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Research article

Comparative analysis of antioxidant and antiproliferative activities of crude and purified flavonoid enriched fractions of pods/seeds of two desert legumes *Prosopis cineraria* and *Cyamopsis tetragonoloba*

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ABSTRACT

Cyamopsis tetragonoloba and *Prosopis cineraria* are two legumes of the semi-arid region of Indian subcontinent which are unexplored with respect to their medicinal potential. Moreover, there is considerable lack in the comparative analysis of the biological properties of crude and enriched fractions obtained from the pods and seeds. Therefore, this study aims in investigating the effect of purification on the antioxidant and anticancerous activities of the extracts from the two legumes. This is the first study to purify an enriched methanolic fraction using Amberlite XAD7HP column chromatography followed by analysis using Thin Layer Chromatography. This matrix provided an economic and time efficient isolation of flavonoids and isoflavonoids from the seeds and pods of the above mentioned legumes. In addition, antioxidant activity carried out using DPPH assay showed that purification process did not contributed to enhanced antioxidant potential. However, inverse results were obtained during anticancerous activity assay on Huh-7 cell lines.

1. Introduction

The family Leguminosae is an important family of the edible plants, boasts of representing the third largest family in the plant kingdom with 745 genera and over 19,500 species and hence serves as a large reservoir of the natural products [1]. One of the crucial categories of the natural products in legumes is represented by the flavonoids and isoflavonoids. They belong to sub-class of phenolics compounds and constitute approximately 50% of the known phenolics compounds in plants [2]. Flavonoids are low molecular weight compounds consisting of two aromatic rings (A & B) joined by a 3-carbon bridge (C6–C3–C6 structure) [3]. The enormous diversity present in the metabolites from the Legume family should open numerous possibilities of their usage in medicinal and other applications. The different plant parts such as leaves, bark, seeds, fruits, root, flowers, etc. are enriched with many active compounds viz. phenolics, alkaloids, steroids, tannins, glycosides, volatile oils, and resins.

Plant-based natural products have contributed immensely in case of therapeutic agents. More than one-third of the FDA sanctioned drugs are derived from natural products. The health-promoting medicinal effect of plants is a resultant effect of either one or the combination of different compounds present. These drugs include either pure phytochemicals or their semi-synthetic derivatives based on the active compounds extracted from plants. Investigation of plants capable of surviving in extremely hostile environments may be considered as a vital strategy in screening for the speedy development of new drugs.

Cancer is a disease that is marked by the tuning out of the signals of the proliferation and dedifferentiation, which make the cells to sustain proliferation, overcome apoptosis, and giving them the power of angiogenesis and invasion. The existing treatment for cancer is associated with certain limitations, which have resulted in extensive research to look for alternatives to drugs having harmful side effects. Synergistic effect between the plant-derived compounds and the conventional chemotherapeutic drugs could help in achieving a decreased dosage of the drugs given during treatment and hereby, lowering the harmful side effects caused on the normal cells in the body. In this regard, studying the anticancerous properties of the extracts obtained from edible plants goes a step further since the dietary intake of the putative compounds can significantly affect the overall execution of the treatment.

The antioxidant and anti-cancerous activities of purified and flavonoid enriched fractions of *Prosopis cineraria* and *Cyamopsis tetragonoloba*, which are legumes localized to a great extent in the arid/semi-arid northwestern regions of India, have not been researched in depth, unlike other legumes which are the predominant part of the human diet.

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Chickpeas (*Cicer arietinum*), Peas (*Pisum sativum*), Pigeon pea (*Cajanus cajan*), Mung bean (*Vigna radiata*), Soya (*Glycine max*), Peanut (*Arachis hypogaea*), etc. are few of the species belonging to Leguminosae family about which several reports reflecting their antioxidant and anti-cancerous effects are present [4].

In this regard, the current study was undertaken where the phytochemical analysis of the crude methanolic and flavonoid enriched fractions obtained from the pods/seeds of *Prosopis cineraria* and *Cyamopsis tetragonoloba* was carried out to identify the putative flavonoid and isoflavonoid using column chromatography and thin layer chromatography techniques. This purification was followed by a comparative analysis of the antioxidant and anti-cancerous activities between the crude methanolic and flavonoid enriched fractions, which threw light on the effect of extraction procedures on the compounded effect of the extracts.

2. Material and methods

2.1. Chemicals and reagents

All the organic solvents hexane, toluene, chloroform, ethyl acetate, methanol, acetone, formic acid and glacial acetic acid used for the experiments were of analytical or High-Performance Liquid Chromatography (HPLC) grade and were supplied by SRL Laboratories and HiMedia. The standards of the flavonoids/isoflavonoids daidzein, daidzin, genistin, genistein, hesperidin, hesperetin, naringin, naringenin, rutin hydrate, and quercetin used were obtained from Sigma Chemical Company. The Thin Layer Chromatography (TLC) plates (precoated with aluminum-backed silica gel 60 F_{254}) were procured from Merck (Darmstadt, Germany).

2.2. Crude extract preparation

The pods/seeds of *Prosopis cineraria* and *Cyamopsis tetragonoloba* were purchased from Pilani region of Rajasthan. For the crude extract preparation from seeds/pods of *Prosopis cineraria* and *Cyamopsis tetragonoloba*, 250 g of the ground dry powder of pods/seeds was taken and initially defatted thrice using 3–5 volumes of hexane by stirring. This was followed by centrifugation at 3000 g for 5 min. Supernatant was decanted and the pellet was dried thoroughly at room temperature. The lipophilic (hydrophobic) compounds were extracted in hexane, and the dried pellet was then extracted using 80% methanol by following the similar extraction procedure as defatting. The final crude preparation was concentrated to dryness using rotary evaporator and stored at 4 °C until further use.

2.3. Flavonoids extraction from pods of P. cineraria and C. tetragonoloba

In the current work, secondary metabolites (particularly flavonoids/isoflavonoids) from *P. cineraria* and *C. tetragonoloba* have been isolated using different column chromatography techniques. To isolate the compounds, crude extract preparation was subjected to column chromatography which included usage of matrix Amberlite XAD7HP. This flavonoid enrichment protocol was a modified version of that reported by Ferres *et.al.* [5] The matrix procured was washed thoroughly using distilled water and then soaked in excess of methanol. The Amberlite XAD7HP matrix was packed in glass column with dimensions 1.8 cm (internal diameter) and 30 cm height. The packed column was equilibrated again using 100% methanol and 10 g of the crude extract of the *P. cineraria* was dissolved in absolute methanol and then loaded on the column. The flow through fraction containing the unbound compounds was collected post which furthermore unbound compounds were eluted out using water.-This elution helped in avoiding the interference with the elution of the phenolics. The final elution was done using absolute methanol until discoloration of the matrix was observed. The eluent was collected in a flask and stored at 4 °C until further use. (The column was washed, equilibrated and regenerated for further experimentation, viz. the isolation of flavonoids from the *C. tetragonoloba* crude extracts). The eluent obtained post-Amberlite column chromatography was concentrated to dryness using rotary evaporator and stored at 4 °C.

