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Research article

Evaluation of potential inhibitory effects on acetylcholinesterase, pancreatic lipase, and cancer cell lines using raw leaves extracts of three fabaceae species

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ABSTRACT

The present study examined the biological potential and phytochemicals of *Sophora mollis, Mucuna pruriens*, and *Indigofera atropurpurea* methanolic leaf extracts. *In vitro* anti-acetylcholinesterase and anti-lipase assays were performed using different concentrations of plant extracts, and the IC_{50} values were determined. The cytotoxic potential of the selected plant extracts was assessed against HeLa, PC3, and 3T3 cell lines using an MTT assay. *S. mollis* leaf extract displayed the highest inhibition percentage (114.60% ± 19.95 at 1000 μg/mL) for the anti-acetylcholinesterase activity with a prominent IC_{50} value of 75.9 μg/mL. The anti-lipase potential was highest with the *M. pruriens* leaf extract (355.5 μg/mL IC_{50}), followed by the *S. mollis* extract (862.7 μg/mL IC_{50}). Among the cell lines tested, the cytotoxic potential of the *I. atropurpurea* extract (91.1 ppm IC_{50}) against the PC3 cell line was promising. High-performance liquid chromatography revealed gallic acid, chlorogenic acid, caffeic acid, vanillic acid, rutin trihydrate, and quercetin dihydrate in varying concentrations in all plant species. The concentration of chlorogenic acid (69.09 ppm) was highest in *M. pruriens*, and the caffeic acid concentration (45.20 ppm) was higher in *S. mollis*. This paper reports the presence of bioactive therapeutic compounds in selected species of the Fabaceae family that could be micropropagated, isolated, and utilized in pharmaceutical industries.

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1. Introduction

Approximately 25% of currently used therapeutical drugs are derived from plants as numerous low-molecular weight compounds known as 'natural products' or 'secondary metabolites' are produced by various plants. The polyphenols are the most common secondary metabolites in plants and possesses ability to interact with enzymes and other biological macromolecules depending on the composition, molecular weight, and hydroxyl groups [1]. Many studies have evaluated traditionally claimed applications of *Mucuna pruriens*, *Indigofera atropurpurea*, and *Sophora mollis* belonging to the family Fabaceae. For example, Mohanasundari et al. [2], Gulati et al. [3], and Katzenschlager et al. [4] demonstrated the antimicrobial, hypoglycemic, antioxidant, and the anti-Parkinson potential of *M. pruriens*. Similarly, *S. mollis* extracts and its chemical constituents possesses significant antibacterial, anthelmintic, anti-inflammatory, and cytotoxic potential [5,6]. Moreover, *I. atropurpurea* is used to treat diarrhea and dysentery [7]. Nevertheless, the scientific data regarding their ability to inhibit enzymes and the growth of cancer cells is entirely lacking.

Alzheimer's disease (AD) is a neurodegenerative disease that reduces the cognitive, behavioral, and social activities that ultimately leads to the death of brain cells [8]. Approximately 55 million people suffered from AD globally in 2020, which has been projected to increase to 78 million in 2030 [9]. The degenerating cholinergic neurons in the forebrain and other parts of the brain cause cognitive decline in AD that can be treated by increasing acetylcholine (ACh) levels in the brain [10]. The ACh-hydrolyzing enzyme is associated with the termination of nerve impulse transmission [11]. Therefore, cholinesterase inhibitors are effective remedies for the asymptomatic treatment of AD. In addition, commonly used synthetic anti-AD drugs, such as tacrine, donepezil, and rivastigmine [12], herbal medicines might be exploited to treat this disease without causing adverse effects [13].

Obesity is another risk factor for hypertension, diabetes, cancer, and other disorders [14]. The World Health Organization (WHO) reported that ~ 650 million adults are obese [15], but obesity can be cured by reducing the absorption of lipids inside the body. Recent studies suggested that pancreatic lipase absorbs lipids in the digestive tract via hydrolyzing triglycerides into fatty acids and glycerol. Thus, lipase inhibition is effective in decreasing caloric yield and weight loss [16]. Powerful lipase inhibitory drugs, such as orlistat currently available in the market, exhibit fecal urgency, oily spotting, flatulence, insomnia, liver problems, and many other diseases [17,18]. Hence, there is a dire need to investigate naturally occurring compounds and plant extracts that can potentially inhibit pancreatic lipase with low toxicity effects [19].

