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Original Article

ANTIOXIDANT, PTP 1B INHIBITION AND A-AMYLASE INHIBITION PROPERTY AND GC-MS ANALYSIS OF METHANOLIC LEAVES EXTRACT OF *ACHYRANTHES ASPERA* AND *CATHARANTHUS ROSEUS* OF NEPAL

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ABSTRACT

Objective: The present study was designed to study phytochemicals and biological activities of the methanolic extracts of two traditional medicine plants *Achyranthes aspera* and *Catharanthus roseus* of Nepalese origin.

Methods: Plant extracts were prepared by cold percolation method. Antioxidant activity, brine shrimp lethality assay, and analysis of phytochemical constituents were carried out using standard methods. The dinitro salicylic acid (DNS) method was used to study the inhibition effect of extracts on α-amylase enzyme. Furthermore, PTP 1B inhibitory activity was evaluated using *p*-nitrophenyl phosphate (*p*-NPP) as substrate.

Results: Phytochemical analysis showed the presence of phytochemicals like alkaloids, flavonoids, glycosides, reducing sugars, etc. in both plants. Brine shrimp lethality assay suggested the presence of pharmacologically active compounds. Total phenolic content and total flavonoid content of *C. roseus* were found to be higher with 73.21 mg GAE/g and 33.15 mg Q/g respectively than that of *A. aspera,* which was found to be 57.09 mg GAE/g and 28.96 mg Q/g respectively. Similarly, the α-amylase inhibition of *A. aspera* and *C. roseus* was found to be 97.60±1.11 µg/ml and 94.05±1.18 µg/ml comparative with IC_{50} 68.13±0.46 µg/ml of standard acarbose. Protein tyrosine phosphatase 1B (PTP1B) inhibition showed IC_{50} for *A. aspera* and *C. roseus* to be 48.72±0.46 and 50.21±1.03 µg/ml, respectively. Qualitative GC-MS analysis of both plant hexane fractions showed acid and ester type of phytoconstituents.

Conclusion: These results suggested that both plants i. e *A. aspera* and *C. roseus,* Nepal origin showed biological activity by targeting multiple drug targets which justifies their traditional uses.

Keywords: *Achyranthes aspera, Catharanthus roseus*, Antioxidant, Total phenolic content, Total flavonoid content, α-amylase inhibition, PTP1B inhibition, GC-MS

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INTRODUCTION

Diabetes mellitus is a group of metabolic diseases which is characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both [1]. The classical classification of diabetes as proposed by the American Diabetes Association in 1997 as type 1, type 2, other specific types, and gestational diabetes. Normally when there is an elevated level of glucose in the bloodstream, the pancreas releases insulin which then binds a membrane protein of a cell and causes a series of reactions that induce glucose transporters to move into the membrane and facilitate the movement of glucose into the cell [2, 3]. Type 2 Diabetes (T2D) is characterized by an increase in insulin resistance and decreased beta-cell function and chronic hyperglycemia [4, 5]. One of the control pathways for postprandial hyperglycemia in diabetic patients is the inhibition of the activity of *α*-amylase and PTP 1B enzymes.

Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of insulin receptor signaling [6]. PTP 1B dephosphorylates and inactivates insulin receptor (IR), thereby switching off insulin signaling. During the combination of insulin and its receptor, PTP1B directly catalyzes the dephosphorylation of the phosphorylated tyrosine residues, insulin receptor, and insulin receptor substrates to negatively regulate insulin signal transduction. High expression of PTP1B influences the activity of Protein Tyrosine Kinases, which results in insulin failing to combine with IR, induces insulin resistance and leptin resistance, and causes T2D and obesity [7, 8]. PTP 1B inhibition has emerged as a validated therapeutic target for the treatment of T2D and related metabolic abnormalities. Inhibition of digestive enzymes, alpha-amylase is also one of the effective methods for the control of the blood glucose level (postprandial hyperglycemia) in diabetes mellitus.