2.4. TLC fingerprint profile for the purified plant extracts

The crude and purified fractions were analyzed using thin layer chromatography. All the extracts to be tested were dissolved in 100% methanol at a concentration of 1 mg/mL and were filtered using 0.45μm filters. For developing the TLC plates, a cylindrical glass chamber (10 cm × 11 cm; D x H) was used; its temperature was kept at 30 ± 1 °C. 5–10 μg of the methanolic extract was spotted near the bottom of the TLC plate. The samples were subjected to analysis using solvent system Butanol: Acetic acid: Water = 7:2:2. Post development, the TLC plates were immediately dried at 30–40 °C and were visualized under UV light at 254 nm and 365 nm. Images of the developed TLC plates were captured using a TLC Visualizer (CAMAG).

2.5. Determination of antioxidant activity using the DPPH radical-scavenging activity method

Antioxidant potential of the plant extracts was calculated using the α, α-diphenyl-β-picrylhydrazyl (DPPH) assay method [6] 0.1 mM solution of DPPH was prepared in 95% Methanol and stored in the dark at -20 °C until further use. For performing the reaction, 1 ml of the DPPH solution was added to 3 ml of solution of extracts in methanol at different concentration (25 μg/mL, 50 μg/mL, 75 μg/mL &100 μg/mL).The reaction was incubated at room temperature for 30 min in the dark. The absorbance was then measured at 517 nm using UV-VIS spectrophotometer (Jasco). A standard curve was plotted using ascorbic acid at a concentration of (5 μg/mL, 10 μg/mL, 15 μg/mL, 20 μg/mL). 95 % methanol was used as a blank solution. While plotting the standard curve, a time-dependent assay was also set up using incubation time of 10, 20, and 30 min. Post completion of reaction, the absorbance of yellow color was measured. Lower values of absorbance of reaction mixture indicate higher activity of radical scavenging. The scavenging potential towards DPPH radical was calculated by using the formula as given below:

$$\text{DPPH scavenging effect}(\%\ \text{inhibition}) = \{(A_0 - A_1)\,/\,A_0 * 100\} \quad (1)$$

Here, A_0 indicates absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and ascorbic acid. The IC_{50} value which is defined as the concentration of the sample resulting in 50% reduction of the initial DPPH concentration, was obtained from the linear regression of plots of mean percentage of the antioxidant activity against the concentration of the test extracts and ascorbic acid (μg/ml) obtained from three replicate assays.

2.6. Anticancerous activities of the purified extracts of pods/seeds of P. cineraria and C. tetragonoloba

2.6.1. In vitro cytotoxicity assay

In vitro Cytotoxicity assay was performed according to the methods previously mentioned by [7] Briefly, cells were seeded at a density of 8×10^4/well in a 96 well plate. After overnight incubation at 37 °C and 5% CO_2, cells were treated with different doses of extracts obtained from the seeds/pods of the *P. cineraria* and *C. tetragonoloba*. The plant extracts were prepared by dissolving 1 mg of dried powder in 30 μl of organic solvent DMSO and making up the final volume to 1 ml using Phosphate Buffer Saline. The concentration of DMSO reaching the cells was non-toxic.

The treatment of the extracts was given for 48 h. Thereafter, MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] (SRL) was added equally in all the wells and incubated for 4 h, following which the formazan crystals formed by the mitochondrial activity of live cells were dissolved in DMSO. Absorbance was measured at wavelength

570nm with a differential filter of 630nm using Multiskan Micro plate Spectrophotometer (Thermo Scientific). % of viable cells was calculated using formula:

$$\text{Viability}(\%) = (\text{mean absorbance value of treated cells})/(\text{mean absorbance value of control})*100 \quad (2)$$

2.6.2. Bright field imaging and dye exclusion assay

For bright field microscopic imaging, cells were cultured at density of 2×10^4 cells/plate in 6 cm culture dishes, treated with flavonoids enriched fractions from both the plants and then images were captured using Olympus (CKX41) microscope at 20 X magnification. To assess the number of dead cells versus the number of live cells, dye exclusion assay using trypan blue was performed. This assay is built on the principle that living cells possess intact cell membranes that exclude certain dyes such as Trypan Blue, whereas dead cells lose this capacity and hence retain the dye within the cell [8]. 0.4% of the concentration of Trypan Blue (procured from HiMedia) was prepared using molecular grade water. Equal volumes of cell suspension (diluted) and 0.4% of trypan blue solution were mixed in a tube and incubated at room temperature for 5–10 min. 20 μl of this mixture was loaded onto the Biorad Chamber slide and the number of live and dead cells were analyzed using Biorad TC20 Automated cell counter.

2.6.3. Flow cytometric analysis of apoptotic cells

To calculate the percentage of apoptotic cells post-treatment by different extracts, flow cytometric analysis was done. HuH7 cells were seeded in 6 wells plate at a seeding density of $1*10^6$. Treatments with the flavonoid enriched plant extracts of concentration 200 μg/ml was given to cells for 24 h. Cells were then harvested and sequential washes with 1X PBS was given. Cells were further resuspended in 500 μl of 1X Binding Buffer (BD BioSciences). Thereafter, 4μl of Annexin-V-FITC and 10μl of Propidium Iodide (PI) were added to cells and incubated in dark for 20 min at room temperature. The samples were then acquired to a flow cytometer (Cytoflex, Beckmann Coulter) and analysis of acquired data was executed using CytExpert software. For the detection of early and late apoptotic cells, the lower (LR) quadrant cells which are representative of only Annexin, and upper right (UR) quadrant which is representative of Annexin V and Annexin V-PI positive cells both, were considered. The percentage of apoptotic cells was represented via bar diagram.

