Chapter 4

Chapter 4
Extraction, Activity guided fractionation and characterization of the crude extract from *Bauhinia variegata* L.

4.1 Introduction

Bauhinia variegata traditionally known as 'Kanchnar' is a medium sized deciduous tree which grows up to 50 ft tall. It is found widely across India, China, Colombia, Myanmar, Nepal, Pakistan, Thailand, and Vietnam (Orwa, Mutua, Kindt, Jamnadass, & Simons, 2009).

Figure 4.1: Documented distribution of *Bauhinia variegata* throughout world (Adapted from Orwa et al., 2009)

In India the bark has been used to treat ulcers and skin diseases. The dried buds have been used in treatment against piles, worms, diarrhoea and dysentery, whereas the roots are used as remedies for snake bites (Sahu & Gupta, 2012). In native countries, flowers, buds, pods and seeds of kanchnar are cooked and used to make pickle (R. Gupta, Paarakh, & Gavani, 2009). On the other hand, different parts of the plant like flowers, stem, buds, stem bark, leaves, roots and seeds are used in various system of medicines like Unani, Ayurveda and Homeopathy in India for the cure of various ailments (Koti, Biradar, Karadi, Taranalli, & Benade, 2009). An emulsion prepared from the bark is

used along with ginger against scrofulous enlargement on glands of the neck (K, 1954) Kanchnara guggula is an ayurvedic preparation made from the bark of *Bauhinia variegata* against scrofulous tumors (B Rajkapoor, B Jayakar, & N Murugesh, 2003). In Asia leaves provide natural casings for 'home'-rolled cigarettes. The leaves are also cut for fodder in some areas. The bark of *B. variegata* have been reported to show the anti-tumorigenic activity therefore, it is likely that the leaves of *B. variegata* could exert similar activity. These preliminary reports on bark of *Bauhinia variegata* showing anti-tumorigenic activity stipulated the need to explore leaves of *Bauhinia variegata* for similar activity.

4.1.1 Classification of *Bauhinia variegata:*

Family: Fabaceae
Subfamily: Caesalpinioideae
Class: Cercideae
Subclass: Bauhiniinae
Genus: Bauhinia
Species: variegata

4.1.2 Vernacular names of *Bauhinia variegata:*

Ayurvedic- Kaanchanaara, Kaanchanaaraka, Kanchanak, Kaanchana, Gandhaari, Sonapushpaka, Ashmantaka.

English- Mountain Ebony.

(a) (b)

Figure 4.2: *Bauhinia variegata* tree and leaves. (a) *Bauhinia variegata L.* is a medium sized deciduous tree. (b) It has broad rounded bilobed leaves. Adapted from (Malarkodi Velraj & Sowmya, 2016).

4.1.3 Morphology of leaves:

Leaves are in alternate arrangement, long petioled (to 10-15 cm long and broad), thin-leathery, simple but deeply cleft at apex, forming 2 large rounded lobes; blades with 11-13 veins extending from heart-shaped or rounded base.

4.1.4 Phytochemicals present in *B. variegata* leaves:

The leaves of *Bauhinia variegata* contain vitamin C, reducing sugars and are used to grow tasar silk worms. It has been reported in a phytochemical study that catechol, tannins, ellagic acid and sterol are also present in leaves (Kirtikar & Basu, 1935). Two compounds namely; hepatotriacontane-12,13-diol and dotetracont-15-en-9-ol have been isolated from the leaves of *Bauhinia variegata*. Structures of these compounds have been elucidated by spectral data analysis and chemical studies (RS Singh & Pandey, 2006). The leaves contain volatile oil, composed of germacrene D, spathulenol, δ- and γ-cadinene (Duarte-Almeida, Negri, & Salatino, 2004). Leaves have flavonoids like quercetin, rutin, kaempferol (Panche, Diwan, & Chandra, 2016). Insulin like-proteins have also been found to be present in this plant. Aqueous extract of leaves of *Bauhinia variegata* can effectively decrease the elevated plasma glucose level and can be evolved as a phytomedicine in treatment of type I diabetes (Garud & Kulkarni, 2014). A new triterpene saponin, named as 23-hydroxy-3alpha-[O-alpha-L-1C4-rhamnopyranosyl-(1"g 4')-O-alpha-L-4C1-arabinopyranosyl-oxy]olean- 12-en-28-oic acid O-alpha-L-1C4-rhamnopyranosyl-(1"'g 4"')-O-beta- D-4C1-glucopyranosyl-(1"'g6"')-O-beta-D-4C1-glucopyranosyl ester, is isolated from the leaves (Mohamed, Mammoud, & Hayen, 2009). Several studies have shown the presence of lupeol, alkaloids, oil, fat glycoside, phenolics, lignin, saponins, terpenoids, β -sitosterol, tannins, kaempferol-3-glucoside, rutin, quercetin, quercitrin, apigenin,apigenin-7-O-glucoside, amides, carbohydrates, reducing sugars, protein, vitamin C, fibers, calcium and Phosphorus (D. Sharma, Chawla, & Negi, 1968). Thus, literature suggests the presence of several phytocomponents of medicinal and therapeutic importance in *B. variegata leaves*.

Cancer research depends either on cell cultures (primary or cancer cell lines), tumours samples, or animal models depending on the purpose of the study. Manipulations for genetic alterations and drug testing are ethically, and in practice, difficult to perform in

animals making cell lines emerge as a feasible alternative to animal studies. Cell lines are exceptionally good for the fundamental study of the cellular pathways and for disclosing critical genes involved in cancer. MCF-7 (Michigan Cancer Foundation-7), is a human epithelial breast cancer cell line established from pleural effusion of breast cancer patient. It has been used extensively in basic research and have provided valuable insight into many aspects of breast cancer biology. This cell line retains several characteristics of differentiated mammary epithelium. It is estrogen receptor (ER) positive and progesterone receptor positive cell line. MCF-7 cell line is an interesting model for studying the efficacy of anticancer drugs because this cell has a high apoptotic threshold due to caspase 3 gene deletion (Engels et al., 2009) & Bcl-2 overexpression (Akar, Durdu, & Baran, 2008). Another cell-line of interest was MDA-MB-231 (M. D. Anderson-Metastasis breast cancer 231) which is also a human epithelial breast cancer adenocarcinoma, established from pleural effusion of a patient. Compared to MCF-7 cell line, it is highly aggressive, invasive and poorly differentiated. It differs from MCF-7 as it lacks estrogen, progesterone receptor expression as well as HER2 amplification hence it is a triple negative breast cancer cell line. In addition to these features, it has also got mutated tp53 gene. Two breast cancer cell lines (with difference in molecular characteristics) MCF-7 and MDA-MB-231 were used in this study with the purpose better understanding of their differential response to extract and phytocomponents from *Bauhinia variegata* leaves as earlier two individual studies using the aqueous extract of *Bauhinia variegata* leaves displayed cytotoxicity on breast cancer cell lines MCF-7 (A. Mishra, Sharma, Kumar, Saxena, & Pandey, 2013) and T47D (K. R. Sharma, Kalauni, & Awale, 2015). However, no mechanism has been reported. Also, the effect has not been studied on triple negative breast cancer cell line MDA-MB-231, which is highly proliferating and metastatic cells. It is important to understand the effect of extract/fraction on these cell line as it will provide conclusive evidence if the phytocomponent can inhibit the proliferation of highly metastatic breast cancer cells and inhibit metastasis. The present chapter is aimed to identify the active phytochemicals from *B. variegata* extract and explore their anti-cancer properties.

Results:

4.1.5 Phytochemical Analysis of *B. variegata* leaves extract:

Fresh and disease-free *B. variegata* leaves were collected from Waghai Botanical Garden, Dang, Gujarat, air dried, powdered, and processed using different solvents in increasing polarity. After 8-12 hours of the extraction process, the extracts were obtained by the procedure mentioned in chapter 3. The percentage yield of the extracts from 42.8 gm of powdered leaves was calculated and the obtained yield is presented in Table 4.1.

Table 4.1: Percentage yield obtained from powdered leaves.

Solvent	% Yield (w/w)	Dried Extract (g)
Petroleum Ether	3.542 %	1.516 g
n- Hexane	0.173 %	0.074 g
Chloroform	1.910 %	0.817 g
Ethyl acetate	0.674 %	0.288g
Methanol	4.98 %	2.131g
Water	7.03 %	3.008g

4.1.6 Qualitative analysis of phytocomponents from *B. variegata* leaves extract:

Different extracts showed presence of different phytocomponents depending upon the polarity of the solvent. Petroleum ether and n-hexane extract showed presence of non-polar phytocomponents. Chloroform and ethyl acetate indicated presence of mid-polar compounds whereas Methanol and aqueous extracts showed polar compounds (Table 4.2).

Table 4.2: Phytochemical Profile of *B. variegata* leaves extracts. (+) indicates presence and (-) indicates absence of phytocomponent.