Cancer is an anomalous state in which the mass of cells does not respond to the normal cell signaling because of the higher self-sufficiency and causes uncontrolled growth and proliferation, leading to the transformed cells [20]. Cervical cancer is the second most common malignant tumor, affecting 527,624 women annually [21], and prostate cancer is prevalent in men [22]. Cancer therapy is performed to kill the affected cells without influencing the normal cyto-physiological processes. The commonly used anticancer agents are toxic at effective doses and may develop multiple drug resistance (MDR) in cells [23].

This study examined the anti-acetylcholinesterase, pancreatic lipase, and cancer-inhibitory activities of three selected species of Fabaceae and determined the concentration of six phenolic and flavonoid compounds present in these species using high-performance liquid chromatography.

2. Material and methods

2.1. Extracts preparation

The fresh leaves of selected Fabaceae species were collected, and their voucher numbers were taken from the Herbarium of the National Agricultural Research Center (NARC), Islamabad. The leaves were powdered and the extracted with methanol (20 g in 200 mL each) for two days. Subsequently, extracts were filtered using whatman filter paper and then evaporated using rotary evaporator (BUCHI rotavapor R −220). The crude extracts were weighed to determine the extract yield (Table 1).

2.2. Anti-acetylcholinesterase activity

The acetylcholinesterase inhibitory potential was assessed using the standard method, as described by Hasnat et al. [24]. In this assay, 140 μL of 0.1 M sodium phosphate buffer (pH 8.0), 20 μL of plant extract, 15 μL of AChE enzyme solution (0.2 units/mL), and 10 μL of 15 mM 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB) were added. After 10 min, 10 μL of the 15 mM AChI substrate was mixed, and the solution was incubated (15 min) at 25 °C. The absorbance at 412 nm was measured, and 100, 500, and 1000 μg/mL of galantamine were then evaluated as standard. The enzyme inhibition was measured as

$$\text{Enzyme inhibition } \% = 100 - \% \text{ enzyme activity}$$

$$\% \text{ enzyme activity} = 100 \times V/V_{max}$$

Table 1
Accession numbers, extract weight, and extract yield of the selected Fabaceae species.

Plant species	Accession number	Weight of extract (g)	Extract yield (%)
Indigofera atropurpurea Buch.-Ham.ex Horn.	RAW101503	6.30	31.50
Sophora mollis (Royle) Baker	RAW101499	5.95	29.75
Mucuna pruriens (Linn.) DC.	RAW101497	4.85	24.25

Where V_{max} is the enzyme activity observed without adding an inhibitor.

2.3. Anti-lipase assay

The lipase inhibition activity was assessed by measuring the 4-nitrophenol produced by the lipase action [25]. Initially, 5 mg/mL lipase from porcine pancreatic type II was dissolved in 50 mM of Tris-HCl (pH 8.0) and centrifuged at 5500 rpm for 5 min. Subsequently, 50 μL of each 100, 500, and 1000 μg/mL of methanolic extracts were added in 100 μL of lipase enzyme solution and then left for incubation (5 min) on ice. Subsequently, 50 μL of *p*-nitrophenyl butyrate (pNPB) was added and incubated (20 min) at 37 °C. Orlistat and PBS were used as the control, and the absorbance was observed at 405 nm. The inhibitory activity was then determined.