3. Results and discussion

3.1. Purification of flavonoids from the selected plants using Amberlite XAD-7HP

Isolation of pure compounds (as possible active components) from plant sources has always been of immense interest to the researchers because of the possibilities of isolation of potential novel and better drugs. The recent advances in the separation processes have significantly decreased the difficulties faced during the extraction procedures, but still, there are many species of plants that are entirely unexplored (or relatively less explored) with respect to their secondary metabolite content. One of the many reasons behind this is their geographical (and endemic at times) distribution which limits the probability of local plants being chosen as the experimental material. Purification of natural products is an essential area of research in phytochemistry as it is a prerequisite for detailed insights into structural aspects and biological activities. In the present work, in order to purify flavonoids/isoflavonoids from the resource material, the defatted crude extracts obtained from the pods/seeds of *P. cineraria* and *C. tetragonoloba* were subjected to the column chromatography using the Amberlite XAD7HP matrix. This matrix is specialized in retaining the phenolics/flavonoids, which can be eluted out using the polar solvent methanol. The usage of the Amberlite XAD 7HP matrix has an advantage over other conventional separation techniques like liquid-liquid chromatography, which offers relatively less degree of purification of the defatted extract. The usage of the Amberlite extraction aided recovery values of 80–90% for flavonoids, allowing a more effective elimination of sugars, acids, pigments, and other interfering compounds. The application of Amberlite resin was first applied to isolate the flavonoids from honey samples by Ferreres et al. in 1991.This technique allowed them to identify 16 flavonoids from the honey samples, some of them include quercetin, kaempferol, 8-methoxykaempferoI, quercetin 3-methyl ether, isorhaminetin, kaempferol 3-methyl ether, galangin, luteolin, and apigenin. Since then, it has been adapted as the most widely used method for polyphenol extraction from samples obtained from different sources. Albeit, modifications may be incorporated in the methodology as per the requirement of the experiments.

Figures 1 and 2 show the TLC analysis of the purification profile of the extracts obtained at different steps from the edible portions of *P. cineraria* and *C. tetragonoloba*. It can be observed from Figure 1 where purification profile of *P. cineraria* indicates that when the equal concentration of all the samples was loaded on to the plate, the defatted methanolic crude fraction (CE) and the unbound fraction (UB; unbound meaning the fractions that eluted out of the column after the phenolics/flavonoids had adsorbed onto the column matrix) showed significantly less intense bands as compared to that of the flavonoid enriched fraction (FEF). The illumination under 254 nm exhibited clear lanes for the CE and UB fractions, whereas FEF fractions showed the presence of multiple dark bands against the light background. The flavonoids being UV active compounds can be detected in high quantity in the FEF fraction eluted post-Amberlite column run. The same TLC, when illuminated at 365 nm, revealed that the bands observed in FEF fractions are of flavonoids nature since the characteristic property of the flavonoids compounds is to get illuminated as yellow, orange, or bluish colors under 365 nm [9]. Hence the isolation of the flavonoids enriched fraction was obtained through 2 steps purification procedure.

Similarly, when extracts from different purification steps obtained for *C. tetragonoloba* were analyzed using TLC, illumination at 254 nm showed that the band patterns of CE and UB fractions were identical to each other, and in the lane, for FEF fraction, novel bands at R_f value 0.58 and 0.67, different from the previous ones were observed (Figure 2). This depicts the usage of the Amberlite XAD7HP matrix has resulted in partial purification of the defatted methanolic crude extract and fractions enriched in flavonoids/isoflavonoids were obtained.

Flavonoids/isoflavonoids in their conjugated forms are highly water-soluble. This has been proved beneficial for the plants where they can be stored in the plant vacuoles at higher concentrations because of the increased polarity. However, when the flavonoids/isoflavonoids have to be separated from the crude extracts which contain other water-soluble compounds as well, it becomes difficult to employ an economical and less tedious separation procedure. Here, Amberlite XAD- 7HP offers an exceptional ability to distinguish between the adsorption of the flavonoids/isoflavonoids on its matrix while at the same time letting the other water-soluble compounds such as sugars, proteins, and other polar metabolites to go as an unbound fraction. A comparative analysis of using different Amberlite matrices such as XAD-2HP, XAD-4HP, XAD-16HP, XAD-7HP, and XAD-8HP for purifying flavonoids showed that XAD-7HP offers a higher recovery rate of flavonoids and their conjugates.

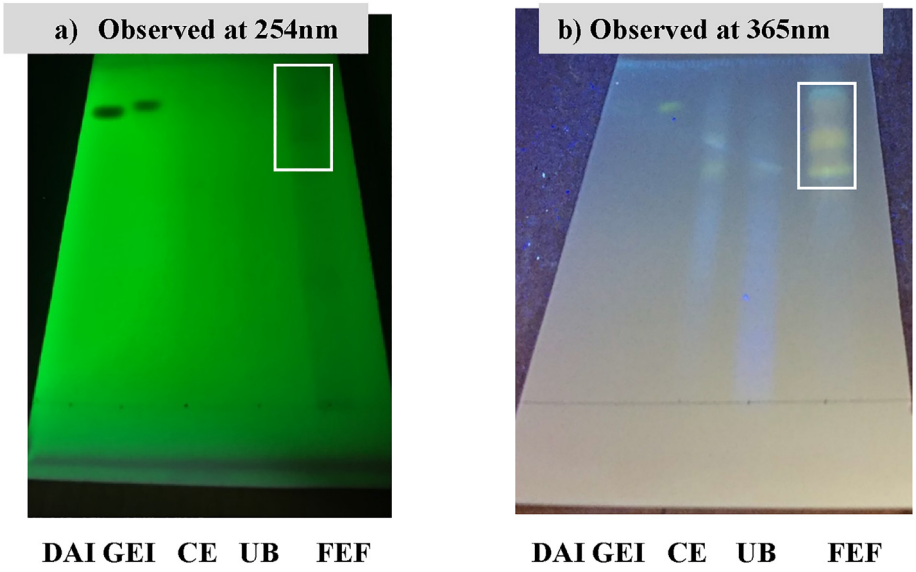

Figure 1. TLC analysis of flavonoids purification from pods/seeds of *P. cineraria*. Solvent system used is as follows: n Butanol: Acetic Acid: Water: 7:2:2. 2.5 μg of standards and 25 μg the purified samples loaded. DAI- Daidzein, GEI-Genistein, CE- Crude extract, UB- unbound, FEF- eluted sample in 100% Methanol. a) observed at 254 nm and b) observed at 365 nm.

