



SIMULTANEOUS QUANTIFICATION OF FLAVONOIDS BY USING RP-HPLC, PHYTOCHEMICAL PROFILING AND CYTOTOXIC ACTIVITY OF *HIPPOPHAE RHAMNOIDES* BERRIES.

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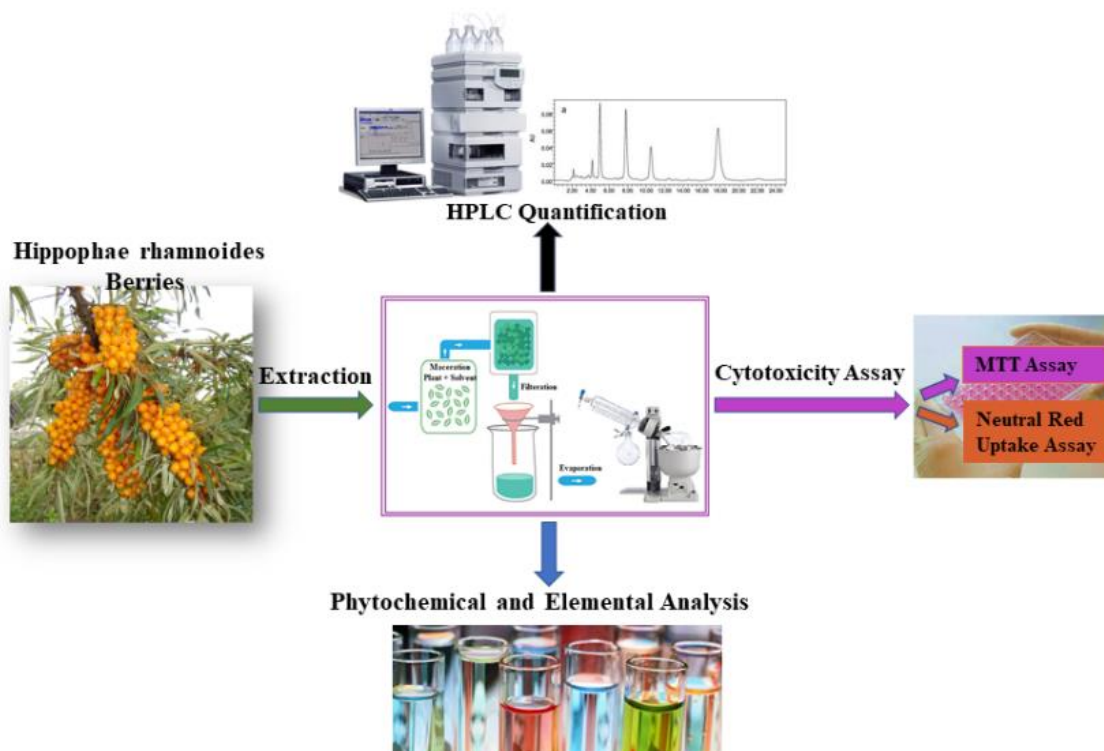
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Graphical representation of the abstract



Abstract

Hippophae rhamnoides commonly known as Seabuck thorn is native to Europe and Asia. This plant has been reported for the treatment of pulmonary, gastrointestinal, cardiac and blood disorders. The

present study, for the first time, was undertaken to explore the biological and phytochemical profiling of the methanol, ethanol and aqueous extracts of berries of *H. rhamnoides*. Phytochemical analysis revealed the presence of alkaloids, glycosides, tannins, flavonoids and terpenoids. Proximate analysis i.e. moisture content, pH and ash values were in normal range indicating the stability of crude powder. Elemental analysis indicated the presence of essential mineral nutrients in trace amounts. RP-HPLC based quantification revealed the presence of significant amount of flavonoids ranging from 0.005 - 5.450 µg/ml. The plant exhibited considerable levels of cytotoxicity against BHK-21 cell line with the LC₅₀ values ranging between 75-330 µg/ml, while extracts at concentration of 400 µg/ml showed significant cytotoxicity for Vero cell line.

