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Original Article

Biochemical profiling and antioxidant evaluation of *Martynia annua* and *Polygonum viviparum*: investigating hepatoprotective properties against abiotic stress

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Abstract

The liver is the second largest organ in the body, playing a crucial role in maintaining homeostasis and regulating metabolism. However, the prevalence of liver diseases has been rising steadily due to an increase in both infectious and non-infectious factors. Medicinal plants offer a remarkable opportunity to enhance our healthcare system by addressing various diseases through their ability to control oxidative stress and support metabolic processes. This revision improves readability while retaining the original meaning. In the present study, purified methanol extracts of *Martynia annua* and *Polygonum viviparum* at different concentrations were used to explore the antioxidant and hepatoprotective potential against abiotic stress on liver cells. Firstly total flavonoids and total phenolic contents of both plants were measured and then their antioxidant activities were determined through DPPH radical scavenging activity and FRAP assay. Moreover, bioactive compounds and in vitro hepatoprotective potential among these plants were determined through LC-MS and Liver Slice Culture assay respectively. In *P. viviparum* high amounts of TPC and TFC were observed with maximum antioxidant potential in terms of DPPH inhibition at 84% and high ferric-reducing ability at 240 mg/mL. The presence of different phytoconstituents like Myricetin, Gallic acid, Ferulic acid, Chlorogenic acid, Sweroside, Morroniside, Echonoside, Swertiamarin and Protocatechuic acid were confirmed in *M. annua* and *P. viviparum* in LC-MS study. The maximum hepatoprotective potential in terms of minimum cytotoxicity 6% and 9% were observed in *M. annua* and *P. viviparum* respectively. It was revealed that both plants have stunning hepatoprotective properties to prevent liver from toxicants and their related complications due to high antioxidant potential and active metabolites.

Keywords: Metabolic processes, Oxidative stress, Phytoconstituents, LC-MS, Cytotoxicity, Liver Slice culture.

1. Introduction

Oxidation is a natural phenomenon that occurs in different metabolic processes and is controlled by several enzymes to maintain homeostasis of the body Genestra (1). High production of ROS disrupts intracellular signalling, metabolic processing and induced oxidative stress in the cells (2) Different metabolic process of the body takes place in the liver and also maintain the homeostasis of the body (3). The increasing prevalence and progression of liver diseases are also associated with oxidative stress (4). While many synthetic medications are used to treat various liver disorders, they are not always fully effective in stimulating liver function and regenerating hepatic cells (5). For that reason, current situations require searching and exploring alternative solutions to form new effective medicines for the treatment of various liver disorders (6). Medicinal plants are important for human health due to their pharmacological and biological properties and are

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used to cure many diseases that originate from oxidative stress (7).

Lactate dehydrogenase enzyme is present in many organs including liver, kidney and cardiac tissues (8). Lactate dehydrogenase catalyses the interconversion of pyruvate to lactate and vice versa in glycolytic pathways (9). In necrosis or damage of the cell, LDH is released by rupturing of the plasma membrane into extracellular fluid and through its concentration hepatoprotective effects of herbal medicines against hepatotoxicants can be measured easily (10).

Martynia annua (herbaceous annual plant), belongs to the family Martyniaceae and is commonly known as Devil's claw(11). Leaves and seeds of *M. annua* are commonly used for the treatment of inflammation, epilepsy and tuberculosis (12). The leaves, fruits and seeds extracts of *M. annua* have exhibited antioxidant, antibacterial, antifungal, anticonvulsant and wound-healing properties (13). *Polygonum viviparum* is a perennial herb, that belongs to the family Polygonaceae (14). *P. viviparum* is used as a folk medicine traditionally for the treatment of gastrointestinal disorders, dysentery, tonsillitis and pharyngitis (15).