Inhibition % = (1-Abs_{sample} / $Abs_{control}$) x 100

2.4. Cytotoxicity assays

2.4.1. Cell culture

HeLa (cervical), PC3 (prostate), and 3T3 (mouse embryonic fibroblasts) cell lines were obtained from the National Cancer Institute Hospital in Karachi. HeLa and 3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL of each penicillin and streptomycin in flasks. On the other hand, the PC3 cells were maintained in DMEM, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1.5 g/L sodium carbonate, and 1% antibiotic solution. All cells were then incubated at 37 °C with 5% CO_2 and were sub-cultured once they produced a monolayer on the flask. The cells were detached by adding 0.25% trypsin consisting of 0.01% EDTA for 10 min.

2.4.2. MTT assay

The cytotoxic potential of the plant extracts was evaluated against three cell lines using an MTT (3-[4, 5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) assay [26]. The dilutions (30, 60, 90, 120, and 150 μg/mL) of each extract were prepared from the stock solutions (20 mg/mL) using 2% DMSO. 100 μL of HeLa, PC3, or 3T3 cells (1×10^5 cells/mL) were added to a 96-well plate along with different concentrations of plant samples. Subsequently, the samples were incubated at 37 °C with 5% CO_2. The cells exposed to 2% of DMSO without plant extracts were used as the negative control, while doxorubicin was measured as a standard drug. After 24 h, 10 μL of MTT (0.5 mg/mL) was added and re-incubated for 4 h at 37 °C. Subsequently, 100 μL of DMSO was added, and the absorbance was read at 490 nm. The percentage inhibition and IC_{50} values of the cell population were determined.

2.5. HPLC analysis

Each plant extract (1 mg) was dissolved in 10% methanol (5 mL) and filtered through membrane filters (0.45 μm). An Agilent 1260 HPLC series equipped with a pump and C18 column (100 mm × 4.6 mm) was run at 30 °C, and the mobile phase was filtered (0.45 μm membrane filter) and degassed by sonication in an ultra-sonic bath. The separation was achieved using an elution gradient of 0.2% H_3PO_4, methanol, and acetonitrile with a flow rate of 1 mL/min. The mobile phase was increased from 0 to 5% in 5 min, 50% in the next 15 min, 70% in 25 min, 100% in 30 min, and then kept isocratic for the next 5 min. The detection wavelength was set at 210 nm and the total running time was 35 min. The standards (gallic acid, chlorogenic acid, caffeic acid, vanillic acid, rutin trihydrate, and quercetin dihydrate) were run at 10, 20, 30, 40, 50, and 70 ppm. The autosampler was used to inject 5 μL of plant sample and the selected standards. The peak areas and retention times of standards were used to plot the calibration curve. A linear equation was obtained using the respective standard curves, and the concentrations of different phenolics/flavonoids were calculated [27].

2.6. Statistics

All assays were conducted three times, and the mean values were determined. The standard deviation was evaluated as a measure of dispersion, and the data distribution was interpreted using the mean and standard deviation by plotting bar charts in Microsoft Excel. The least significant difference (LSD) was determined using statistix 8.1. The IC_{50} values were calculated in all bioassays using Graphpad prism software.

3. Results and discussion

3.1. Acetylcholinesterase inhibitory activity

The anti-acetylcholinesterase activity of plant extracts was evaluated at 100, 500, and 1000 μg/mL, and results were compared with the standard galantamine, which showed and IC_{50} value of <1 μg/mL. Among the species, the *S. mollis* leaf extract indicated the highest inhibition percentage (114.60 ± 19.95%) at the highest concentration of 1000 μg/mL. The efficacy of extracts was recorded as *S. mollis* (IC_{50} value of 75.96 μg/mL) > *M. pruriens* (IC_{50} value of 508.20 μg/mL) > *I. atropurpurea* (IC_{50} value of 560.0 μg/mL) (Fig. 1). A comparison with published reports is difficult because of the lack of studies on acetylcholinesterase inhibition with the leaf extracts

of selected species. Kamkaen et al. [28] and Bhat et al. [29] performed anticholinesterase tests with *M. pruriens* seed extracts and reported that it was active for acetylcholinesterase inhibition, suggesting that the application of *M. pruriens* seed extract could be more effective for treating neurodegenerative disorders compared to the leaf extract observed in the present study.