Keywords: Hippophae; Phytochemicals, HPLC; flavonoids; cytotoxicity.

1. Introduction

Fruits are the crucial part of human diet. They provide health benefits and helps in preventing illness. Fruits contain variety of nutrients including vitamins, minerals, bioactive compounds, and phytochemicals, especially antioxidants which help in reducing risk of chronic diseases. Fruits are naturally rich in fiber, potassium, iron, vitamin C and low in sodium, calories and fat [1]. Other than the conventional source of foods which are known as the staple foods, some foods are taken less frequently and/or on certain occasions and are called as non-conventional foods e.g., wild fruits [2]. Phytochemical and nutritional composition of these common conventional food sources have been studied extensively and their nutritive value is well established [3]. Despite the fact that non-conventional food plants are widely consumed and are likely to be nutritious [4, 5], sufficient information on the phytochemical composition of the wild fruits are still missing. Practically very less information available on the nutritive value of the wild non-conventional fruit plants which significantly contribute to the nutrient uptake of the local population [3]. Thus, exploring and understanding the phytochemical composition and antioxidant potential of these non-conventional plants may encourage utilization of these plants as a source of antioxidants and their acceptability for nutraceutical and pharmaceutical purposes.

H. rhamnoides belonging to family Elaeagnaceae is native to the cold temperate regions of Europe and Asia [6]. Its fruits is primarily valued as a rich source of vitamins, flavonoids, lycopene, carotenoids, and phytosterols which are therapeutically important and contains potent antioxidants [7-9]. Literature search showed very less information on this highly valuable fruit. It is, therefore, the objective of this study was to determine the secondary metabolites, proximate analysis, elemental nutrients, RP-HPLC flavonoids quantification and cytotoxic potential of this fruit. The outcomes emerging from the current study will evaluate the potential use of *H. rhamnoides* fruit in nutraceutical and pharmaceutical formulations as dietary supplements for humans.

Experimental

Plant material.

H. rhamnoides L. berries were collected from the northern areas of Pakistan and identified by Prof. Dr. Zaheer-ud-Din, Department of Botany, G.C University Lahore, and Pakistan. The sample was deposited in herbarium, and a Voucher no 3421/A was issued.

Extraction

The fresh berries were shade dried for 15 days, then pulverized to a fine powder and stored in amber color bottles. The powder (500g) was extracted separately with methanol, ethanol and distilled water respectively, by using 3 L of each solvent and soaked for 72 h with occasional shaking. The resultant extracts were dried by using rotary evaporator and the residues of each extract were weighed and stored at 4°C in a refrigerator.

Proximate Analysis

Moisture content, pH, total ash contents, acid insoluble ash, water insoluble ash, water soluble ash and sulphated ash were performed according to the standard protocols of AOAC (2006).

Elemental analysis

Lead, cadmium, copper, zinc, manganese, magnesium, cobalt and chromium was measured by using atomic absorption spectroscopy using method given in the literature [10].

Simultaneous quantification of flavonoids by using reversed phase high performance chromatography (RP-HPLC)

Flavonoids were estimated by using developed method with little modifications [11]. The analysis were performed using RP-HPLC model (LC- 10A, Shimadzu, Kyoto, Japan) equipped with vacuum degasser (DGU-20A5), two quaternary pumps (LC-20AT), auto sampler (SIL-20AC), column oven (CTO-20AC) and UV-DAD detector (SPD-M20A). The detector was operated in a sensitivity range of 0.005 AUFS. Output of the range was 15mV and the data acquisition was performed with LC-10 software. All the samples as well as standard solution were filtered by using 0.45µm PTFE syringe filters. Each sample/standard solution (20µL) were eluted through the column C18 Merck (5µm, 4.6 x 250 mm particle size) using isocratic mobile phase containing trifluoroacetic acid 50%: acetonitrile and methanol 50%, at flow rate of 1.0ml/min. The temperature of the column was maintained at 30 °C and detection was carried out using DAD detector, at wavelength 360nm. The peaks obtained were compared to the standard using retention time. The concentrations of the standards in the different extracts can be calculated using the formula

$$\text{Concentration of unknown} = \frac{\text{peak area of sample solution}}{\text{peak area of standard solution}} \times \text{conc. of standard}$$

