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Research Article

Isolation and Identification of Flavonoid Fractions from the Leaves of *Volkameria inermis* and its *In-vitro* Cytotoxic Study

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ABSTRACT

The current study aims to optimize the solvent for the isolation of flavonoid fractions and identification of flavonoid components through HPTLC and HPLC techniques and it's *in vitro* cytotoxic study against Ehrlich Ascites Carcinoma (EAC) cell lines. *In vitro* cytotoxic assay tryphan blue dye exclusion was carried out against EAC cell lines. The results revealed that ethyl acetate was found as the best solvent for the isolation of flavonoid fractions and several kinds of flavonoids such as quercetin and kaempferol were found as the major flavonols in this plant. *In vitro* cytotoxic study suggests that the flavonoid fractions of this plant possess potent antitumor activity against EAC cell lines.

Keywords: High Performance Liquid Chromatography (HPLC), High Performance Thin Layer Chromatography (HPTLC), Ehrlich Ascites Carcinoma (EAC) cell lines, Flavonols, Kaempferol, Quercetin.

INTRODUCTION

Medicinal plants have gained importance in past decades due to their extensive continuum of pharmacological effects. Specific concentration of phytochemicals from the plants produces cancer chemopreventive effects with no significant toxicity[1]. Flavonoids are polyphenolic compounds that are ubiquitously found in plants. These compounds possess many mechanisms of actions such as carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis, antioxidation and reversal of multidrug resistance or a combination of these mechanisms[2]. Based on this significance, flavonoid fractions are isolated from the leaves of *Volkameria inermis* and its *in vitro* cytotoxic property was studied against EAC cell lines. *Volkameria inermis* is an evergreen shrub of 1-1.8m tall, that adapts a climbing habit. Traditionally it was used as an ornamental plant in home gardens. This plant was rich in secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids etc[3].

MATERIAL AND METHODS

Plant Collection

Fresh leaves of *Volkameria inermis* were collected from Tamil Nadu Agriculture University (TNAU) Coimbatore, India. Authentication was done at Botanial Survey, Tamil Nadu Agriculture University (TNAU) Coimbatore, India (BSI/SRC/5/23/2015/Tech/2082).

Extraction of Flavonoids

The collected leaves were washed thoroughly in tap water, shade dried and pulverized. Flavonoids were extracted using 10g of pulverized plant material and were extracted with 100ml of petroleum ether (b.p. 40- 60 C) in a shaker for 24 hours. The solid residue obtained was then treated with ethyl acetate for 24 hours and filtered. The resulting filtrate was concentrated using flash evaporator for complete solvent removal. The freeze dried material was extracted with boiling acetone and the residue was concentrated at atmospheric pressure. This concentrated residue was extracted successively with light petroleum ether (b.p. 40- 60°C) and benzene to remove non flavonoid and other matter. This protocol was repeated for the preparation of flavonoid fractions in aqueous, chloroform and diethyl ether[4].

Determination of Total Flavonoid Contents

The reaction mixture consists of 1ml of plant extracts, 0.6 ml of sodium nitrite (5% w/v), 0.5 ml of aluminium chloride (10% w/v), 3ml of sodium hydroxide (4.3%w/v), and distilled water is added to make the volume to 10ml. The reaction mixture was allowed to stand for 15 minutes before reading the absorbance. Absorbance was measured at 500nm in a UV- Vis spectrophotometer[5]. Quercetin was used as a standard and results were calculated as quercetin equivalents (Quercetin eq., mg/ml) of *Volkameria inermis.*

Analysis of Flavonoids

The flavonoid fractions in the sample was analysed using HPTLC and HPLC.

HPTLC

High Performance Thin layer chromatography (HPTLC) studies were carried out using the optimized solvent system chloroform: ethylacetate: glacial acetic acid in a ratio 12:7:1v/v. Chromatography was performed at 25±2°C on precoated aluminium plates as mentioned above of size 10x10cm and 0.2mm thickness were used. The standards quercetin and kaempferol in the concentration of 1mg/ml were applied for the

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Table 1: Total Flavonoid Content in Different Solvent Systems.