Compound	Petroleum ether	n-Hexane	Chloroform	Ethyl Acetate	Methanol	Water
Alkaloids	-	-	-	-	-	+
Flavonoids	-	-	-	+	+	+
Saponins	-	-	-	-	+	+
Quinones	-	-	+	-	+	-
Phenols	+	+	+	+	+	+
Steroids	+	+	+	-	-	-
Proteins	-	-	+	-	-	-
Carbohydrate	-	-	-	-	+	+
Glycoside	-	-	-	+	+	-
Terpenoids	-	-	+	+	+	-

4.2 Evaluation of Antioxidant Activity: Antioxidants significantly prevents oxidation of oxidizable substrates through scavenging the free radicals, quenching singlet and triplet oxygen, or decomposing peroxides, by absorbing and neutralizing them. The antioxidative effect is attributed to phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes. It is a well-established fact that the medicinal properties of phytochemicals is directly proportional to the antioxidant potential they possess. The dose dependent inhibition of DPPH radical was carried out and it was observed that the methanolic and aqueous extract showed maximum antioxidant activity with the strongest DPPH radical scavenging activity out of all the fractions. N-hexane and Petroleum ether fraction showed least antioxidant activity compared to other fractions. Ascorbic acid was taken as a standard (Figure 4.3).

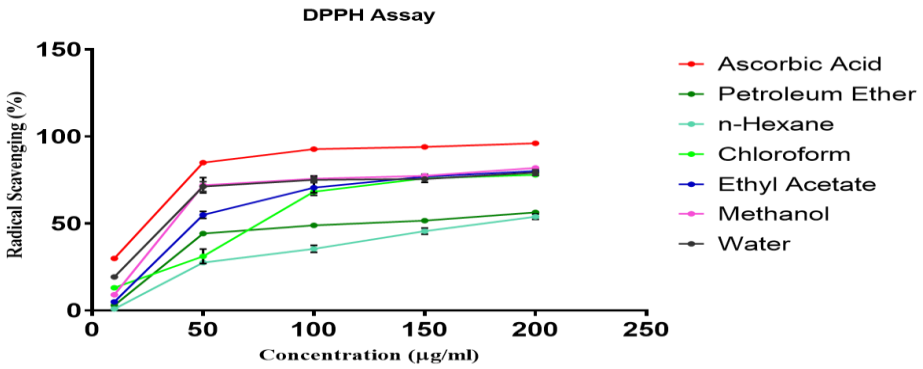
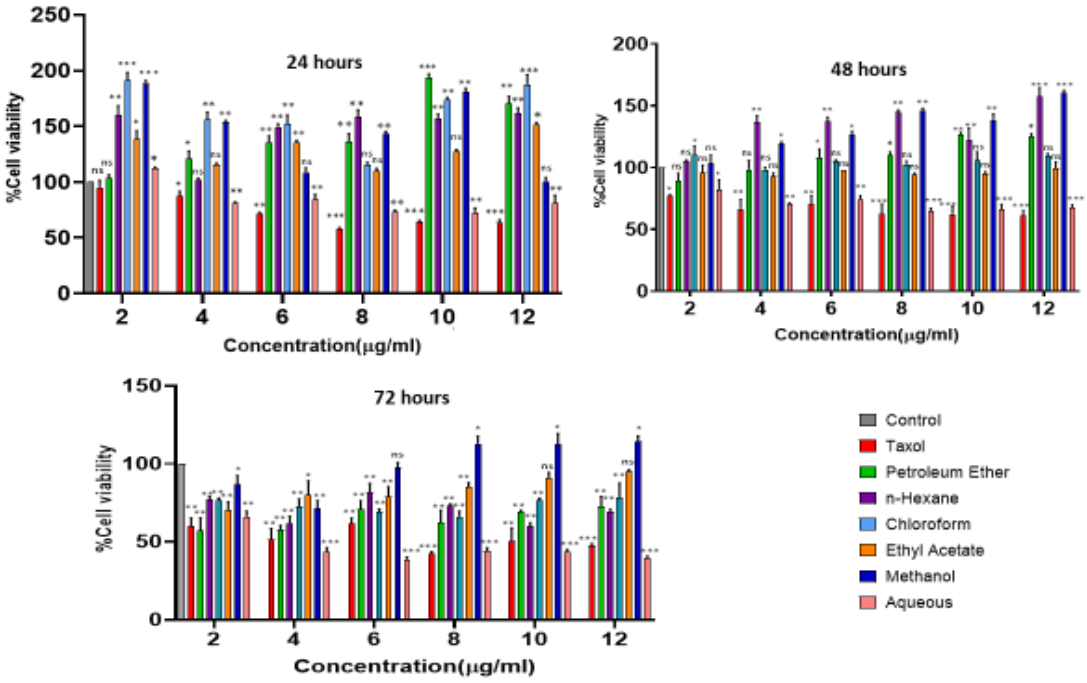


Figure 4.3: Percentage scavenging of DPPH free radical by different extracts of *Bauhinia variegata* L.

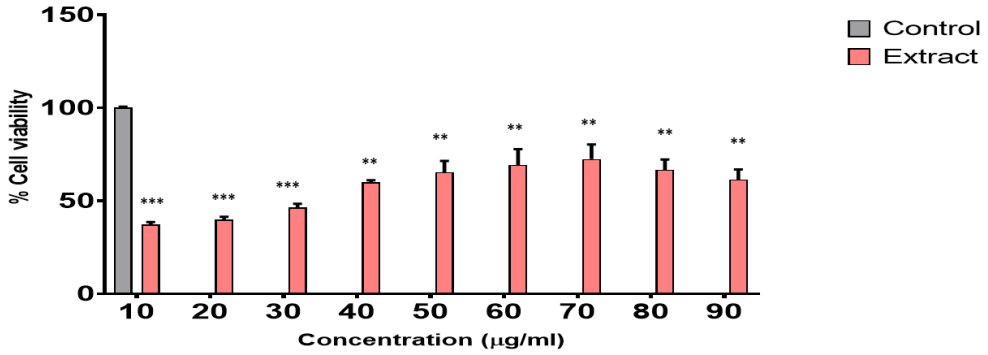
4.3 To check the cytotoxic effect of *Bauhinia variegata L.* extracts on MCF-7 and MDA-MB-231 cells in time and dose dependent manner.

Cytotoxic effect of each extract was checked by MTT assay. MCF-7 is estrogen and progesterone receptor positive cell-line and represents benign conditions for the breast tumor whereas MDA-MB-231 cells are triple negative for hormone receptors and are highly metastatic. ER/PR and Her2 negative and represent malignant conditions for breast tumor.

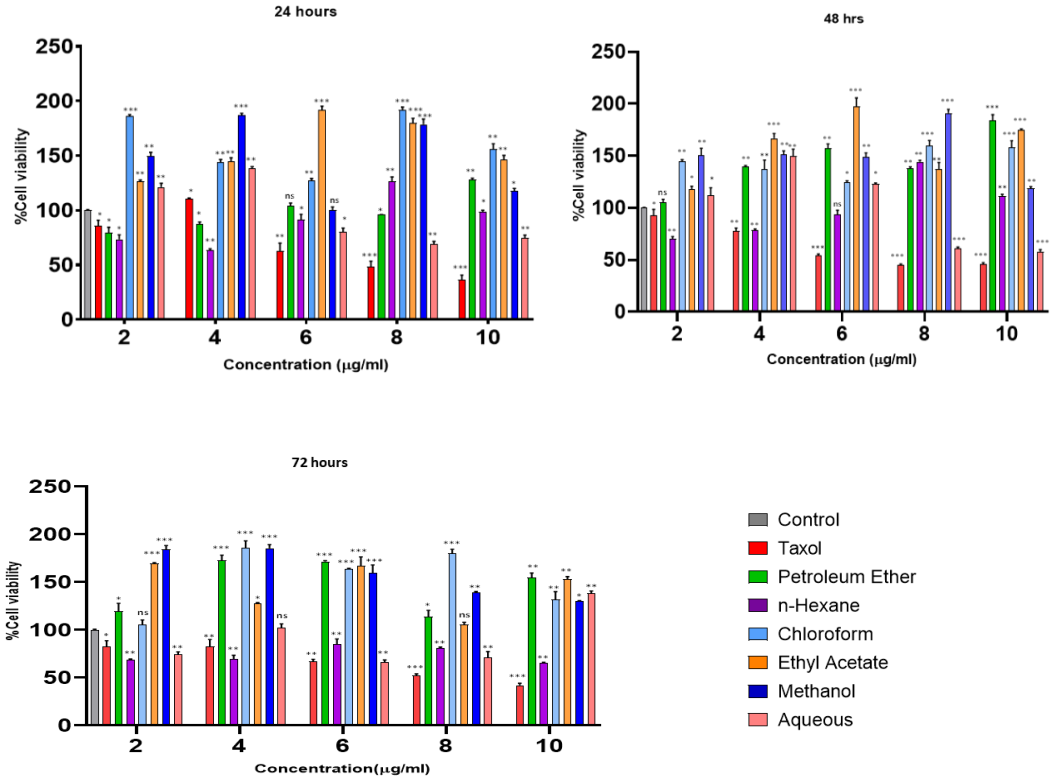
(a)



(b)



(c)



(d)