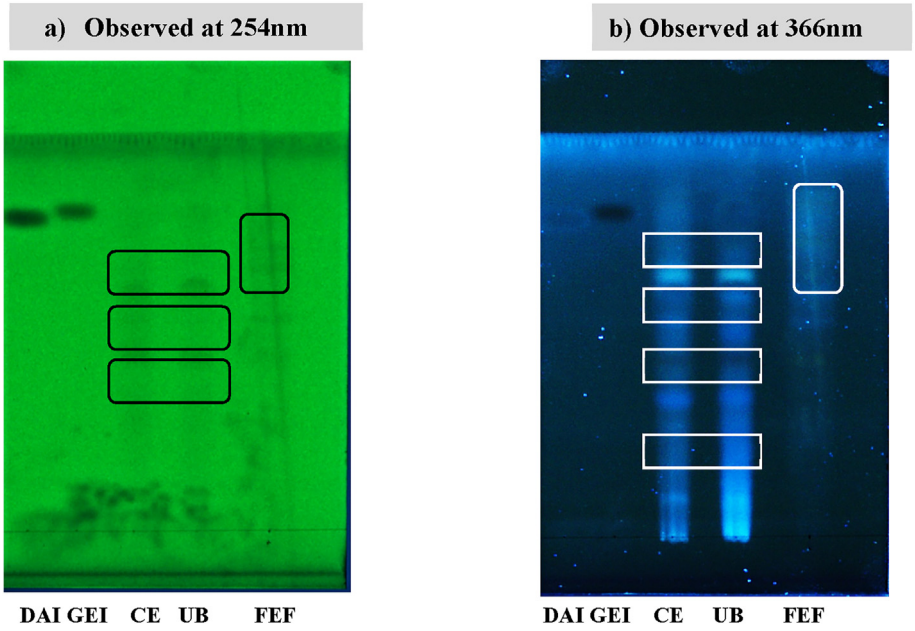

Figure 2. TLC analysis of flavonoids purification from pods/seeds of *C. tetragonoloba*, Solvent system used is as follows: n Butanol: Acetic Acid: Water: 7:2:2. 2.5 μg of standards and 25 μg of the purified samples loaded. DAI- Daidzein, GEI-Genistein, CE- Crude extract,UB- Unbound, FEF- eluted sample in 100% Methanol. a) Observed at 254 nm and b) Observed at 366 nm.

3.2. Antioxidant activities of extracts purified from pods/seeds of P. cineraria and C. tetragonoloba

Free radical scavenging potentials of test extracts which include crude and enriched fractions of *P. cineraria* and *C. tetragonoloba,* at different concentrations (25, 50, 100, and 200 μg/ml) was tested by the DPPH method and calculated as per Eq. (1). The results are shown in Figure 3 A-E. The standard curve was plotted using ascorbic acid and the graph is shown in Figure 3 A. IC_{50} values of the standard and plant extracts which indicates the concentration of the extract at which 50% radical scavenging activity is observed, is shown in Table 1. IC_{50} value for ascorbic acid was observed as 18.64 μg/ml.

From the results obtained, it is clear that the crude as well as enriched fractions, both showed considerable radical scavenging activities. The crude extract of *P. cineraria* showed high radical scavenging activity as compared to that of the enriched fraction. The IC_{50} value for crude extract was observed as 30.0 μg/ml (Figure 3 B). The enriched fraction, which was obtained after the column chromatography step, showed an increased IC_{50} value of 40.63 μg/ml (Figure 3 C). The activity observed in case of enriched fraction was in dose dependent manner. At highest concentration (200 μg/ml), 86.36% of scavenging potential was observed. The difference observed in the scavenging potential of crude and enriched fractions could be accounted to the fact that the crude extracts consists of vast number of secondary compounds which were

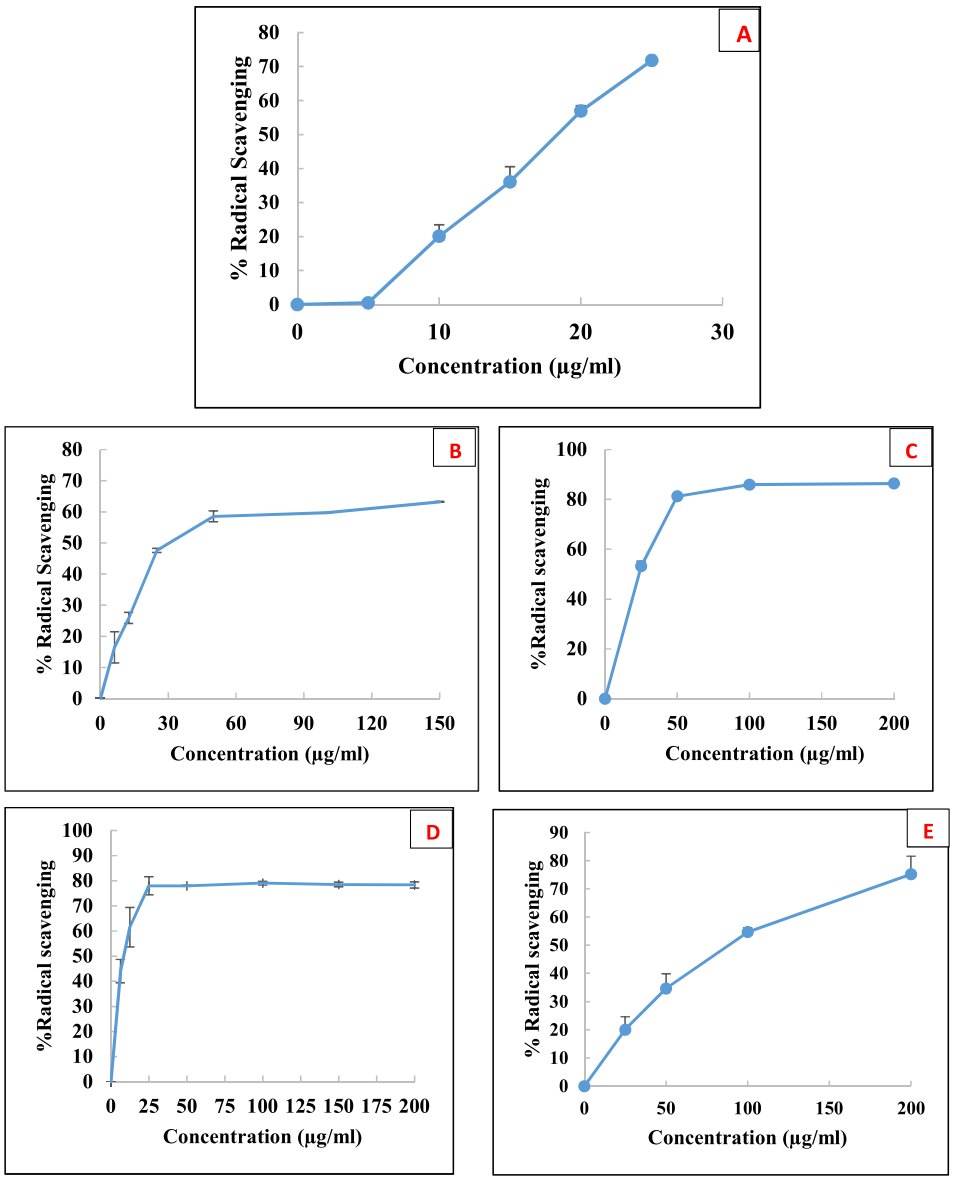

Figure 3. DPPH radical scavenging activity of A) Ascorbic acid, B) *P. cineraria* pods CE, C) *P. cineraria* pods FEF, D) *C. tetragonoloba* seeds CE, and E) *C. tetragonoloba* seeds FEF. The results are expressed as mean ± SD (n = 3).