Cytotoxicity Assay

Neutral Red Uptake assay:

Cell viability was determined by neutral red uptake test (NRU) [12]. Vero Cells were maintained in the Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1x Penicillin-Streptomycin (PS) and seeded in 10,000 cells per cm² density in 24-wells culture plate by adding 1.5ml of media containing cells. The micro titer plates were incubated for 24 hours at 37 °C, humidified atmosphere with 5% CO₂ incubator to ensure proper cell attachment. After incubation of 24 hours, cells were treated with the sample solution by adopting two fold serial dilutions (400µg/ml, 200µg/ml, 100µg /ml). The samples were in four replicates of each extract and control. The plates were placed in incubator again for 24h. After incubation media was removed and cells were washed with PBS (Phosphate Buffer Saline) to remove non adherent and dead cells. After above, 200 µl of neutral red media was added (3.3%w/v). The plates were incubated again for 2 hours in CO₂ incubator (5%) at 37°C allowing the lysosomes of viable cells to take up the dye. Cells were then washed with PBS to remove any extracellular dye and 200µl of extraction solution (H₂O: acetic acid: ethanol) (49: 1: 50) was added in each well. The plates were again incubated with 5% CO₂ for 10mins. Once the cells release their color the plates were placed in the spectrophotometer. The optical density was taken at 570nm. The readings were obtained in four replica for each concentration which were averaged and absorbance was plotted against concentration of test samples [13].

MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cytotoxicity assay was performed according to the method Nemudzivhadi *et al* [14] with slight modifications. BHK-21 Cells were maintained in the DMEM supplemented with 10% fetal bovine serum (FBS) and 1x Penicillin-Streptomycin (PS). 100 µl of cells (1.5×10⁴) were seeded in each well of 96 well plate and were incubated at 37°C in 5 % Co₂ for overnight. Cells with above 80 % confluency were

treated with the H.R extracts at a concentration range of (0.1-400 ug/ml). After 48h incubation, the medium was aspirated and MTT solution (5mg/ml) was added, and incubated again for 4h at 37°C in 5 % CO₂. After 4h, the wells were solubilized with 100ul DMSO per well and the absorbance was measured by microplate reader infinite@pro 200 Tecan (Switzerland) at a primary wavelength of 570 nm and reference wavelength of 670nm. Each plate contained the sample, negative control and blank. DMSO (1% V/V) was used as a negative control. The % of cell viability and LC50 were calculated as:

$$\% \text{ cell viability} = \frac{\text{Absorbance sample} - \text{Absorbance control}}{\text{Absorbance control}} \times 100,$$

Statistical analysis

The obtained results were expressed as mean ± SEM. Statistical analyses were performed on SPSS version 24 by using one way ANOVA followed by Dunett's test.

2. Results and Discussion

2.1. Phytochemical/Proximate and Elemental Analysis:

Secondary metabolites are considered as bioactive chemical constituents of plants origin [15]. The results of phytochemical screening revealed the presence of various medicinally important phytochemicals such as alkaloids, glycosides, flavonoids, tannins and terpenoids (Table. 1). Carbohydrates, proteins, amino acids, fats and fixed oils were also present. These results are consistent with previously reported findings [16].

The objective of physicochemical analysis was to standardize the natural medicinal plant. Stability of natural products is greatly dependent on moisture contents. Less moisture content is needed for prevention of chemical decomposition and microbial contamination in the natural products. The quality and purity of powdered crude drugs are determined by estimating ash value. Physicochemical properties i.e. moisture content, pH and ash values of crude powder of *H. rhamnoides*. and were found within the normal recommended range (Table. 2) and indicates stability of these crude powder because fluctuating values are reprehensive of degradation of phytochemical constituents [17].