According to nutritionists [30], vitamin C helps reduce neurodegenerative diseases. The noradrenaline-producing neurons are the areas affected by Alzheimer's disease; this neurotransmitter is interlinked with stress and other environmental reactions, along with the changes that cause memory loss and inflammation [31]. Vitamin C is a co-factor of dopamine that synthesizes noradrenaline [30]. AD also causes plaque accumulation, leading to oxidative stress and other adverse effects of the disease. Therefore, diets rich in vitamin C and other antioxidant substances also inhibit neurological disturbance caused by oxidative stress [31]. In the current study, *S. mollis* extract exhibited some positive effects in the anti-acetylcholinesterase assay in comparison to the other species. It can be proposed that further purification of *S. mollis* extract and its fractionation could be effective in enhancing anti-acetylcholinesterase activity. Optimization of different parameters that may increase the anti-acetylcholinesterase activity could be examined in future. Moreover, further studies are recommended to elaborate the precise nature of samples, such as scrutiny for extended durations, increased concentrations and different *in vivo* assays for its possible application in drug industries.

3.2. Porcine pancreatic lipase inhibitory assay

Pancreatic lipase is secreted by the pancreas and is involved in the digestion of fats and hydrolysis of 50–70% of dietary lipids. The anti-lipase activity is a widely examined mechanism to determine the efficiency of plant-based anti-obesity drugs [32]. The present study showed that *M. pruriens* exhibited the lowest IC_{50} value, *i.e.*, 355.50 μg/mL, followed by the *S. mollis* leaf extract (IC_{50} value of 862.70 μg/mL) and is effective in inhibiting the lipase activity. The *M. pruriens* and *S. mollis* leaf extracts also showed the highest inhibition (IC_{50} values of 68.49 ± 0.67 μg/mL and 50.71 ± 1.85 μg/mL) against porcine pancreatic lipase at 1000 μg/mL, suggesting that these species are effective at the highest concentration (Fig. 2).

Orlistat, obtained from *Streptomyces toxytricini,* is a clinically approved drug used to reduce obesity in individuals [33]. Akhtar et al. [34] reported that *M. pruriens* seeds help decrease the blood glucose level in alloxan-diabetic rabbits by stimulating insulin release. On the other hand, the anti-lipase activity of the raw leaf extracts of selected species has been investigated for the first time. The current data show that the selected extracts can be utilized effectively as anti-obesity agents.

Previous studies [35,36] also proposed that phenolic and flavonoid compounds from medicinal plants inhibit lipase activity. Hence, phenolic and flavonoid compounds play a crucial role in lipase inhibition. On the other hand, a plant extract is a mixture of a variety of active substances that contribute to the bioactivities altogether, suggesting that the composition of active substances that inhibit pancreatic lipase activity needs to be analyzed further.

3.3. Anticancer assays

The effects of the selected methanol extracts on the viability of HeLa, PC3, and 3T3 cells were determined using an MTT assay. The percentage inhibition was recorded at five different concentrations, and IC_{50} values were then determined. Doxorubicin (standard drug) revealed 100%, 89.90%, and 96.20% inhibition against HeLa, PC3, and 3T3 cells at 30 ppm. *M. pruriens* extract showed significant activity against 3T3 cells by displaying an IC_{50} value of 281.40 ppm, while the *I. atropurpurea* extract was effective in inhibiting PC3 cells (IC_{50} value of 91.10 ppm), and *S. mollis* were potent against HeLa cells (IC_{50} value of 146.50 ppm). On the other hand, *S. mollis* extract showed the lowest cytotoxicity potential against PC3 cells (IC_{50} value of 1256.00 ppm), followed by *I. atropurpurea* and *M. pruriens* methanol extracts against HeLa cells (IC_{50} values of 1066.00 ppm and 743.80 ppm) (Table 2).

