4. Results

4.1 Extraction of plant material

The air-dried plant of *A. aspera* (roots), *B. diffusa* (roots), and *E. littorale* (whole plant) was powdered and extracted with petroleum ether, ethyl acetate, alcohol, hydro-alcohol, and water as a solvent with a soxhlet apparatus. All the extracts were concentrated under reduced pressure and controlled temperature (40–50ºC) in a rotary evaporator. The extracts were stored at -20ºC in airtight containers for further experimental purposes. Phytochemical screening and organoleptic properties of the extracts were evaluated for characterization and identification of phytoconstituents. Organoleptic evaluation refers to evaluation of the extracts by color, odor, consistency, and percentage yield.

A. aspera extracts have a characteristic odor and are semisolid in consistency, with yields ranging from 3 to 7% w/w (Table 4.1). Preliminary photochemical screening of an alcoholic extract of an *A. aspera* showed the presence of carbohydrates, proteins, amino acids, steroids, flavonoids, glycosides, alkaloid, phenolic compounds, and saponins (Table 4.5) which are matched as per the literature (30).

B. diffusa extracts have characteristics of odor with 5 to 7% w/w yield and semisolid consistency (Table 4.2). Preliminary photochemical screening of the alcoholic extract of *B. diffusa* confers the presence of carbohydrates, proteins, amino acids, flavonoids, glycosides, alkaloids, and phenolic compounds which are as per the reported literature (Table 4.6) (214).

The alcoholic extract of *E. littorale* has the highest yield of 12% w/w. All other extracts of the *E. littorale* have yields range from 4 to 8% w/w (Table 4.3). The alcoholic extract showed the presence of carbohydrates, proteins, amino acids, flavonoids, glycosides, steroids, alkaloids, phenolic compounds, and saponins (Table 4.7) by phytochemical screening, which are as per the literature (215). Alcoholic extracts of *A. aspera*, *B. diffusa,* and *E. littorale* were found to have extractive values of 27.5%, 25.0%, and 21.0% w/w, respectively.

All three plant alcoholic extracts comply with the heavy metal and microbial counts as per the USFDA guidelines (216-217). Data on heavy metals and microbial analysis profile is presented in Table 4.4.

Table 4.1: Organoleptic properties of *A. aspera* extracts

Specification	Petroleum ether	Ethyl acetate	Alcoholic	Hydro alcoholic	Aqueous
Color	Green	Dark green	Pale yellow	Brown	Black
Consistency	Semisolid-liquid	Semisolid	Semisolid	Semisolid	Semisolid
Odor	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
Yield (% w/w)	3.0	5.0	6.5	6.2	7.0

Table 4.2: Organoleptic properties of *B. diffusa* extracts

Specification	Petroleum ether	Ethyl acetate	Alcoholic	Hydro alcoholic	Aqueous
Color	Dark green	Blackish green	Brownish green	Brown	Black
Consistency	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid
Odor	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
Yield (% w/w)	5.0	5.8	7.0	6.8	6.5

Table 4.3: Organoleptic properties of *E. littorale* extracts

Specification	Petroleum ether	Ethyl acetate	Alcoholic	Hydro alcoholic	Aqueous
Color	Green	Blackish green	Dark green	Brown	Blackish green
Consistency	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid
Odor	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
Yield (% w/w)	4.0	8.0	12.0	7.0	6.8

Table 4.4: Heavy metals and microbial analysis of extracts

Tests	**Specifications**	**Alcoholic extract of *B. diffusa***	**Alcoholic extract of *A. aspera***	**Alcoholic extract of *E. littorale***
Heavy metals				
Leads	NMT 1 ppm	Complies	Complies	Complies
Arsenic	NMT 1 ppm	Complies	Complies	Complies
Cadmium	NMT 3 ppm	Complies	Complies	Complies
Mercury	NMT 1 ppm	Complies	Complies	Complies
Microbial profile				
Total aerobic count	NMT 1000 CFU/gm	340 CFU/gm	370 CFU/gm	360 CFU/gm
Yeast and moulds	NMT 100 CFU/gm	Absent	Absent	Absent
E coli	Absent	Absent	Absent	Absent
Salmonella	Absent	Absent	Absent	Absent

Data is as per the certificate of analysis (CoA) of the extracts

Table 4.5: Preliminary phytochemical screening of *A. aspera* (root) extract

Class	Tests	Petroleum ether	Ethyl acetate	Alcoholic	Hydro alcoholic	Aqueous
Carbohydrate	Molisch Test	-	-	+	+	+
	Fehling's Test	-	-	+	+	+
	Benedict's Test	-	-	+	+	+
Protein	Biuret Test	-	-	+	+	+
	Millon's Test	-	-	+	+	+
Amino acid	Ninhydrin's Test	-	-	+	+	+
Steroids	Salkowski Test	-	+	+	+	+
Glycoside	Keller-kiliani Test	-	-	+	+	+
	Liebermann's Test	-	-	+	+	+
Saponins	Foam Test	-	-	+	+	+
Flavonoids	Alkaline Reagent Test	-	+	+	+	+
Alkaloids	Dragendorff's Test	-	-	+	+	+
	Mayer's Test	-	-	+	+	+
	Wagner's Test	-	-	+	+	+
Tannins and phenolic compounds	Ferric chloride Test	-	-	+	+	+

+ present, - absent

Table 4.6: Preliminary phytochemical screening of *B. diffusa* (root) extract

Class	Tests	Petroleum ether	Ethyl acetate	Alcoholic	Hydro alcoholic	Aqueous
Carbohydrate	Molisch Test	-	-	+	+	+
	Fehling's Test	-	-	+	+	+
	Benedict's Test	-	-	+	+	+
Protein	Biuret Test	-	-	+	+	+
	Millon's Test	-	-	+	+	+
Amino acid	Ninhydrin's Test	-	-	+	+	+
Steroids	Salkowski Test	+	-	-	-	-
Glycoside	Keller-kiliani Test	-	-	+	+	-
	Liebermann's Test	-	-	+	+	-
Saponins	Foam Test	-	+	-	+	-
Flavonoids	Alkaline Reagent Test	-	+	+	+	+
Alkaloids	Dragendorff's Test	-	+	+	+	+
	Mayer's Test	-	+	+	+	+
	Wagner's Test	-	+	+	+	+
Tannins and phenolic compounds	Ferric chloride Test	+	+	+	+	+

+ present, - absent

Table 4.7: Preliminary phytochemical screening of *E. littorale* (whole plant) extract