Organic solvents	Total flavonoid content of *Volkameria inermis* (mg/g leaf)
Ethyl acetate	1.88±0.08
Aqueous	1.67±0.11
Chloroform	0.47±0.03
Diethyl ether	0.7±0.174

The values are mean ± SD of triplicates

quantification. A volume of 20µl of samples dissolved in HPLC grade methanol along with the standards were applied to the plates as 6/8mm bands, 8mm from the bottom, 15mm from the side, under a stream of nitrogen, by means of a CAMAG (Switzerland) Linomat V semiautomatic sample applicator fixed with a 100µl Hamilton HPTLC syringe. The spraying rate was 150 nLs^{-1}. Linear ascending development to a distance of 80mm was carried out on 10x10 cm twin trough chamber saturated with the mobile phase, pre-saturated with the solvent for 30min. After run, the plates were removed from the chamber, air dried and visualized at 254 and 366nm. Densitometric scanning was performed with
Camag TLC scanner III controlled by CAMAG CATS 4 integration software at 254nm. The slit dimensions were 4x0.3/6x0.3mm and the scanning speed was 20 mm s^{-1}. The Rf values of the resolved spots were noted. Evaluation was by peak areas with linear regression[6].

High Performance Liquid Chromatography

The analysis of flavonoids in the samples was carried out by using HPLC. The HPLC conditions adjusted were as follows. The samples were filtered through a 0.45µm PTFE syringe tip filter and were injected (10µL) through the Column: Dynamax C18 (250 x 4.6 mm, 5 µm) BDS Hypersil RP-C18 column (Thermo, 5µm, 120Å, 250mm × 4.6mm) at column temperature 40˚C. The liquid chromatography system was equipped with the photo diode array detector (PDA) and a vacuum degasser and analytical data was evaluated by using X-Caliber data processing system (2.0 SR2). The mobile phase composed of methanol:water (7:3) was eluted at a flow rate of 1mL/min and the effluent was monitored at 339 nm by UV detector[7].

Cell Line

EAC cells were obtained from Amala Cancer Research Institute, Thrissur, Kerala, India and used for *in vitro* cytotoxic study.

In vitro Cytotoxicity

The *in vitro* cytotoxic effect of the flavonoid fractions of *Volkameria inermia* was evaluated against EAC cell lines using trypan blue dye exclusion method.

Trypan Blue Dye Exclusion Method

The EAC tumor cells propagated in the peritoneal cavity of the mice were taken and washed with saline Phosphate Buffered Saline thrice by centrifuging at low speed. 0.1ml containing $1x10^6$ cells was used for the *in vitro* assay. Various concentrations of the sample were incubated with EAC cell lines at 37˚C for three hours. At the end of the incubation period 0.1 ml trypan blue was added and layered the cells on the haemocytometer for counting. The dead cells were blue in colour and counted to calculate the percentage of dead cells[8].

Per cent Cytotoxicity = Dead cell count / (Dead cell count + Viable cell count) × 100

RESULTS AND DISCUSSION

The total flavonoid content of the residues of ethyl acetate, aqueous, chloroform and diethyl ether solvents of *Volkameria inermis* were assessed by aluminium chloride method and are shown in Table 1.

Total flavonoid contents of *Volkameria inermis* were found to be more in ethyl acetate fraction than that found in aqueous, chloroform and diethyl ether. So, the flavonoid fraction of ethyl acetate of *Volkameria inermis* was used for the following *in vitro* and *in vivo* studies.

The total phenol, tannin, alkaloid and flavonoid in *Hibiscus tiliaceus* Linn. wood extracts were evaluated using petroleum ether, ethyl acetate and methanol as solvents. The results showed that ethyl acetate extract has high concentration of total phenol, tannin, alkaloid and flavonoid contents[9].

Analysis of Flavonoids

High Performance Thin Layer Chromatography

The analysis of flavonoid components such as quercetin and kaempferol was found to be highly reproducible in the mobile phase chloroform: ethyl acetate: acetic acid (12:7:1). The calibration curves for the compounds were found to be linear (Fig 1,2,3,4 and Plate 1, 2). A peak purity test was performed by comparing the peaks of the standard with that of the sample peaks.

Quercetin and rutin was present in the ethanolic leaf extract of *Acacia catechu*[10]. The presence of alkaloids and phenolic compounds (Quercetin) of *Barleria cristata* Linn. leaves and Ferulic acid in *Lycopodium clavatum* was reported in HPTLC fingerprint[11-12].