Sinha et al. [37] and Yadav et al. [38] also determined *M. pruriens* mediated anti-cancerous effect against human breast cancer cells and hepatic (Huh-7) cells. This study is also coherent with the studies of Gupta and Patel [39], who observed mild cytotoxicity (IC_{50} values of 36.74 μg/mL and 39.42 μg/mL) of *M. pruriens* on MCF-7 (breast cancer) and A549 (lung cancer) cell lines. Abd-Alla et al. [40] reported that the active compounds in *S. mollis*, such as scopoletin and β-sitosterol glucoside, exhibit some cytotoxic effects against HeLa and 3T3 cell lines. Furthermore, the isoquinoline alkaloid occurring in *M. pruriens* also possesses cytotoxic activity [41]. Overall, the plant extracts showed significant cytotoxic potential when examined against HeLa, PC3, and 3T3 cell lines, which can be attributed to the presence of various cytotoxic compounds. On the other hand, the precise mechanism of apoptosis should be explored using more

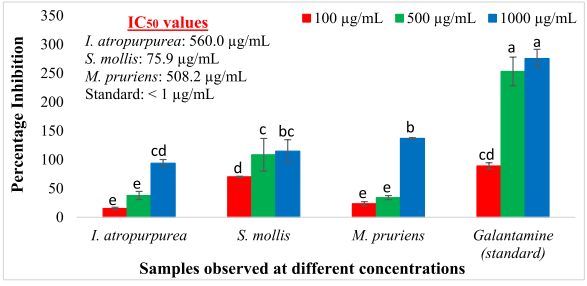

Fig. 1. Anti-acetylcholinesterase activity of raw leaf extracts of three Fabaceae species. The data indicate mean ± SD (3n), and each letter (a–e) indicates a significant difference at $P < 0.05$.

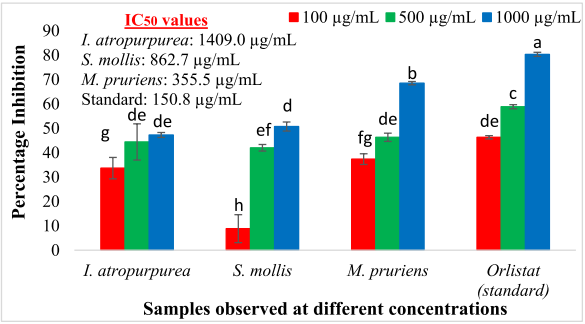

Fig. 2. Anti-lipase activity of the leaf extracts of selected species. The data are reported as the mean ± SD (3n), and each letter (a–h) indicates a significant difference at $P < 0.05$.

Table 2
Cytotoxicity potential of three Fabaceae species determined against HeLa, PC3, and 3T3 cell lines.

Plant species	Cell lines	Percentage Inhibition at Different Doses (ppm)					IC_{50} (ppm)
		30	60	90	120	150	
M. pruriens	HeLa	1.8	3.6	5.4	7.8	11.0	>150
	PC3	0	1.1	3.3	5.8	7.8	>150
	3T3	0	0	0	2.5	4.8	>150
I. atropurpurea	HeLa	0	2.1	4.2	5.6	6.3	>150
	PC3	15.3	30.6	45.9	61.2	76.5	98.03
	3T3	0	0	3.2	6.6	12.1	>150
S. mollis	HeLa	0	0	14.3	30.4	52.1	146.5
	PC3	1.9	3.8	5.7	7.6	9.5	>150
	3T3	0	0	0	5.2	7.7	>150

IC_{50}: Half-maximal inhibitory concentration.

sensitive assays like flow cytometry and other microscopy techniques.

3.4. HPLC analysis

The HPLC chromatograms indicating the presence of some selected compounds are shown in Fig. 3a to c. Selected plant species exhibited chlorogenic acid, caffeic acid, and quercetin in higher concentrations than vanillic acid, gallic acid, and rutin trihydrate when analyzed by HPLC. *M. pruriens* was the richest source of chlorogenic acid (69.09 ppm), caffeic acid (17.01 ppm), and quercetin dihydrate (16.87 ppm). Similarly, *S. mollis* also possesses the highest concentration of caffeic acid (45.20 ppm) and quercetin dihydrate (14.37 ppm), while *I. atropurpurea* consists of chlorogenic acid (16.87 ppm) and quercetin dihydrate (16.79 ppm) in higher concentrations. The remaining compounds were detected at the lowest concentration (<6 ppm) in examined plant species. The calibration curve validation data of six (6) compounds showed satisfactory linearity with a correlation coefficient (R^2) values of 0.942–0.995 (Table 3).

