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IN VITRO MECHANISTIC APPROACH AND EVALUATION OF ANTIDIABETIC ACTIVITY OF *ATROPA ACUMINATA*

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ABSTRACT

Objective: The purpose of this study was to look into the antidiabetic and antioxidant properties of hydroethanolicextract of *Atropa acuminata* (HEEAA). Experimental research was conducted to furnish the scientific evidence of antioxidant activity against various stable free radicals like nitric oxide, superoxide, and lipid peroxidation (measures the radical scavenging activity of antioxidants) and ant diabetic potential against different enzymes like alpha-amylase, alphaglycosidase, dipeptidyl peptidase IV (DPP-IV), pancreatic lipase,etc. of Atropa plant (*Atropa acuminata)*and also evaluate their maximum inhibitory value (IC_{50} values).

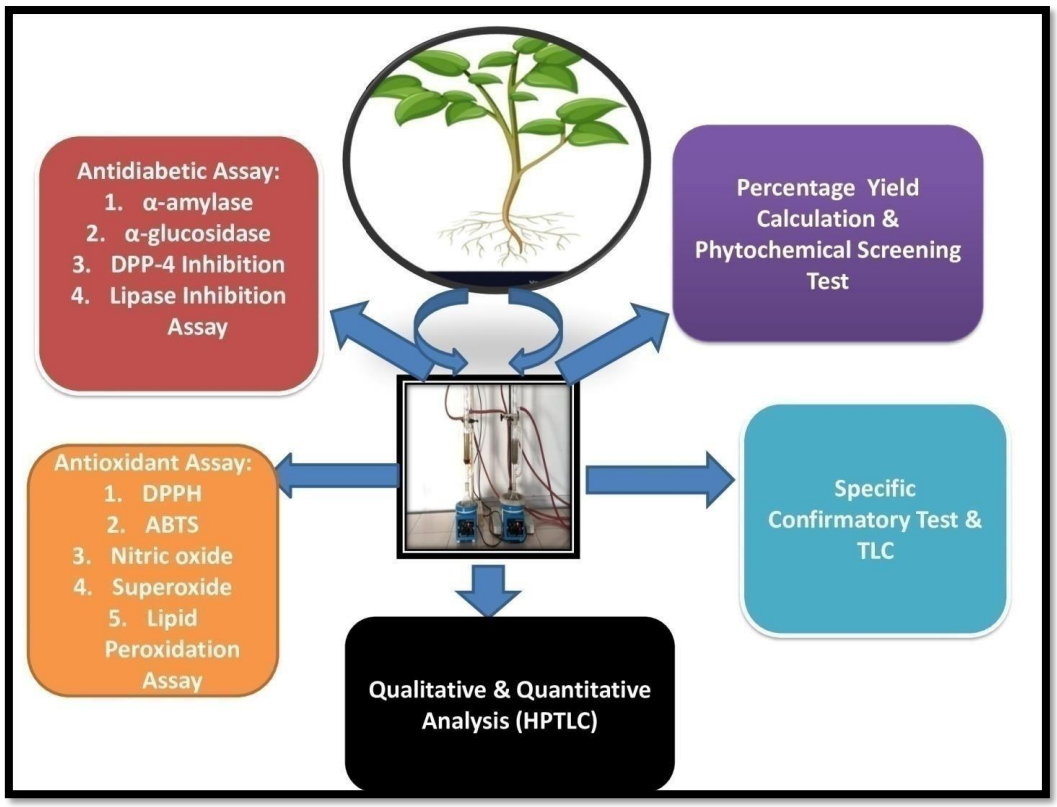
Methods: Shade-dried root material was pulverized, homogenous material was passed through sieve number 40, using solvent ethanol: water in a ratio of 50:50 soxhletextraction was performed. Thick semi-solid material was vacuum dried using a rotary vacuum flash evaporator and then concentrated in a desiccator, stored in a refrigerator at 40°C for evaluating maximum inhibitory concentration (IC50 values) in both activities (free radical scavenging and enzyme inhibitory antidiabetic activity).

Results: High-performance thin layer chromatography **(**HPTLC) results confirmed the presence of atropine in the extract withan R_F valueof 0.37 and the percentage yield calculated as per standard calculation for atropine was 0.553 % w/v respectively. Among the different antioxidant studies, the maximum inhibitory concentration value in lipid peroxidation assay for plant extract was greater (170.3±0.6 and 152.4±0.5) in comparison to other antioxidant assays performed. Although,the extract showed no or very weak significant inhibition against dipeptidyl peptidase IV and pancreatic lipase.The extract displayed an appreciable effect on alpha-amylase and alphaglycosidase enzyme inhibition at the highest investigated concentration (200 µg/ml).

Conclusion: The results showed that *A. acuminata* hydroethanolic extract has anti-oxidant activity and appears to exert a hypoglycemic impact.

Keywords: Diabetes, *Atropa acuminata,* antioxidant, HPTLC, Lipid peroxidation, alphaamylase

Graphical Abstract



INTRODUCTION

Diabetes is a prevalent and persistent metabolic illness that has rapidly emerged as a significant global issue, carrying substantial social, health, and economic implications [1, 2]. Diabetes mellitus (DM) encompasses a diverse range of conditions characterized by elevated blood glucose levels resulting from either a complete or partial deficiency in insulin synthesis or impaired insulin function. Diabetes mellitus is characterized by persistent high levels of blood glucose, which is linked to detrimental effects on various organs and systems, such as the retina, kidney, neurological system, heart, and blood vessels [3]. Diabetes ranks as the fourth most prominent cause of mortality in developed nations, and empirical research indicates its widespread occurrence in numerous emerging and newly industrialized nations. According to the International Diabetes Federation (IDF), the prevalence of diabetes mellitus was estimated to be 366 million in 2011, with a projected increase to 552 million by the year 2030 [4]. The mortality rate attributed to diabetes in India had a notable rise, escalating from 0.98 percent in 1990 to 3.1 percent of total deaths in 2016. According to a study conducted in 2016, diabetes accounted for a total of 27.5 million years of disability-adjusted life, with 10 million of those years attributed directly to the condition [5].