Therefore, in the present study, *M. annua* and *P. viviparum* were used to evaluate their hepatoprotective potential in the liver slice culture model against hepatotoxicity induced by CCl4. Liver Slice Culture assay is in vitro technique that provides a suitable environment for liver cells and valuable approach for the evaluation of hepatoprotective potential of medicinal plants (16)

2. Materials and methods

2.1. Collection and extraction of plant

The seeds of *M. annua* and rhizomes of *P. viviparum* were taken from local market and identified by plant taxonomist (senior botanist) Department of Botany, University of Agriculture Faisalabad, Pakistan. The issued voucher specimen numbers were 53-2-18 and 53-1-18 of *M. annua* and *P. viviparum* respectively. The selected parts of plants were ground into fine powder and prepared their extract in methanol through maceration process.

2.2.Total phenolic content

The total phenolic content of indigenous medicinal plants was measured following the protocol outlined in reference (17). Methanolic extracts of the plant solutions were prepared in DMSO at concentrations of 100, 300, and 500 µg/mL. Subsequently, 20 µL of each plant extract and 100 µL of Folin-Ciocalteau reagent were added to a microwell plate and incubated in the dark for 3 minutes. After incubation, 100 µL of a 10% Na_2CO_3 solution was added to the mixture of plant extracts and Folin-Ciocalteau reagent, followed by an additional incubation in the dark for one hour. The absorbance was then measured at 765 nm using an ELISA plate reader. The results for total phenolic content (TPC) were expressed as mean ± SD in terms of Gallic acid equivalents (GAE µg/g). This revision improves readability, corrects grammatical errors, and ensures clarity in the methodology.

2.3. Total flavonoid content

The aluminum chloride ($AlCl_3$) colorimetric method was used to determine the total flavonoid content of indigenous medicinal plants (18). For this purpose, 1 mL of each plant extract (prepared in DMSO) and 300 µL of a 5% $NaNO_2$ solution were combined in test tubes and incubated at room temperature for 5 minutes. After incubation, 300 µL of a 10% $AlCl_3 \cdot 6H_2O$ solution was added to each test tube mixture, and the tubes were kept at room temperature for an additional 5 minutes. Following this, 1 mL of 1 M NaOH solution was added, and the total volume in each test tube was adjusted to 3 mL by adding deionized water. The absorbance of the resulting mixture was measured at 510 nm. The results for total flavonoid content were expressed as mean ± SD in terms of catechin equivalents (CE µg/g plant). This revision improves readability, corrects grammatical errors, and ensures clarity in the methodology.

2.4. Antioxidant activities

2.4.1. DPPH radical scavenging activity

DPPH free radical scavenging activity was performed by following the method (19). The reaction mixture was prepared by adding 195 µL of DPPH solution and 5 µL of each plant extract with 100, 300 and 500 µg/mL concentrations. The reaction mixture was incubated in the dark, at room temperature for 30 min and then absorbance was noted at 517 nm. Ascorbic acid was used as a reference standard. The percentage inhibition of DPPH was calculated by following Equation 1.

DPPH inhibition (%) = [(Ac − As)/Ac] × 100 (Equation 1)

Where "As" presents the absorbance of DPPH with plant extracts and "Ac" is the absorbance of DPPH without plant extracts.

2.4.2. Ferric reducing antioxidant power (FRAP) assay

Ferric-reducing antioxidant power assay was performed by following the protocol (20). The FRAP working reagent was prepared through 300 mM acetate buffer (pH 3.6), and 20 mM FeCl3. H2O and 10 mM 2, 4, 6 – tripyridyl -S- triazine (TPTZ) in a ratio of 10:1:1. The reaction mixture was prepared by adding 2.8 mL of FRAP reagent and 200 µL of each plant with 100, 300 and 500 µg/mL concentration in test tubes and incubated on water bath at 37°C for 30 minutes. The change in absorbance was measured at 593 nm against the FRAP reagent. Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) was used as the standard, and a standard curve was prepared to quantify the antioxidant content, expressed as ferrous (Fe^{2+}) equivalents in mg/g of plant material.