Trace element plays a crucial role in the medicinal value of a plant, in health and to cure disease. They play a nutritive, catalytic and balancing function in plants [18]. Magnesium is very important for regulating electrical potential in nerves and membranes. It also plays an important role in improving insulin sensitivity, protect against diabetes and its complications and also reduce blood pressure [19]. Zinc is an essential trace element for humans and other animals required for the function of over many enzymes. It is the second most abundant trace metal in humans after iron and it is the only metal which appears in most of enzyme classes [20]. Manganese is an important element for human health, essential for development, metabolism, and the antioxidant system. [21]. Elemental analysis of the berries shown in Table S3, indicates the presence of Zinc, Manganese and Magnesium in trace amounts. All these elements are imperative for muscles structures, bone functioning, cell signaling and apoptosis [22, 23].

Table 1: Qualitative Phytochemical screening of different extracts

Phytochemical constituents	Methanol extract	Ethanol extract	Aqueous extract	n-hexane extract	Chloroform extract	Ethyl acetate extract	n-butanol extract
Alkaloids							
Dragendroff s' test	+	+	-	+	+	+	+
Mayer s' test	+	+	-	+	+	+	+
Hager s' test	+	+	-	+	+	+	+
Wagner s' test	+	+	-	+	+	+	+
Glycosides							
Killer killiani test	+	+	-	+	+	+	+

Tannins							
Ferric chloride test	-	+	-	-	-	+	+
Flavonoids							
Lead acetate test	+	+	+	-	-	+	+
Alkaline reagent test	+	+	+	-	-	+	+
Carbohydrates							
Molish s' test	+	+	+	-	-	+	+
Seliwanoffs' test	+	+	+	-	-	+	+
Fehling s' test	+	+	+	-	-	+	+
Proteins and amino acids							
Millon s'test	+	+	+	-	+	-	-
Biuret test	+	+	+	-	+	-	-
Fats and fixed oils							
Stain test	-	+	-	-	+	+	+
Saponification test	-	+	-	-	+	+	+
Terpenoids							
Salkowski test	+	+	+	+	+	+	+

Table 2: Proximate analysis of crude powder of *Hippophae rhamnoides* berries

Sr. No	Physicochemical properties	Percentage (%)
1	PH	5.2
2	Moisture content	8
3	Total ash	5.9
4	Acid insoluble ash	3.70
5	Water insoluble ash	3.2
6	Sulphated ash	27

Table 3: Elemental analysis of crude powder *Hippophae rhamnoides* berries

Sr. No	Elements	Concentration in mg/kg
1	Lead	-
2	Cadmium	-
3	Copper	-
4	Zinc	0.9148
5	Manganese	0.015
6	Magnesium	0.626
7	Cobalt	-
8	Chromium	-

2.2. Simultaneous Quantification of Flavonoids by RP –HPLC analysis :

The antioxidant and free radical scavenging activities of most plants extract were mainly due to the presence of flavonoids [24]. Flavonoids are the secondary metabolites which play an immensely important role in human health and nutrition. RP -HPLC analysis on the *H. rhamnoides* methanol, ethanol and aqueous extracts was performed to obtain insights into the possible chemical composition of as indication whether they contain flavonoids as possible contributor to cytotoxic and antioxidant activity of the extracts. Reverse phase HPLC-PDA based profiling was used for quantitative analysis of selected plant flavonoids and the chromatographic fingerprinting was done by comparing the retention time and UV spectra of the reference compounds with those of the test samples. It was observed that all these three flavonoids (Myricetin, Quercetin, Kaempferol) were

present in all extracts. as shown in Table 4. A significant amount of quercetin (1.318 $\mu\text{g}/\text{mg}$) and myricetin (0.645) was quantified from crude methanol extract. Typical chromatogram of standard as well as compounds detected from various fractions are presented in (Fig. 1-4).