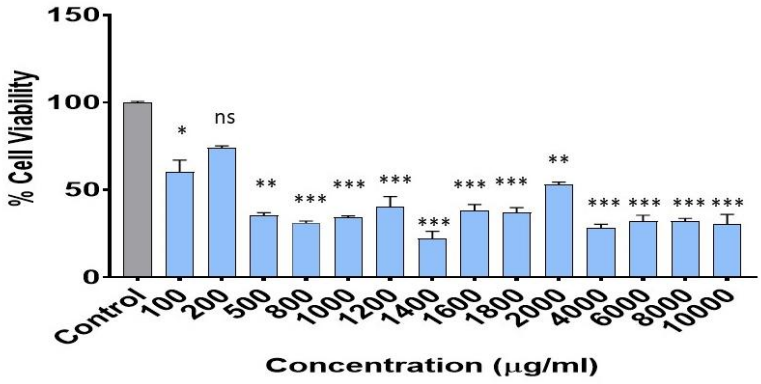


Figure 4.4: Anti-proliferative activity of *Bauhinia variegata* extracts towards breast cancer cell-lines MCF-7 and MDA-MB-231. Taxol was used as standard reference drug. (a) effect of low concentration on MCF-7 cell-lines at 24, 48 and 72 hours. (b) effect of high concentration on MCF-7 cell-lines for 24 hours. (c) effect of low concentration on MDA-MB-231 cell-lines at 24, 48 and 72 hours. (d) effect of high concentration on MDA-MB-231 cell-lines for 24 hours. Cell viability is represented as mean ± standard deviation of three independent experiments (each with 6 parallel measurements). p value is represented as ns- nonsignificant, * p < 0.05, ** p < 0.01, *** p < 0.001.

MCF-7 (ER/PR positive) and MDA-MB-231 (triple negative breast cancer cells) cells were exposed to varying concentration of extracts for 24 hours, 48 hours, and 72 hours. The aqueous extract showed good cytotoxicity against both the cell-lines. IC50 was found to be 10µg/ml for MCF-7 cells and for MDA-MB-231 cells at 72 hrs treatment and at 48 hrs treatment respectively (Figure 4.4 a & 4.4 c). To decrease the incubation time, cells were exposed to higher concentration of aqueous extract and a IC50 of 35µg/ml for MCF-7 cells and 1300µg/ml for MDA-MB-231 cells were obtained at 24 hours (Figure 4.4 b & 4.4 d). As aqueous extract showed cytotoxicity, hence it was used to carry out for further experiments.

4.4 Isolation and characterization of the phytocomponents from aqueous extract

4.4.1 Characterization of aqueous extract of *Bauhinia variegata* L. using High Resolution Liquid Chromatography- Mass Spectrometry (HRLC-MS) analysis:

In order to analyse the constituents of the aqueous extract for phytochemicals having anti-cancer activity, High resolution Liquid chromatography Mass spectroscopy (HRLC-MS) was performed as described in chapter 3. The list of probable phytocomponents identified in the aqueous extract of *Bauhinia variegata L.* is mentioned in the table below along with the name of the compound, molecular formula, molecular weight, retention time and peak area (Table 4.3).

HRLC-MS analysis revealed the presence of three known anti-cancer compounds: Berbamine, Rhapontin and 4'-Desmethylpapaverine in the aqueous extract (figure 4.5). Berbamine is a part of the bisbenzylisoquinoline class of alkaloids, Rhapontin belongs to stilbene glucoside, whereas Papaverine is a non-opioid alkaloid which also belongs to benzylisoquinoline-alkaloid class of compounds. These compounds are known to possess cytotoxic property against various cancer cells including breast cancer. Based upon the peak area (which indicates their abundance), the amount of Berbamine, Rhapontin and 4'-Desmethylpapaverine in the aqueous extract of *Bauhinia variegata* leaves was found to be 50.09 µg/mg, 0.648 µg/mg and 0.05605 µg/mg respectively. The standards Berbamine dihydrochloride, Rhapontin and Papaverine hydrochloride were used as a positive control. They were also sent for the LC-MS analysis (Figure 4.7).

The mass spectra of the constituents were compared with the MassBank library, the phytocompounds were characterized and identified (Table 4.3). The name, molecular weight, and structure of the components of the test materials were ascertained. The important molecular structures of the compounds present in *B. variegata* extract are shown in Figure 4.6.

Table 4.3: List of probable phytocomponents detected in the aqueous extract of *Bauhinia variegata* L. leaves. *indicates anti-cancer compound

No.	Compound Name	Chemical formula	Molecular weight	Retention Time	Peak Area
1.	Tebuthiuron	$C_9 H_{16} N_4 O S$	228.0995	0.865	-
2.	Methyl N-(a-methylbutyryl) glycine	$C_8 H_{15} N O_3$	173.1056	1.147	231970
3.	Terbinafine metabolite	$C_{19} H_{23} N O_3$	313.1674	3.774	44054+56942
4.*	**4'-Desmethylpapaverine**	**$C_{19} H_{19} N O_4$**	**325.131**	**4.612**	**21579**
5.	Carboxyterbinafine derivative	$C_{20} H_{23} N O_4$	341.1628	4.829	210605+151703
6. *	**Berbamine**	**$C_{37} H_{40} N_2 O_6$**	**608.2886-608.2879-608.2887**	**5.821-5.84-6.051**	**67661+65680+58647**
7.	Hydroxy-3-O-methyl-6-beta naltrexol	$C_{21} H_{27} N O_5$	373.1882	5.825	38457
8.	Pyrrhoxanthin	$C_{39} H_{48} O_6$	612.3455-612.346	6.232-6.646	130137+204636
9.	Proansamycin X	$C_{35} H_{47} N O_{10}$	640.3126-640.3104	6.341-6.41	65051+38542
10.	Swietenine	$C_{32} H_{40} O_9$	568.2732-568.2729	9.05-10.17	211764+136009
11.*	**Rhapontin**	**$C_{21} H_{24} O_9$**	**420.1415**	**10.315**	**1544**

12.	b-D-Glucopyranosiduronic acid, 6-(3-oxobutyl)-2-naphthalenyl	$C_{20}H_{22}O_8$	390.131-390.1313	11.075-11.446	86904 - 58469
13.	3alpha,6beta,7alpha-Trihydroxy-5beta-cholan-24-oic acid.	$C_{24}H_{40}O_5$	408.2875	19.672	-
14.	14-hydroxy-5Z-tetradecenoic Acid	$C_{14}H_{26}O_3$	242.1881	26.679	53053
15.	Prometon	$C_{10}H_{19}N_5O$	225.1619	26.719	181526

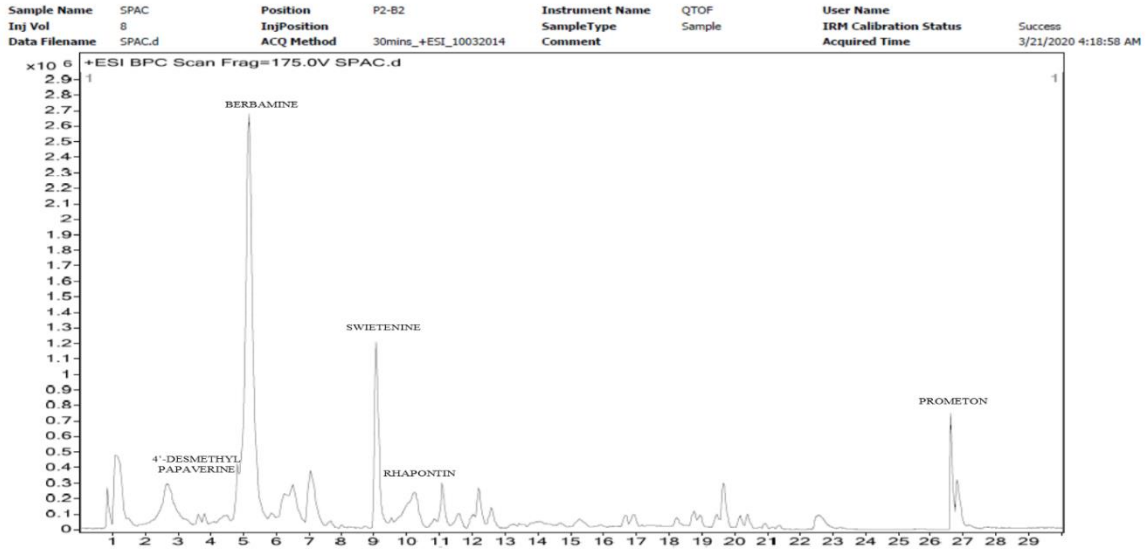


Figure 4.5: HRLC-MS chromatogram of *Bauhinia variegata* L. aqueous extract.

