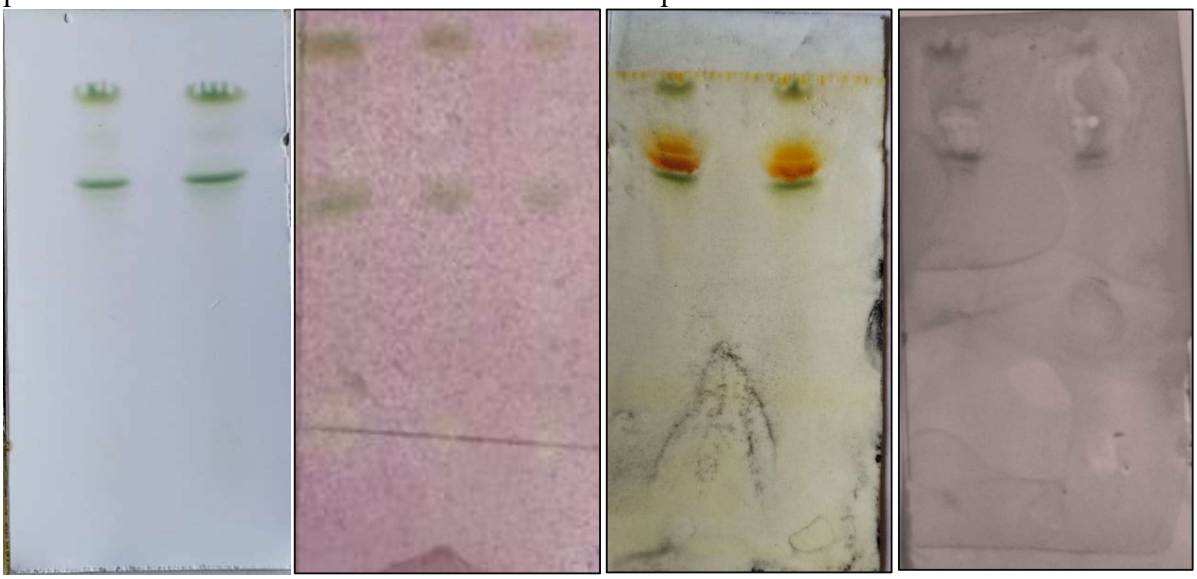


Fig:1.3 Graph of concentration vs %inhibition of α-Glucosidase

1.3.2 TLC fingerprinting:TLC fingerprinting profiling is a valuable technique for evaluating herbal products and identifying and characterizing constituents. This technique involves separating compounds in extracts at different retention factors, allowing for visual representations of chemical composition. In the case of Pippali, TLC fingerprinting was performed using a specific solvent system, namely Toluene: Ethyl acetate: Formic acid: Methanol (6:6:1.8:0.25 v/v/v/v), revealing multiple constituents within the ethanolic extract. This technique helps researchers gain insights into the chemical profile of herbal products, enabling identification, comparison, and quantification of constituents, ultimately contributing to a better understanding of their overall composition and potential biological activities.

1.3.3 TLC-Bioautography assay: The TLC-bioautography assay and MS analysis of Pippali's

active spots revealed the presence of Piperine(m/z = 286.85), a free radical scavenging compound. Piperine plays a significant role in Pippali's antioxidant properties, neutralizing free radicals involved in oxidative stress and cellular damage. This information helps us understand Pippali's mechanisms and potential health benefits associated with its consumption.



(a) (b) (c) (d)

Fig:1.4 (a) Normal Tlc Plate for scrapping of active spots, (b) Tlc-Bioautogram for Antioxidant Activity, (c) Tlc-Bioautogram for α-amylase activity, (d) Tlc-Bioautogram for α-glucosidase activity.

Piperine, a bioactive compound found in herbal products, has shown promising potential as a nutritional supplement for combating various diseases. Its antioxidant potential supports its use in experimental models. However, the effects of herbal products extend beyond antioxidant properties, addressing conditions like diabetes, cardiac disorders, and cancer. In diabetes, herbal products have antidiabetic properties, regulating blood glucose levels and inhibiting key enzymes. In cardiac disorders, some bioactive compounds have cardioprotective effects, reducing the risk of cardiovascular diseases and promoting heart health. In cancer research, some compounds have anticancer properties, inhibiting tumor growth and promoting apoptosis. Further research is needed to validate their efficacy and safety in clinical settings, as well as individual variations, dosage, and potential interactions with other medications. Furthermore, in α-amylase TLC bioautography, the MS analysis of the scrapped bioactive bands from the controlled TLC revealed one prominent bioactive compound as Piperine
Piperine is a well-known alkaloid having four isomers and here even after scrapping off the different spots same Piperine was responsible for both the activities of Antioxidant as well as α-amylase.
Moreover in the case of α-Glucosidase TLC-Bioautography, one white active spot appeared on the TLC-bioautogram. The corresponding spot from the active zone control plate was scrapped, and MS analysis was performed and MS revealedPiperlongumine.