Table 4: Concentration of Myricetin, Quercetin and Kaempferol in different extracts *Hippophae rhamnoides* berries

Sr. No.	Sample	Concentrations in $\mu\text{g}/\text{ml}$			Concentrations of compounds(g)		
		Myricetin	Quercetin	Kaempferol	Myricetin	Quercetin	Kaempferol
1	Methanol extract	0.645	1.318	0.043	0.008	0.004	0.117
2	Ethanol extract	0.731	1.577	0.049	0.003	0.013	0.407
3	Aqueous extract	0.157	0.102	0.005	0.796	1.227	23.481

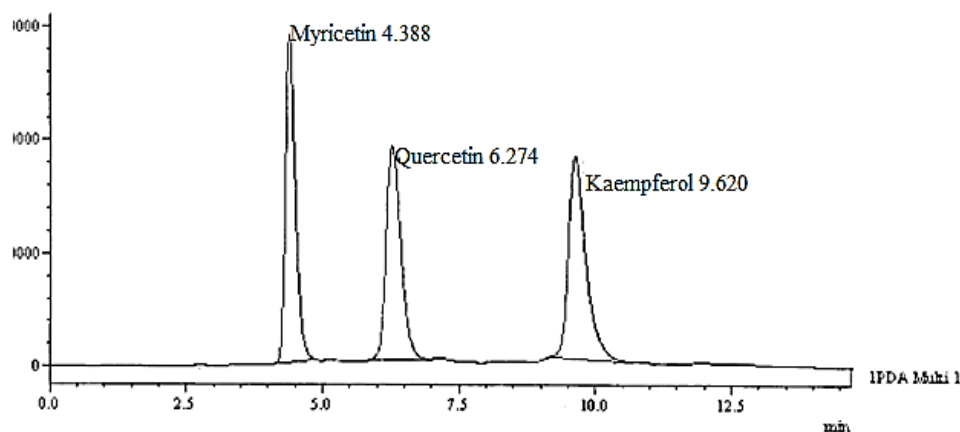


Figure 1: Standard HPLC chromatogram of flavonoids

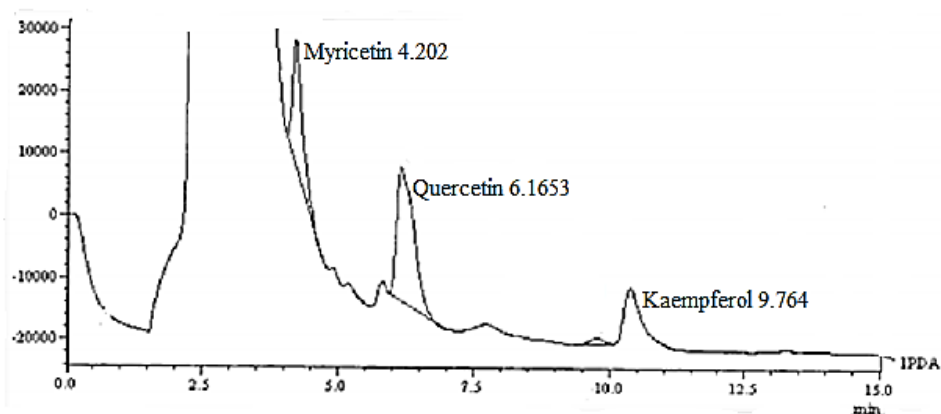


Figure 2: HPLC chromatogram of *Hippophae rhamnoides* berries methanol extract

2.3. Cytotoxicity Assays:

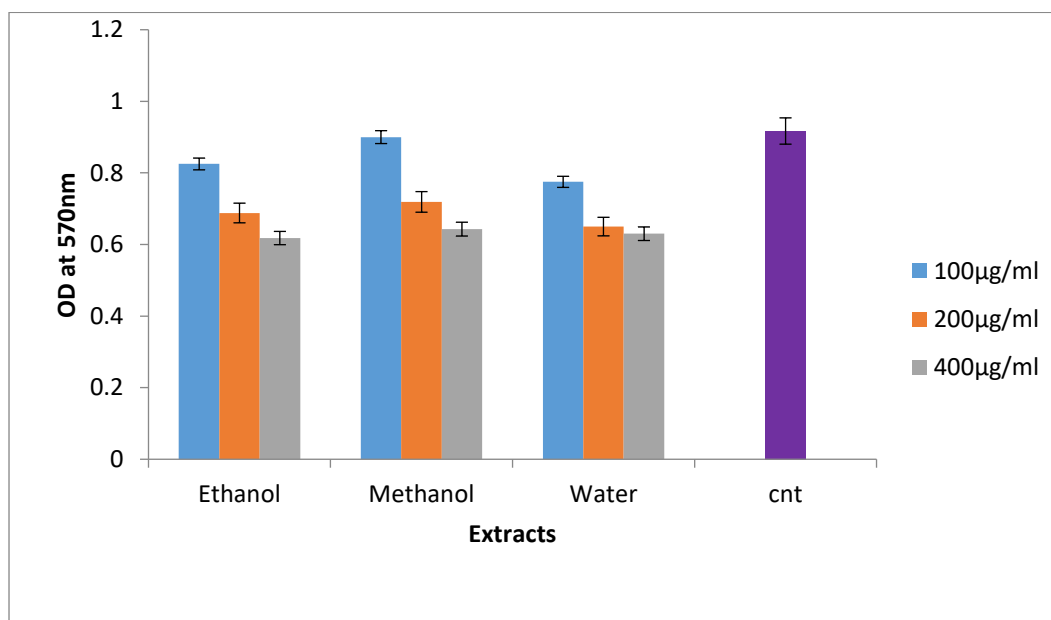
The cytotoxic potential was evaluated by MTT and Neutral red uptake assay. MTT assay measures the cell viability based on the reduction of yellow tetrazolium MTT dye to purple formazon crystals by mitochondrial dehydrogenase enzymes. The amount of formazon crystals reflects the number of metabolically active cells [25]. While, Neutral red uptake assay uses dye that accumulates in the lysosomes of the cells. Surplus amount of dye washed off and cell damage exhibited by bound dye in cell sheets calculated calorimetrically [13, 26]. Cytotoxic potential of all extracts was evaluated against BHK-21 cell line by performing MTT assay. All extracts showed cytotoxic effect in dose dependent manner. Among all tested extracts, cytotoxic potential of ethanol was strongest with highly significant decline