High Performance Liquid Chromatography

Identification of the constituents was performed by HPLC. The resulting chromatogram (Fig 5) is a plot of time vs area. Identification of individual flavonoids was carried out on the basis of retention time. Figure 6, represents the presence of quercetin and kaempferol with their retention time corresponding to 3.7 and 4.2 respectively

The presence of three flavonoids namely quercetin, kaempferol and myricetin in the methanolic extract of *Euphorbia wallichii* using HPLC was identified[13].

In vitro cytotoxic effect of the flavonoid fractions on EAC cells (Tryphan blue method)

The flavonoid fraction of *Volkameria inermis* was found to be more cytotoxic against Ehlrich Ascites Carcinoma. The 60µg/ml concentration produced 50% *in vitro* cytotoxicity (Fig 7). The effect of *in vitro* cytotoxicity increased with increase in the concentration of flavonoid fractions.

The ethanolic and water extracts of *Ocimum basilicum* possessed high cytotoxic and antioxidant properties against EAC cell lines[14]. Similarly, *in vitro* cytotoxic study was carried on the ethanolic extract of aerial parts of *Salvia*

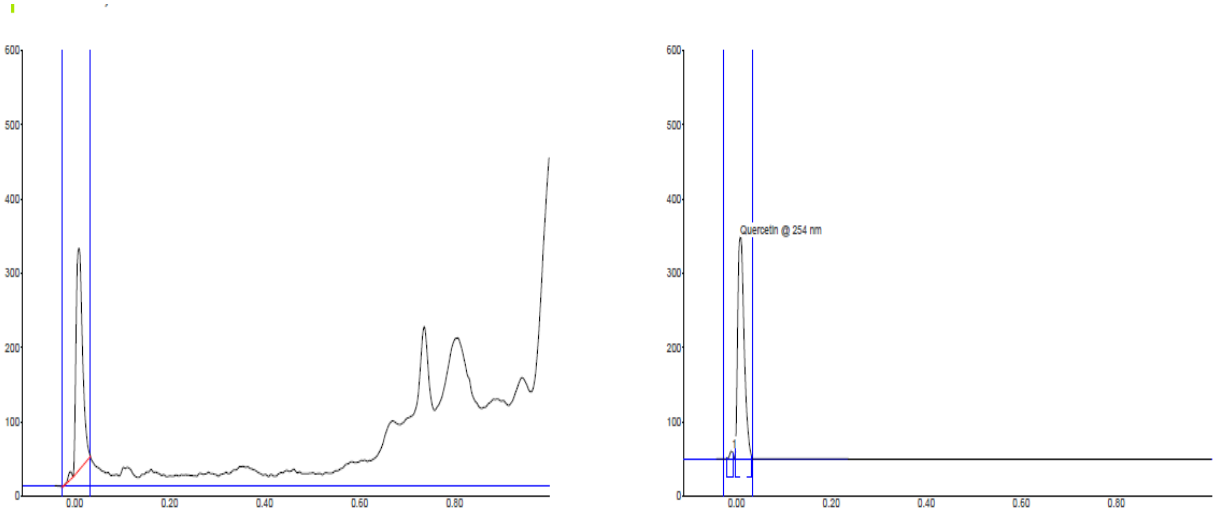


Figure 1: HPTLC chromatogram of Quercetin in *Volkameria inermis* recorded at 254 nm.