Class	Tests	Petroleum ether	Ethyl acetate	Alcoholic	Hydro alcoholic	Aqueous
Carbohydrate	Molisch Test	-	-	+	+	+
	Fehling's Test	-	-	+	+	+
	Benedict's Test	-	-	+	+	+
Protein	Biuret Test	+	-	+	+	+
	Millon's Test	+	-	+	+	+
Amino acid	Ninhydrin's Test	-	+	+	+	+
Steroids	Salkowski Test	-	+	+	+	+
Glycoside	Keller-kiliani Test	-	-	+	+	+
	Liebermann's Test	-	-	+	+	+
Saponins	Foam Test	-	+	+	+	+
Flavonoids	Alkaline Reagent Test	-	+	+	+	+
Alkaloids	Dragendorff's Test	-	+	+	+	+
	Mayer's Test	-	+	+	+	+
	Wagner's Test	-	+	+	+	-
Tannins and phenolic compounds	Ferric chloride Test	+	+	+	+	+

+ present, - absent

4.2 MTT (cytotoxicity) assay

All the extracts and reference standard showed dose dependent cytotoxicity on the HepG2 cell line. All the extracts of *A. aspera* showed statistically significant (P<0.001) cytotoxicity above 80 µg/mL concentration as compared to the untreated cells. The alcoholic extract of *A. aspera* showed statistically significant cytotoxicity as compared to petroleum ether extract (P<0.1).

The percentage cells viability at 640 µg/mL (highest concentration) was 20%, 63%, 50%, 39%, 68%, respectively for alcoholic, aqueous, hydro alcoholic extract, ethyl acetate and petroleum ether extract (Figure 4.1). The IC_{50} value of alcoholic extract was found to be 192 µg/mL. Thus, alcoholic extract was selected for further studies.

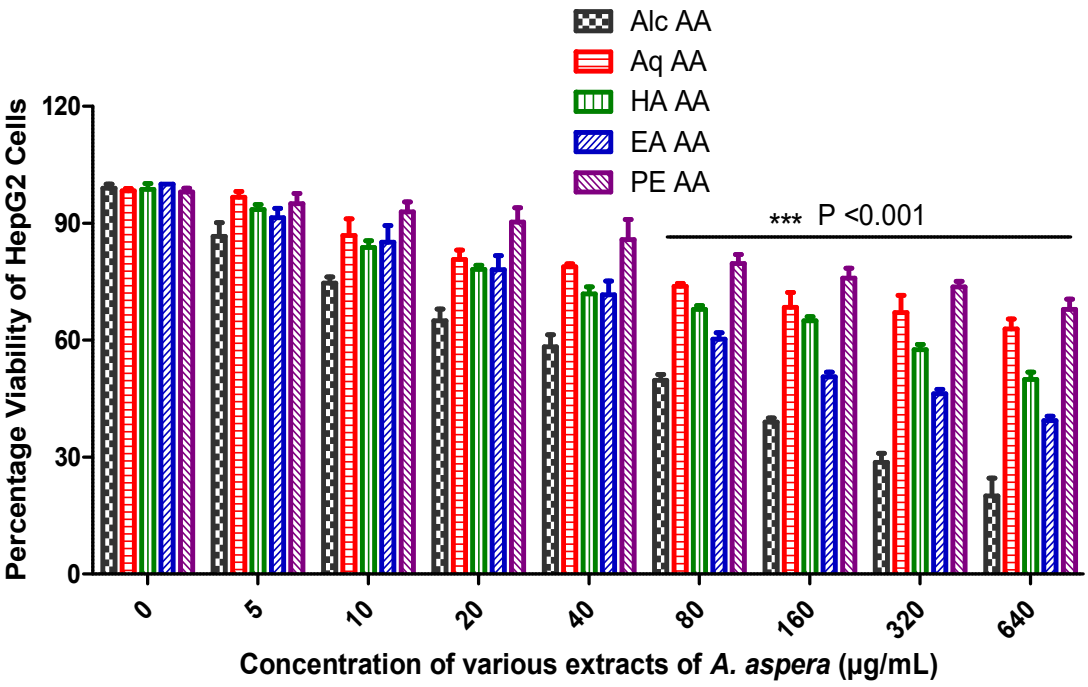


Figure 4.1: Percentage cell viability with *A. aspera* extracts in MTT assay

Following treatment with *A. aspera* (AA) extracts at the indicated concentration, MTT assay was used to assess the viability of the HepG2 cells. Each value (n = 3) is mean ± SD. Alc-alcoholic extract, Aq-aqueous extract, HA-hydro alcoholic extract, EA-ethyl acetate extract, PE-petroleum extract. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. *** p<0.001 as compared to the control cell without treatment.

The alcoholic extract of *B. diffusa* showed potent cytotoxicity, which leads to significantly ($P<0.01$) less formazan crystal formation measured by the MTT assay (cytotoxicity) with an IC_{50} value of 141 µg/mL compared to its aqueous, and petroleum ether extract. The cytotoxicity of hydro-alcoholic (with 48% viable cells) and ethyl acetate extract (54% viable cells) was not significant as compared to alcoholic extract, but it had only 12% of viability of cells at 640 µg/mL concentration. All the extracts of *B. diffusa* above 40 µg/mL showed statistically significant ($P<0.001$) cytotoxicity as compared to the untreated cells (Figure 4.2). Hence, alcoholic extract was selected for further studies.