Natural products-based therapeutic approaches provide a fruitful source for searching for safe, effective, and relatively inexpensive new remedies for diseases [9]. Instead of using the side effects causing chemical drugs, the ancient medicine could be explored to identify the novel drug formulations that are more effective with lesser side effects and also cheaper cost. The active compound that is responsible for the pharmacological effect could be found very easily and also commercialized as a drug product itself with proper approval from the respective organizations [10]. Therefore, natural products are considered as important sources for new drugs or lead optimization for PTP1B inhibition and digestive enzymes inhibition, for the management of diabetes and obesity [11, 12]. In Nepal, about 1600-1900 species of plants are commonly used in traditional medicinal practices. Only a few of them are explored scientifically [13-15].

Achyranthes aspera Linn. Belongs to the amaranthaceae family [16]. The major chemical constituents are carbohydrates, protein, glycosides, alkaloids, tannins, saponins, flavonoids, lignin, etc. [17]. It is known for its uses as antimicrobial and antidiabetic, anti-phlegmatic, antiperiodic, diuretic, like toothbrush, mildly astringent, for the cure of night blindness, for treatment of malarial fever, anti-peroxidative, abortifacient, anti-leprotic, in treatment of asthma, cough, piles, dropsy, oedema and also for the cure of bites of a poisonous snake. It is used to cure scabies, rheumatism, and hemorrhoids [18-20].

Catharanthus roseus L. (G.) Don belongs to the Apocynaceae family. Detailed phytochemical studies showed several highly glycosylated flavonoids, mainly quercetin, kaempferol, and isorhamnetin derivatives. Other classes of phenolic compounds, namely benzoic acids, and phenylpropanoids have also been described [21]. It is known for its uses as antileukemic and antimutagenic, antioxidant, antidiabetic, antimicrobial, for improving blood circulation, as

vasodilatory, transient depressor, to kill some intestinal parasites. It possesses mild antibiotic effects and is antihypertensive [10, 22].

Many research articles had been published reporting the biological activities of these plants. The chemical constituents of the plants are highly influenced by the variation in genetic, geographical, and seasonal factors as well as the developmental stages of the concerned plant and their parts/tissues. Unlike synthetic drugs, which usually contain a single active compound targeting a specific drug target, plant extracts may contain various active ingredients aiming at multiple drug targets.

Therefore, taking account of these reports, the present study aims to quantify the polyphenols and flavonoids in the methanolic extract from leaves of *A. aspera* and *C. roseus* to determine their antioxidant potential and to evaluate its inhibitory properties on the *α*-amylase and PTP1B activities as well as their GC-MS analysis.

MATERIALS AND METHODS

Collection of plant materials and preparation of methanolic extract

The leaves of *A. aspera* and *C. roseus* were collected from Kavre district and identified at the Central Department of Botany, Kirtipur. Leaves were cleaned, chopped into small pieces, and shade dried for 10-15 d. Then it was ground into a powder and stored. Then methanolic leaves extract of both the plants was prepared by cold percolation method.

Chemicals and reagents

The chemicals used in this study were methanol (Merck, Germany), porcine pancreatic α-amylase, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ascorbic acid, PTP1B (human, recombinant) dithiothreitol (DTT), para-Nitrophenylphasphate (*p*-NPP) were purchased from Sigma-Aldrich, USA. All additional chemicals used in this research work were of the commercially available analytical grade.

Phytochemical screening

Phytochemicals present in both the plant extracts were identified by various color reactions with different reagents based on standard protocol along with some required modifications according to our laboratory facility [23, 24].

Brine shrimp lethality assay

This assay was performed according to the procedure given by Pisutthanan *et al.* [25, 26].

Total phenolic content and total flavonoid content

Total phenolic content in plant extract was calculated by Folin-Ciocalteu Colorimetric method based on oxidation-reduction reaction, whereas Total Flavonoid Content was determined by Aluminium Chloride Colorimetric Assay [26, 27].

Antioxidant assay

The ability of plant samples to scavenge 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radicals was estimated by the method as described by Paudel *et al.* [28, 29].

Alpha-amylase inhibition assay

Dinitrosalicyclic acid (DNS) method was used with certain modifications to determine the effect of crude extracts on α-amylase inhibition [30, 31].