2.4.3. Biochemical characterization of plants through LC-MS

Biochemical characterization of seeds of *M. annua* and rhizomes of *P. viviparum* was performed by Liquid chromatography Mass spectrometry (LC-MS). The separation of the phytoconstituents was performed on the HPLC Surveyor Plus System. An equipped pump of Luna RP C-18 analytical column with 4.6 × 150 mm length and 3.0 µm particle size was used for HPLC. For elution, two solvents were used including LCMS grade methanol (mobile phase A) and acidified water 0.5% formic acid v/v (mobile phase B). On the base of the gradient system solvent, elution was executed with the flow rate of 0.3 mL/ min. The gradient elution was programmed as follows: from 10% A in 5 minutes, form 20 % B in 20 minutes and maintain it till the end of analysis. The injection volume was 5.0 µL and temperature of the column was maintained at 25 °C. The effluent

from HPLC column was directed to electron spray ionization mass spectrometer. Negative ion mode with spectra acquired over a mass range of m/z 200 to 2000 were used for LCMS analysis. The optimum values of ESI-MS parameters of sheath gas and auxiliary gas were 45 and 5 units/min respectively, with spray voltage +4.0 kV, Capillary temperature 320 °C, capillary voltage -20 V and tube lens -66.51 V. For the interpretation of mass spectra data of the molecular ions X- caliber software was used (21)

2.5. In vitro hepatoprotective potential through Liver Slice Culture Model

The hepatoprotective potential of *M. annua* and *P.viviparum* was explored through Liver Slice Culture assay by following the method after slight modification(22)

2.5.1. Preparation of Liver Slice Culture Model

The liver of rats was removed after dissection and transferred into pre-warmed Krebs–Ringer – Hepes (KRH) medium (pH 7.4). KRH medium was comprised of HEPES 2.5 mM, KCl 2.85 mM, NaCl 118 mM, CaCl2 2.5 mM, KH2PO4 1.15 mM, MgSO4.7H2O 1.18 mM, β Hydroxy butyrate 5 mM and Glucose 4 mM. The liver was sliced into thin sections using a sharp scalpel blade and transferred to a capped Eppendorf flask containing KRH medium. The flask was then incubated at 37°C in a shaking water bath for 1 hour. To maintain the physiological conditions of the liver cells, the KRH medium was replaced with fresh medium every 10 minutes. After incubation, these liver slices were used to evaluate the hepatoprotective potential of the plants.

2.5.2. Hepatoprotective potential of plants

Various concentrations (50, 100, 300, 500, 700 and 1000 µg/mL) of *M.annua* and *P. viviparum* methanolic were prepared in DMSO. For each concentration, 20-22 liver slices were taken into falcon tubes that contained 2 mL KRH media already. The liver slices were incubated at 37°C for 30 minutes in a shaking water bath. After this initial incubation, the KRH medium was replaced with 2 mL of fresh KRH medium containing each concentration of the plant extracts. Ascorbic acid (10 mM) was used as a reference standard. The liver slices with plant extracts were then incubated for an additional hour at 37°C. Following this, 1 mL of CCl_4 (40 mM) was added to each Falcon tube to induce hepatotoxicity, and the samples were incubated again for 2 hours in the shaking water bath at 37°C. During these 2 hours, the liver slices were aerated every 10 minutes by removing the caps of the Falcon tubes to allow oxygen exchange. After incubation, the culture medium was collected to measure the lactate dehydrogenase (LDH) level, which serves as a cytotoxic marker of liver injury. For the measurement of LDH, a commercial LDH Cytotoxicity Assay Kit II from Abcam (ab65393) was used. This revision improves readability and ensures clarity in the methodology while maintaining all essential details.

The following formula was used to calculate the percentage cytotoxicity of LDH released from liver slices (Equation 2):

$$Cytotoxicity\ (\%) = \frac{Test\ samples_{Abs} - Low\ control_{Abs}}{Low\ control_{Abs} - High\ control_{Abs}} \times 100 \quad \text{(Equation 2)}$$

Here, "Test samples Abs" indicated liver slice culture medium of plant extract and 40 mM CCl4, "High control Abs" showed absorbance of liver slice culture medium incubated with only 40 mM CCl4 and "Low control Abs" desiginated as culture medium of liver slices incubated with only KRH medium.

2.6. Statistical analysis

The collected data of all the recorded traits were analyzed by using one-way analysis of variance (ANOVA). The differences were analyzed by t-test, which were significant. SAS software (Version 9.1; SAS Institute, Cary, NC, USA) was used for statistical analysis. Least significant difference post hoc test at 95% probability was used to separate treatment means where ANOVA denoted significant differences [23]. SigmaPlot was used for the graphical presentation of the data (SigmaPlot 2008).