exhibiting antioxidant properties. The purification step which was specific for the separation of flavonoids/isoflavonoids from the crude extracts, might have steered the elimination of potential scavengers. Similar pattern of results was observed with the DPPH assay carried out using crude and enriched fractions of *C. tetragonoloba.* The crude extracts showed a maximum of 78.85% of radical scavenging at a concentration of 200 µg/ml whereas the enriched fraction showed a maximum of 75.21% radical scavenging potential. However, the IC_{50} values showed that the crude extract was significantly more efficient as a scavenger as compared to the enriched fraction. The IC_{50} of crude extract for *C. tetragonoloba* seeds was obtained as 7.25 µg/ml (Figure 3 D) whereas for enriched fraction, 111.75 µg/ml was observed as the concentration to express 50% radical scavenging activity (Figure 3 E). This indicates the presence of strong scavengers in the crude extracts as against in the enriched fraction. A differentiation expression of phytochemicals in both the plants fostered the different antioxidant potential in the extracts which is evident from Table 1.

Table 1. IC_{50} values of ascorbic acid and different purified extracts.

S.No.	Name of the Extract	IC_{50} Value (µg/ml)
1	Ascorbic Acid	18.64
2	*P. cineraria* crude extract	30.0
3	*P. cineraria* flavonoid enriched fraction	40.63
4	*C. tetragonoloba* crude extract	7.25
5	*C. tetragonoloba* flavonoid enriched fraction	111.75

The association between the role of diet and some chronic human diseases have been studied thoroughly for a very long time. Several epidemiologic and related experimental investigations have established that the antioxidants present in diet effectively neutralize the harmful free radicals [10]. These free radicals are detrimental to cells, and initiating the diseases. Antioxidants, therefore, offer a protective mechanism for cells against a wide range of radicals and in long term can significantly decrease the disease occurrence [11]. This is the reason that current markets are now filled with the antioxidants supplements which have found an enormous consumer base and people are taking the supplements as preventive measure. Besides this, antioxidants are also crucial in the food industry since they are added as natural or synthetic preservatives to weigh down the effect of oxidative deterioration on

storage and processing [12]. Because of their low volatility and highly stable chemical nature, the antioxidants assist in preserving the level of nutrients, the color, freshness, taste, aroma texture, and functionality of the food. But there have been reports indicating that some of the commonly used synthetic antioxidants such as butylhydroxyanisole and butylhydroxytoluene (BHA and BHT, respectively) need to be swapped with natural antioxidants because of their impending health threats and harmfulness [13]. Considering the vast applications of antioxidants in general, the quest for isolating them from natural resources has garnered much attention, and apparent efforts have been made to identify new natural resources for active antioxidant compounds.

The antioxidant efficacy of two edible legumes *C. tetragonoloba* and *P. cineraria* of semi-arid regions was studied using DPPH assay. As observed from Table 1, the IC_{50} values for the crude defatted extract were found to be lower as compared to that of the enriched fractions for both the plants. This could be because of the loss of some key compounds during the purification process which are present in their active form in the crude defatted extract but couldn't be obtained in the enriched fractions.

The antioxidant potential of the aerial parts such as barks, leaves (mostly inedible for humans) of *P. cineraria* and *C. tetragonoloba* has been studied previously by many researchers. Soni *et al.* investigated the antioxidant potential in the chloroform, ethyl acetate and methanolic crude extracts of the bark of *P. cineraria*. There the IC_{50} values were observed as 28.87 μg/ml, 26.20 μg/ml, and 25.50 μg/ml, respectively [14]. Napar *et al.*(2012) reported that methanolic crude extract of *P. cineraria* leaves was capable of exhibiting 60% radical scavenging activity for DPPH [15]. Hydroethanolic extract of bark of *P. cineraria* was also found to increase the activity of catalase and glutathione peroxidase enzyme in diabetic mice, indicating that the phytochemicals present in the plant extracts can also induce antioxidant activity via inducing these enzymes [16]. A comparative analysis of the radical scavenging activity of the leaf extracts of *P. cineraria* obtained in different solvents like petroleum ether, benzene, chloroform, ethyl acetate, methanol and water showed that the methanolic and ethyl extract showed the highest values viz 84.06% and 84.63 % respectively [17]. A preliminary analysis that was done at the beginning of the current study to deduce the extraction capacity of various solvents showed that 80 % methanol offers the best solvent for crude preparations. The values of the antioxidant potential obtained for the crude and flavonoid enriched fraction of pods of *P. cineraria* were found to be significant compared with those of the previous reports. It is evident that methanol is the best choice of solvents to extract the antioxidants from different aerial parts of the plant. One interesting observation that was perceived during the review studies related to biological activities is the lack of in-depth studies carried out with the seeds/pods of *P. cineraria* which are its edible portion for humans. Nevertheless, the current study showed that the seeds/pods of this edible legume are prominently rich in antioxidants properties which could be eventually probed for isolating the more purified, individual natural radical scavengers.

Similarly, the crude and enriched fractions of *C. tetragonoloba* also showed the presence of the radical scavenging properties. *C. tetragonoloba* has a long tradition of being used in folklore medicine and has been known to act as cooling agent, appetizer, laxative, and digestive aid [18]. Besides this, it is a commercially important plant with respect to guar gum production. Most of the biological activity studies have focused on crude extracts (mainly ethanolic) for e.g. Kobeasy and El-sala, (2011) investigated the antioxidant activity of the crude ethanolic extract obtained from seeds where a maximum scavenging of 30% was observed at a concentration of 100 ppm [19]. Another study involved the usage of ethanolic extract of fresh, young and tender pods of *C. tetragonoloba* where it showed radical scavenging activity of 51.6% [20]. Dietary intervention of tender pods was observed in high-cholesterol-fed rats where elevated ascorbic acid and glutathione concentrations, and increased activities of antioxidant enzymes, both in blood and liver were observed [21]. The level of antioxidants activities was found more in crude extracts as compared to that of the enriched fraction suggesting a synergistic and additive effect of all the compounds present in the crude extracts.