Protein tyrosine phosphatase 1B inhibition assay

PTP 1B inhibitory activity was evaluated using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate following a standard protocol with certain modifications [8, 32, 33].

GC-MS analysis of hexane fraction

Further fractionation of methanol extract of both samples was done to get hexane fraction, chloroform fraction, and ethyl acetate fraction. Then the phytochemicals present in the hexane extract were identified by using GC-MS QP 2010.

RESULTS AND DISCUSSION

Phytochemical screening

The results obtained from the phytochemical screening indicating the presence and absence of different types of phytoconstituents are tabulated below in table 1.

Table 1: Phytoconstituents of *A. aspera* and *C. roseus*

S. No.	Phytochemicals	Results	
		AA	CR
1.	Alkaloids	+	+
2.	Flavonoids	+	+
3.	Terpenoids	+	+
4.	Coumarins	-	+
5.	Glycosides	+	+
6.	Quinones	+	+
7.	Reducing sugars	+	+
8.	Polyphenols	+	+
9.	Saponins	+	+

Where (+) = Present and (-) = Absent

Almost all tested phytochemicals were found in both plant extracts except coumarin in *A. aspera*. The result is well supported by previously reported results of these plants [17, 34, 35]. Plants having vitamins (C, E), carotenoids, flavonoids (flavones, isoflavonones, flavonones, anthocyanins, and catechins), and polyphenols (ellagic 7 acid, gallic acid, and tannins) had been reported with their remarkable antioxidants activity, and alpha-amylase inhibitory activity [36]. Berberine, papaverine, 2-arylbenzofuransare, cinnamic acid, flavonoids, terpenes, proteoglycan, quinolone, steroids, N-or S-containing compounds, phenolics, etc. are some natural products that have been reported with PTP1B enzyme inhibition action [11].

Brine shrimp bioassay

Results obtained from the brine shrimp lethality assay are presented in table 2.

Table 2: Calculation of LC_{50} value of *A. aspera* and *C. roseus* leaves

S. No.	Plant extracts (methanolic)	LC_{50} value
1.	*A. aspera*	681.29 µg/ml
2.	*C. roseus*	464.16 µg/ml

IC_{50} values are expressed as mean±SD (n = 3)

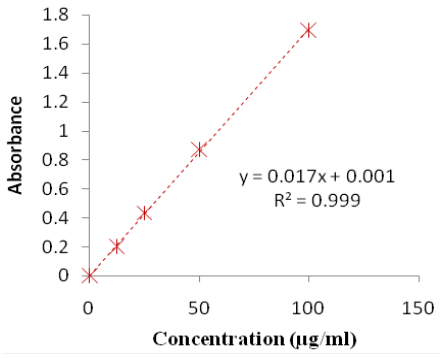

Fig. 1: Calibration curve of gallic acid

The methanolic extract of *A. aspera* and *C. roseus* were found to be toxic against brine shrimps as shown by their LC_{50} values of 681.29 µg/ml and 464.16 µg/ml, respectively, much below the accepted maximum potential value of 1000 µg/ml. These results showed the

presence of pharmacologically active compounds in the methanol fraction of both plants. Literature survey revealed the cytotoxic activity *C. roseus* and *A. aspera,* where superior results were reported than the results found here [37, 38]. This can be attributed due to the variation in altitude of plants, extraction procedure, etc.

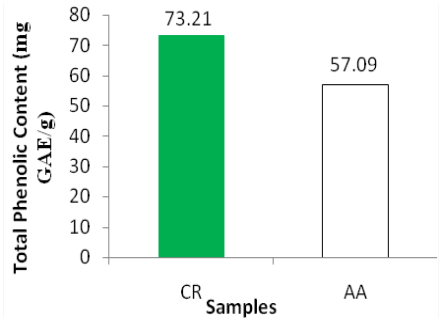

Fig. 2: TPC values of both samples Where CR = *C. roseus* and AA = *A. aspera*

The concentration of gallic acid at the different absorbance of plant extracts was used to evaluate the total phenolic content of the plant extracts, which is shown in fig. 1 & 2.