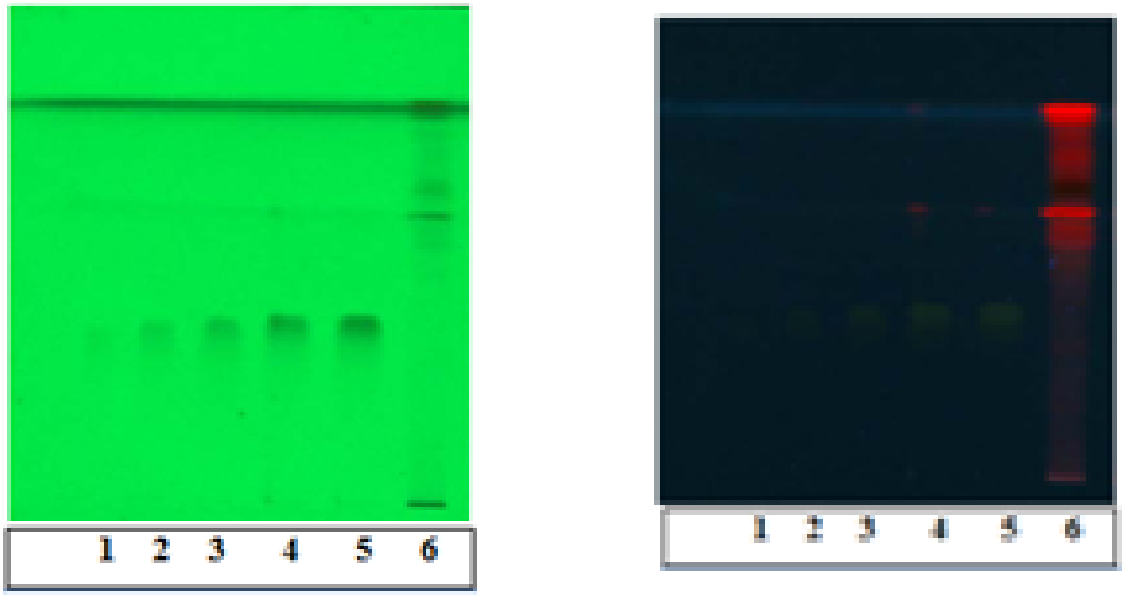


Plate 1: Visualization of Quercetin band at 254 and 366nm
Lane 1- 5 = Standard quercetin, Lane 6 = Flavonoid fractions
Lane 1 – 0.2µg Lane 2 – 0.4 µg Lane 3 – 0.6 µg Lane 4 – 0.8 µg Lane 5 – 1.0 µg

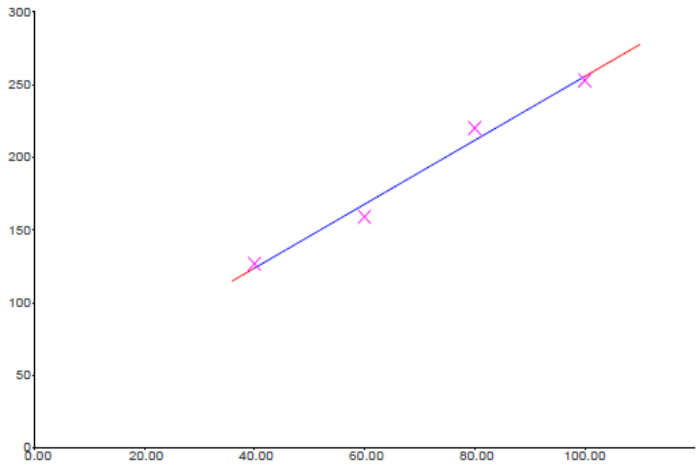


Figure 2: Linear Calibration Curve for Quercetin Standard.

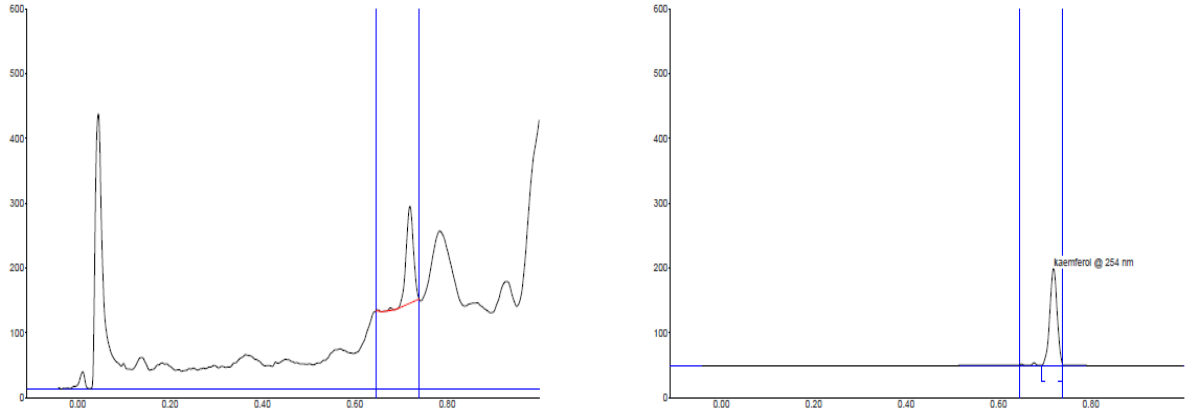


Figure 3: HPTLC chromatogram of Kaempferol in *Volkameria inermis* recorded at 254 nm.