Previous studies indicated the presence of a phenolic amino acid (levo-3,4-dihydroxyphenylalanine) and cholorogenic acid (49.20 mg/100 g) in *M. pruriens* seeds [42,43]. This study confirmed the presence of chlorogenic acid in *M. pruriens* leaves extracts (16.87 ppm). Moreover, some bioactive compounds, such as tannins, triterpenes, isoflavones, and coumarins, have also been detected in the roots and aerial parts of *S. mollis* previously [5,44]. This study corroborates the existence of six compounds in the leaf extracts of the three species responsible for their biological activities.

Rutin, a polyphenolic compound, possesses anti-inflammatory, anti-tumor, antioxidant, anti-asthma, and antimicrobial potential [45]. Caffeic and vanillic acid are used as antioxidant agents to reduce the proliferation of cancer cells. Quercetin can suppress the growth-signaling pathways of cancer cells [46,47]. Chlorogenic and gallic acid exhibit antioxidant, antipyretic, anti-ulcer, anti-obesity, and neuroprotective activities. Chlorogenic acid also helps regulate the lipid and glucose metabolism and treat ailments, such as diabetes, obesity, and cardiovascular disease [48,49]. The reported biological activities of these compounds further potentiate the medicinal value of selected species. Therefore, the present study recommends the micropropagation, isolation, and characterization of these compounds for future industrial applications.

4. Conclusion

This paper reported the anti-acetylcholinesterase, anti-lipase, and anticancer activities of three Fabaceae plants leaf extracts. *S. mollis* extract showed significant (114.60 ± 19.95%) acetylcholinesterase inhibitory potential, *M. pruriens* extract indicated the highest (IC_{50} value of 355.50 µg/m) anti-lipase activity, while *I. atropurpurea* extract revealed the highest (IC_{50} value of 91.10 ppm)