A slightly higher concentration of phenols was measured in methanolic fraction of *C. roseus* than that of *A. aspera* fraction i.e. 73.21 mg GAE/g and 57.09 mg GAE/g, respectively.

The literature revealed that TPC ranged from 8.18±1.61 to 21.46±2.39 mg GAE/g for methanolic extract of *C. roseus* [39]. Also, TPC extracted from its shoots in different solvent systems were in the ranges 3.2 to 8.5 GAE (g/100g per dry matter) [39]. Another study showed *A. aspera* Linn. Possessed phenol constituent's 9.16±0.84 mg/g in the leaves of methanolic extract [41].

Total flavonoid content

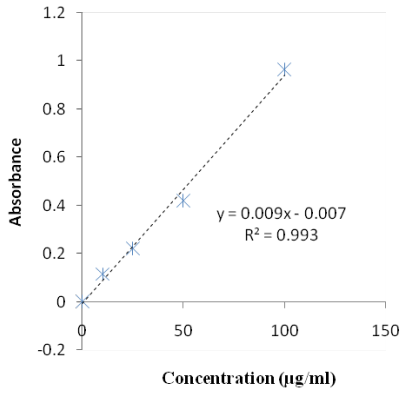

Fig. 3: Calibration curve of quercetin

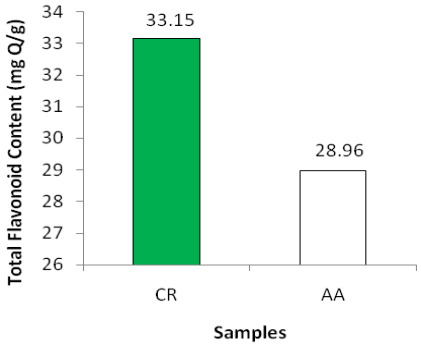

Fig. 4: TFC of samples, CR and AA

The concentration of quercetin at the different absorbance of plant extracts was used to evaluate the total flavonoid content of the plant extracts, which is shown in fig. 3 & 4.

A slightly higher concentration of flavonoid was measured in methanolic fraction of *C. roseus* than that of *A. aspera* fraction i.e. 33.15 mg Q/g and 28.96 mg Q/g, respectively.

The literature revealed that methanolic extract of leaves of hexaploid plants of *A. aspera* Linn. had the flavonoid 78.36±1.63 mg/g[40]. Roots contained 8.3 mg/g and 13.5 mg/g of QE respectively [41]. TFC extracted from *C. roseus* shoots in different solvent systems were in the ranges 1.8 to 5.4 (g/100g per dry matter). 100% methanolic extract of it showed the highest TFC 19.8 g/100 g [40]. The results revealed that *C. roseus* and *A. aspera* are rich sources of phenolic and flavonoids. The reported traditional usage of these plants can be attributed to these phenolic and flavonoids content. Variation of TPC and TFC from plants to plants and parts to parts can be justified as the fact that the time of maturity at harvest, growing condition, soil condition, altitude of plant growth, and post-harvest treatment [41].

DPPH free radical scavenging activity

% DPPH free radical scavenging activity at different concentrations of ascorbic acid, *A. aspera* and *C. roseus* is tabulated below in table 3:

MeOH extract of leaves of *C. roseus* had the IC_{50} value of 49.74±0.52 µg/ml very close to the IC_{50} value of standard ascorbic acid 32.58±0.25 µg/ml than IC_{50} value of *A. aspera* i.e. 53.54±0.40 µg/ml. table 4. The antioxidant property of both plants might be due to the phytoconstituents such as flavonoids, polyphenols, acids, esters, alcoholic groups, etc. This result is comparable to the data reported previously. The previous data obtained revealed that the 100 µg/ml extracts of *C. roseus* possessed 45.7±3.4 % inhibition in the methanolic extract [36]. DPPH assays at different concentrations (200 to 1000 µg) in methanol showed 81.70% at 800 µg [34]. Antioxidant activity of extracts of *A. aspera* by DPPH showed IC_{50} of the ethanolic extract showed 556.07 µg/ml [15]. Its roots and leaves possessed IC_{50} values of 241.86 µg/ml and 129.91 µg/ml, respectively [19].