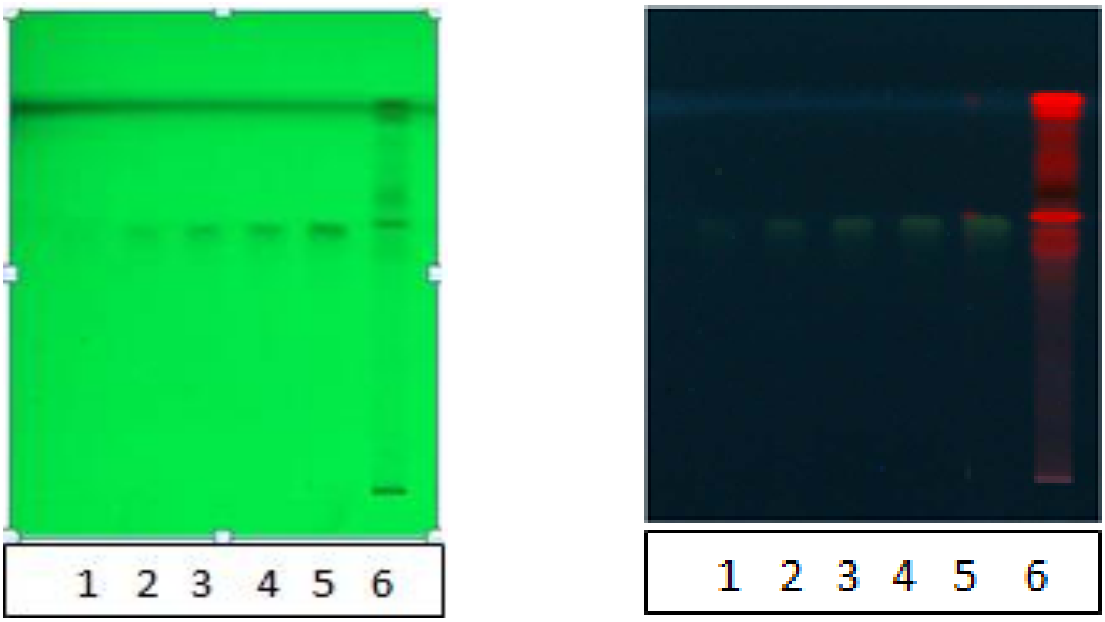


Plate 2: Visualization of Kaempferol band at 254 and 366nm
Lane 1- 5 = Standard Kaempferol, Lane 6 = Flavonoid fractions
Lane 1 – 0.2µg Lane 2 – 0.4 µg Lane 3 – 0.6 µg Lane 4 – 0.8 µg Lane 5 – 1.0 µg