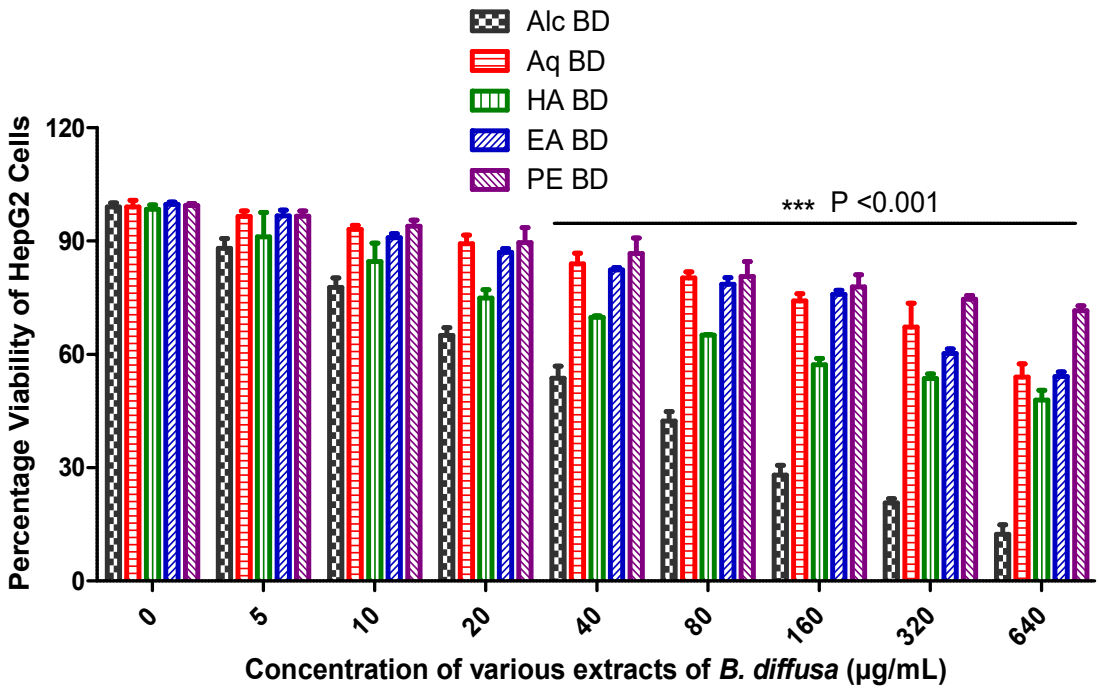


Figure 4.2: Percentage cell viability with *B. diffusa* extracts in MTT assay

Following treatment with *B.diffusa* (BD) extracts at the indicated concentration, MTT assay was used to assess the viability of the HepG2 cells. Each value (n = 3) is mean ± SD.). Alc-alcoholic extract, Aq-aqueous extract, HA-hydro alcoholic extract, EA-ethyl acetate extract, PE-petroleum extract. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test. *** $p<0.001$ as compared to the control cells devoid of any treatment.

When compared to other extracts of *E. littorale*, the alcoholic extract exhibited the maximum cytotoxicity, with an IC_{50} value of >373 µg/mL. When compared to alcoholic extract, the percentage cell viability of aqueous, hydro-alcoholic, and ethyl acetate extracts at 640 µg/mL

concentration was nearly identical (40–60% cell viability) and exhibited no discernible activity. At the same concentration petroleum ether extract was found to have 77% cell viability. All the extracts of *E. littorale* above 40 µg/mL concentration showed statistically significant (P<0.001) cytotoxicity as compared to the untreated cells (Figure 4.3). Thus, alcoholic extract was selected for further studies.

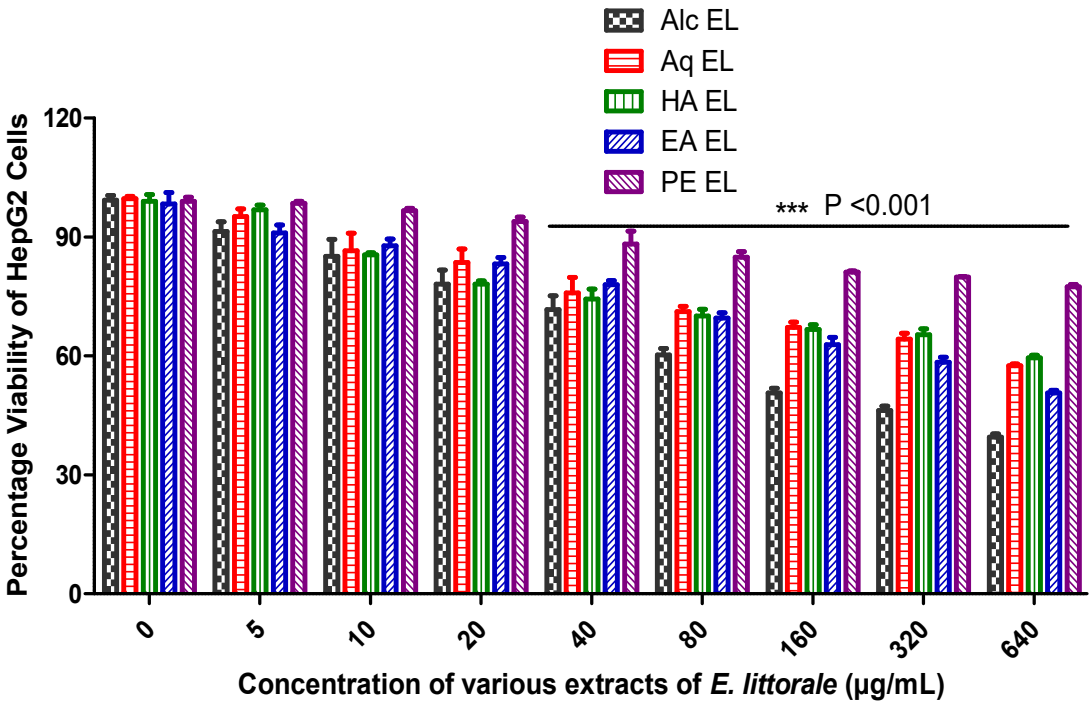


Figure 4.3: Percentage cell viability with *E. littorale* extracts in MTT assay

Following treatment with *E. littorale* (EL) extracts at the indicated concentration, MTT assay was used to assess the viability of the HepG2 cells. Each value (n = 3) is mean ± SD. Alc-alcoholic extract, Aq-aqueous extract, HA-hydro alcoholic extract, EA-ethyl acetate extract, PE-petroleum extract. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. *** p<0.001 as compared to the control cells without treatment

Sorafenib showed dose-dependent cytotoxicity (Figure 4.4). Sorafenib above 0.312 µM concentration showed statistically significant (P<0.001) cytotoxicity as compared to the untreated cells. Sorafenib showed 22% viability at highest concentration (20 µM) with IC_{50} value of 6.68 µM. The IC_{50} value of sorafenib was obtained from combining the data from different set of experiments done with the extracts.

The alcoholic extracts of the three plants showed the highest activity as observed with a lower IC_{50} value compared to their other (aqueous, hydro alcoholic, ethyl acetate and petroleum ether) extracts. Thus, the alcoholic extract was (graphical abstract Figure 4.5) selected for further screening by invitro and invivo experiments. The IC_{50} values of extracts and sorafenib are shown in Table 4.8.