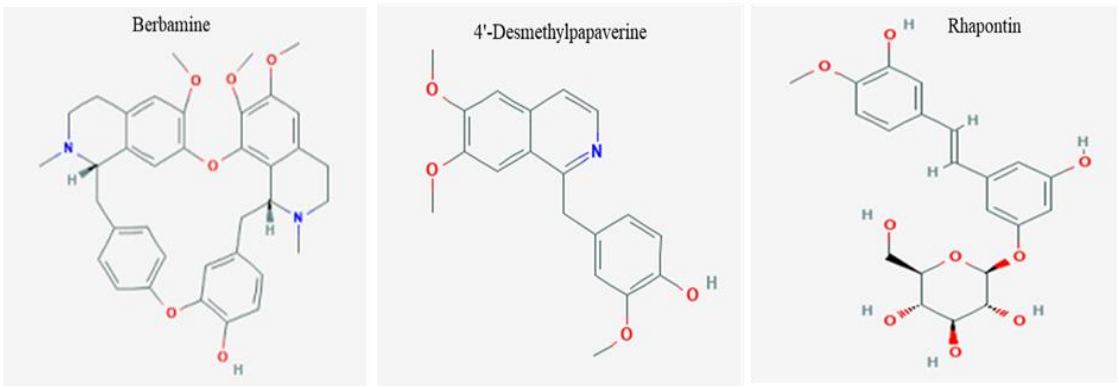


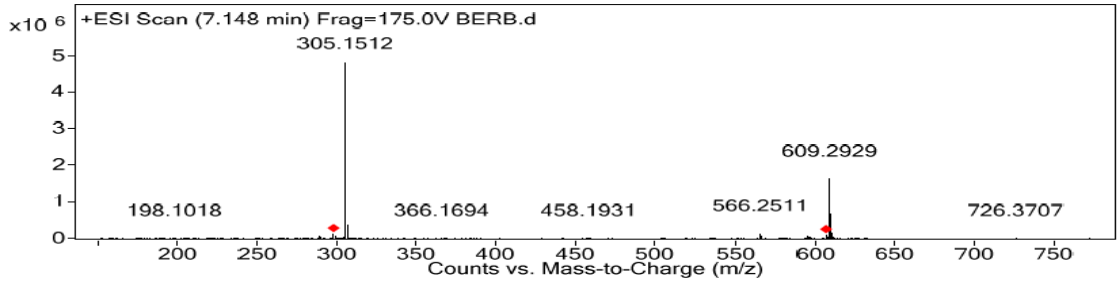
Figure 4.6: The molecular structures of major compounds from *Bauhinia variegata* L. aqueous extract.

Berbamine has molecular formula- C_{37} H_{40} N_2 O_6, molecular weight- 608.2887, retention time-6.603 to 7.065. The LC/MS fragment showed the peak at 5.821, 5.84 and 6.051 minutes with a mass [M+] 608.2886, 608.2879 and 608.2887 respectively. The daughter ion spectra of these compound (B1) exhibited the characteristic fragments m/z 89.0592, 192.1017, 367.1657, 609.2925 in EI pattern. The daughter ion spectra of these compound (B2) exhibited the characteristic fragments m/z 107.0478, 305.1523, 444.1777, 566.2539 in EI pattern. The daughter ion spectra of these compound (B3) exhibited the characteristic fragments m/z 105.0683, 192.0980, 256.1075, 367.1663, 472.2101 in EI pattern (Figure 4.7.1).

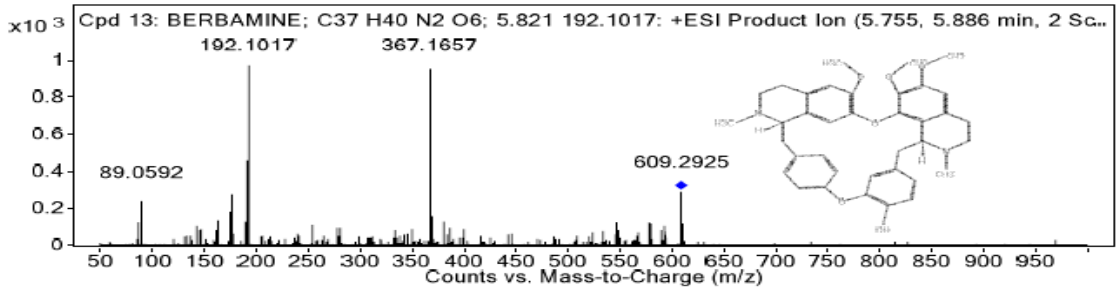
Another important phytocomponent present was 4'-Desmethylpapaverine. 4'-Desmethylpapaverine has molecular formula- C_{19} H_{19} N O_4, molecular weight-325.131, retention time-5.795 to 5.993. The LC/MS fragment showed the peak at 4.612 minutes with a mass [M+] 325.131. The daughter ion spectra of these compound exhibited the characteristic fragments m/z 178.0859 and 311.1142 in EI pattern (Figure 4.7.2).

Rhapontin was also found to be present in the aqueous extract. Rhapontin has molecular formula- C_{21} H_{24} O_9, molecular weight-420.1415, retention time-8.76 to 8.961. The LC/MS fragment showed the peak at 10.315 minutes with a mass [M+] 420.1415. The daughter ion spectra of these compound exhibited the characteristic fragments m/z 158.1542, 279.0933, 568.2967, 640.3166, 816.4233 in EI pattern (Figure 4.7.3).

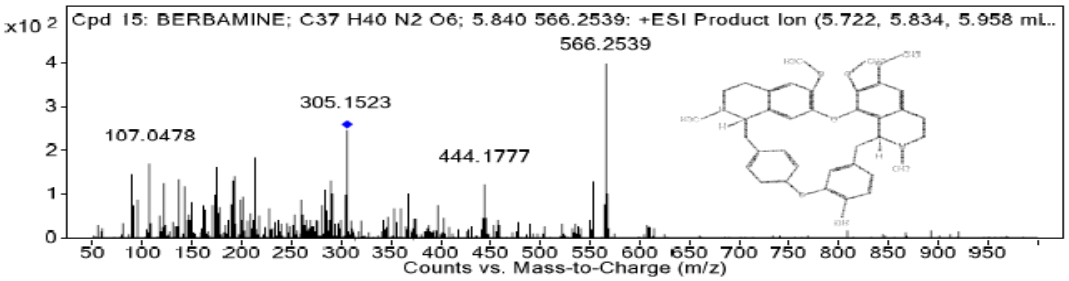
4.7.1 LC-MS chromatogram of Berbamine dihydrocholoride (standard).



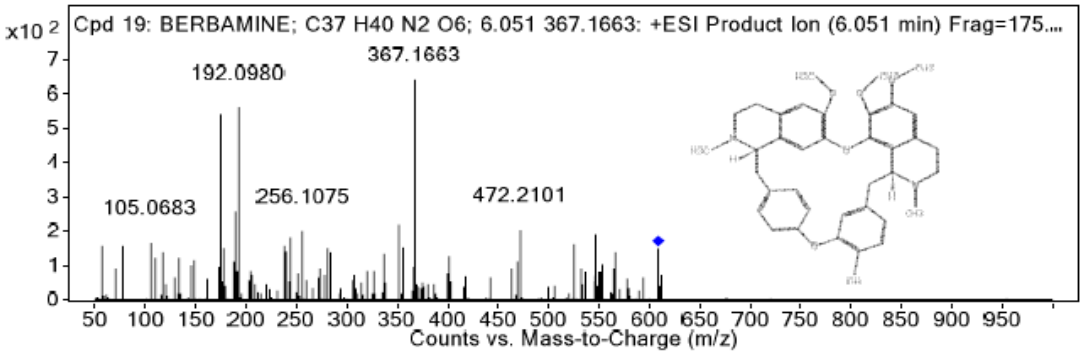
Berbamine peak (A1)



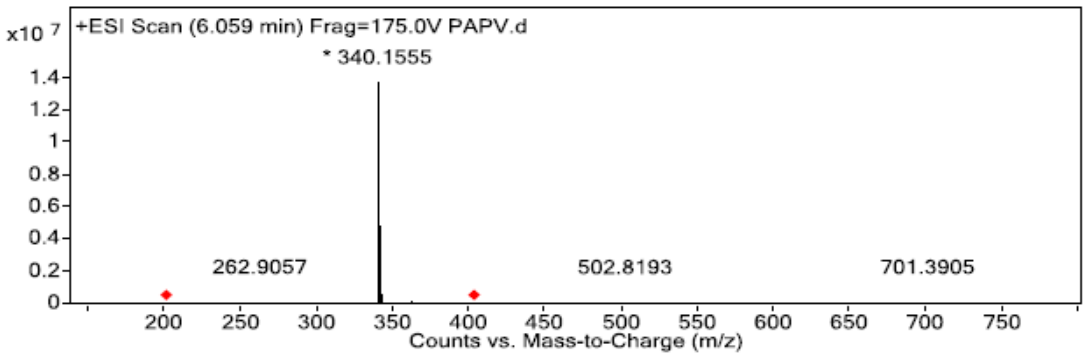
Berbamine peak (A2)



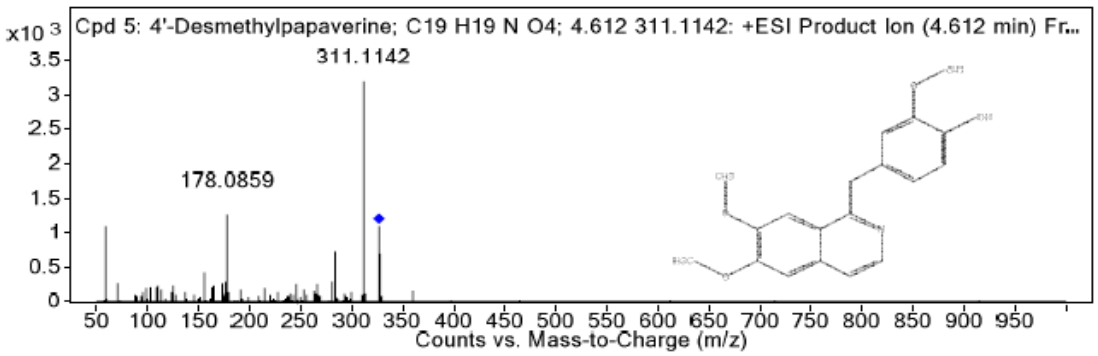
Berbamine peak (A3)