Alpha-amylase inhibition test

Alpha-amylase inhibition % values at different concentrations of standard acarbose and both plant extracts are shown in the below table 5.

Table 3: % inhibition of ascorbic acid, AA and CR at different concentrations

Concentration (µg/ml)	% Inhibition		
	Ascorbic Acid	AA	CR
20	49.48±1.21	37.41±1.23	39.31±0.85
40	75.51±0.92	52.63±1.79	51.75±1.30
60	80.52±1.08	59.48±1.31	62.01±1.37
80	90.08±1.47	64.17±1.57	70.43±1.48
100	92.59±0.45	72.81±1.49	77.78±1.04

Where CR = *C. roseus* and AA = *A. aspera,* Mean % Inhibition are expressed as means±SD (n = 3), IC_{50} values of the plant extracts along with the standard Ascorbic acid is tabulated below in table 4:

Table 4: Comparison of IC_{50} values of both extracts with ascorbic acid

Sample	IC_{50} (µg/ml)
Ascorbic acid	32.58±0.25
A. aspera	53.54±0.40
C. roseus	49.74±0.52

IC_{50} values are expressed as mean±SD (n = 3)

Table 5: % Inhibition at different concentrations

Concentration (µg/ml)	% Inhibition		
	Acarbose	AA	CR
10	19.75±0.81	13.09±0.65	11.67±0.67
20	23.41±1.03	24.76±0.43	23.96±0.43
40	30.06±0.35	31.61±0.60	28.07±0.43
60	39.74±0.27	36.48±0.64	33.32±0.48
80	58.28±0.80	38.61±0.67	41.12±1.14
100	72.15±0.55	49.33±0.62	52.75±0.62

Where CR = *C. roseus* and AA = *A. aspera,* Mean % Inhibition are expressed as means±SD (n = 3), IC_{50} values of both plant extracts along with the standard acarbose is tabulated below in table 6:

Table 6: Comparison of IC_{50} values obtained from α-amylase inhibition

Samples	IC_{50} (µg/ml)
Acarbose	68.13±0.46
AA	97.60±1.11
CR	94.05±1.18

Where CR = *C. roseus* and AA = *A. aspera,* Mean % Inhibition are expressed as means±SD (n = 3)

Here, IC_{50} value of standard acarbose was found to be 68.13±0.46 µg/ml. *A. aspera* and *C. roseus* showed α-amylase inhibitory activity with IC_{50} value 97.6±1.11 µg/ml and 94.05±1.18 µg/ml, respectively table 6. The result of phytochemicals analysis supported the alpha-amylase inhibitory activities of plant extracts. There was a dose-dependent increase in percentage inhibitory activity against α-amylase by these two plant extracts. The results obtained here are in good correlation with previously reported results [29, 44].

Protein tyrosine phosphatase 1B inhibition activity

% inhibition value of PTP1B inhibition activity at different concentrations is shown in table 7.

Table 7 % Inhibition of PTP1B at different concentrations

Concentration (µg/ml)	% Inhibition		
	Ursolic acid	AA	CR
5	52.98±0.84	2.65±0.48	2.33±0.82
10	57.11±0.95	14.29±1.14	11.92±0.79
20	65.79±0.73	29.87±1.50	35.23±0.87
35	89.53±0.70	54.55±0.53	51.29±1.27
70	98.07±0.52	59.76±0.43	57.72±1.39

Mean % Inhibition are expressed as means±SD (n = 3), IC_{50} values of the plant extracts along with the standard Ursolic acid is tabulated below:

Table 8: Comparison of IC_{50} values obtained from PTP1B inhibition

Samples	IC_{50} (µg/ml)
Ursolic acid	13.58±0.23
AA	48.72±0.46
CR	50.21±1.03

Where CR = *C. roseus* and AA = *A. aspera,* IC_{50} values are expressed as mean±SD (n = 3)

IC_{50} value of standard ursolic acid was found to be 13.58±0.23 µg/ml. *A. aspera* showed higher PTP1B inhibitory activity with IC_{50} value 48.72±0.46 µg/ml table 8. A higher amount of saponin, reducing sugars, and glycoside in a phytochemical test is in agreement with this result. To the best of our knowledge, there are no previous reports related to the PTB1 inhibitory activity of *A. aspera* was exhibited.