Table 4.8: IC_{50} value of extracts and reference compound

Extract/reference compound	IC_{50}
Alcoholic extract of *A. aspera*	192 ± 2.2 µg/mL
Alcoholic extract of *B. diffusa*	141 ± 2.7 µg/mL
Alcoholic extract of *E. littorale*	373 ± 3.0 µg/mL
Sorafenib	6.68 ± 0.3 µM

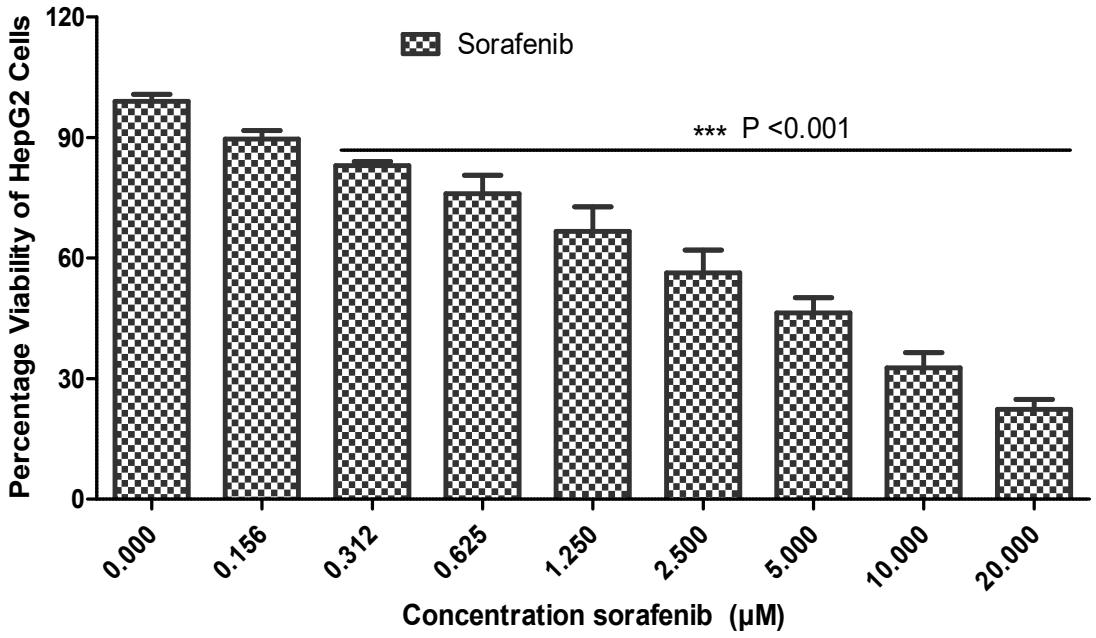


Figure 4.4: Percentage cell viability with sorafenib in MTT assay

MTT test was used to assess the viability of HepG2 cells after they were treated with sorafenib at the indicated concentrations. All values mean ± SD (n = 3). Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. *** $p<0.001$ as compared to the untreated cells.

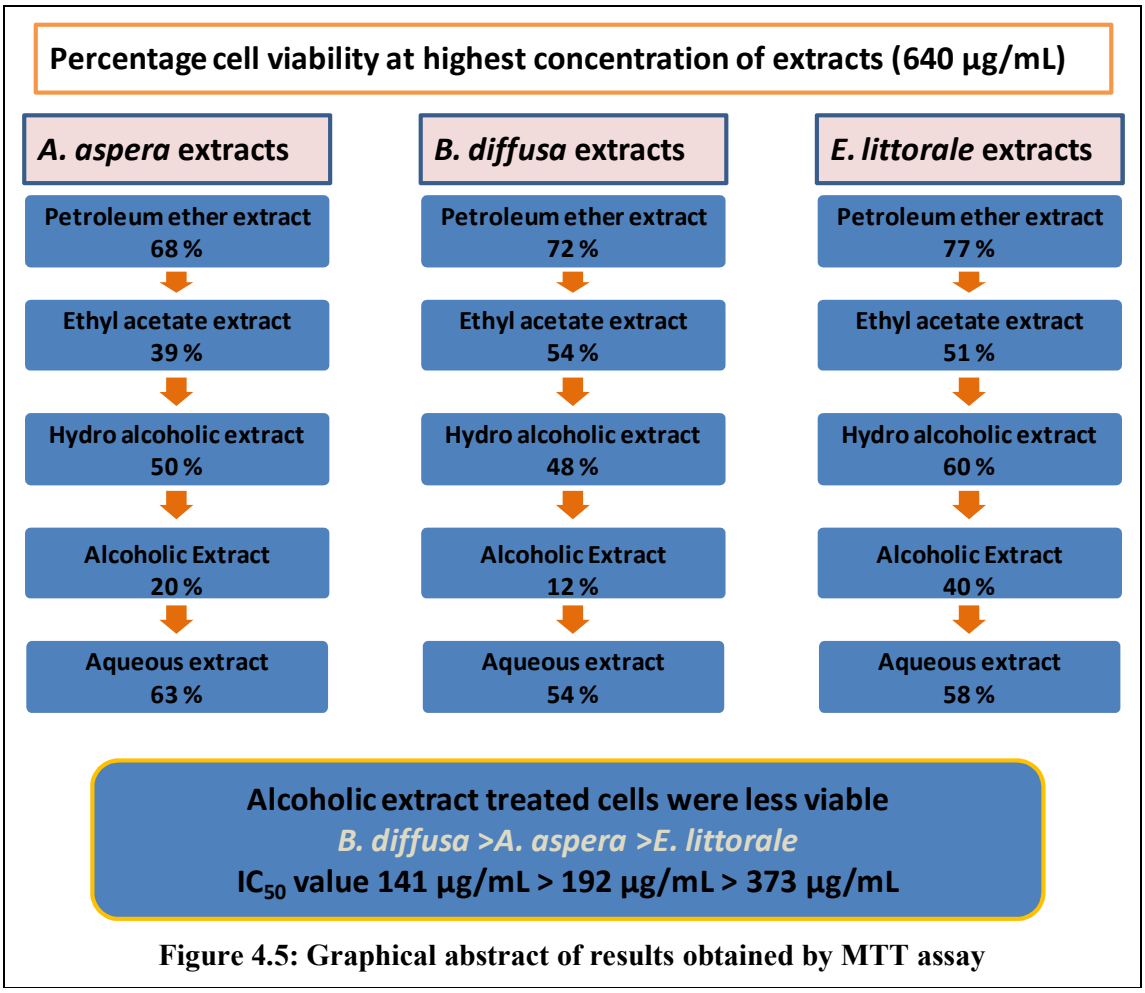


Figure 4.5: Graphical abstract of results obtained by MTT assay

4.3 Scratch motility (wound healing) assay

Alcoholic extracts of *B. diffusa* and *A. aspera* were assessed for scratch motility assay on HepG2 cells by *in vitro* experiment. The HepG2 cells were exposed to the IC_{50} concentrations of the extracts and sorafenib. The cells crossing the scratch area were measured in terms of wound healing.