Particularly in type 2 DM, oxidative stress is thought to have a major role in the development of vascular problems [6]. Damage to enzymes and cellular machinery, as well as a rise in insulin resistance due to oxidative stress, are all consequences of free radical generation in diabetes, which occurs as a result of non-enzymatic glycation of proteins, glucose oxidation, and enhanced lipid peroxidation. Antioxidants in the diet have received a lot of attention because of their potential to ward against free radicals and hence a host of human diseases [7, 8]. These diseases include diabetes, obesity, asthma, cancer, atherosclerosis, stroke, rheumatoid arthritis, and neurodegeneration. Inadequate health care systems, a lack of sufficient facilities, drug toxicity, etc., all contribute to making it difficult to effectively treat diabetes and its accompanying problems in India and elsewhere. In recent years, researchers have shifted their attention to alternative and complementary medicine. Herbal medicines are growing in popularity because they are safer and more effective than conventional pharmaceuticals, while also being more affordable [8, 9]. The alkaloids, flavonoids,

phenolic, and tannins found in herbs increase the efficiency of pancreatic tissues by increasing insulin secretion or decreasing intestinal absorption of glucose, making them a valuable resource for treating a wide range of gastrointestinal disorders. The condition is caused by free radicals in the body's tissues, which can be absorbed and neutralized by phenolic and alkaloid components.

These antioxidant-rich phytochemical compositions were found in the *Solanaceae* family plant species *A. acuminata*. This plant can be found in the Western Himalayan Mountains, specifically in Kashmir between 1800 and 3600 meters above sea level and up to 2500 meters above sea level in the neighboring Himachal Pradesh highlands. It is located close to Chakrata, Muzaffarabad, and Kashmir in the Northwest Himalayas, India. *A. acuminata* is a perennial plant that can grow up to 0.9 meters tall and 0.75 meters wide [9]. Tropane alkaloids, particularly NMethyl ornithine, are highly oxygenated triterpenes found in *A. acuminata* root extracts, according to a previous phytochemical analysis that was reported in the scientific literature. By using CE-ESI-TOF-MS, direct examination in spectrometric method, and high-performance liquid chromatography (HPLC), alkaloids including hyoscyamine N-oxide, norhyoscyamine, apoatropine, hyoscyamine, 6-hydroxyhyoscyamine, and scopolamine were found to be present in high concentrations in *A. acuminata* root extracts. Atroposides A, B, C, D, E, F, G, and H are eight important steroidal glycosides that have been discovered in the seeds of *Atropaacuminata*. All of these atroposides have a relatively low number of investigated phytoconstituents. C (3-Obeta-D-glucopyranosyl (1-->4)-beta-D-galactopyranoside), E (3-O-beta-D-glucopyranosyl (1->2)-beta-D-galactopyranoside),-beta-D-glucopyranosyl (1-->4)-beta-D-galactopyranoside), G (3O-alpha-L-rhamnopyranosyl(1-->4)-beta-D-galactopyranoside),(1-->2)-beta-D-glucopyranosyl (1-->4)-beta-Dgalactopyranoside) [9-10]. The phytochemistry of *A. acuminata* has been well developed and demonstrated in various literature. We have expanded our investigation because of the structural variety and complexity of *A. acuminata*, but phytochemistry is still unknown. The present study was undertaken to evaluate the antioxidant and anti-diabetic activities of *A. acuminate* hydroethanolic extract in vitro, this work aimed to investigate the molecular approach, which is not reported so far. After an initial assessment, additional study is needed to identify the phytoconstituents from this plant and create an NDDS formulation of the extract and their extracted ingredient.

MATERIAL AND METHODS

Plant material Collection

Atropa acuminata roots were taken in September 2020 from dense forests in Mandi and Kullu, Himachal Pradesh. Dr. MadhavaChetty, botanist, Sri Venkateswara University, Tirupati-517 502, A.P., authenticated the plant sample. Our lab has saved voucher specimens in the herbarium (SVU/SC/76/321/20-21) for future reference.

Chemicals

2,2-Diphenyl-2-picryl hydrazyl (DPPH), ρ-nitro phenyl-α-D-glucoside solution (PNPGLUC), Metformin, DMSO, stock solution, petroleum ether, ethanol, ethyl acetate, glibenclamide, EZ-cytox Kit, benzyl alcohol, poly vinyl alcohol, DPPH, nitric oxide, superoxide, lipids, Hydrazine, ethanol or methanol, Ascorbic acid, ABTS, rutin, potassium per sulfate, Nitric oxide,sodium nitroprusside, 1-napthylamine (5%), Griess-Ilosvay reagent, naphthyl ethylene diamine dihydrochloride (0.1% w/v), Purple azo dye, sodium nitroprusside, 50% glacial acetic acid, Sulphanilic acid reagent (0.33% w/v), sulphanilic acid, NEDD, sodium hydroxide, nitro blue tetrazolium (NBT), 5 mMNaOH, poly unsaturated fatty acids (PUFA), egg lecithin, 0.25 N HCl, Sodium phosphate buffer, DNS color reagent, sodium di-hydrogen orthophosphate, disodium hydrogen orthophosphate, 3,5 Dinitro salicylic acid, sodium potassium tartrate, phenol and sodium metabisulfite, Starch solution, phosphate buffer, phosphate buffered saline (20 mM), Tyrode solution, Insulin solution (0.4 U/mL), sodium carbonate, Sodium phosphate buffer 80mM, pH 6.8. Sodium dihydrogen orthophosphate (1.9095 g), disodium hydrogen phosphate (3.42 g) 80mM sodium phosphate buffer, 1mM p-nitro phenyl glucopyranoside, and Acarbose. Metformin tablets and chemicals (500mg) were collected from *Franco-Indian Pharmaceuticals Pvt. Ltd*

Extraction of *Atropa acuminata* root Powder

The roots were shade-dried and further pulverized to a homogeneous powder using an electric blender and passed through sieves with a mesh size of 40 and kept in an airtight container. The coarsely powdered material (200g) was further extracted using Soxhlet with ethanol and water in a 50:50 ratio, then concentrated and evaporated the solvent further, and stored the extract. The hydroethanolic extraction of *A. acuminata* (HEEAA) then yielded a 6% thick, black, semisolid residue. After the solvent was removed by reduced pressure distillation, the semisolid material was vacuum dried using a rotary flash evaporator (Rota vapour, R-210/215, Buchi, Switzerland). The concentrated semi-solid substance was dried in a desiccator and the dried extract was then stored in the refrigerator at 40^0C.