3.3. Anticancerous activities of extracts purified from pods/seeds of *P. cineraria* and *C. tetragonoloba*

3.3.1. In vitro cytotoxicity assay of purified extracts against Huh7 cell line

The lack of any published data regarding the effect of these compounds on the cell viability of the Hepatocellular carcinoma (Huh7) cell line generated our interest in performing the current study. Despite the fact that *P. cineraria* and *C. tetragonoloba* have been an integral part of folk medicine for a long time, negligible reports are present where a comparative analysis of the effect of crude as well as flavonoid enriched fraction on the cancer cell line was studied.

Figure 4 A-D show the effect of different extracts on the cell viability of Huh7 cells which was calculated using Eq. (2). Different doses of PC-CE (*P. cineraria* Crude Extract), PC-FEF (*P. cineraria* Flavonoids Enriched Fraction), CT-CE (*C. tetragonoloba* Crude Extract), and CT-FEF (*C. tetragonoloba* Flavonoids Enriched Fraction), were used to treat the cancerous cells. This concentration was optimized between 50-200 μg/ml. All four extracts viz. PC-CE, PC-FEF, CT-CE, and CT-FEF were found to inhibit the proliferation of Huh7 cells in a dose-dependent manner. At the initial concentration which was taken as 50 μg/ml, PC-CE as well as PC-FEF showed absence of any cytotoxicity on Huh7 cells (Figures 4 A-B). Whereas at 50 μg/ml of CT-CE, there was 5 % decrease in cell viability and for CT-FEF there occurred a 10% decrease (Figure 4 C–D). Highest cytotoxicity was observed at 200 μg/ml of PC-CC treatment to cells where a decrease in 60% of cell viability was recorded. At 200 μg/ml of PC-FEF treatment, 63 % of cell death was seen. Treatment given using CT-CE and CT-FEF at concentration of 200 μg/ml resulted into decrease of 52% and 63% of cell viability respectively. This is a significant decrease in the cell viability obtained for the Huh7 cell line. The IC_{50}, which is defined as the concentration of the drug or treatment at which 50% of the maximum inhibition occurs under *in vitro* conditions, was calculated for all the extracts. IC_{50} is an indication of the effectiveness of a drug or compound in inhibiting a specific biological function (Table 2). The IC_{50} values for the extracts obtained from the experimental plants are as follows:

3.3.2. Bright field imaging and dye exclusion assay

The MTT results indicated lower IC_{50} values for the flavonoid enriched fractions; hence for dye exclusion assays, Huh7 cells were treated for 48 h with the enriched fraction using a concentration of 200 μg/ml Figure 5 A-B show the bright field image of the live and dead cells in the untreated sample and samples treated with PC-FEF, whereas Figure 5 C depicts the image of live and dead cells in the CT-FEF treated samples. As expected, the number of dead cells (indicated by rounding up of cells) increased in the case of the treated cells as compared to that of the untreated cells. This shows that flavonoid enriched fractions of both the plants were able to induce cell death in Huh7 cells after treatment given for 48 h. Only a single dose was chosen to conduct the dye exclusion study since the dose-dependent study of MTT analysis showed that maximum cell death occurred at concentration of 200 μg/ml. The decrease in cell viability observed during MTT assay and trypan blue staining indicate that exposure of Huh7 cells to flavonoids enriched fractions might have triggered the pathways which results in apoptotic death. To confirm whether the cell death occurred because of apoptosis or necrosis, flow cytometric analysis was carried out with the treated and untreated samples which have been discussed in later sections. The number of the live cells and dead cells obtained after the treatment as assessed by the trypan blue staining are shown in Figure 5 D. The graphical representation clearly indicates that there is significant decrease in the viability of the Huh7 cells after 48 h treatment of the flavonoids enriched extracts. This shows that the compounds which have been extracted out from the experimental plants *P. cineraria* and *C. tetragonoloba* are capable of inhibiting proliferation in the cancer cells.

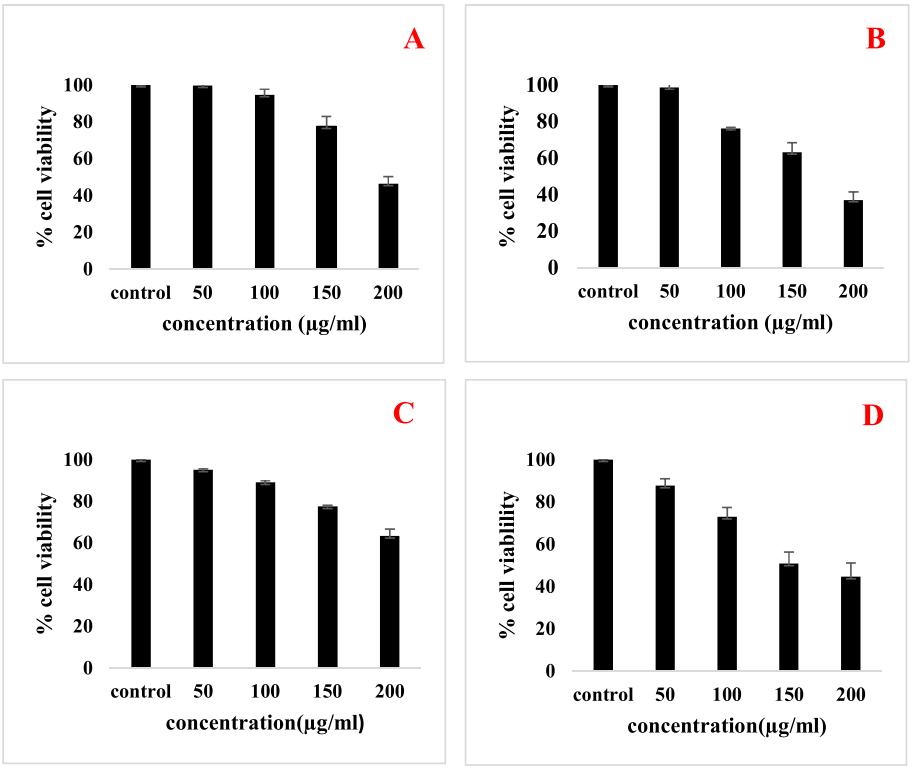

Figure 4. Cytotoxic effect of (A) *P. cineraria* crude, (B) *P. cineraria* flavonoid enriched fractions, C) *C. tetragonoloba* crude, D) *C. tetragonoloba* flavonoid enriched fractions against Hepatocellular carcinoma cell line Huh7. Percentage growth inhibition of Huh7 cells was assayed at different concentrations of extracts (50–200 µg/ml). Data represent mean ± SD of three replicates ($p < 0.05$).