As migration is a primary step in cancer metastasis, the study focused on determining the effect of both the extracts (*A. aspera*, *B. diffusa)* and sorafenib on the migration of HepG2 cells using a wound healing assay. In the wound healing assay, the scratch area of the control (untreated) well showed filling of the gap after 48 hrs of scratching. The untreated cells were able to split and replenish the cell population of the scratch area by about 67% of the wound, which indicates the strong migration capacity of HepG2 cells. On the other hand, cells treated with *A. aspera* and *B. diffusa* showed significantly ($p< 0.01$ to 0.001) less filling of scratch

area as compared to untreated cells. *B. diffusa* and *A. aspera* extract-treated cells in the wells showed migration of only 45% and 35% of cells, respectively, in the scratched area as compared to untreated cells, indicating inhibition of cell migration by both extracts. Moreover, sorafenib also showed significant ($p < 0.001$) wound healing as the scratch area was only filled by 29% of the cells. No significant difference was found in the percentage wound healing activity of sorafenib and *A, aspera,* while the sorafenib activity was significantly ($p<0.1$) better than that of *B. diffusa*. The inhibitory percentage and scratch area are shown in Figure 4.6 and 4.7.

Overall, the result showed that both the extracts and sorafenib significantly reduced the rate at which the cells were able to divide and inhibited the migration ability (motility) of the cancer cells.

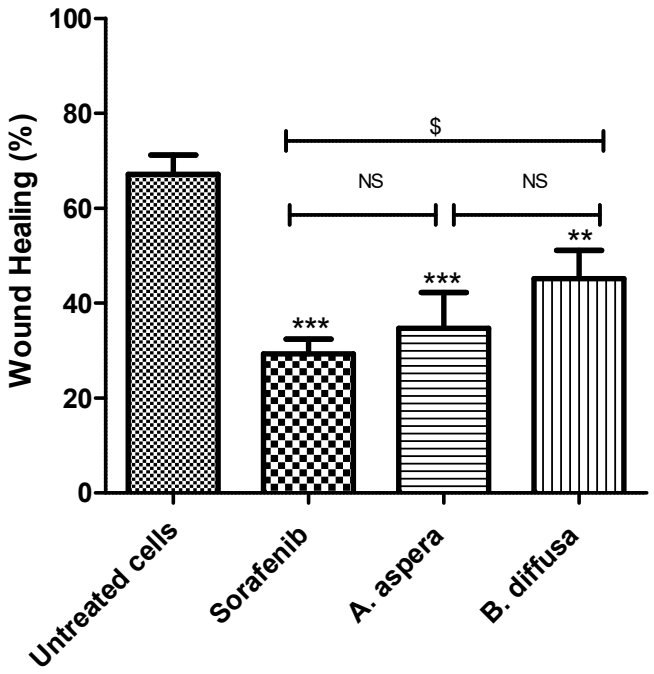


Figure 4.6: Percentage wound healing with *A. aspera*, *B. diffusa* extracts and sorafenib on the HepG2 cells scratch motility assay

HepG2 cells were treated with *A. aspera*, *B. diffusa* and sorafenib for 48 hr. A percentage scratch wound healing assay was then conducted to examine the invasive and metastatic capabilities of the cells. Each number (n = 3) is mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons ** $p<0.01$, *** $p<0.001$ as compared to untreated cells. $ $p<0.1$ as compared to sorafenib treated cells

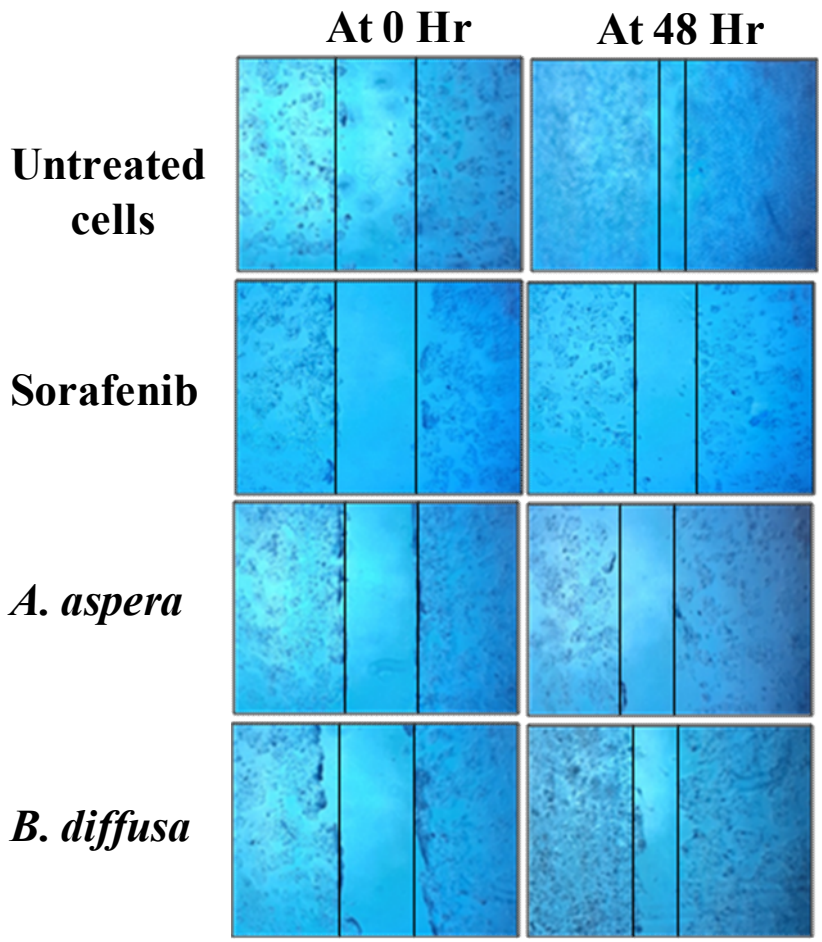


Figure 4.7: Inhibitory effects of *A. aspera*, *B. diffusa* extracts and sorafenib on the migration of HepG2 cells

4.4 Colony formation (clonogenic cell survival) assay

The clonogenic cell survival assay assesses the capacity of a cell for its unrestricted proliferation and maintaining its capacity for self-reproduction to generate a sizable colony. This cell is then termed as a clonogenic cell (218).

Untreated cells showed healthy colonies, which were well proliferated in two weeks. A significant ($p< 0.01$ to 0.001) decrease in colony-forming ability of the cells was observed with the cells treated with *A. aspera* (74%), *B. diffusa* extract (68%), and sorafenib (16%) post 2 weeks of incubation period as compared to the untreated (control) cells (Figure 4.8 and 4.9). Sorafenib had significantly ($p<0.001$) inhibited the colony formation as compared to

both the extracts. In all the treatment group cells were remarkably hampered for survival and proliferation.