in cell numbers at concentration at 50 $\mu\text{g}/\text{ml}$ with LC_{50} of 75 $\mu\text{g}/\text{ml}$ (Fig. 9) while significant decline in cell numbers was observed in aqueous extract at 200 $\mu\text{g}/\text{ml}$ having LC_{50} at 330 $\mu\text{g}/\text{ml}$ (Fig. 10). The least significant cytotoxic effect was showed by methanol extract

at concentration of 400 µg/ml with LC₅₀ at 170µg/ml (Fig. 6). The comparison of the cytotoxic potential of all these extracts against vero cell line can be seen in Fig 9. For neutral red uptake assay, the comparison of different extracts showed that, at a dose of 400µg/ml ethanol and aqueous extract showed significant decline in absorbance than methanol when compared with the control. All the extracts at doses of 200 µg/ml and 100 µg/ml showed same significant results in comparison to control as shown in table. 5 and Fig. 9. Aqueous extract at all doses showed highest cytotoxic potential as compare to other extracts Fig 8.

Table 5: Cytotoxic effects of *Hippophae rhamnoides* berries extracts on VERO cell line

DOSE	Aqueous extract	Methanol extract	Ethanol extract
Control	0.919±0.001		
100 µg/ml	0.775±0.004	0.901±0.001	0.823±0.001
200 µg/ml	0.650±0.001	0.710±0.001	0.688±0.001
400 µg/ml	0.630±0.001	0.643±0.001	0.618±0.002

Values are expressed as mean ± Standard mean error (n=3).



5: Graphical presentation of absorbance of control and extracts on Vero cell line

Table 61: Cytotoxic potential of *Hippophae rhamnoides* berries extracts on BHK-21 cell line