3.3.3. *Flow cytometric analysis of apoptotic cells*

Bright field images of cells treated with the flavonoid enriched fractions PC-FEF and CT-FEF showed discrete rounding up of cells post-treatment, which is an indication of the apoptotic cells. Therefore, we got interested in analyzing the occurrence of the apoptosis in Huh7 cells by other methods. Figure 6 represents the results obtained after the AnnexinV-PI staining assay carried out to study the apoptosis in the selected cells. The four sections of the quadrant in each sub figures of Figure 6 from the upper left in a clockwise direction represent necrotic cells, late apoptotic cells, early apoptotic cells, and live cells, respectively. Of these four kinds of cell modes, necrosis and late apoptosis are generally considered as cell death, and thus, their values were omitted during calculating apoptotic cells.

The number of the live cells in untreated samples as observed from the lower left quadrant accounted for the 94.4% of the total count. The total cells that underwent necrosis are 4.82% whereas, as expected, only 1.04% of the cells comprised of the early and late apoptotic cells. When the number of the live cells was evaluated in case of the Huh7 cells treated with flavonoid enriched fraction, CT-FEF led to a drastic decrease to 67.98% in live cells whereas PC-FEF led to a decrease (87.63%, lower left quadrants). Similarly, when the number of necrotic cells was compared in all the three samples, it was found to be 4.82%, 30.28%, and 2.67 % in Untreated, CT-FEF, and PC-FEF respectively. The right upper and right lower quadrants represent the early and late apoptotic cells correspondingly. The CT-FEF treated cells showed that 1.74% of cells were undergoing apoptosis post treatment for 48 h whereas the PC-FEF treated cells accounted for total 9.7% apoptotic cells. This was a significant increase in the total fold increase in the apoptotic cells as compared to the untreated cells. Taking into consideration into only the early apoptotic cells from the above data, the CT-FEF showed a 2.2-fold increase and PC-FEF showed a substantial 9.3 times fold increase as against that of the untreated cells. The data obtained here implies that the compounds (mainly flavonoids) in the CFE were causing more of necrosis whereas those of the PC-FEF were able to cause cell death by inducing apoptosis.

Evaluating the apoptosis inducing potential of the extracts showed that the PC-FEF was more efficient in inhibiting the Huh7 cells by apoptosis as compared to that of CT-FEF. Hepato cellular carcinoma is the fifth most common cancer in the world, causing more than 1 million casualties in world in a year [22]. The conventional therapeutic regime is associated with an array of side effects which has generated the interest to look for the alternative treatments. A remarkable number of epidemiological investigations suggest that consumption of diet rich in fruits and vegetables can reduce the risk of various cancers, and Hepato cellular carcinoma is no exception to this [23]. An intensive review on the role of phtyochemicals in prevention and treatment of liver cancer by Bishayee *et al*) states that phenolics especially flavonoids decrease the tumor cell viability by inducing apoptosis [24]. The phytochemical profiling of the crude and enriched fractions of *P. cineraria* showed that purification process has enriched the flavonoids concentration in the flavonoids enriched fractions and multiple flavonoids/isoflavonoids be present in the pods. Flavonoids are reported to induce apoptosis in not only hepatocarcinoma but also other cancer cells by one of the several mechanisms which include, inhibition of expression of Ras proteins, inhibition of cell cycle progression, regulation of cyclin D1 expression, as inhibition of STAT1 etc.

The cytotoxicity effect of the extracts obtained from different aerial parts (excluding pods) of the *P. cineraria* plant has been studied

Table 2. IC_{50} of the purified extracts of pods/seeds of *P. cineraria* and *C. tetragonoloba* against Huh7 cell line.

Name of the extract	IC_{50} value in µg/ml
C. tetragonoloba Crude (CT-CE)	>200
C. tetragonoloba flavonoids enriched (CT-FEF)	150
P. cineraria Crude (PC-CE)	175
P. cineraria flavonoids enriched (PC-FEF)	150

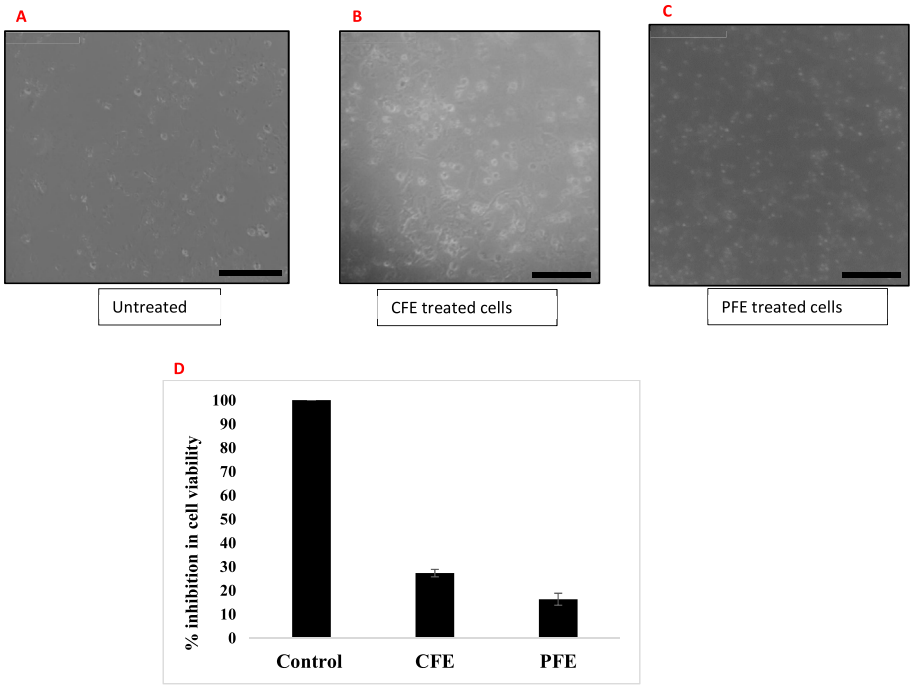

Figure 5. Bright field images of the A) untreated Huh7 cells, B) cells treated with CT-FEF at a concentration of 200 μg/ml, and C) cells treated with PC-FEF at a concentration of 200 μg/ml. Post-treatment for 48 h, cells were stained with trypan blue stain. Scale bar = 100 μm. D) % inhibition in cell viability of Huh7 cells in untreated (control) vs treated cells using flavonoids enriched fraction of P.cineraria (PFE) and *C. tetragonoloba* (CFE) was checked using Trypan blue dye exclusion assay. The concentration of extracts was chosen at 200 μg/ml. Data represent mean ± SD of three replicates ($p < 0.05$).