3. Results

3.1. Total phenolic and flavonoid contents

The highest total phenolic content 267.43 ± 20.06 was recorded in *P. viviparum* at 500µg/mL while at the same concertation *M. annua* showed 236.62 ± 9.74 amount of total phenolic contents in terms of Gallic acid equivalents microgram per gram (Figure 1). The total flavonoid content in both studied plants was measured in terms of Catechin equivalents (CE µg/g). In *M. annua* and *P. viviparum* flavonoid content 131.86 ± 1.13 and 130.46 ± 2.13 were observed respectively at 500µg/mL concentration (Figure 2).

3.2. Antioxidant potential

Antioxidant activity of *M. annua* and *P. viviparum* at different concentrations was measured in terms of percentage inhibition of DPPH and ferric-reducing antioxidant ability. The obtained result showed that the antioxidant abilities of studied plants increased in concentration concentration-dependent manner. At 500 µg/mL maximum percentage inhibition of DPPH 84.73 ± 2.65 and ferric reducing power 240.54 ± 17.67 was observed in *P. viviparum*. However, *M. annua* showed 83.17 ± 7.05% as DPPH inhibition and 237.54 ± 2.12 as a ferric reducing power at 500 µg/mL

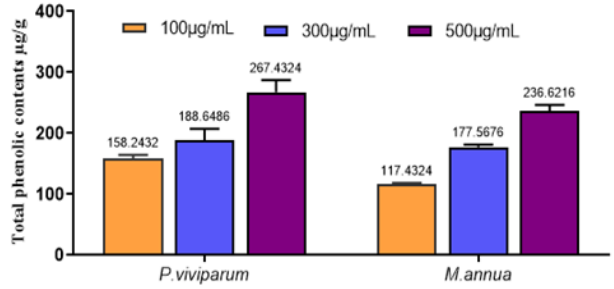

Fig. 1. Total phenolic content in *P. viviparum* and *M. annua.*

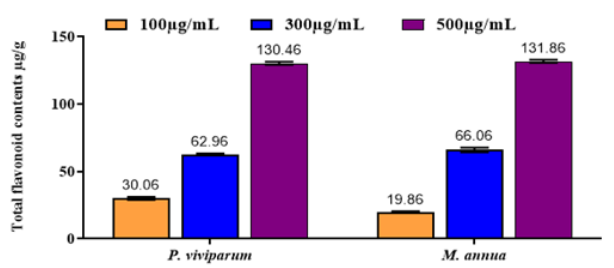

Fig. 2. Total flavonoid content in *P. viviparum* and M. annua.

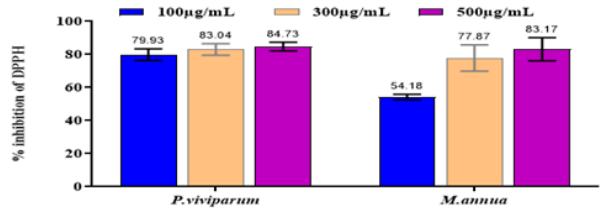

Fig. 3. Percentage inhibition of DPPH in *P. viviparum* and M. annua.

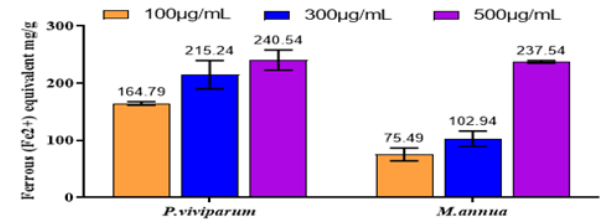

Fig. 4. FRAP values (Fe (II) mg/mL) of *P. viviparum* and M. annua.

concentration as is presented in Figures 3 and 4.