Concentrations (µg/ml)	OD(optical density)±SEM			Percentage survival		
	Methanol	Ethanol	Aqueous	Methanol	Ethanol	Aqueous
0.19	2.06±0.012	1.25±0.101	2.16±0.022	93	96	97
0.39	2.03±0.012*	1.26±0.066	2.14±0.014	92	93	97
0.78	2.03±0.012	1.29±0.052	2.11±0.007	87	90	90
1.56	1.89±0.035	1.34±0.045	2.04±0.024	82	88	89
3.12	1.72±0.017*	1.35±0.017*	2.18±0.094	65	78	84
6.25	1.65±0.046*	1.75±0.099	1.97±0.059	70	77	87
12.5	1.60±0.034*	1.79±0.089*	2.02±0.014*	61	67	78
25	1.52±0.024*	2.05±0.021*	1.84±0.062*	59	52	73
50	1.57±0.015*	2.02±0.022*	1.75±0.064*	55	46	62
100	1.59±0.009*	2.11±0.068*	1.64±0.079*	54	43	56
200	1.47±0.005*	2.05±0.019*	1.42±0.045*	52	44	50
400	1.48±0.024*	2.12±0.065*	1.37±0.033*	50	42	46

Values are expressed as mean \pm Standard mean error (n=3). *p value less than 0.05 (P<0.05), significant value.

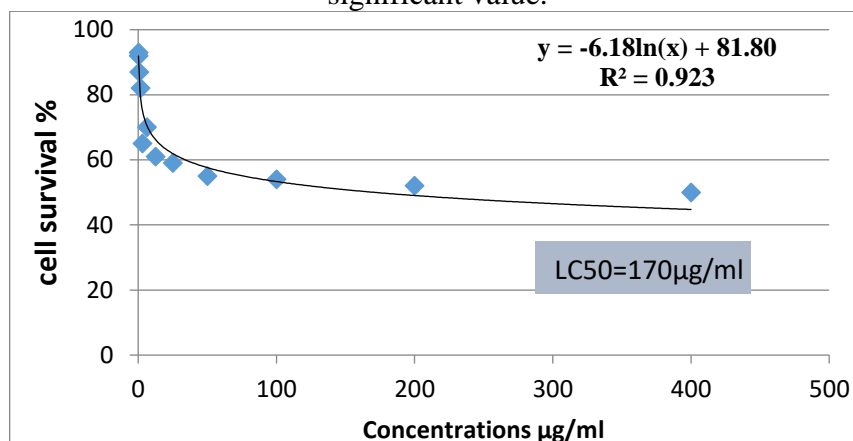


Figure 61: Cytotoxic potential of *Hippophae rhamnoides* berries methanol extract (BHK-21 cell line)

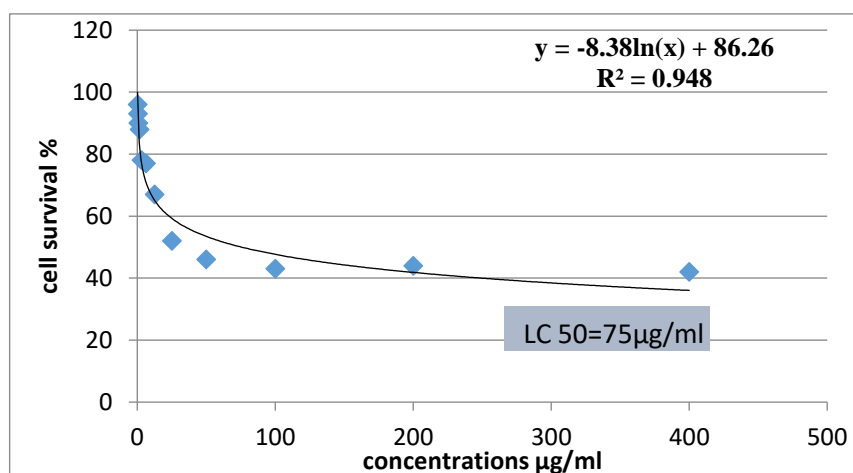


Figure 7: Cytotoxic potential of *Hippophae rhamnoides* berries ethanol extract (BHK-21 cell line)