Thin Layer Chromatography

The extracted material was put on TLC plates where numerous solvent ratios were selected on the premise of their upward nature of polarity (Toluene: Ethyl acetate= 1:9/ 9:1, Ethyl acetate: Butanol: Distilled water= 2:4:4, Toluene: Ethyl acetate: Formic acid= 1:1:1, Ethyl acetate: chloroform: formicacid: water = 7:1.5:1.5:1 etc.) to customize the solvent system for the elution of phytochemicals effectively on TLC plate. The silica plate yielded yellowish-green dots, which show that phenols, alkaloids, and flavonoids are present in the sample. The most efficient method for eluting phytochemicals is 75:15:10:1.8 v/v chloroform, methanol, acetone, and 25% ammonia. This mixture will be utilized for both qualitative and quantitative examination.

For high-performance thin-layer chromatography (HPTLC) analysis

Preparation of Standard Solution: Weigh 4 mg Standard Atropine in a 2ml volumetric flask. Add 1ml methanol to dissolve the standard and make up the volume up to mark with methanol. Filter the Standard with a syringe filter. Use the standard solution thus obtained for HPTLC Quantification.

Preparation of Test Solution: Weigh accurately 1.014 gm of Sample (HEEAA) in an Iodine Flask. Add 25 ml methanol to it and reflux it for 30 minutes. Cool down the sample and filter the sample with Whatmann Paper No. 1 and evaporate till 5 ml remains. Pour this 5ml sample intoa 10 ml Volumetric flask and make up the volume up to mark with Methanol. Use the Test solution thus obtained for HPTLC Quantification.

Application of spots, chromatographic conditions, and TLC densitometric scanning The sample (HEEAA) was prepared as explained above and 10 µL samples were applied in the form of a band. The extract was applied on precoated silica gel 60 F_{254} on an aluminum TLC plate at a speed of 70 nL/s and with a space of 16 mm between two bands using CAMAG Linomat 5 – Applicator. The development of the plate was carried out in a twin trough glass chamber pre-saturated for 30 min using mobile phase Chloroform:Methanol:Acetone: 25% Ammonia (75:15:10:1.8 v/v). After the development of the TLC plates, the plates were dried in a current of hot air, and densitometric scanning was performed at a wavelength of 190 nm using TLC Scanner III in the absorbance/reflectance mode.

Qualitative Phytochemical Screening

The phytochemical tests were performed on *A. acuminata* extract (HEEAA) to determine the presence of key phytochemical constituents such as carbohydrates, proteins, amino acids, alkaloids, glycosides, saponins, flavonoids, phenolic compounds, and tannins using standard test procedures followed by specific confirmatory test [11].

Antioxidant Assay

***In vitro* Free Radical Scavenging Activity**

The extract's (HEEAA) *in vitro* antioxidant activity was determined using standard techniques. The suppression of free radicals is the foundation of *in vitro* techniques. After samples are added to a system that generates free radicals, the suppression of free radical activity is determined [12].

$$\% \text{ inhibition} = \frac{[\text{Absorbance of Control- Absorbance of Test}]}{[\text{Absorbance of Control}]} \times 100$$

The IC50 values calculated represent the sample concentrations required to scavenge 50% of free radicals.

DPPH (2, 2-Diphenyl-1-picryl-hydroxyl) assay

The DPPH radical scavenging activity was quantified using a spectrophotometric approach. 1 mg of ethanol-dissolved test extract was added to a 200µMetabolic DPPH solution, along with various concentrations (ranging from 31.25 to 1000 g/ml) [13]. Each solution's absorbance at 517 nm was measured after the combination was incubated at 37 degrees Celsius for 20 minutes in the dark. Ascorbic acid, a synthetic antioxidant, was utilized as the benchmark. The extract or standard-containing blank solution that didn't include any reagents was used to test absorbance. The percentage scavenging and IC50 values calculated are included in the results (the IC50 value is the concentration of the sample required to block 50% of the radical) [14].

ABTS (2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt) assay

By reacting an ABTS solution (7 mM) with 2.45 mM ammonium per sulfate, radical captions were formed. Before use, the mixture was allowed to stand in the dark at room temperature for 12-16 hours. 1mg of test extract dissolved in ethanol was added to 0.3 ml of ABST solution and the final volume was made up to 1 ml with ethanol and incubated for 20 minutes. At 734 nm, the absorbance of these solutions was measured spectrophotometrically. As a positive control, synthetic antioxidant ascorbic acid was used [15].

Nitric oxide radical assay

The nitric oxide reaction mixture (6 ml) was then incubated at 25^0C for 150 minutes with various amounts of the extract or standard in DMSO, phosphate buffer saline (PBS, pH 7.4), and sodium nitroprusside (10 mM, 4 ml). After incubation, 0.5 ml of the reaction mixture containing the nitrite ion was taken out [16]. After thoroughly mixing the mixture, 1 ml of sulphanilic acid reagent was added to complete the diazotization procedure. Following that, 1 ml of NEDD was added, mixed, and left to sit for 30 minutes under diffused. A chromophore with a pink hue was created. At 540 nm, absorbance was measured[17].

Superoxide radical assay

To test the superoxide anion radical scavenging activity, a reaction mixture containing 0.3 ml of the extracts, substance, and standard in DMSO, 0.1 ml of NBT (1 mg ml-1 solution in DMSO), and 1 ml of alkaline DMSO (5 mMNaOH in 1 ml water) was used [29]. In this assay, superoxide radicals were produced in 3.0 ml of Tris-HCl buffer (16 mM, pH 8.0), which also contained 1 mg of test extract dissolved in 50% ethanol, which was added at various concentrations (31.25-1000 µg/ml), 78 µM of β-nicotinamide adenine dinucleotide (reduced form NADH), 50 M of nitro blue tetrazolium[18].

Lipid Peroxidation (LPO) assay

Three extracts, a chemical, and a standard were evaluated for their ability to reduce lipid peroxidation under the process. Egg lecithin (3 mg/ml, phosphate buffer, and pH). This 7.4) was ultrasonically processed [19]. Test extracts (100µl) with concentrations ranging from 31.25 to 1000 µg/mL. Liposome mixture; the test sample was not present in the control. Lipid Peroxidation was initiated by the addition of 10 µl of $FeCl_3$. Following incubation, add (400 mM) 10 µl of L-ascorbic acid and (200 mM). After 1 hour at 37°C, the process was stopped by adding 2ml of 0.25 N HCl containing 15% trichloroacetic acid (TCA) and 0.375% thiobarbituric acid (TBA) [20]. The reaction mixture was heated for 15 minutes and then cooled. Absorbance was measured at 532nm. Synthetic antioxidant ascorbic acid was used as the positive control.