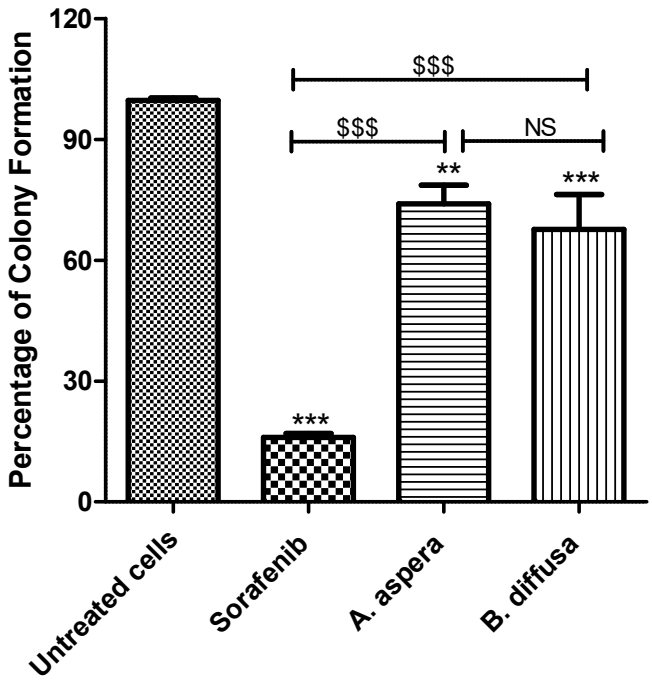


Figure 4.8: Percentage colony formation assay following treatment with *A. aspera, B. diffusa* extracts and sorafenib on HepG2 cells

Percentage of HepG2 cells colony formation on treatment with *A. aspera*, *B. diffusa* extract and sorafenib. The data are presented as the mean ± SD from at least three independent experiments. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. ** $p<0.01$, *** $p<0.001$ as compared to untreated cells. $$$ $p<0.001$ as compared to sorafenib treated cells. NS: non significant

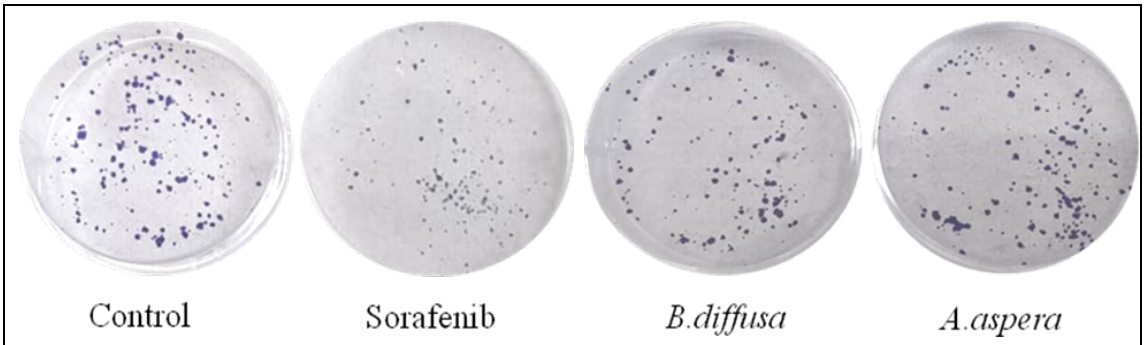


Figure 4.9: Colony formation following treatment with *A. aspera, B. diffusa* extracts and sorafenib on HepG2 cells colony formation assay

4.5 Apoptosis assay

To investigate the impact of *A. aspera* and *B. diffusa* extracts on HepG2 cell apoptosis, flow cytometric analysis was carried out. Propidium iodide (PI) and Annexin V-FITC labeling allowed for the differentiation of intact (living) cells from early, late, and dead apoptosis cells. Exposure of both the extract and sorafenib for 48 hr with the cells caused a significant ($p< 0.001$) cell death as compared to untreated cells. The treatment with *A. aspera* extract and sorafenib led to a significant ($p< 0.01$) reduction in the necrosis of the cells. *A. aspera* extract exposure caused more damage to the cancer cells than sorafenib, however, both extracts and sorafenib led to a considerable ($p<0.001$) amount of cells in the early and late apoptosis stages as compared to untreated cells. Treatment of extract leads to significantly ($p<0.1$) fewer live cells and more cells in early apoptosis ($p<0.001$) as compared to sorafenib-treated cells. BD FACS Calibur and Cell Quest Pro Software (version: 6.0) were used to analyze the data. Figure 4.10 and Table 4.9 display the percentage of cells in each stage of cell death. This FACS experiment shows that the extract caused early and late stage apoptosis in the cells, which results in cell death.

Table 4.9: Percentage of cell population in different stages of cell death

Stages of cell	Untreated cells	*A. aspera* extract	*B. diffusa* extract	Sorafenib
Live (%)	98 ± 2.31	54 ± 1.91***$^{\$}$	66 ± 4.37***$^{@}$	63 ± 2.81***
Early apoptosis (%)	0.06 ± 0.01	21 ± 0.63***$^{\$\$\$}$	16 ± 0.21***$^{@@@}$	17 ± 0.49***
Late apoptosis (%)	0.4 ± 0.1	25 ± 0.67***$^{\$\$\$}$	18 ± 1.02***$^{@@@}$	20 ± 1.24***
Necrosis (%)	0.07 ± 0.01	0.02 ± 0.01**	0.06 ± 0.02$^{\$\$@}$	0.01 ± 0.01**

Data represented as mean ± SD, n = 3. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons

** $p<0.01$ and *** $p<0.001$ as compared to untreated cells

$^{\$}$ $p<0.1$, $^{\$\$}$ $p<0.01$ and $^{\$\$\$}$ $p<0.001$ as compared to sorafenib treated cells

$^{@}$ $p<0.1$, and $^{@@@}$ $p<0.001$ as compared to *A. aspera* extract treated cells