3.3. LC-MS results of *M. annua* and *P.viviparum*

Biochemical characterization of M. annua was performed through LC-MS and the full mass spectrum is presented in Figure 5A. The highest peaks at 319.33, 337.42, 377.42 and 393.42 m/z were observed with positive mode ESI. The mass spectrum of *M. annua* from 150 to 400 m/z revealed Gallic acid and Myricetin at 170 and 319.33 m/z respectively (Figure 5B). The MS-MS of peak 170 m/z CID (27:00) with positive mode of ESI confirmed Gallic acid presence at 127.83 and 169. 92 m/z (Figure 5C). The mass spectrum in Figure 5D depicted the presence of Ferulic acid, Chlorogenic acid and Swertiamarin at 194.17, 377.42 and 397.42 m/z respectively with positive ESI. Moreover, Hydroxymethylfurfural (C6H6O3) and Myricetin (C15H10O8) at 127 and 317.42 m/z respectively were also identified with negative mode of ESI in *M. annua* (Figure 5E). The MS-MS of peak at 153 m/z designated Protocatechuic acid (C7H6O4) at CID 27:00 with negative mode of ESI (Figure 5F).

The full mass spectrum of *P. viviparum* was presented at high peaks of 130.08, 219.08, 266.25, 337.33 381.25 and 402.33 m/z (Figure 6A). The mass spectrum with positive ESI showed a polyphenolic compound Caffeic acid (C9H9O4) and natural iridoid sweroside (C16H22O9) at 180.08 and 381.25 m/z respectively (Figure 6B). A glycoside Morroniside (C17H26O11) at 428.25 m/z with positive mode of ESI was found in *P. viviparum* (Figure 6C). The LC-MS analysis of *P. viviparum* revealed the presence of Gallic acid (C7H6O5) at 169.08 m/z and Quinic acid at 191.08 m/z with negative ESI (Figure 6D). Chlorogenic acid (C16H18O9) by negative ESI was found at 353.25 m/z. The MS-MS of peak 353.17 m/z with CID 20:00 into three peaks 173, 179 and 191 m/z indicated Chlorogenic acid presence in *P. viviparum*. The MS-MS of peak 154 m/z by CID 27:00 negative mode ESI into 109.83 and 123.75 m/z confirmed Protocatechuic acid presence in *P. viviparum* (Figure 6E & F).

3.4. Hepatoprotective potential

The results of the liver slice culture assay indicated that the hepatoprotective potential, measured as the percentage of cytotoxicity, increased in a dose-dependent manner. The untreated control group exhibited 4% cytotoxicity, while the high control group, which was treated with

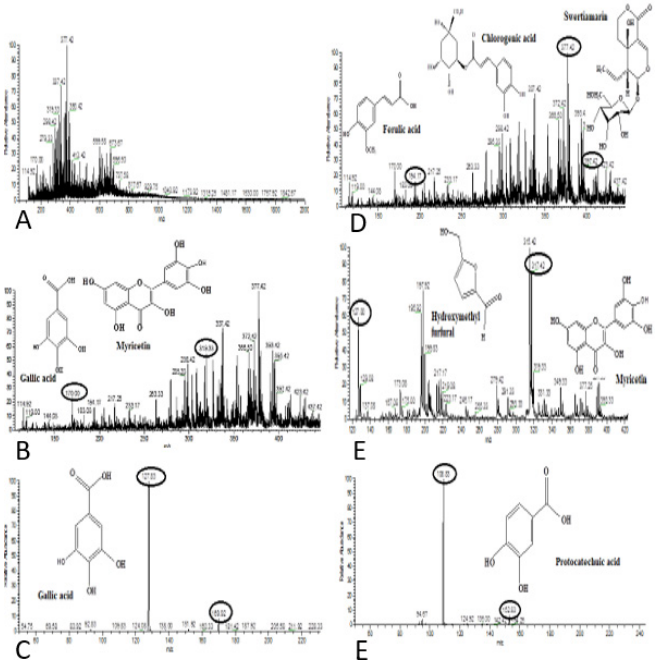

Fig. 5. **A**. Full mass spectrum of *M. annua*, **B**. Mass spectrum of Gallic acid at 170 m/z and Myricetin at 319.33 m/z in M. annua, **C**. MS-MS confirmed Gallic acid at 170 m/z, **D**. Mass spectrum of Ferulic acid, Chlorogenic acid and Swertiamarin in *M. annua*, **E**. Mass spectrum of Hydroxymethylfurfural and Myricetin correspondingly at 127 and 317.42 m/z in *M. annua*, **F**. MS-MS of peak at 153 m/z of Protocatechuic acid in *M. annua.*