***In vitro* Antidiabetic Activity of *Atropa acuminata* plant Extract Pancreatic Glucose Release studies α-amylase Inhibitory Activity**

The α-amylase assay was performed according to the method described by Odeyemi [21].

Briefly 15µlof the plant extract at different concentrations (50 µg/ml – 200 µg/ml) (diluted in a phosphate buffer) was added to 5 µl of enzyme porcine pancreatic solution into a 96-well plate. After 10 min of incubation at 37°C, the reaction was initiated by adding 20 µl of starch solution and further incubated for 30 min at 37°C [22]. The reaction was then stopped by adding 10 µl1M of HCl to each well followed by 75 µl of iodine reagent. A blank containing phosphate buffer (pH 6.9) instead of the extract and positive control (acarbose, 64µg/ml) was prepared. No enzyme control and no starch control were included for each test sample. The absorbance was measured at 580 nm and the percentage inhibitory activity was calculated by using the following equation:

$$\% \text{ inhibition} = \frac{[\text{Absorbance of Control- Absorbance of Test}] \times 100}{[\text{Absorbance of Control}]}$$

α-Glucosidase inhibition activity

The α-glucosidase inhibition assay was determined using a method described by Sancheti et al., 2010 [23] with slight modification. Briefly, 5µl of the plant extract (prepared at concentrations of 50 µl/ml, 100 µl/ml, and 200 µl/ml) was added to 20 µl of 50 µl/ml alpha-glucosidase solution into a well of a 96-well plate. Thereafter, 60 µl of 67 mM potassium phosphate buffer
(pH 6.8) was then added. After 5 min of incubation, 10 µl of 10 mM ρ-nitrophenyl-α-Dglucoside solution (PNP-GLUC) was then added and further incubated for 20 min at 37°C.
After incubation, 25 µl of 100 mM Na_2CO_3 solution was added and the absorbance was measured at 405 nm. A blank and sample blank were also prepared by adding 5 µl of deionized water instead of plant extract and 20 µl of deionized water instead of the enzyme, respectively.
Epigallocatechin gallate (10 µl/ml) was used as a positive control.
The percentage inhibition was calculated using the following equation:

$$\% \text{ inhibition} = \frac{[\text{Absorbance of Control- Absorbance of Test}] \times 100}{[\text{Absorbance of Control}]}$$

Under the assay conditions specified, the IC50 value is defined as the concentration of extract that inhibits 50% of alpha-glycosidase activity. When significant inhibition was observed, the IC50 values were calculated using linear regression, and a sigmoidal dose-response equation with a variable slope was obtained. The Mean Standard Deviation is used to represent all values.

DPP-IV Inhibition activity

DPP-IV inhibition assay was carried out according to the method described by *Almasri et al. 2009*[24] with slight modification. The assays were conducted using the DPP IV Drug
Discovery Kit (Biomol, Germany), which is based on the cleavage of a chromogenic substrate(H-Gly-Pro-para-Nitro aniline) by DPP IV to release para-nitro aniline (pNA) measured at 405 nm. 15 µl of human recombinant DPP-IV enzyme solution (50mM in Tris buffer) was added into 10L of plant extract in wells of a 96-well plate. After 10 min of incubation at 37° C, 50 µl of 20 mMρNA substrate (GlyPro- ρNA) dissolved in Tris buffer was added to initiate the reaction and further incubated for 30 min at 37° C. After incubation, 25 µl of 25% acetic acid solution was added to stop the reaction and the absorbance was measured at 410 nm. A blank and sample blank were also prepared by adding 35 µl of the buffer instead of plant extract and 15 µl of the buffer instead of the enzyme, respectively.
The percentage inhibition was calculated using the following equation:

$$\% \text{ inhibition} = \frac{[\text{Absorbance of Control- Absorbance of Test}] \times 100}{[\text{Absorbance of Control}]}$$

Lipase Inhibition Assay

The lipase inhibition assay was determined according to the method described by Lewis and Liu

[25]. Briefly, 10µL of the plant extract and distilled water was added to the well of 96-well, clear, flat bottom plates. Thereafter, porcine pancreatic solution (10 mg/ml) was freshly prepared in 50 mMTris-HCl buffer (pH 8.0) and centrifuged to remove insoluble material and was then added at 4 times the volume to each of the samples (40 µl). After 15 min of incubation, 170 µl of substrate solution (20 mg pNPP in 2ml isopropanol added to 18 ml 50 Mm Tris-HCl buffer (pH
8.0) containing 20 mg gum Arabic, 40 mg sodium deoxycholate, and 100 µl Triton X-100) was then added and incubated for 25 min at 37^{o} C; the absorbance was then measured at 410 nm using a spectrophotometer and the percentage inhibition was calculated using the following equation:

$$\% \text{ inhibition} = \frac{[\text{Absorbance of Control- Absorbance of Test}]}{[\text{Absorbance of Control}]} \times 100$$

Statistical Examination

For statistical analysis of the three data groups, results were expressed as the mean SEM, and means were compared using one-way analysis of variance (ANOVA) followed by Dennett's ttest for multiple comparisons. The value $p < 0.001$ was deemed statistically significant.

RESULTS

Phytochemical Screening

Preliminary phytochemical screening revealed that it contains alkaloids, phenolic compounds, and flavonoids, but no carbohydrates, glycosides, tannins, amino acids, or proteins, as explained in Table 01.

Table 01: Preliminary Phytochemical screening of HEEAA plant extract

S.No	Phytochemicals/ Extract Constituents	*Present/ Absent*
1.	Carbohydrates	-
2.	Phenolic compounds	++
3.	Flavanoids	+
5.	Alkaloids	+++
6.	Glycosides	-
7.	Tannins	-
8.	Amino Acid	-
9.	Proteins	-

$^{(+)}$ indicates the present, ($^{-}$) indicates the absent of Phytoconstituents.

$^{(+)} < {}^{(++)} < {}^{(+++)}$ indicates the intensity of the respective color in screening methods.