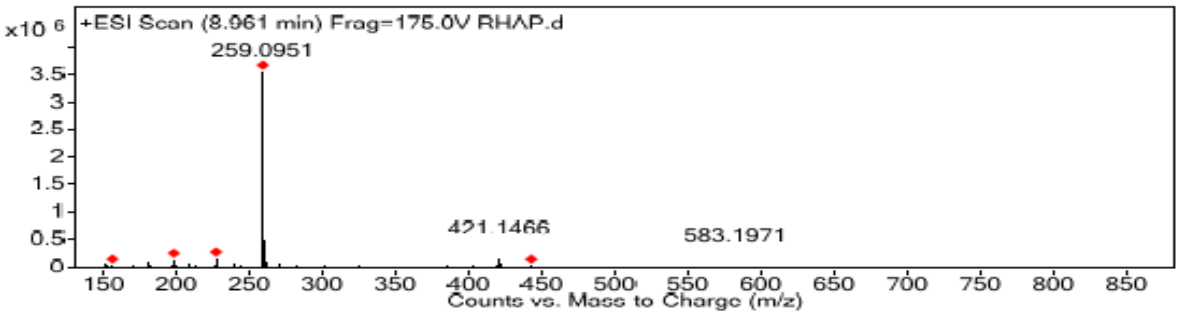
4.7.2 LC-MS chromatogram of Papaverine hydrocholoride (standard).



4'-Desmethylpapaverine peak



4.7.3 LC-MS chromatogram of Rhapontin (standard).



Rhapontin peak

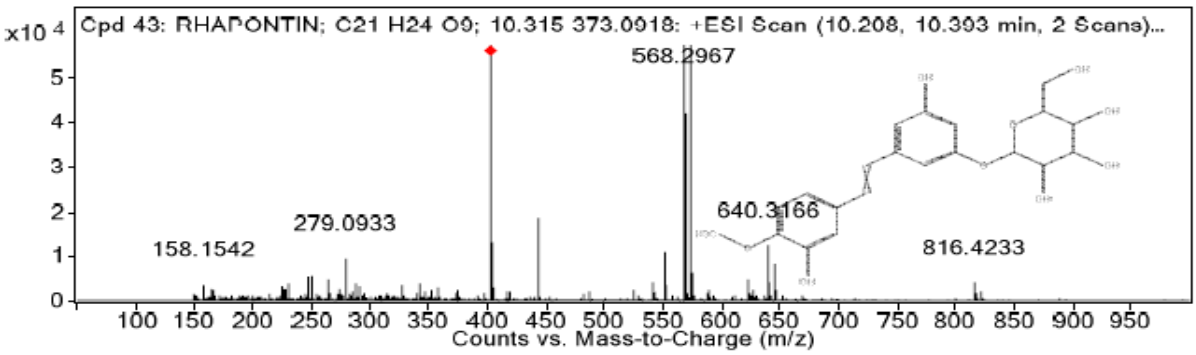


Figure 4.7: HRLC-MS analysis of standards. The structure of the major phytocomponents Berbamine, Rhapontin and Papaverine identified from aqueous extract of *B. variegata L.* 4.7.1 Berbamine dihydrocholoride (standard) 4.7.2 Papaverine hydrocholoride (standard) 4.7.3 Rhapontin (standard).

4.4.2 Thin Layer Chromatography (TLC) of the aqueous extract of *B. variegata* leaves.

With the aim to separate the phytocomponents, TLC of the aqueous extract was done with various solvent systems on TLC plates (silica gel 60 F_{254}). The solvent system that gave best chromatographic separation was Chloroform: Ethyl acetate: Methanol (4:6:5) (Figure 4.8). The spots were visualized at short uv wavelengths and long uv wavelengths in UV chamber (254 nm & 365 nm respectively) and later derivatized by 5% anisaldehyde sulphuric acid spraying reagent.

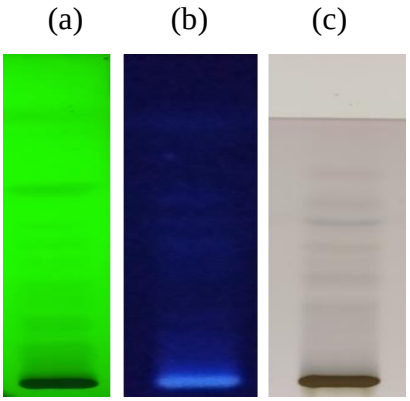


Figure 4.8: TLC analysis of aqueous extract of *Bauhinia variegata L.* (a) 254 nm (b) 365 nm, (c) post derivatization with 5% anisaldehyde-sulphuric acid reagent (under visible light).

4.4.3 Flash Chromatography of aqueous extract of *B. variegata* L. Leaves.

Different fractions of aqueous extract were subjected to Flash chromatography. Eluents collected from flash chromatography were further separated by TLC separation and rest of the samples collected as eluents were dried and preserved for future assays. TLC was performed using Chloroform, Ethyl acetate and Methanol (4: 6: 5 respectively) as solvent system (figure 4.9). Sixteen fractions were run along aqueous extract and with standards Berbamine dihydrochloride, Rhapontin and Papaverine hydrochloride. The RF values of aqueous extract were found to be 0.141, 0.22, 0.49, 0.66, 0.82, 0.84, 0.93, 0.96. The % yield and the retention factor (R.F.) value of the fractions obtained from

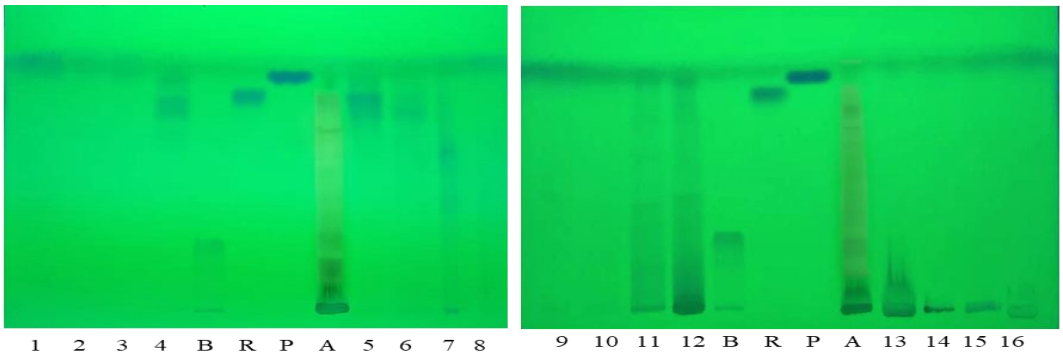
the aqueous extract of *Bauhinia variegata L*. by Flash chromatography are given in Table 4.4.

Table 4.4: The % yield and the R.F. value of the fractions obtained from the aqueous extract of *Bauhinia variegata* L.by Flash chromatography.

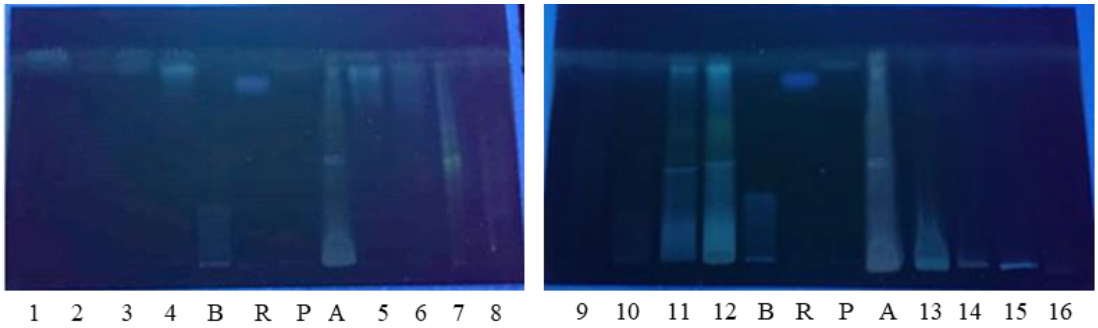
Track No.	% Yield (w/w)	Total Bands observed	RF VALUE	Track No.	% Yield (w/w)	Total Bands observed	RF VALUE
1	4.7	1	0.96	11	5.145	2	0.47, 0.93
2	11	1	0.94	12	11.7	2	0.49, 0.93
3	14.9	1	0.94	13	27.65	1	0.14
4	9.6	3	0.8, 0.82, 0.91,	14	5.55		
5	9.85	3	0.8, 0.84, 0.94	15	10.95		
6	10.3	1	0.8	16	8.05		
7	9.5	3	0.49, 0.66, 0.8	B	-	1	0.23
8	4.75	1	0.96	R	-	1	0.84
9	14.4	1	0.96	P	-	1	0.93
10	9.7	1	0.96				

The spots observed were compared with the standards (Berbamine, Rhapontin, Papaverine) to confirm their presence (figure 4.9). Fractions 1-4 shows presence of papaverine at both 254 nm and 366 nm. Fraction 4 also shows presence of one additional band almost similar to Rhapontin at 254 nm. Fraction 5 and 6 shows similar band pattern like fraction 4. Fraction 7 shows one unique band at 254 nm and 366 nm each. Fractions 11 and 12 shows band at similar position to papaverine at both 254 nm and 366 nm. Apart from that, fraction 11 and 12 shows a unique band at 366 nm. Similar retention time suggests that the two samples could be the same.