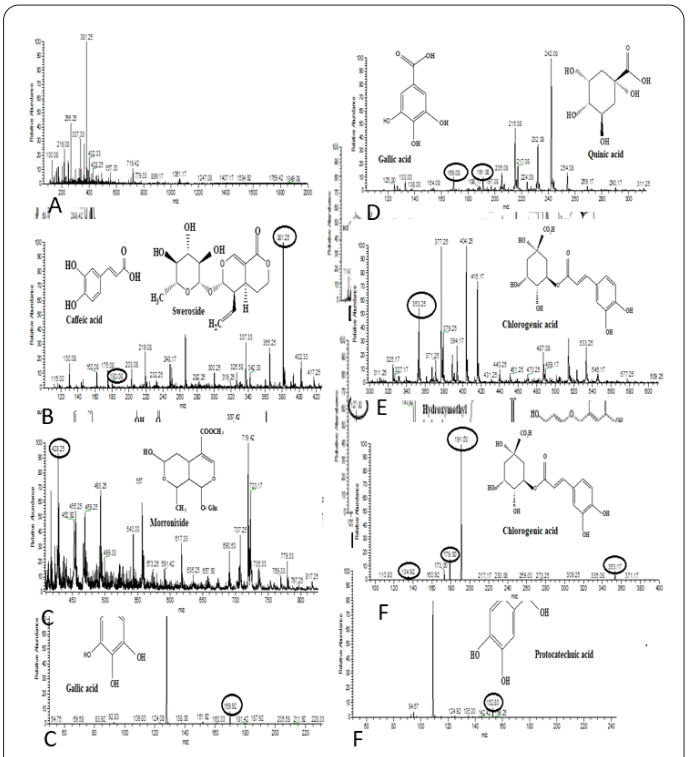

Fig. 6. **A**. Full mass spectrum of *P. viviparum*, **B**. MS of *P. viviparum* showed Caffeic acid at 180.08 m/z and Sweroside at 381.25 m/z, **C**. MS of *P. viviparum* revealed Morroniside at 428.25 m/z, **D**. *P. viviparum* showed Gallic acid at 169.08 m/z and Quinic acid at 191.08 m/z, **E**. Mass spectrum of *P. viviparum* indicated Chlorogenic acid at 353.25 m/z, **F**. MS- MS at 353.17 m/z confirmed the presence of Chlorogenic acid.

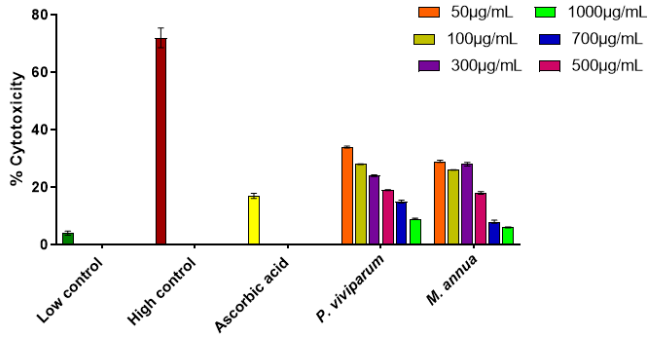

Fig. 7. Comparison of hepatoprotective potential of *M. annua* and *P. viviparum* with high, low and standard groups.

40 mM CCl_4, showed 72% cytotoxicity. At the minimum studied concentration of 50 µg/mL, *M. annua* showed 29% cytotoxicity as compared to *P. viviparum* in which 34% cytotoxicity was observed. However, at maximum studied concentration (1000 µg/mL) *M. annua* and *P. viviparum* exhibited maximum hepatoprotective potential in terms of minimum cytotoxicity 6% and 9% respectively (Figure 7).