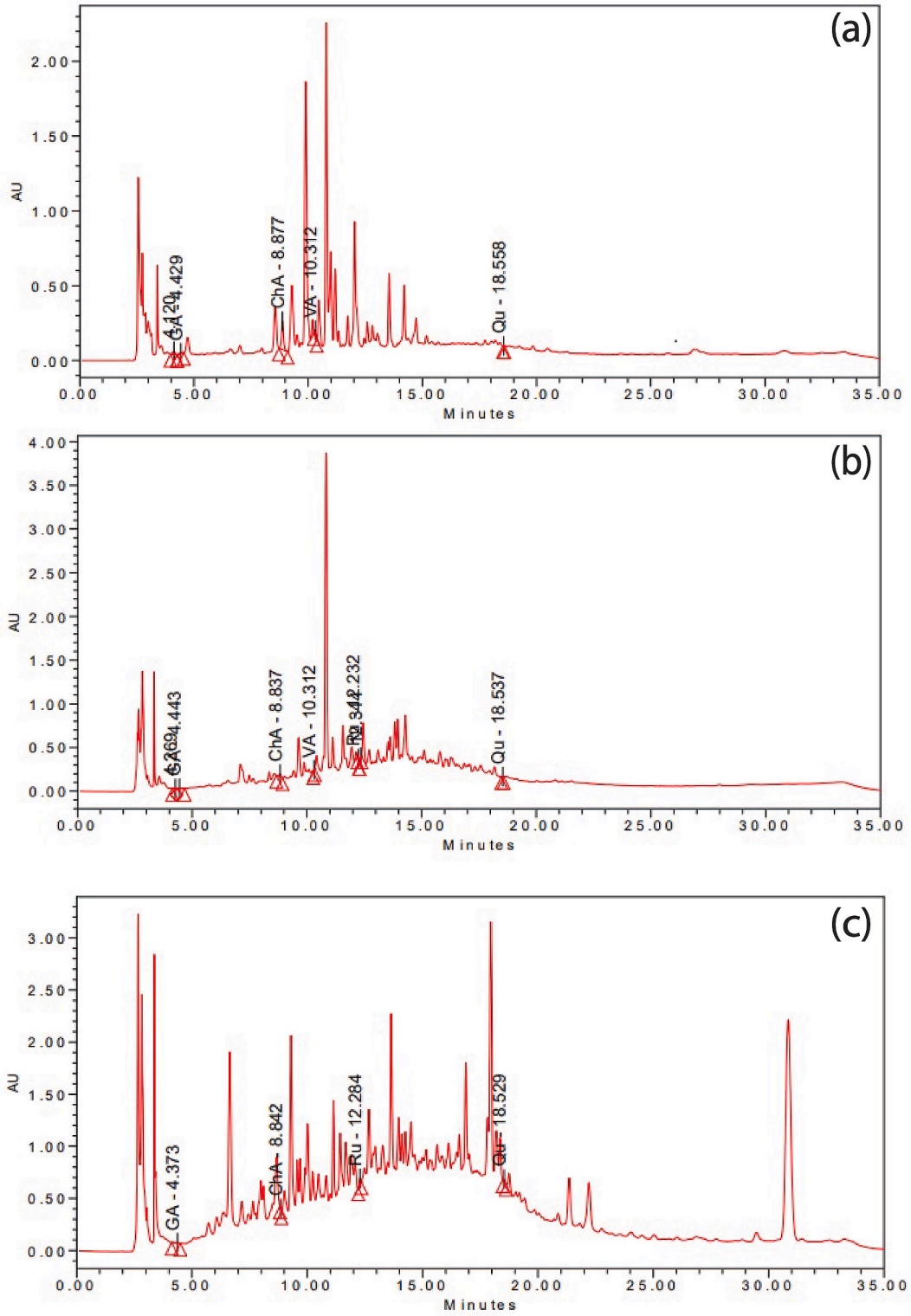

Fig. 3. HPLC chromatograms indicating some selected phenolics and flavonoids at the respective retention times **(a)** *M. pruriens* **(b)** *I. atropurpurea* **(c)** *S. mollis* (GA: Gallic acid; ChA: Chlorogenic acid; Ru: Rutin trihydrate; Qu: Quercetin dihydrate; VA: Vanillic acid).

Table 3
Quantification of six compounds determined by HPLC.

Retention time	Compounds	Linear equation	Correlation coefficient (R^2)	Concentration (ppm)		
				M. pruriens	I. atropurpurea	S. mollis
4.2	Gallic acid	y = 86488× - 140,627	0.942	2.25	2.03	2.33
8.9	Chlorogenic acid	y = 17624× - 75,341	0.967	**69.09**	**16.87**	5.14
10.2	Caffeic acid	y = 34331x + 7815.6	0.995	17.01	4.75	**45.20**
10.3	Vanillic acid	y = 99047× - 428,208	0.969	5.85	4.47	ND
12.2	Rutin trihydrate	y = 33754x + 36,891	0.990	1.09	0.76	5.57
18.4	Quercetin dihydrate	y = 42276x + 713,406	0.955	16.87	16.79	14.37

*ND: Not detected; The bold values represent the maximum concentration of bioactive compounds identified in testing plant samples.

anticancer ability against the PC3 cell line. HPLC revealed six compounds in varying concentrations associated with these biological activities. This study strongly supports using selected Fabaceae extracts as potential drugs, but further molecular studies are needed to validate the underlying pharmacology.

Author contribution statement

Iram Fatima: Conceived and designed the experiments; Performed the experiments; Wrote the paper.
Naila Safdar: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Wasim Akhtar: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Ammara Munir: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Saddam Saqib, Asma Ayaz, Saraj Bahadur, Abdulwahed Fahad Alrefaei, Fazal Ullah: Analyzed and interpreted the data; Wrote the paper.
Wajid Zaman: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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