(a)



(b)



(c)

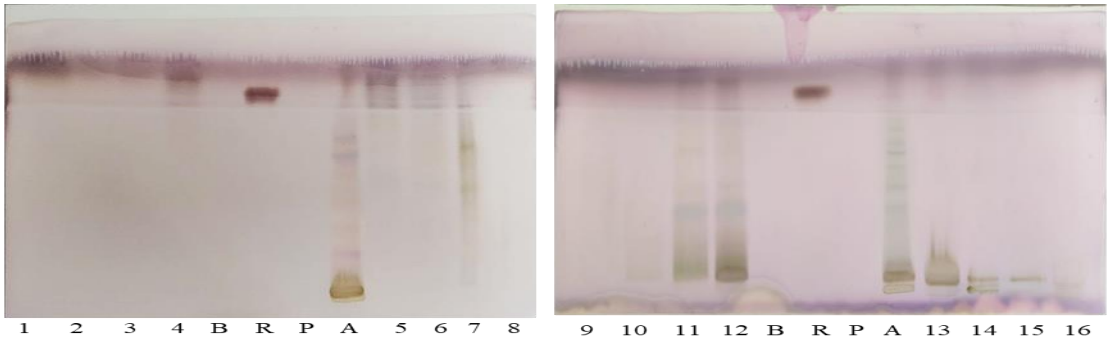


Figure 4.9: Thin Layer Chromatography of aqueous extract fractions of *Bauhinia variegata* L. TLC plates observed at 254nm, 366 nm and post derivatization with 5% anisaldehyde sulphuric acid reagent. 1 to 16 lane Samples, B-Berbamine standard, R-Rhapontin std, P-Papaverine std, A- Aqueous extract.

4.4.4 Fourier-transform infrared spectroscopy (FTIR) of aqueous extract of *B. variegata* L. leaves.

FTIR is a powerful tool to identify the chemical bonds and functional groups present in compounds. Figure 4.10 shows the peak values and the possible functional groups present in the aqueous extracts of *Bauhinia variegata leaves.*

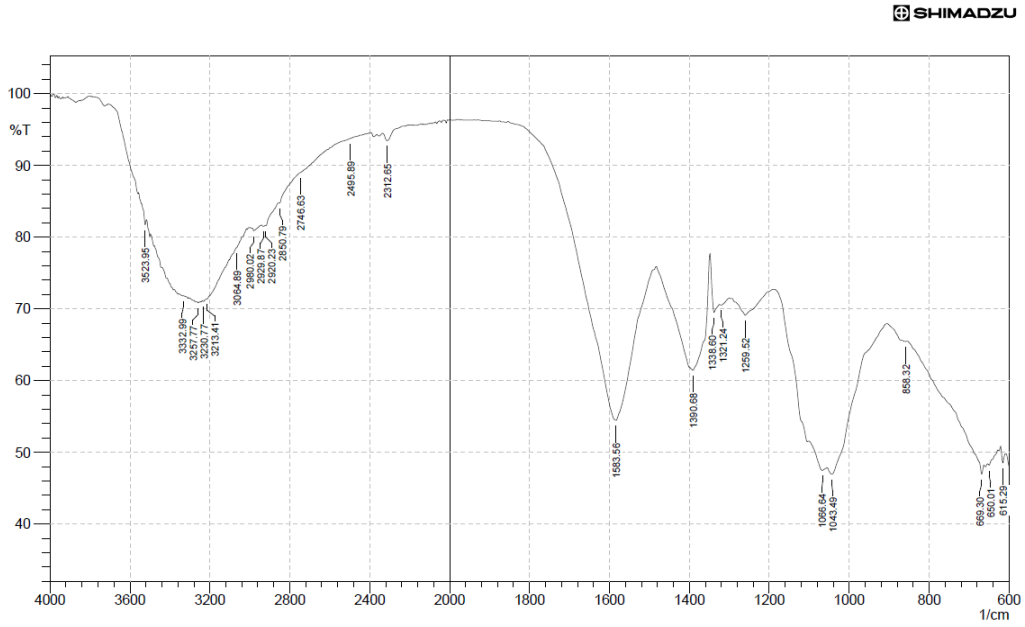


Figure 4.10: FTIR spectra of aqueous extract of *Bauhinia variegata L. leaves.*

The IR spectrum of the aqueous extract of *B. vareigata L.* leaves has common absorption bands at ~1583 cm^{-1} for *v*(C=C) of the phenyl rings, at ~ 1553 and ~ 1467 cm^{-1} due to v(C=N) + (C=C) of the pyridine ring. Broad absorption band at ~ 3250 cm^{-1} indicate the presence of -OH group in the Aqueous extract. Spectra of pure Berbamine showed the characteristic absorption bands at 1515, 1275 and 1117 cm^{-}. The absorption bands obtained at 1259 and 1117 cm^{-} indicates the probable presence of Berbamine in aqueous extract of *Bauhinia variegata* L. leaves.

4.4.5. Cytotoxicity assay of the probable anti-cancer compounds found in aqueous extract by HRLC-MS.

MTT was performed with the standards of Berbamine, Rhapontin and Papaverine. Both the cell-lines were susceptible to the inhibitory effect of Berbamine (Figure 4.11 a). The IC50 value of Berbamine for MCF-7 was 50 μg/ml and for MDA-MB-231 was 75 μg/ml. Rhapontin also showed anti-proliferative activity against breast cancer cell-lines (Figure 4.11 b). In case of Rhapontin, IC50 for MCF-7 was found to be 75 μg/ml and IC50 for MDA-MB-231 was found to be 850 μg/ml. Papaverine also showed anti-proliferative activity against both the cell-lines (Figure 4.11 c). Dose dependant decrease in cell viability was observed when breast cancer cells were given treatment with Papaverine. IC50 of MCF-7 cells treated with papaverine was found to be 100 μg/ml and for MDA-MB 231 cells it was 75 μg/ml.

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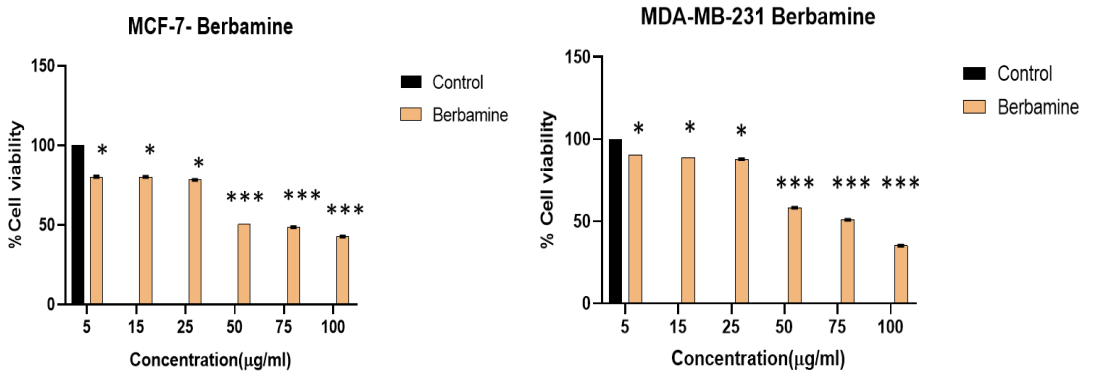
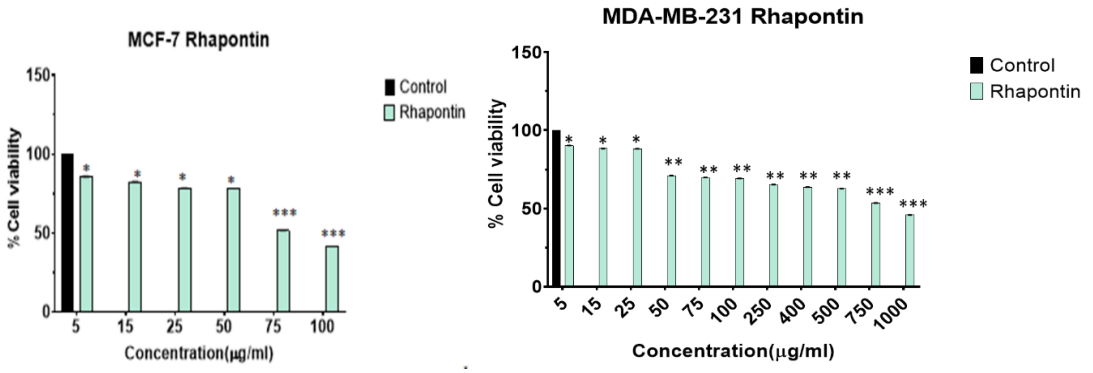
(a)