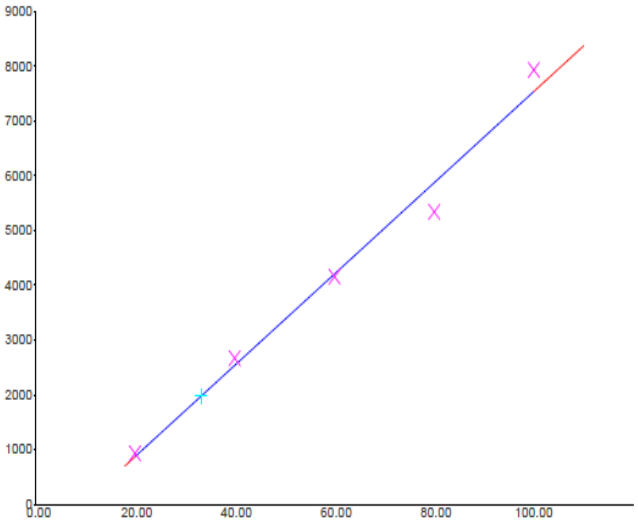
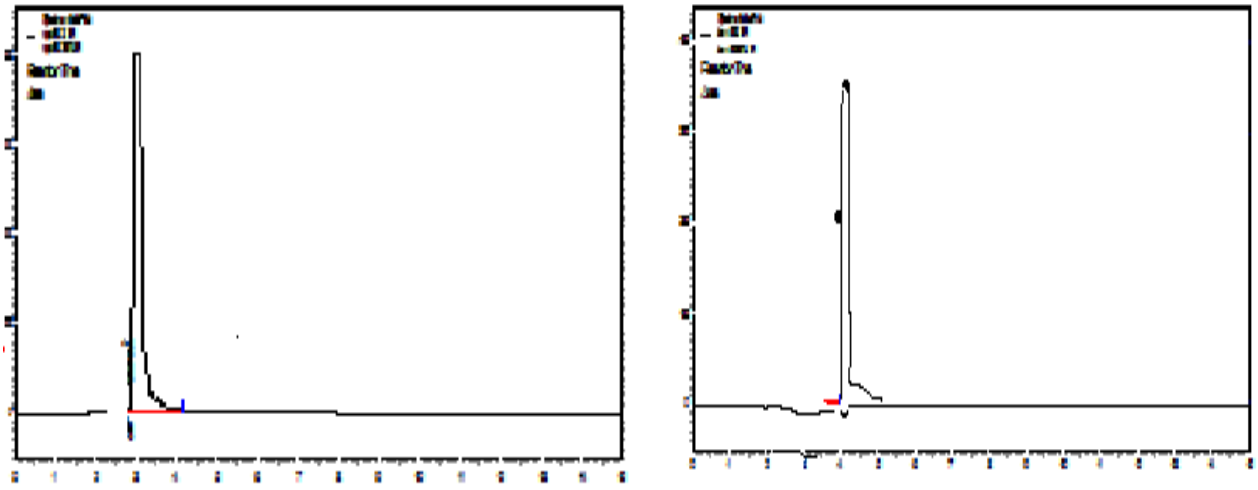


Figure 4: Linear Calibration Curve for Kaempferol as Standard.



A) Quercetin B) Kaempferol

Figure 5: HPLC Chromatogram of Standards.

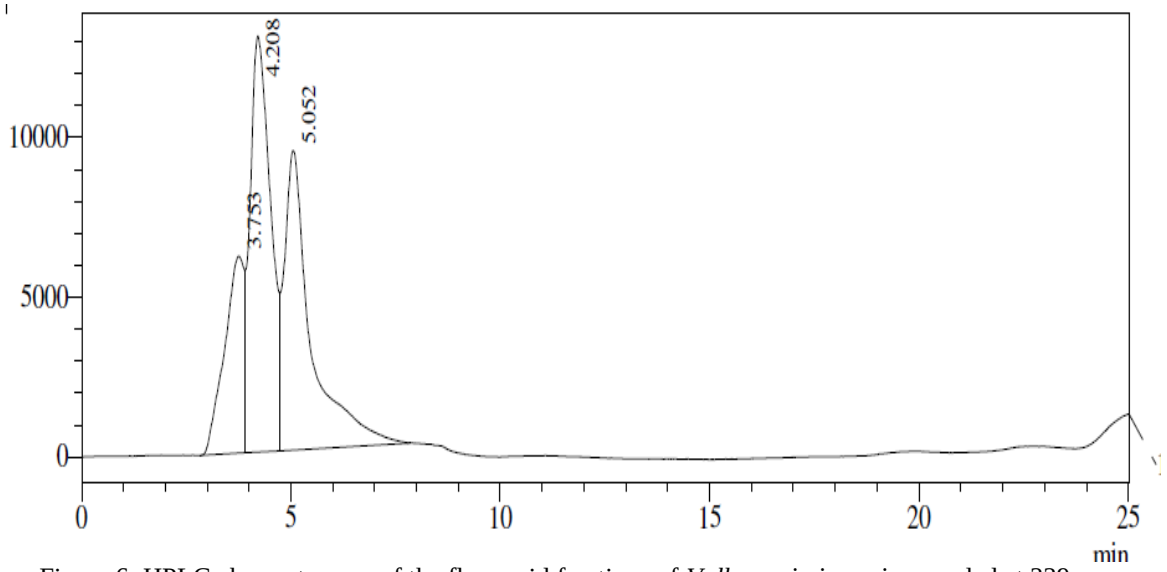


Figure 6: HPLC chromatogram of the flavonoid fractions of *Volkameria inermis* recorded at 339 nm.