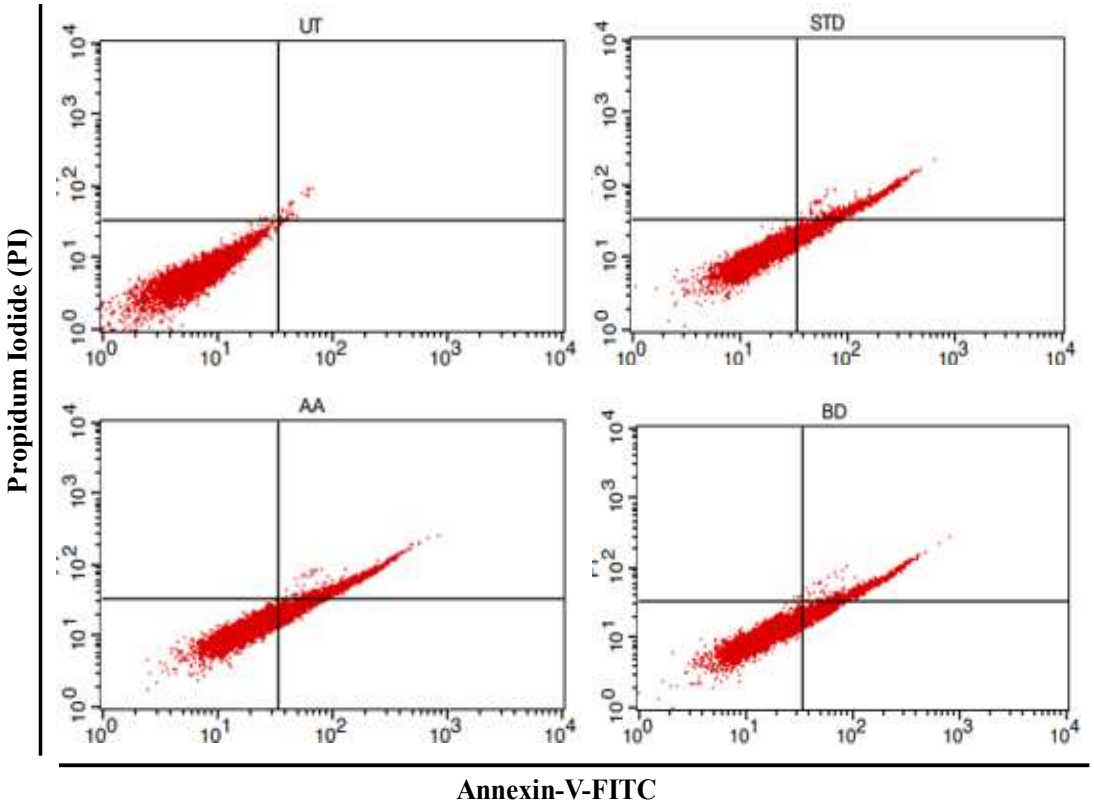


Figure 4.10: The alcoholic extract of *A. aspera* and *B. diffusa* induced early and late stage apoptosis in the HepG2 cell line

Annexin V and PI-stained HepG2 cells were evaluated using flow cytometry. In each panel, viable cells (negative for both annexin V and PI) are shown in the lower left quadrant, only annexin V+ cells (early apoptotic) are shown in the lower right quadrant, annexin V+ and PI+ cells (late apoptosis) are shown in the upper right quadrant, and only PI+ cells (necrotic) are shown in the upper left quadrant. UT-untreated cells, AA-*A. aspera*; BD-*B. diffusa*, STD-standard sorafenib

4.6 Phytochemical analysis of extracts by LCMS

The qualitative screening of alcoholic extracts of *A. aspera* and *B. diffusa* by LCMS leads to the identification of 12 (Table 4.10) and 14 (Table 4.11) phytoconstituents, respectively. Each identified chemical component contains the following information: compound name, retention time (Rt), ionization mode, adduct ions, and fragment ions m/z.

By comparing the fragmentation pattern in the positive and negative modes against the spectra of the literature, phytocompounds were identified in both the extracts.

Table 4.10: Compounds identified in alcoholic extract of *A. aspera* by LCMS

Sr. No.	Phytocompounds	M/Z	Mol Wt.	Rt	Mode	Adduct
1	Gentisic acid	155.1	154.1	17.28	ES+	M+H
2	Azelaic acid	189.0	188.2	21.81	ES+	M+H
3	Undecanedioic acid	218.2	216.2	4.63	ES-	M+2H
4	Linolenic acid	283.3	280.4	29.09	ES+	M+2H
5	Chlorogenic (5-caffeoylquinic) acid	352.2	354.3	7.37	ES+	M-2H
6	4-caffeoylquinic acid	413.1	354.0	3.57	ES-	M+CH3COOH-H
7	Kaempferol-3-o-glucoside	447.1	448.1	5.31	ES-	M-H
8	20-hydroxyecdysterone	514.0	480.6	6.79	ES+	M+CH3OH+H
9	4,5-dicaffeoylquinic acid	534.3	516.4	5.34	ES+	M+NH4
10	Apigenin-7-o-hexuronide-4'-o-rhamnoside	591.1	592.1	6.13	ES-	M-H
11	3,4,5-tricaffeoylquinic acid	682.1	681.0	5.40	ES+	M+H
12	Oleanolic acid	458.1	456.7	6.52	ES+	M+H

Chromatogram of alcoholic extract of *A. aspera* at positive and negative ion mode is shown in Figure 4.11 and 4.12 respectively. Mass spectrum and structure of phytoconstituents detected by LCMS in alcoholic *A. aspera* extract is shown in Figure 4.13.

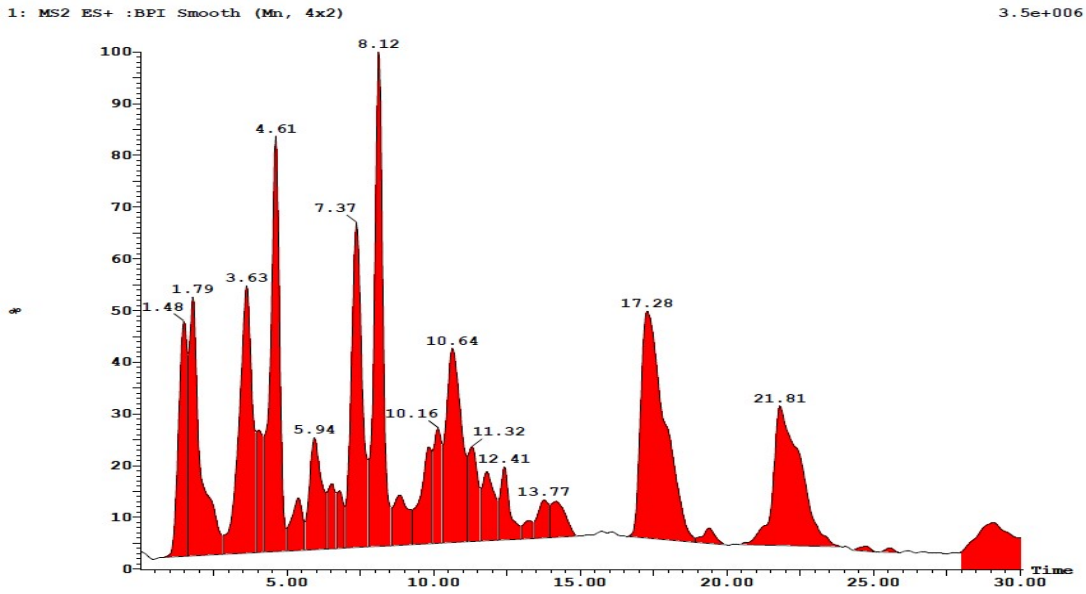


Figure 4.11: LCMS chromatogram of alcoholic extract of *A. aspera* (ES+ ve)