(b)



(c)

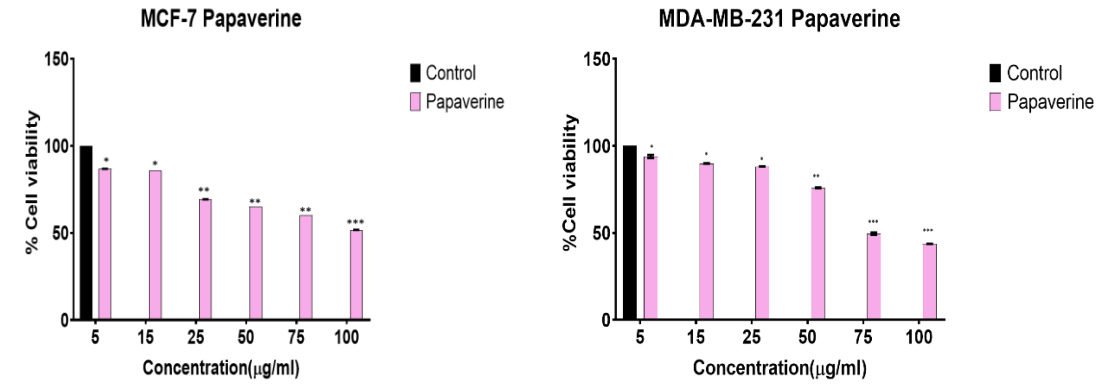
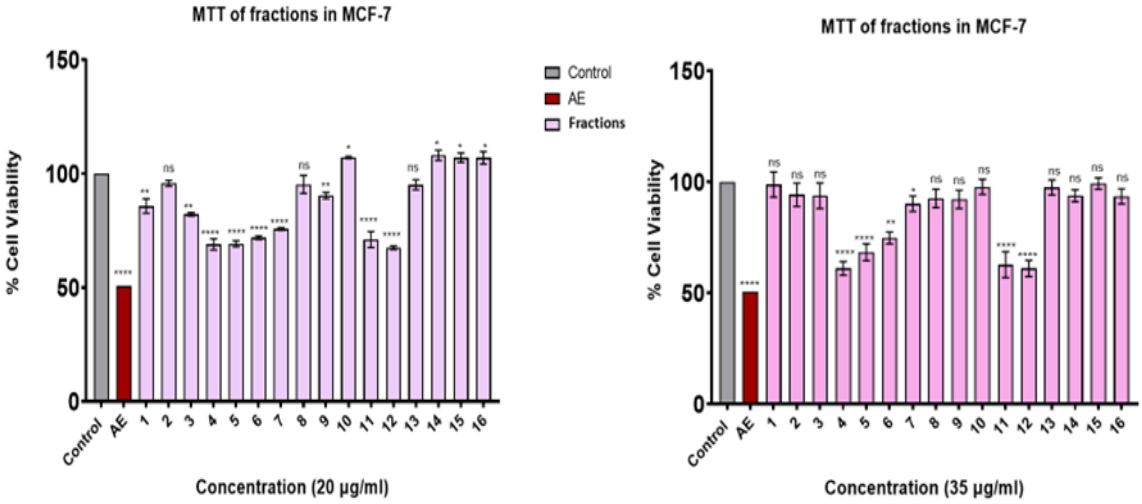


Figure 4.11: Cytotoxicity assay of standards against breast cancer cell lines. (a) cytotoxicity by Berbamine in breast cancer cells for 24 hours. (b) cytotoxicity by Rhapontin in breast cancer cells for 24 hours. (c) cytotoxicity by Papaverine in breast cancer cells for 24 hours. Data representation mean *p < 0.05; **p < 0.01; ***p < 0.001

4.4.6. Cell cytotoxicity assay of the fractions of aqueous extract obtained after flash chromatography on breast cancer cell lines.

(a)



(b)

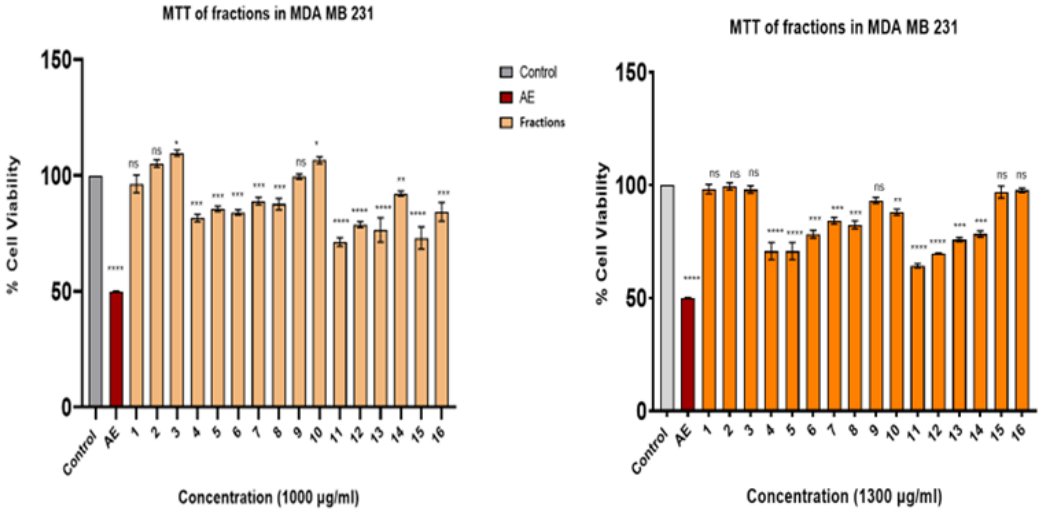


Figure 4.12: Cytotoxicity assay of fractions of aqueous extract of *B. variegata* leaves against breast cancer cell lines (a) Cytotoxicity on MCF-7 cells for 24 hours. (b) Cytotoxicity on MDA-MB-231 cells for 24 hours. Data representation mean *p < 0.05; **p < 0.01; ***p < 0.001.

The fractions obtained from the aqueous extract of *B. variegata* leaves showed cytotoxicity against MCF-7 and MDA-MB-231 breast cancer cell lines. Fraction no. 4 ,5 ,6 ,7, 11 and 12 showed inhibition of cell proliferation in MCF-7 cells at 35 μg/ml concentration whereas at 20 μg/ml in MCF-7 fractions 1, 3, 4, 5, 6, 7, 9, 11 and 12 showed cytotoxicity. In MDA-MB-231 fraction no. 4, 5, 6, 7, 8, 11, 12, 13, 15 and 16 showed effectiveness at 1000 μg/ml and fraction no. 4, 5, 6 ,7, 8, 11, 12, 13 and 14 were effective at 1300 μg/ml. This suggests that the fractions of aqueous extract of *Bauhinia variegata* L. also possess cytotoxic activity (figure 4.12)

The aqueous extract of *B. variegata* was checked for anti-cancer properties and it is shown to be potent towards both MCF-7 and MDA MB231 cells. This is the first time that phytocomponents from *B. variegata* leaves have been reported to have-anti cancer effects against breast cancer cell lines MDA-MB-231. The HRLC-MS analysis revealed the presence of three known compounds possessing anti-cancer activity. Hence this plant can be further explored for anti-tumorigenic properties. Also, the efficacy of these compounds individually or synergistically needs to be investigated.

4.5 Discussion:

Several parts of *Bauhinia variegata* have been exploited for its medicinal use due to abundance of phytocomponents present in the plant but no elaborate study on specific role of a particular phytocomponent is reported. Being a plant with mention of medicinal use this study was taken up to identify phytocomponents for the anticancer activity on breast cancer. Preliminary phytochemical screening of the crude ethanolic extracts of *B. variegata* leaves showed the presence of steroids, alkaloids, glycosides, saponins, flavonoids, tannins and carbohydrates(N. Gupta, Bhattacharya, Sharma, & Narang, 2011) The aqueous extract of *B. variegata* leaves, showed presence of carbohydrates, tannins, phenols, amino acids, alkaloids, flavonoids(Sudheerkumar, Seetaramswamy, Babu, & Kumar, 2015) .The aqueous extract prepared by successive solvent extraction (Rajat Singh, Bachheti, Saraswat, & Singh, 2012) was rich in

phytoconstituents like phenolic compounds, protein and amino acids, carbohydrates and glycosides, saponins, flavonoids. (A. Mishra et al., 2013) performed extraction of powdered leaf using sequence of solvents with increasing polarity, where the aqueous extract tested positive for phenols, flavonoids, tannins and saponins. The plant powder was initially defatted using petroleum ether, followed by eluotropic series of solvents consisting of n-hexane, chloroform, ethyl acetate, methanol, and water. All the extracts tested positive for phenols which is similarly reported by (A. Mishra et al., 2013). In this study, the aqueous extract from *B. variegata* leaves showed presence of phytocompounds such as alkaloids, flavonoids, saponins, phenols and carbohydrates which is almost similar to the aforementioned literature work on *B. variegata* leaves.