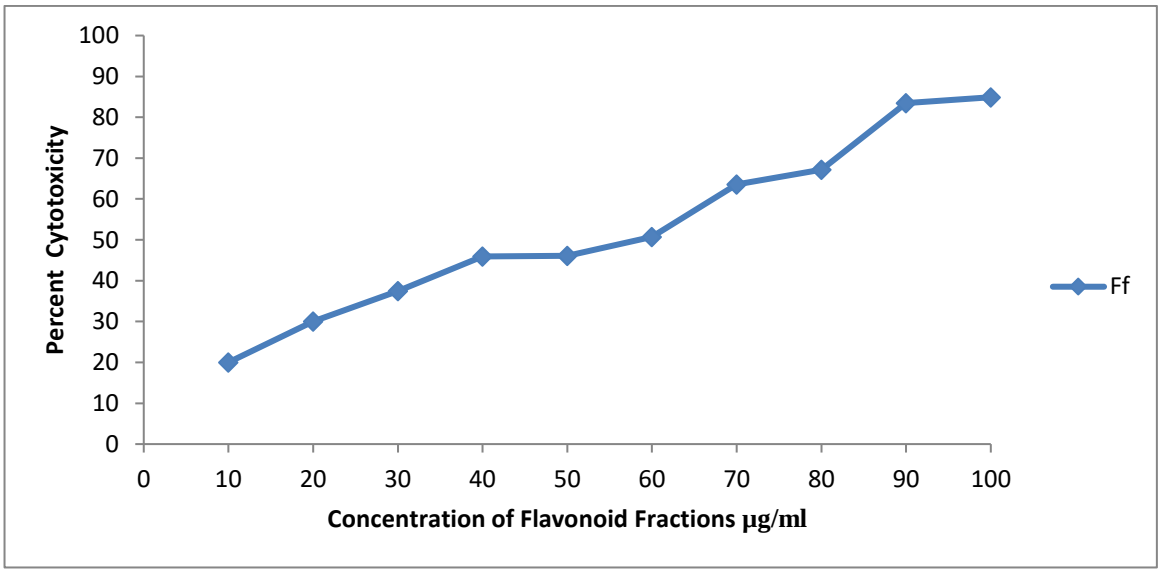
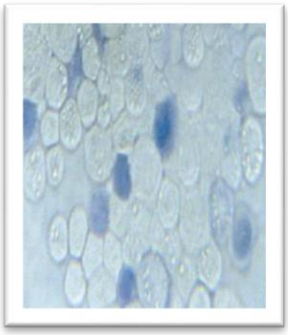
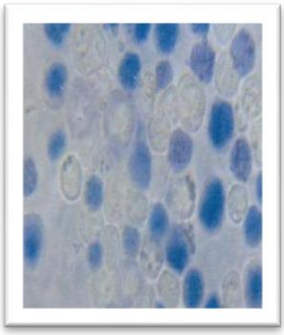


Figure 7: Effect of *In vitro* Cytotoxicity.



Untreated ELA Tumor Cells



ELA Tumor Cells Treated with Flavonoid Fractions

leucantha cav. The data obtained showed that *Salvia leucantha cav* exhibited increase in cytotoxicity with increase in the concentration of the extract[15].

CONCLUSION

Isolation of flavonoids and its *in vitro* cytotoxic study were carried out in the leaves of *Volkameria inermis*. Different solvent systems were used to extract the flavonoid fractions of which ethyl acetate was found as the best solvent for the isolation of flavonoid. The presence of quercetin and kaempferol was identified by HPTLC and HPLC techniques by comparing with their respective standards. The flavonoid fractions showed increase *in vitro* cytotoxic nature with increase in the concentration of the flavonoid fractions. However further studies are required to evaluate the *in vivo* cytotoxicity, its apoptotic gene expression studies and its application as an anticancer agent is essential.

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