Specific Test for Alkaloids

The presence of phytochemicals was determined by qualitative testing of the freshly prepared crude extract [26]. Using standard test procedures, these were identified by distinctive color changes. The Vitali Morin test revealed a violet color indication, indicating the presence of tropane alkaloids in *A. acuminata* plant extract.

Calculation of Percentage Yield

The percentage yield of extract was determined at 18.85% from the 1000 gm weight of powdered plant material whereas, the weight of crude extract was considered to be 188.5 gm.

Quantitative estimation of Marker compound in HEEAA under HPTLC

HPTLC was performed, and the results confirmed the presence of atropine in the extract with an R_F of 0.37 in comparison to the standard with an RF of 0.38. The standard concentrations applied were 2, 5, and 10µg/ml for the compound, and the sample concentration applied was 1.014 gm/ml. The percentage yield calculated as per standard calculation found for atropine was 0.553 % w/v. The chromatographic conditions and chromatogram are represented in the following Table 02, 03, 04 & Figure 01(a)(b).

Table 02: Rf values for Test sample (HEEAA) and Standard Atropine at different concentrations T1(2µg), T2(5µg), T3(10µg) and T4 (Atropa acuminata)

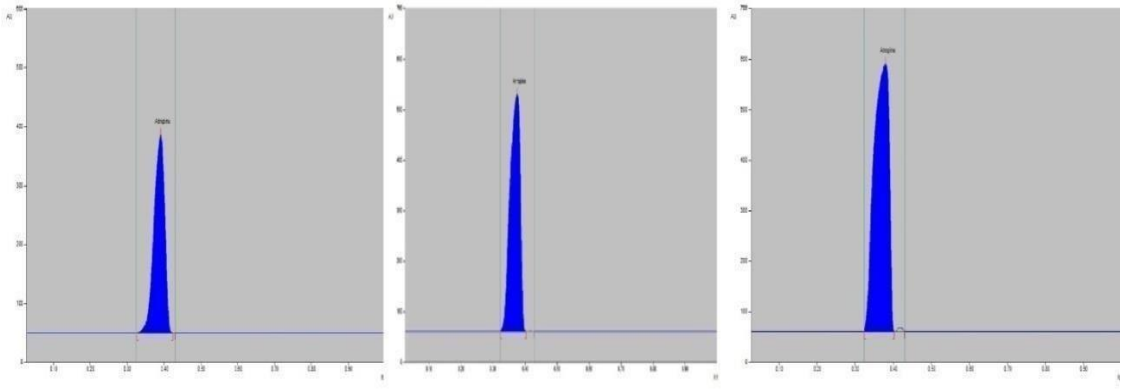
Spot No.	T1	T2	T3	T4
1	--	--	--	0.13
2	--	--	--	0.24
3	--	--	--	0.32
4 (Atropine)	0.39	0.38	0.38	0.37
5	--	--	--	0.45
6	--	--	--	0.53
7	--	--	--	0.72

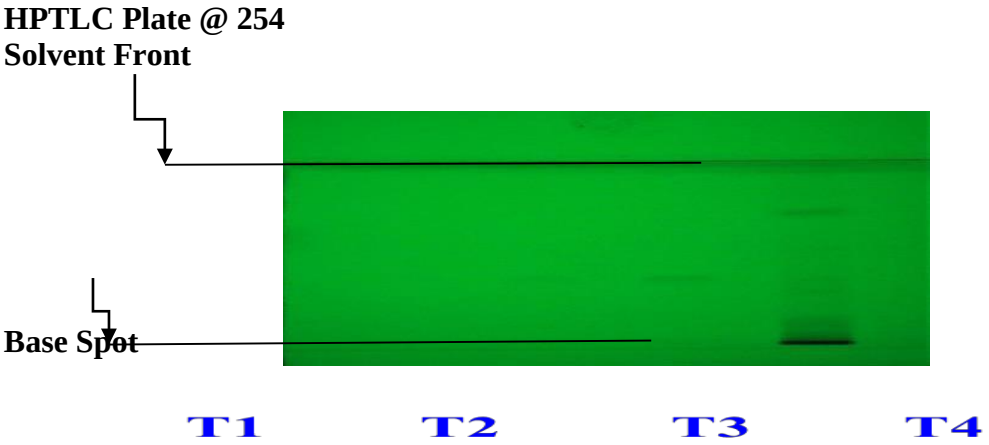
Table 03: Various parameters **(**weight, Rf values, AUC) and Percentage atropine obtained in Atropa acuminata plant Root Extract sample using HPTLC Technique.

Parameters	**Atropine**			**Atropa acuminata**
Weight	2 µg	5 µg	10 µg	1.014 gm
Rf Value	0.39	0.38	0.38	0.37
AUC	9341.1	14156.9	23514.9	392.4
%Atropine	--	--	--	0.553 %

Table 04: Various chromatographic conditions like stationary phase, mobile phase, application mode, chamber saturation time and visualization process during HPTLC qualitative and quantitative analysis.

Chromatographic Conditions	
Application Mode	CAMAG Linomat 5 - Applicator
Filtering System	Whatman filter paper No. 1
Stationary Phase	MERCK - TLC / HPTLC Silica gel 60 F_{254} on Aluminum sheets
Application (Y axis) Start Position	10 mm
Development End Position	80 mm from plate base
Sample Application Volume	10 µL sample and 2,5 and 10 µL of Standard
Distance Between Tracks	16.0 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Chamber Saturation Time	30 minutes
Mobile Phase (MP)	Chloroform: Methanol: Acetone: 25% Ammonia (75:15:10:1.8 v/v)
Visualization	@ 190





Track T1: Atropine 2 µg
Track T2: Atropine 5 µg
Track T3: Atropine 10 µg Track T4: *Atropa acuminata*
Figure 01: (a) HPTLC densitometric chromatogram of different standard atropine concentrations (2 µg/ml), (5 µg/ml), (10 µg/ml) **(b)** Band appearance, Base Spot and Solvent Front in HPTLC plate for T1, T2, T3 and T4 spottings.