4. Discussion

Natural bioactive compounds such as phenolic and flavonoids of plants are antioxidants and are used as therapeutic agents to control many diseases (24). Phenolics are strong reducing agents, due to electron-donating properties that inhibit many free radical reactions, which induce oxidative stress (25). Several diseases including cancer, inflammation, neurodegenerative, cardiovascular and liver infections are associated with increase in oxidative stress that damages DNA and cell membranes and these fetal diseases can be overcome by the use of plant-based medicines (26, 27).

The spectrophotometric DPPH radical scavenging assay and ferric reducing antioxidant power assay are appropriate methods to quantify the antioxidative potential of medicinal plants against free radicals due to their reducing abilities (28). DPPH radicals are reduced by acceptance of electrons and change their color from blue to yellow in the presence of antioxidants, while in the case of ferric reducing antioxidant assay blue color product is formed after the reduction of TPTZ - Fe3+ into TPTZ-Fe2+ (29). The antioxidant potential of *M. annua* and *P. viviparum* increased in a concentration-dependent manner and these findings are aligned with another study in which Drypetes sepiaria Stem was used to see its antioxidant potential.

LC-MS analysis of *M. annua* and *P. viviparum* revealed the presence of Gallic acid, Chlorogenic acid, Protocatechuic acid, Swertiamarin, Sweroside, Ferulic acid, Echinacoside and Morroniside (30) These are powerful antioxidant agents by inhibiting apoptosis and lipid peroxidation through radical scavenging activities (31, 32). Ferulic acid, Protocatechuic acid and Echinacoside are phenolic compounds that are found in many plants with lots of pharmacological effects to cure cancer, and cardiovascular and hepatic, respiratory and neurological disorders. It has also photoprotective effects and biological effects including antioxidant, anti-inflammatory and anti-microbial activities (33, 34, 35). Chlorogenic acid, found in fruits and vegetables and acts as a strong antioxidant agent by increasing the activity of antioxidant enzymes catalase and superoxide dismutase and inhibiting lipid peroxidation in kidney and liver cells (36).

Sweroside is an iridoid, a type of active secondary metabolite involved in various metabolic processes. It is a natural bioactive compound found in Swertia pseudochinensis, which is used as a therapeutic agent for treating diarrhea and jaundice (37). Sweroside has the potential to cure hepatobiliary disorders and possesses antioxidant, cell neuritogenic and wound-healing properties (38, 39). Morroniside is an iridoid glycoside, used as a vegetable drug in China and found in Cornus officinalis (40). It was revealed from previous studies that Morroniside has different biological properties like tissue regeneration and protecting cells against apoptosis (22, 41).

The phytoconstituents of *M.annua* and *P. vivparum* strongly exhibited hepatoprotective potential against hepatotoxicity induced by CCl4. Liver slice culture assay is an in vitro technique that provides a suitable environment for liver cells and valuable approach for the detection of hepatoprotective potential of medicinal plants (42, 43) Lactate dehydrogenase (LDH) is a cytosolic enzyme and in case of hepatotoxicity it released from the necrotic cells and its increased level indicated the cytotoxicity. Plants are used along with hepatotoxicants in liver slice culture assay and the amount of LDH released in the medium indicated the hepatoprotective potential of plants (44, 45).

5. Conclusion

This study highlights the significant hepatoprotective and antioxidant properties of *Martynia annua* and *Polygonum viviparum*, demonstrating their potential as therapeutic agents against liver disorders and abiotic stress. The findings reveal that both plants possess bioactive compounds that effectively mitigate oxidative stress and enhance liver cell protection. Further research is essential to explore additional therapeutic effects and mechanisms of action associated with these medicinal plants, potentially contributing to improved treatments for liver-related health issues. In conclusion, the hepatoprotective and antioxidant bioactive compounds found in *M. annua* and *P. viviparum* demonstrate significant potential to combat liver disorders and abiotic stress. Further investigations on these plants are warranted to explore their additional therapeutic effects.

Consent for publications

The author read and approved the final manuscript for publication.

Ethical disclosure

All the conducted research work was in accordance with the regulations of the International Bioethics Committee (IBC).

Informed consent

The authors declare that no patients were used in this study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of interest

All the authors mentioned in the manuscript have no conflict in the research work and compilation.

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