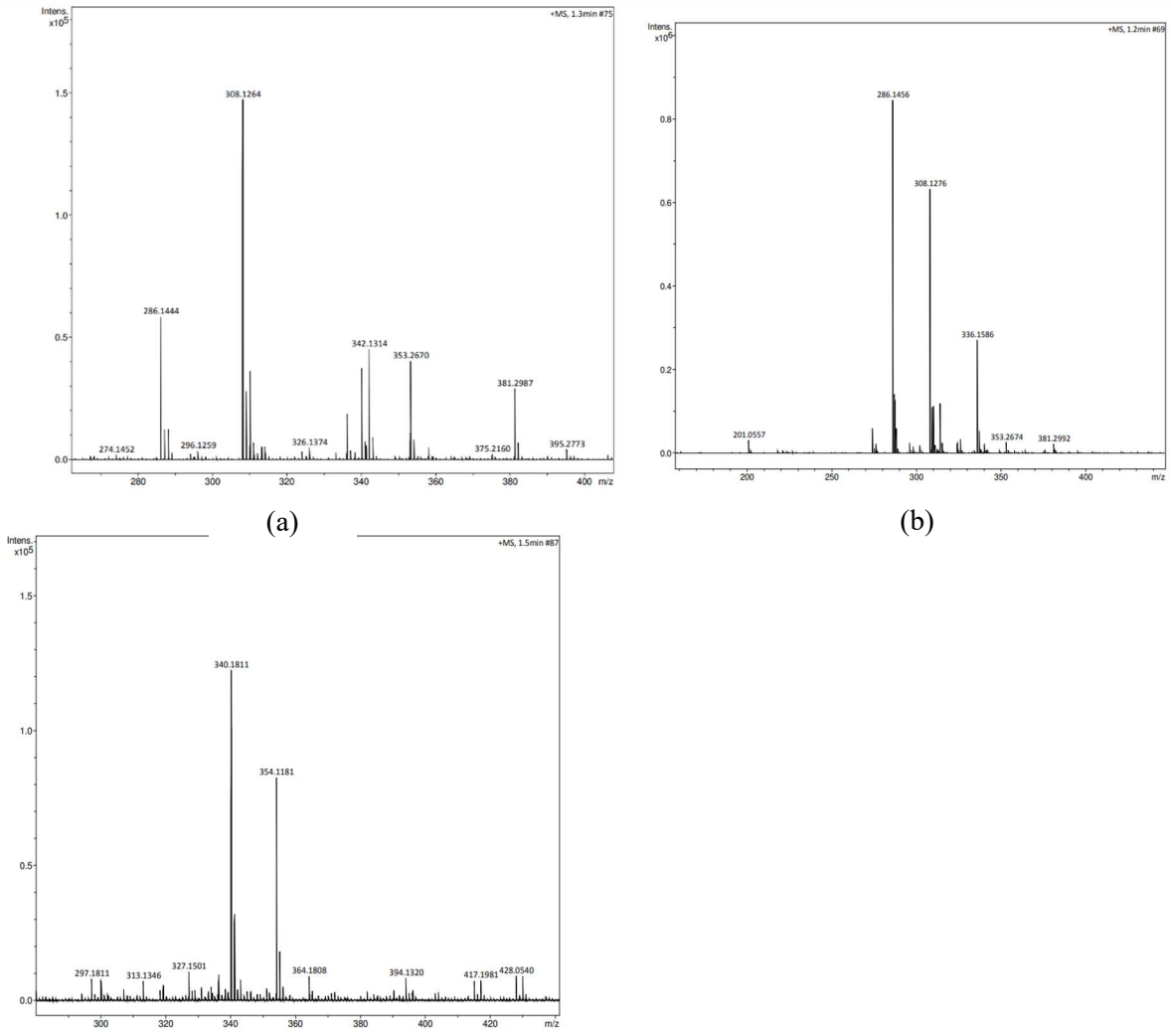


Fig:1.5 (a) Mass Spectra of Antioxıdant, (ɒ) Mass Spectra of α-amylase, Mass Spectra of α-glucosidase

Pippali, a plant used in diabetes treatment, has been found to have multiple physiological effects on diabetic patients. These include reducing glucose production, increasing GLP-1 and GIP hormones, decreasing glucose absorption, increasing insulin secretion, improving insulin sensitivity, enhancing glucose uptake and storage in muscles, reducing diabetic nephropathy risk, providing protection against oxidative damage, controlling blood glucose levels, and inhibiting the DPP4 enzyme's activity. TLC-bioautography and mass spectrometry analysis have identified several chemical constituents in Pippali, which contribute to its antioxidant and antidiabetic properties. This research helps identify and characterize the specific compounds that contribute to its beneficial effects on managing diabetes and combating oxidative stress.

1.4 Conclusion:

Pippali, a medicinal plant, has shown remarkable antidiabetic and antioxidant properties in in vitro studies. Its bioactive compounds interact with enzymes like α-amylase and α-glucosidase, regulating blood glucose levels and reducing postprandial spikes. Pippali's antioxidant properties neutralize free

radicals, which can cause cellular damage and contribute to various diseases, including diabetes. Pippali supplementation significantly reduces fasting blood glucose, postprandial glucose, and glycosylated hemoglobin levels, making it a promising natural remedy for managing diabetes and promoting overall health.

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