***In vitro* antioxidant studies**
The antioxidant activity of *Atropa acuminate* hydroethanolic extract, HEEAA, and standards were evaluated using DPPH, 2,2'-azino-bis (3-ethyl benzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), Nitric oxide radical assay, Superoxide radical assay, and Lipid Peroxidation (LPO) methods. The results of the *in vitro* antioxidant studies are presented below in (Table 03) which indicate that IC50 value in lipid peroxidation assay for both HEEAA and ascorbic acid standard is greater (170.3±0.6 and 145.4±1.5) compared to another antioxidant assay.

Table 03: *In vitro* antioxidant studies of HEEAA

Extract	DPPH	ABTS	Nitric Oxide	Super Oxide	Lipid peroxidation
HEEAA	72.4±0.3	101.3±0.7	71.2±0.7	69.4±0.4	170.3±0.6
Standard					
Ascorbic acid	5.8±0.3	13.2±0.2	48.2±0.6	-	145.4±1.5
Catechin	-	-	-	40.15	-

***Value in the table was obtained by calculating the average of three experiments ± S.E.M**

***IN-VITRO* STUDIES**
α-AMYLASE INHIBITION ACTIVITY
In this study, the results indicated that the HEEAA exhibited no significant effect on α -amylase. At the highest concentration (50µg/ml) investigated, the extract displayed an appreciable effect on α -amylase by 55.06% (Figure 02).

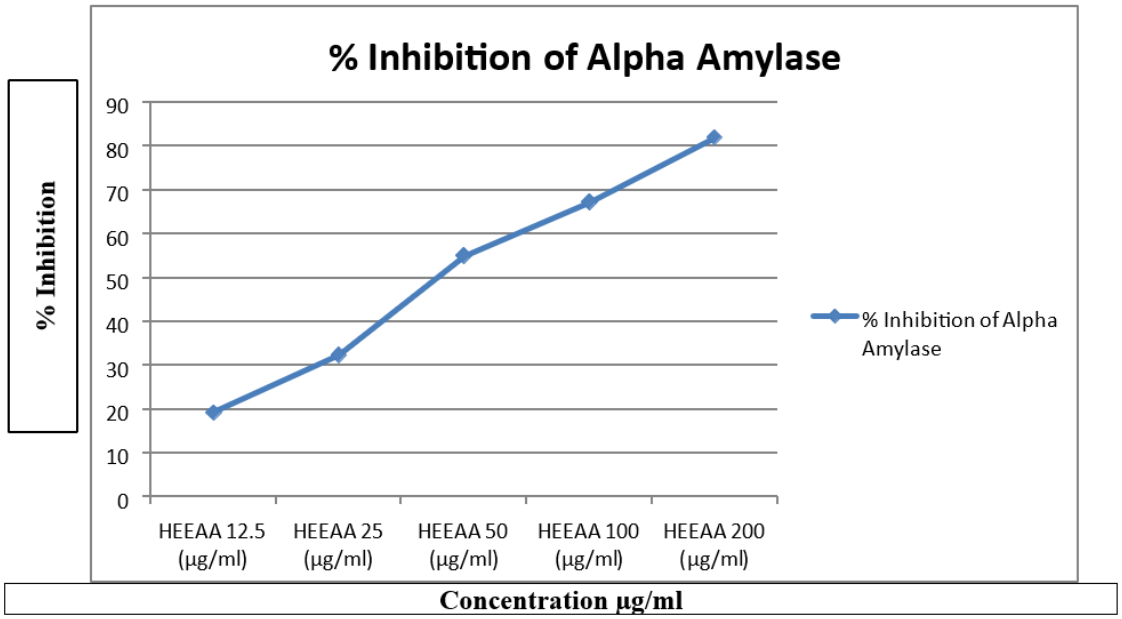


Figure 02: α-Amylase Inhibition Assay Result

α-GLUCOSIDASE INHIBITION ACTIVITY

The HEEAA showed significant inhibition of the α-glycosidase enzyme. At the highest concentration (200µg/ml) investigated, the extract displayed an appreciable effect on alphaglycosidase by 41.23% (Figure 03).

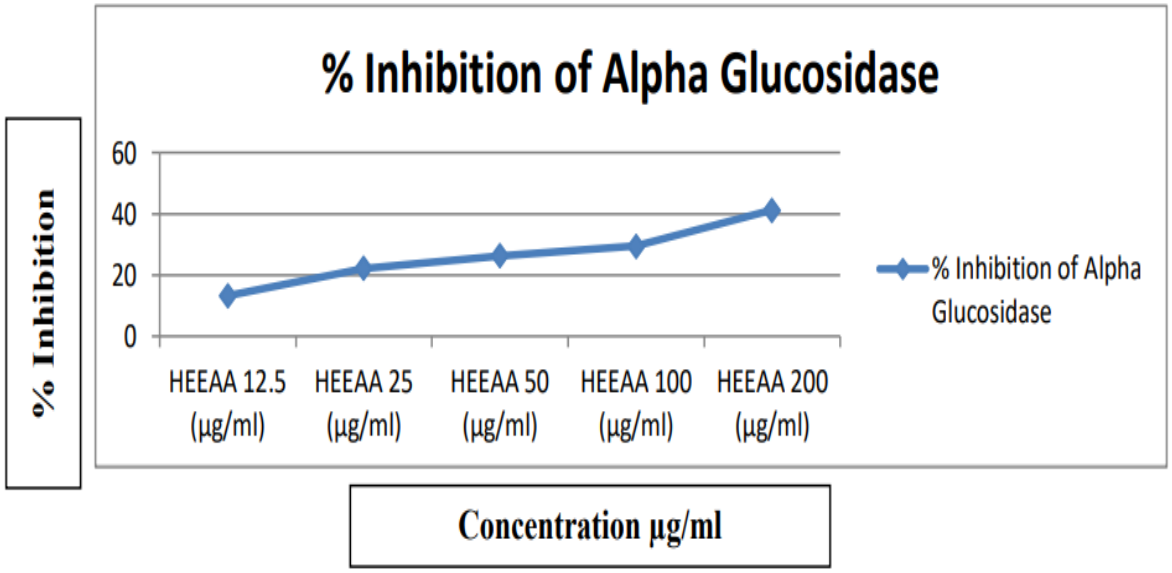


Figure 03: α-Glucosidse Inhibition Assay Result

DPP-IV INHIBITION ACTIVITY

The HEEAA displayed no significant inhibition at all the tested concentrations compared to the positive control (diprotin A) which exhibited 73.8% inhibition of DPP-IV activity. The highest inhibition obtained for *A. acuminata* extract was 79.17% (Figure 04).