Previous studies portray *B. variegata* as a natural source of anti-oxidants. Ethanolic extract of *Bauhinia variegata* leaves showed a free radical scavenging activity which was almost similar to that of ascorbic acid (Bhatia L, 2011). The methanolic extract of *Bauhinia variegata* bark is the source of potent antioxidant with the strongest DPPH radical scavenging activity (K. R. Sharma et al., 2015). Several studies on methanolic bark extract by a different group of researchers revealed its strong antioxidant potential (N. Sharma et al., 2019). A comparative study on methanolic extracts of the aerial parts (leaves, stem bark and floral bud) of *Bauhinia variegata* revealed that the leaves (17.9 µg/ml.) and floral buds (17.2 µg/ml) have almost similar anti-oxidant property with stem-bark (19.5 µg/ml) on slightly higher side (S. Pandey, Agrawal, & Maheshwari, 2012). (Bhaskar, 2013) assessed free radical scavenging activity of aqueous and ethanolic bark extracts of *Bauhinia variegata* to find that aqueous extract shows better anti-oxidant activity in comparison to ethanolic extract. Results of present study, were also in agreement with the literature depicting strong antioxidant potential by methanol and aqueous extracts of *Bauhinia variegata* leaves. This property can be attributed to the phytocomponents present in *Bauhinia variegata* extracts which have proton donors that can scavenge free radical DPPH (Bhatia L, 2011). Thus, in general antioxidant activity of these extracts might be accredited to its alkaloids, flavonoids, phenolic and other phytocompounds.

In present study, the cell-cytotoxicity assay demonstrated that the aqueous extract possesses most cytotoxic activity compared to other extracts. Literature also states that compared to various extracts, aqueous fraction of *B. variegata* was effective on

inhibiting cell proliferation in different cell-lines by different proportions viz. 99 % for prostate cancer cell line (DU-145), 87% for lung cancer cell line (HOP-62), 99 % for ovarian cancer cell line (IGR-OV-1) cell line, 93 % breast cancer cell line (MCF-7), and 94 % for leukemia (THP-1) cell line. Petroleum ether extracts was least effective against most of the cancerous cell (A. Mishra et al., 2013). One more study with sequential solvent extraction showed that the aqueous fraction of *B. variegata* leaves exhibited profound cytotoxic activity compared to other extracts. It displayed cytotoxicity (91–99 %) against colon cancer cell line (Colo-205 and HCT-116) and lung (A549) cancer cell lines and lesser activity was observed against prostate cancer cell line (PC-3), breast cancer cell line (T47D) and lung cancer cell line (NCI-H322) (K. R. Sharma et al., 2015). The difference in the results between the two groups may be due to difference in the origin and differential molecular characteristics of different cell-lines. Hence, *in vitro* studies with the leaves of this plant demonstrates that aqueous extract of *Bauhinia variegata* L. leaves has significant cytotoxic potential on MCF-7 (A. Mishra et al., 2013) and T-47D cell lines (K. R. Sharma et al., 2015) compared to other extracts. To our knowledge, this is the first study to show cytotoxic effect of *Bauhinia variegata* L. leaves on MDA-MB-231 cell-line. Here, the results have been supported by reports showing potent cytotoxic action of aqueous extract against breast cancer cell lines.

Paclitaxel was used as a standard anti-cancer drug for these studies as it is a well-known plant derived anticancer drug used in breast cancer treatment. Liebmann et al. (1993) has revealed that no additional cytotoxicity post 24 hours drug exposure was observed, even by increasing the paclitaxel concentration above 50 nm. and the cells treated with lower concentrations of the drug had poor survival rate compared to the cells exposed to very high concentrations (10,000 nM). Similar kind of cytotoxicity pattern is observed with aqueous extract in present study. The assessment of cell cytotoxic properties of aqueous extract in comparison with paclitaxel, against breast cancer cell lines (MCF- 7 and MDA-MB-231) in this study also substantiates anti-cancer activity of phytoconstituents of *B. variegata* leaves. The presence of different phytocomponents (alkaloids, flavonoids, phenols etc.) might act either individually or in combination for demonstrating anticancer effects (A. Mishra et al., 2013). Different fractions obtained

from aqueous extract by Flash chromatography were also shown to possess cytotoxic potential. The crude aqueous extract itself is displaying promising cytotoxicity. Hence further bioactivity guided fractionation till separation of pure phytoconstituent responsible for cytotoxicity was needed. To identify potential constituents, of aqueous extract, sample was subjected to LC-MS analysis which showed presence of three anti-cancerous compounds: Berbamine, Rhapontin and Papaverine.

Berbamine dihydrochloride (BBM), a natural benzylisoquinoline alkaloid is mostly extracted from the plant, *Berbaris amurensis*. Literature reveals that Berbamine inhibits growth of liver cancer cells (Z. Meng et al., 2013). BBM has been reported to have antitumor activities in various types of cancers, including myeloma, breast and lung cancers, especially targeting cancer stem cells (Gu et al., 2013); (Yang et al., 2014). Berbamine also displays the strong activity of inducing apoptosis in both estrogen receptor-negative MDA-MB-231 cells and estrogen receptor-alpha-positive MCF-7 breast cancer cells, but not in normal human mammary epithelial cell line MCF10A(Shan Wang et al., 2009) The present study data showed the IC50 value of Berbamine was 50µg/ml in MCF7 cells and 75µg/ml in MDA-MB-231 cells. A study showed the IC50 value of Berbamine at 24 hrs was 51.6 µM for MDA-MB-231 cells (Shan Wang et al., 2009). There are evidences of Berbamine inhibiting the NF-kB & Akt pathway suppressing the invasion, proliferation & migration in MDA-MB-231 cells(Shan Wang et al., 2009). Berbamine promoted apoptosis in MDA-MB-231 & MCF 7 cells by inducing p53 expression whereas reduces COX-2, PI3K, LOX, mTOR, MDM2 expression (Liu et al., 2021). Berbamine also blocked the fusion between autophagosome and lysosome fusion by impeding the interaction of SNAP29 and VAMP8 in breast cancer MCF-7 and MDA-MB-231 cell-lines . Berbamine suppresses cell proliferation and promotes apoptosis in ovarian cancer partially via the inhibition of Wnt/β-catenin signaling (T. Zhang et al., 2019). Suppression of human lung cancer, cell growth and migration by Berbamine had also been reported (Duan, Luan, Liu, Yagasaki, & Zhang, 2010).

Reports for Rhapontin (a stilbene glycoside) suggests that it suppresses cell-growth of KATO III by apoptosis (Hibasami et al., 2007). It also decreases the metastatic and angiogenic abilities of MDA-MB-231 breast cancer cells via suppression of the HIF-1α pathway (A. Kim & Ma, 2018). Rhapontin also induces apoptosis in leukemic cancer

cell lines like HL60, HL60/MX1, HL60/MX2 for Human acute promyelocytic leukemia (APL) and CEM/C1, CCRF-CEM cell-lines for acute lymphoblastic leukemia (ALL) (Czop et al., 2019). According to results obtained from cell viability assay in present study, it was observed that Rhapontin significantly reduced the cell viability of MCF-7 cell lines at the concentration of 75µg/ml. However, MDA-MB-231 cell line required high concentration to inhibit it's the cell viability- between 750-1000 µg/ml of concentration. This suggest that Rhapontin does not exhibit any effect against MDA-MB-231 cell line. Even recent studies have shown that treating MDA-MB-231 with Rhapontin does not affect its cell viability, but it does stop invasive and migratory property (A. Kim & Ma, 2018). On the other hand, Rhapontin exhibits cytotoxic effect against MCF-7 cells.

Studies on papaverine showed selective and potential antitumor activity against several types of cancer cells, including breast carcinoma MCF-7 and MDA-MB-231(Sajadian et al., 2015), T47D (Afzali et al., 2015), colorectal carcinoma HT29 (Afzali et al., 2015) , fibrosarcoma HT1080(Afzali et al., 2015) prostate carcinoma PC-3 (H. Huang, Li, Zhang, & Wei, 2017), LNCaP(Goto et al., 1999; Shimizu, Ohta, Ozawa, Matsushima, & Takeda, 2000) hepatocarcinoma HepG2(Noureini & Wink, 2014). The cytotoxic effect of Papaverine was more on MCF-7 cancer stem cells when compared to parental cells, while opposite effect was observed in MDA-MB-231 cells compared to MCF-7 cells (Sajadian et al., 2015). Dose dependant decrease in cell viability was observed when breast cancer cells were given treatment with Papaverine. Surprisingly present study with papaverine, showed IC50 of 100 µg/ml with MCF-7 cells and 75 µg/ml with MDA-MB 231 cells. Thus, papaverine was effective on both the cell-lines.

This study has shown the presence of potential multiple anti-cancer phytocompounds in *B. variegata* leaves. The anti-cancerous property of aqueous extract might be due to synergistic effects of these compounds or either individual effect along with other phytocomponents with different levels of bioactivity. Further this anti-proliferative activity should also be further extended to other breast cancer cell lines also. Hence, various phytochemicals may contribute together and are responsible for the medicinal activities of the plant. Thus, *B. variegata L.* having rich repertoire of bioactives can be

a good source for anti-cancer phytocomponents which can be explored for pharmaceutical development and therapy against several tumors.