Whereas *C. roseus* leaf extract has IC_{50} value 50.21±1.03 µg/ml. The result is well supported by published results [44, 45]. Priyanka *et al.* reported that 3-nitrophthalic acid present in *C. roseus* methnolic extract might be responsible for PTB1B inhibition activity by Docking and by applying "Lipinski Rule of Five" on ligands [47].

The GC analysis of hexane fraction extracted from methanolic extract of *A. aspera* coupled with mass library search facilitated the identification of 14 different peaks constituting 99.99 % area, 8 compounds were identified with more than 90 % accuracy (matching factor), constituting 77.52 % of the total area fig. 5. The

majority of compounds identified were fatty acids, esters of fatty acids, and hydrocarbons. The major phytochemicals analyzed in the hexane fraction of methanolic extract of *A. aspera* leaves along are tabulated in table 9.

The literature revealed that n-Hexadecanoic acid belonging to the Palmitic acid family possessed antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant antiandrogenic properties. Hexadecanoic acid, ethyl ester belonging to fatty acid ester, has similar properties and acts as a 5-Alpha reductase inhibitor. Phytol is a diterpene having antimicrobial, anti-inflammatory, anticancer, and diuretic activities. 9,12-Octadecadienoic acid, methyl ester has the properties of anti-inflammatory, hypercholesterolemic, cancer preventive, hepatoprotective, nematicide, insectifuge, antihistamine, antieczemic, anti-acne, 5-alpha reductase inhibitor, antiandrogenic, anti-arthritic, anti-coronary [46].

GC-MS Analysis of hexane extract of A. aspera

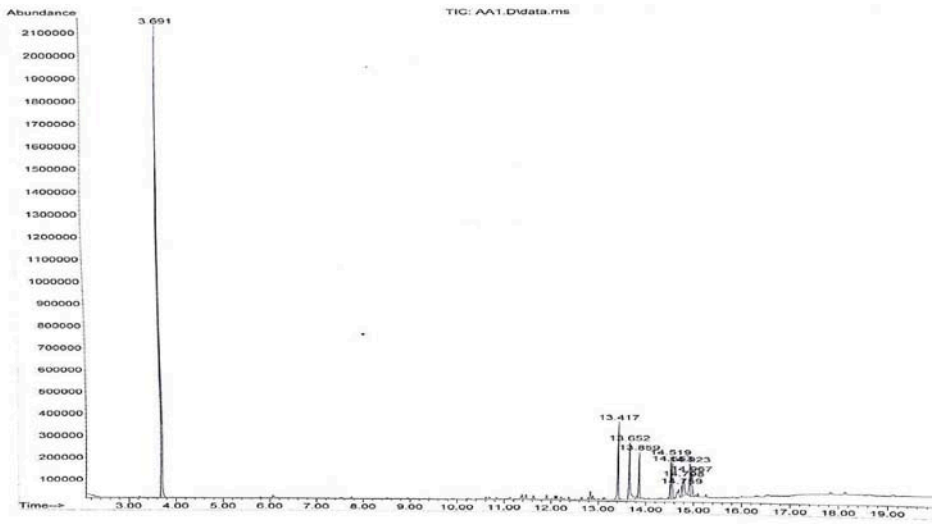

Fig. 5: GC Chromatogram of AA leaves

Table 9: Compounds identified in the hexane extract of *A. aspera* leaves

S. No.	Area %	Retention time (min)	Compound name	Molecular formula	Molecular weight
a.	28.80	13.417	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
b.	15.93	13.652	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270
c.	9.64	13.859	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284
d.	4.09	14.519	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294
e.	5.26	14.563	9,12,15-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	292
f.	8.06	14.661	Phytol	$C_{20}H_{40}O$	296
g.	1.76	14.694	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298
h.	3.98	14.923	Linoleic acid, ethyl ester	$C_{20}H_{36}O_2$	308