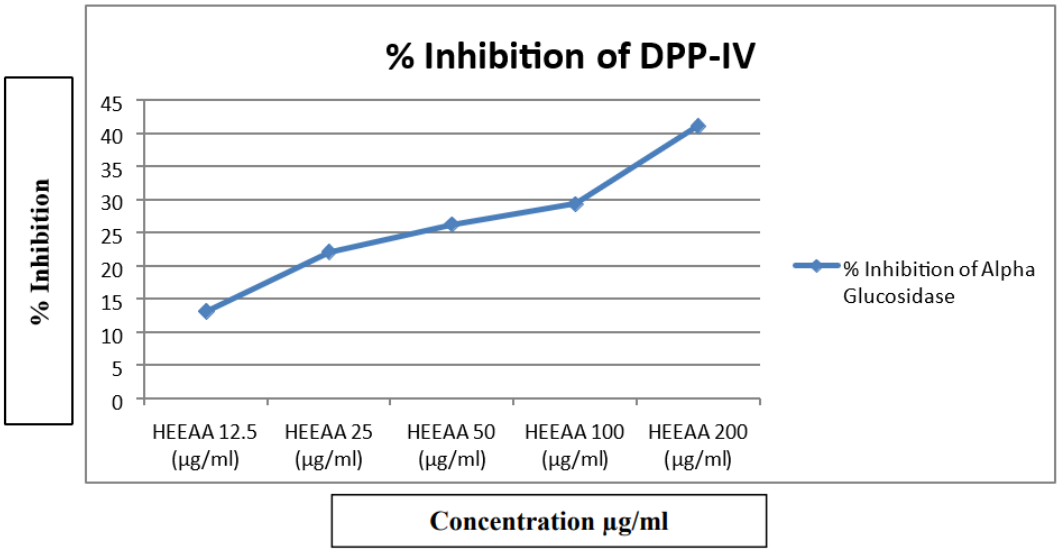


Figure 04: DPP-4 Inhibition Assay Result

LIPASE INHIBITION ASSAY

The HEEAA exhibited weak significant inhibition against pancreatic lipase at all the concentrations investigated in a concentration-dependent manner (Figure 05]. The highest inhibition obtained for HEEAA was 59.41% which was much lower when compared to orlistat (56.5%), a known lipase inhibitor.

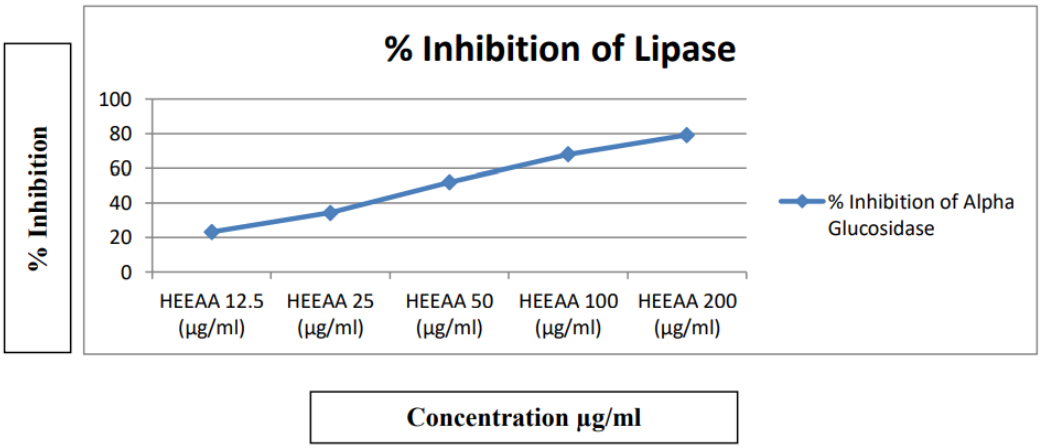


Figure 05: Lipase Inhibition Assay Result

Table 04: *In-vitro* α-amylase, α-glycosidase, DPP-IV, and lipase inhibition activity

Extract/ Standard Drug	% Inhibition of α-glycosidase	% Inhibition of α-amylase	% Inhibition % Inhibition of DPP-IV of Lipase	
Epigallocatechingallate (10µg/ml).	55.3	-	-	-
Acarbose (65 µg/ml)	-	93.9	-	-
Diprotin A(50µg/ml)	-	-	73.8	-
Orlistat(50µg/ml)	-	-	-	56.5
HEEAA 12.5 (µg/ml)	13.25 ±2.14	19.32±1.35	23.25 ±0.97	11.21±2.23
HEEAA 25 (µg/ml)	22.15±2.34	32.39±2.28	34.33±1.75	21.24±1.75
HEEAA 50 (µg/ml)	26.3±1.25	55.06±2.24	52.05±0.27	33.15±2.12
HEEAA100 (µg/ml)	29.45±3.43	67.32±1.15	68.07±1.43	48.09±1.76
HEEAA200 (µg/ml)	41.23±2.12	82.09±3.12	79.17±1.98	59.41±0.34

DISCUSSION

Indian system of traditional medicines has long been used and systematized but still, there are lots of herbs that are unrecognized in the northern region. Alternative herbal medicine has been used past

from thousands of years and these Ayurveda herbs have a greater value in the treatment of lifestyle disorders such as diabetes mellitus, obesity, etc. [27, 28]. The present study has been initiated to study the *A. acuminata* plant as a potential antioxidant and ant diabetic therapeutic agent. For this, hydroethanolic root extract from *A. acuminata* was obtained to evaluate phytochemical screening, *in-vitro* antioxidant, and ant diabetic properties. Furthermore, the presence of alkaloids was determined by HPTLC studies.

The results of Qualitative phytochemical screening showed that the hydroethanolic extract of *A. acuminata* roots has the majority of phytochemicals rich in alkaloids, phenolic compounds, and flavonoids. The reason for the same phytochemicals may be the organic nature of solvents to dissolve the active bioactive compounds. The main therapeutic properties of plants depend upon their secondary metabolites presence. It was noted that the good concentration of phytochemicals in plant extract will make it better for future isolation and purification. The results of the current study are in consistent with the previous study on this plant [29].