previously and reported in literature. Sumathi *et al.* showed that the crude methanolic extract of leaves of *P. cineraria* was showing 70 % decrease in cell viability of MCF-7 cells as compared to untreated cells [25]. Interestingly, the cytotoxicity of the plant extract was in the range of that with the commercial drug tamoxifen implying that the leaves are good source of the phytochemicals which are able to induce cytoxicity. Another study revealed that hydroethanolic extract of leaves was able to reduce tumor cell viability in N-nitrosodiethylamine induced Phenobarbital promoted experimental liver tumors in male Wistar rats [26]. When the efficacy of hydroalcoholic extract of leaf and bark was tested in Swiss albino mice against an Ehrlich ascites carcinoma tumor model, the haematological parameters of the treated mice were comparable with that of the healthy mice [27]. The investigation of anti-cancerous properties of *P. cineraria* was also extended to the green synthesis of silver and copper nanoparticles using the phytoextracts and it was observed that the phytochemicals bound nanoparticles were showing 10 % more cell death as compared to that of cell death induced by crude phytoextract indicating that there is an enhancement of the nanoparticles bioactivity via the compounds present in the *P.cineraria* [28].

Similarly, the anti-proliferative potential of the crude ethanolic extracts of seeds of *C. tetragonoloba* has been previously tested against Acute Myeloblastic Leukemia and at concentration of 100 μg/ml of the extract, approximately 40 % of cell death was observed [19]. Another study showed that hydroethanolic extract of guar seeds collected from the Egypt region, was inducing cytotoxicity on intestinal carcinoma (CACO-2) cell line, colon carcinoma cell line (HCT116) and human prostate carcinoma cell line (PC3) with IC_{50} values of 101.1, 41.0, and 40.5 μg/ml respectively [29]. The lower IC_{50} of the purified flavonoid enriched fraction obtained from the seeds of *C. tetragonoloba* indicates that the cumulative concentration of the flavonoids and isoflavonoids

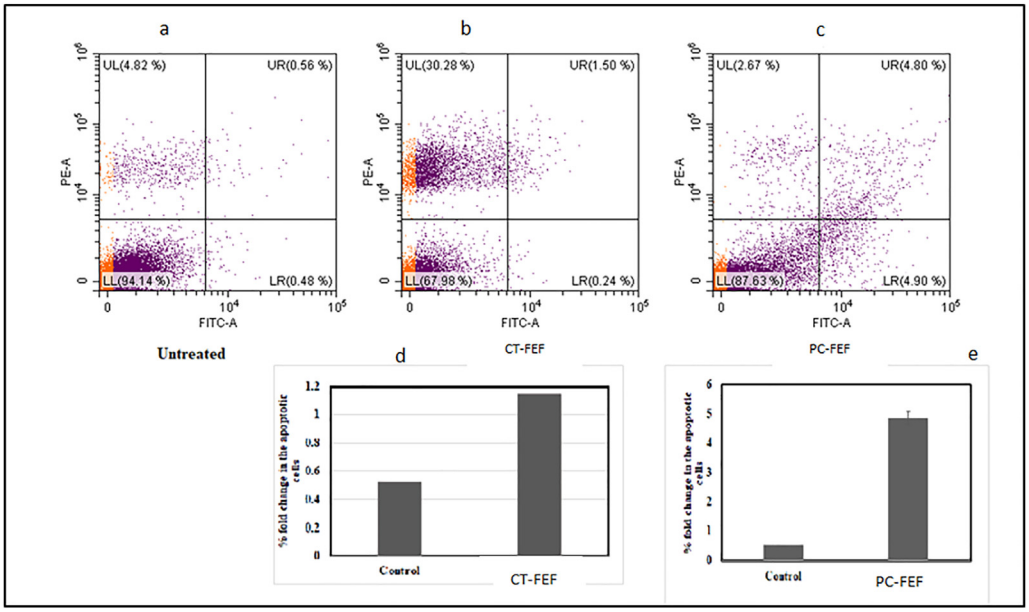

Figure 6. Presence of apoptotic cells was evaluated by AnnexinV-PI staining after treatment of Hepato cellular carcinoma (Huh7) cells with a) untreated, b) CT-FEF and c) PC-FEF at 200 μg/ml dose for 48 h through flow cytometry. In the figure, cells in lower right and upper right quadrant represent early and late apoptotic cells, respectively. A fold increase in apoptotic cells was calculated with respect to untreated control (taken as arbitrary unit "1") and is represented through bar diagram for d) *Cyamopsis tetragonoloba* and e) *Prosopis cineraria*.

have been increased due to the usage of the Amberlite XAD 7 HP column matrix.

4. Conclusions

From the multitude of assays performed and the studies documented previously, it could be said that the *P. cineraria* and *C. tetragonoloba* extracts obtained from seeds/pods are rich source of antioxidants and anticancerous potential. When compared with the previously published data regarding the biological activities of other aerial parts of these plants, the activities present in edible seeds/pods were found to be significantly high. The increased resistance in the cancerous cells against a wide range of conventional treatments and the harmful side effect of the drugs have led the research for new substitutions. The dietary intervention of the phytochemicals on the disease prognosis and treatment has also been demonstrated extensively in diverse reports which makes establishing the phytochemical profile of edible plants much relevant [30]. If investigating non-edible parts of the plants have facilitated in discovering the potential drug molecules against diseases, phytochemical profiling of the edible parts, moreover for the local plant species, play a crucial role in recognizing their importance and extended utility for the local population. The vast flora and fauna of the Indian subcontinent offers a congregation of plant species which are yet to be explored. Thus, the present study provides a substantial evidence on the biological potential of two regional legumes viz *P. cineraria* and *C. tetragonoloba.* Besides this, we have tried to see if the purification process carried out to obtain the flavonoids enriched fraction increases or decreases the biological activities pertaining to anti-oxidants and anti-cancerous strategies. We have observed that flavonoid enriched fraction had higher IC_{50} values as compared to their crude counterpart for antioxidant activities indicating that purification process might have caused the removal of some non-flavonoids compounds which were exhibiting a compounded antioxidant effect. Whereas an inverse observation was recorded while comparing the anticancerous activities of crude and flavonoid enriched fractions isolated from both the plants implying that major flavonoids present in them have a significant anti-cancerous potential against the Huh7 cell lines. Further studies will be required to throw light on the underlying mechanisms via which the flavonoids present in *P. cineraria* and *C. tetragonoloba* induce apoptosis in Huh7 hepatocellular carcinoma cell lines.

Declarations

Author contribution statement

Vidushi Asati: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Abhilasha Srivastava: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sudeshna Mukherjee, Pankaj Kumar Sharma: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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