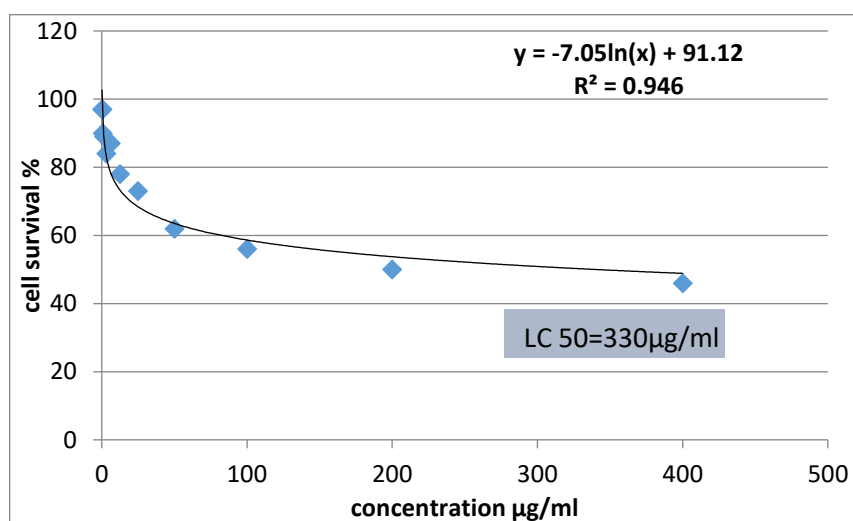


Figure 8: Cytotoxic potential of *Hippophae rhamnoides* berries aqueous extract (BHK-21 cell line)

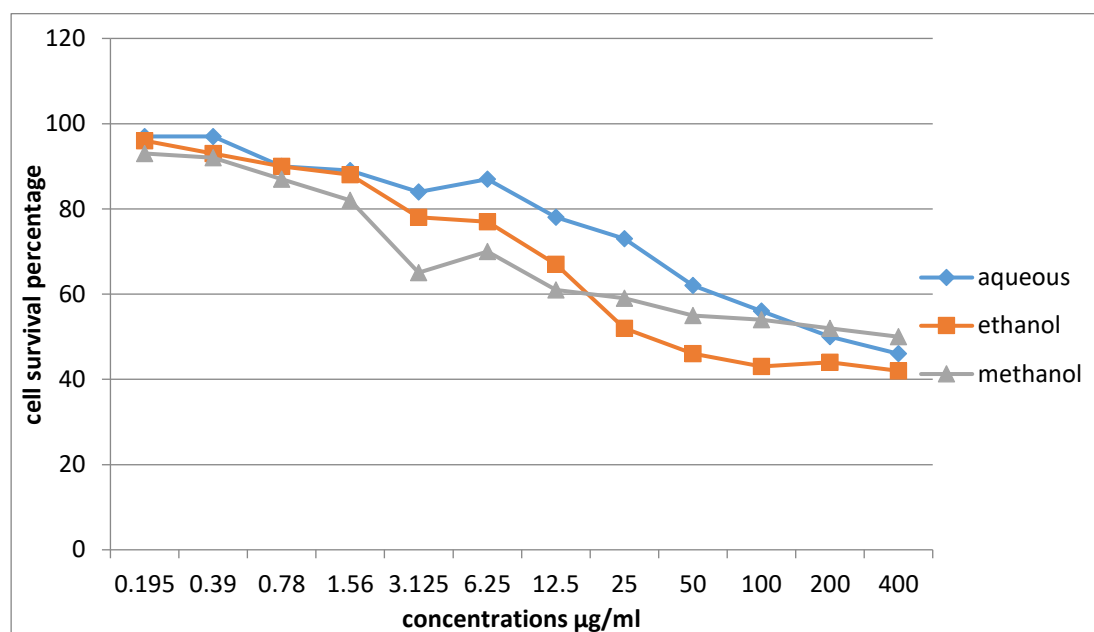


Figure 9: Comparison of cytotoxic potential of different extracts on BHK-21 cell line.

3. Conclusion

The present report is the first comprehensive study of the phytochemical and biological profiling of *H. rhamnoides* berries. The study reveals RP- HPLC has proved to be the method of choice for the separation and quantification of variety of flavonoids in different extracts. The present study may proceed for further investigations for the isolation of bioactive secondary metabolites responsible for the observed activities. The fruit of this plant could serve as novel scaffolds in drug discovery.

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