GC-MS Analysis of *C. roseus*

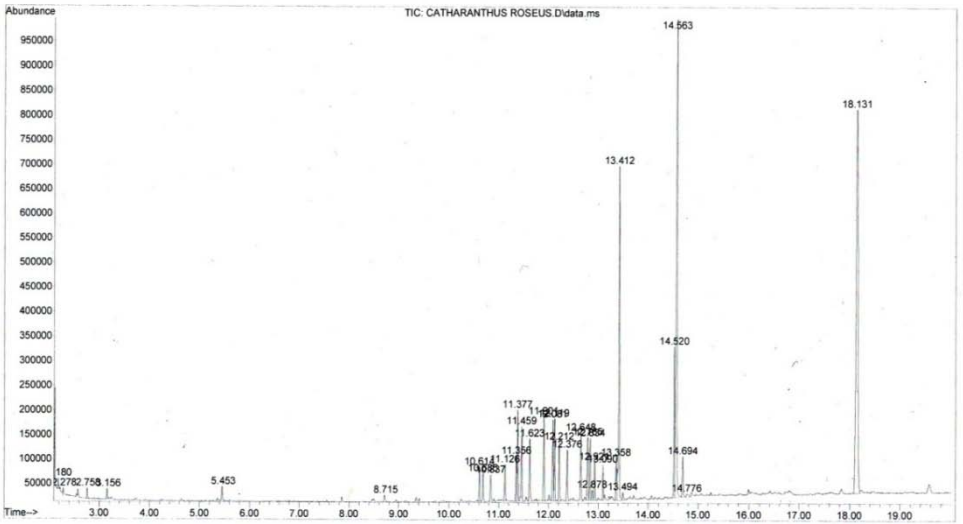

Fig. 6: GC chromatogram of the hexane extract of *CR* leaves

The GC chromatogram analysis of hexane fraction from methanolic extract of *C. roseus* coupled with mass NIST 08 library search facilitated the identification of 13 different peaks corresponding to the 13 different compounds constituting 100.01 % peak area and out of these peaks, 6 compounds were identified with more than 90 % accuracy (matching factor), constituting 76.69 % of the total peak area percentage. The majority of compounds identified were fatty acids, esters of fatty acids and a few of them were hydrocarbons.

A list of six compounds is presented in table 10, along with their area %, retention time, molecular formula, and molecular weight.

Table 10: Compounds identified in the hexane extract of *C. roseus*

S. No.	Area%	Retention time (min)	Compounds	Formula	Molecular weight
a.	9.74	11.476	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270
b.	4.79	11.906	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
c.	4.80	14.520	9,12-Octadecadienoic acid, methyl ester,	$C_{19}H_{34}O_2$	294
d.	1.34	14.694	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298
e.	29.92	14.563	9, 12, 15-Octadecatrienoic acid, methyl ester,	$C_{16}H_{32}O_2$	292
f.	26.10	18.131	1, 2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	$C_{16}H_{23}O_4$	279

CONCLUSION

The present study has shown that the methanolic extract of the leaves of *A. aspera* and *C. roseus* has remarkable antioxidant potential and inhibitory activity of the *α-amylase* and PTP 1B enzymes. These effects would be due to its important phenolic composition, whose quantitative study has revealed the varied presence of polyphenols and flavonoids. These results could justify the use of these plants in traditional medicine for the treatment of type 2 diabetes and complications. Study of chemical constituents of these plants using sophisticated technologies like NMR, HPLC, etc. can provide a way for extensive research that can be used for commercial drug production with lesser or no side effects.

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AUTHORS CONTRIBUTIONS

B. Subba analyzed the data, and wrote the manuscript, supervise, whereas Yashoda Karki carried out the laboratory work under the guidance of Dr. Deegendra Khadka (PTP1B inhibition assay). All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

All authors have none to declare.

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