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 anti-diabetic, anti-pleuritic, antiperiodic, diuretic, like toothbrush, mildly astringent, for the cure of night blindness, for treatment of malarial fever, anti-peroxidative, abortifacient, anti-heat, in treatment of asthma, cough, piles, dropy, 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, andisorhamnetin 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, anti-diabetic, antimicrobial, for improving blood circulation, as
vasodialatory, 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 (human, recombinant) dithiothreitol (DTT), para-Nitrophenylphosphate (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-picrylhydrazyl (DPPH) free radicals was estimated by the method as described by Paudel et al. [28, 29].

**Alpha-amylase inhibition assay**

Dinitrosalicylic 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.

| Phytochemicals | S. No. | Results | Phytoconstituents of *A. aspera* and *C. roseus* | 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, cinnic 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.

| S. No. | Plant extracts (methanolic) | LC50 value |
|-------|---------------------------|------------|
| 1.    | *A. aspera*                | 681.29 µg/ml |
| 2.    | *C. roseus*               | 464.16 µg/ml  |

The methanolic extract of *A. aspera* and *C. roseus* were found to be toxic against brine shrimps as shown by their LC50 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 toxicity of both the plant extracts.
The presence of pharmacologically active compounds in the methanol fraction of both plants. Literature survey revealed the cytotoxic activity of *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 the 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 that *A. aspera* Linn. possessed phenol constituent’s 9.16±0.84 mg/g in the leaves of methanolic extract [41].

**Total flavonoid content**

% 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 IC₅₀ value of 49.74±0.52 µg/ml very close to the IC₅₀ value of standard ascorbic acid 32.58±0.25 µg/ml than IC₅₀ value of *A. aspera* i.e. 53.54±4.0 µg/ml. The antioxidant property of both plants might be due to the phytoconstituents such as flavonoids, polyphenols, acids, esters, alcohols, 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 IC₅₀ of the ethanolic extract showed 556.07 µg/ml [15]. Its roots and leaves possessed IC₅₀ 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.

| 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±1.37 |
| 60                   | 80.52±1.08                | 59.48±1.31 | 62.01±1.37 |
| 80                   | 90.08±1.47                | 64.17±1.57 | 70.43±1.46 |
| 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). IC₅₀ values of the plant extracts along with the standard Ascorbic acid is tabulated below in table 4:
Table 4: Comparison of IC\textsubscript{50} values of both extracts with ascorbic acid

| Sample       | IC\textsubscript{50} (µg/ml) |
|--------------|-------------------------------|
| Ascorbic acid| 32.58±0.25                    |
| A. aspera    | 53.54±0.40                    |
| C. roseus    | 49.74±0.52                    |

IC\textsubscript{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\textsubscript{50} values of both plant extracts along with the standard acarbose is tabulated below in table 6:

Table 6: Comparison of IC\textsubscript{50} values obtained from α-amylase inhibition

| Samples       | IC\textsubscript{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\textsubscript{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\textsubscript{50} value 97.6±1.11 µg/ml and 94.05±1.18 µg/ml, respectively. The result of phytochemicals analysis supported the α-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\textsubscript{50} values of the plant extracts along with the standard Ursolic acid is tabulated below:

Table 8: Comparison of IC\textsubscript{50} values obtained from PTP1B inhibition

| Samples       | IC\textsubscript{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\textsubscript{50} values are expressed as means±SD (n = 3)

IC\textsubscript{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\textsubscript{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 PTP1B inhibitory activity of A. aspera was exhibited.

Whereas C. roseus leaf extract has IC\textsubscript{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 methanolic extract might be responsible for PTP1B 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](image)

**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_{17}H_{34}O_{2} | 284             |
| d.    | 4.09   | 14.519               | 9,12-Octadecadienoic acid, methyl ester | C_{18}H_{36}O_{2} | 294             |
| e.    | 5.26   | 14.563               | 9,12,15-Octadecatrienoic acid, methyl ester | C_{19}H_{38}O_{2} | 292             |
| f.    | 8.86   | 14.661               | Phytol | C_{20}H_{40}O | 298             |
| 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_{42}O_{2} | 308             |

### GC-MS Analysis of *C. roseus*

![Fig. 6: GC chromatogram of the hexane extract of CR leaves](image)
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.

| S. No. | Area% | Retention time (min) | Compounds                                      | Formula           | Molecular weight |
|--------|-------|----------------------|-----------------------------------------------|-------------------|------------------|
| a.     | 9.74  | 11.476               | Hexadecanoic acid, methyl ester                | C_{16}H_{32}O_{2} | 270              |
| b.     | 4.79  | 11.906               | n-Hexadecanoic acid                            | C_{16}H_{32}O_{2} | 256              |
| c.     | 4.90  | 14.520               | 9,12-Octadecadienoic acid, methyl ester        | C_{18}H_{32}O_{2} | 294              |
| d.     | 1.34  | 14.694               | Octadecanoic acid, methyl ester                | C_{18}H_{32}O_{2} | 298              |
| e.     | 29.92 | 14.653               | 9, 12, 15-Octadecatrienoic acid, methyl ester  | C_{19}H_{32}O_{2} | 292              |
| f.     | 26.10 | 18.131               | 1, 2-Benzenedicarboxylic acid, mono-(2-ethylhexyl) ester | C_{18}H_{30}O_{2} | 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 Karhi 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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