Free radicals have a major role in oxidative stress and degeneration of tissues which leads to oxidative damage of cells these radicals may be either ROS or RNS are reactive oxygen and nitrogen species (RNS). The plant extract's antioxidant potential is due to the presence of flavonoids, which exhibit redox and chelation potential. Alkaloids, flavonoids, and tannins are found in various parts of the plant, including phytochemical-rich roots [30-32]. The results of HEEAA's *in vitro* antioxidant activity revealed that the lipid peroxidation assay has the highest scavenging effect on lipid peroxide radicals. The antioxidant activity of plant hydroethanolic extract was compared to that of reference antioxidants. Furthermore, the increasing action of DPPH radical scavenging activity demonstrated the capacity to give hydrogen particles bringing the number of electrons picked up and HEEAA had DPPH activity as its ability to donate hydrogen particles. The chemical reaction between the free radicals and the phytochemicals present in our extract, HEEAA is the reason for the quenching of various radicals. The ABTS radical scavenging, NO, and superoxide radical activities have also been shown by HEEAA.

It was also claimed that antioxidants play a significant role in the prevention of various heart diseases, diabetes, and cancer [33, 34]. This disorder became the fourth leading cause of death worldwide because it complicates cardiovascular health and causes diabetic nephropathy, diabetic neuropathy, and diabetic retinopathy which are inflammatory disorders due to the release of various mediators like IkB, NF-KB, TLR-4, MAPK, TNF, ROS, Macrophage, NF-KB, IL-1β, IL-6 [35, 36]. These mediators cause oxidative stress and mitochondrial dysfunction. Therefore, the antioxidant activity of the plant plays a preventive role on beta cells in the treatment of Diabetes [37].

Literature also revealed that the plant contains multiple phytochemicals like atropine, belladonna, hyoscine, hyoscyamine, etc [38]. Bljajic et al., 2021 investigation also suggested that the plant hydroethanolic extract shows a promising effect in decreasing blood sugar levels by inhibiting α-glycosidase enzyme (oxidative markers) activity [39, 40]. Plant roots scavenge oxidative radicals in rat mesenteric tissue, which is important because beta cells play a role in diabetes mellitus diseases. More research is being conducted to assess the efficacy of *A. acuminata* root in intestinal enzyme assays due to its antioxidant and inhibitory role. Two *In vitro*antidiuretic assays were performed, with the α-amylase enzyme showing better hypoglycemic activity than the α-glycosidase enzyme because the literature shows that plant extract contains atropine, hyoscine, and belladonna. This could be attributed to the presence of rich chemical constituents in plant roots as well as potential anti-diabetic compounds, which will be confirmed by additional molecular and mechanistic approach studies [41]. Presently, there are several antidiabetic drugs used to treat or manage diabetes and the mechanisms of action of these drugs are well known. These include the inhibition of alphaamylase, alpha-glycosidase, lipase, and DPP-IV enzyme. Our results indicated that the extract demonstrated significant inhibition of alpha-amylase, glycosidase, lipase, and DPP-IV when compared to the respective positive controls. However, the effective inhibition of DPP IV observed in this study by the extract is probably physiologically relevant. This suggests that the ant diabetic mechanism of *A. acuminata* is therefore through the inhibition of this enzyme [42].

Studies have shown that one of the consequences of abnormally high blood glucose in patients suffering from diabetes is protein glycation. Protein glycation is the reaction between reducing sugar (galactose, mannose, glucose, fructose, and ribose) and the free amino group of a protein reversibly leading to the formation of adducts (Schiff and Amadori products) and over a long period produces glycation products. These reactions play a significant role in the development of the pathogenesis of diabetic complications [43]. The extract displayed weak significant inhibition on protein glycation and collagenase at the concentration investigated compared to aminoguanidine and EDTA. However, this study represents the first attempt to investigate both protein glycation and ant-collagenase properties of the extract of *A. acuminata. T*herefore, it can be deduced from this study that the Hydroethanolic root extract of *A. acuminata* might not be a good therapeutic agent in alleviating diabetic complications.

CONCLUSION

Even though many medicinal plants have been found to have antihyperglycemic properties, *A. acuminaa* is becoming increasingly important in the management of diabetes because it has long been used as a traditional treatment for the disease and its mechanism of action has not been thoroughly investigated. Therefore, an effort has been made in the present study to assess its antidiabetic potential by utilizing a mechanism-based approach. *Atropa acuminata* roots were hydroethanolically extracted, and their potential as an ant diabetic drug was examined. Using vitro experiments, it was discovered that the plant may influence the α-glycosidase, β-amylase, glucose absorption, and insulin secretion enzymes. Our findings from these investigations suggested that *Atropa acuminata* root extracts are rich in such phytochemical composition which improves diabetic health.

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AUTHORS CONTRIBUTIONS

Mrs. Priyanka Thakur has performed the laboratory research work, prepare the manuscript, also drafted the revised manuscript. All the results of research work was supervised and interpreted by Dr. Vinay Pandit. He also approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

Abbreviations:

AA- *Atropa acuminata*
HEEAA-Hydroethanolic Extract of *Atropa acuminata*
NP- Nanoparticles
SD- Standard Deviation LPO- Lipid peroxidation
DPPH- 2, 2-Diphenyl-1-picryl-hydrazyl

ABTS- 2,2'-azino-bis (3-ethylbenzo- thiazoline-6-sulfonic acid
IC50- 50% Inhibitory Concentration EC50- 50% Effective Concentration
HPTLC- High-Performance Thin Layer Chromatography DMSO-Dimethyl Sulfoxide
NEDD-1-Nephthyl ethylene diamine PUFA- Polyunsaturated Fatty Acid
G60F254-Silica with gypsum binder and fluorescence indicator SEM- Standard Error Mean
SD-Standard Deviation ANOVA-Analysis of Variance HCl- Hydrochloric Acid
DNS-3, 5-dinitrosalicyllic acid PNPG-4-Nitrophenyl-Bd-glucosidase NaOH- Sodium Hydroxide
NBT-Nitrobluetetrazolium
NADH-Nicotinamide adenine dinucleotide TBA-Tertiary Butyl Alcohol
ATP/ADP-Adenosine-Triphosphate/ Adenosine diphosphate
Camp-Cyclic Adenosine monophosphate Rf- Refractive Index
CAMAG-World leader In HPTLC TLC- Thin Layer Chromatography

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