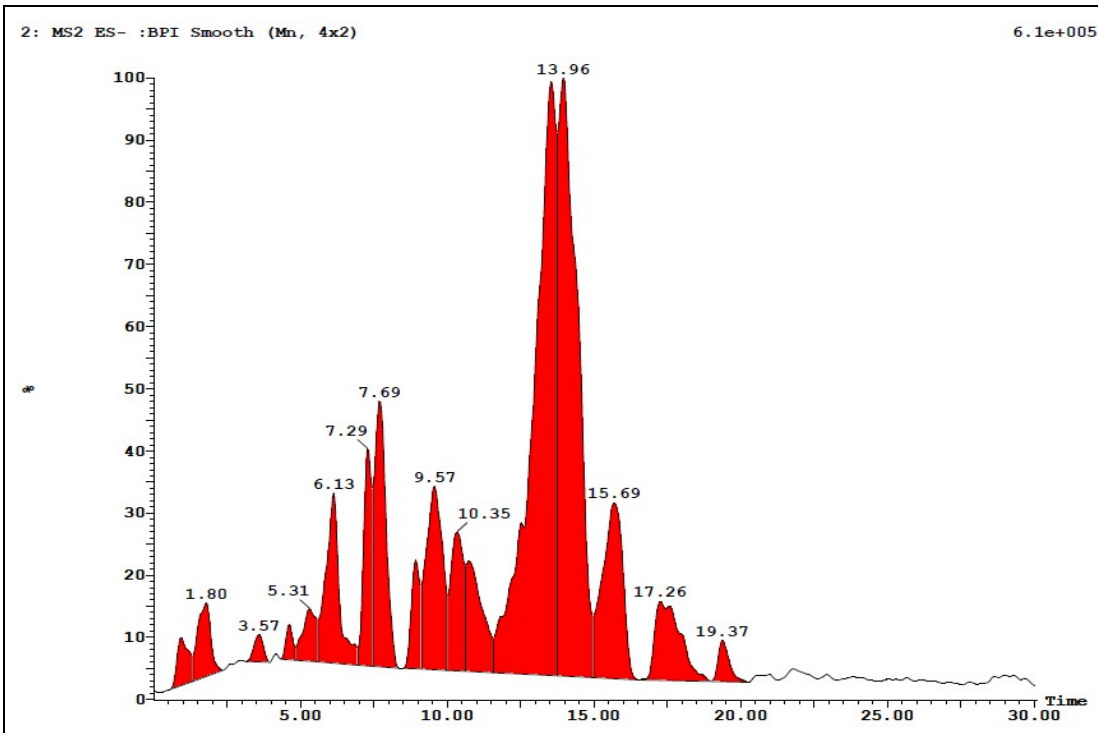
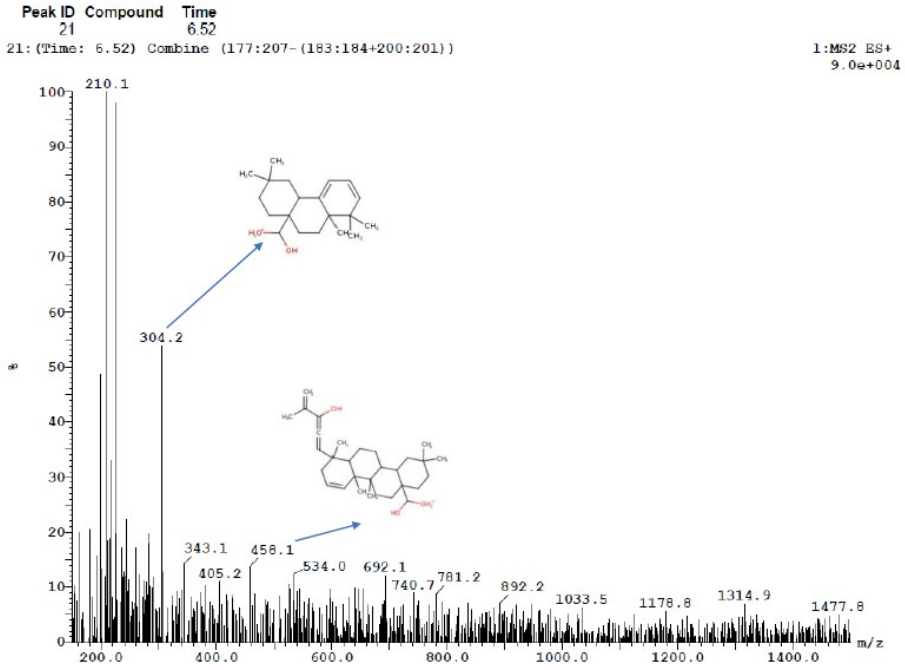


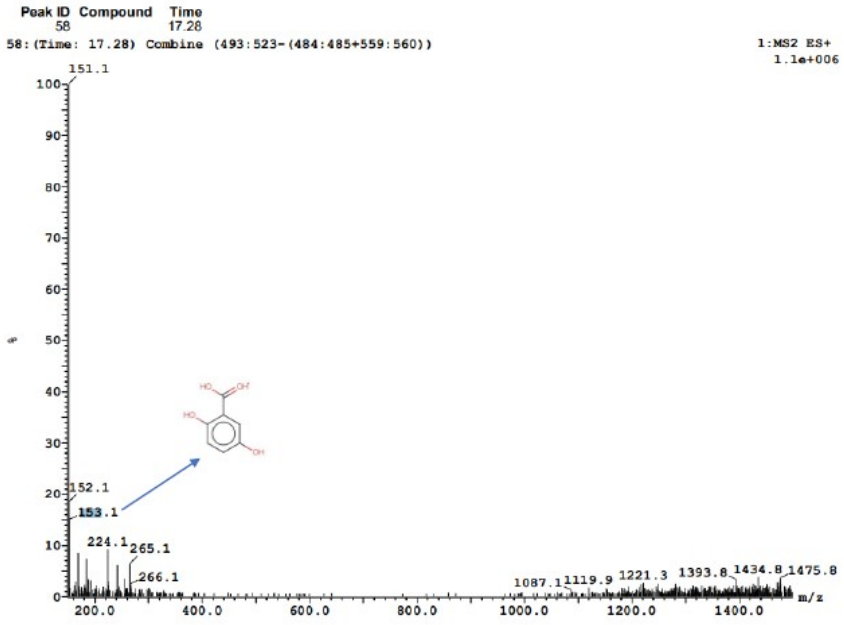
Figure 4.12: LCMS chromatogram of alcoholic *extract of A. aspera* (ES- ve)

Figure 4.13: Mass spectrum and structure of phytoconstituents detected by LCMS in alcoholic *A. aspera* extract

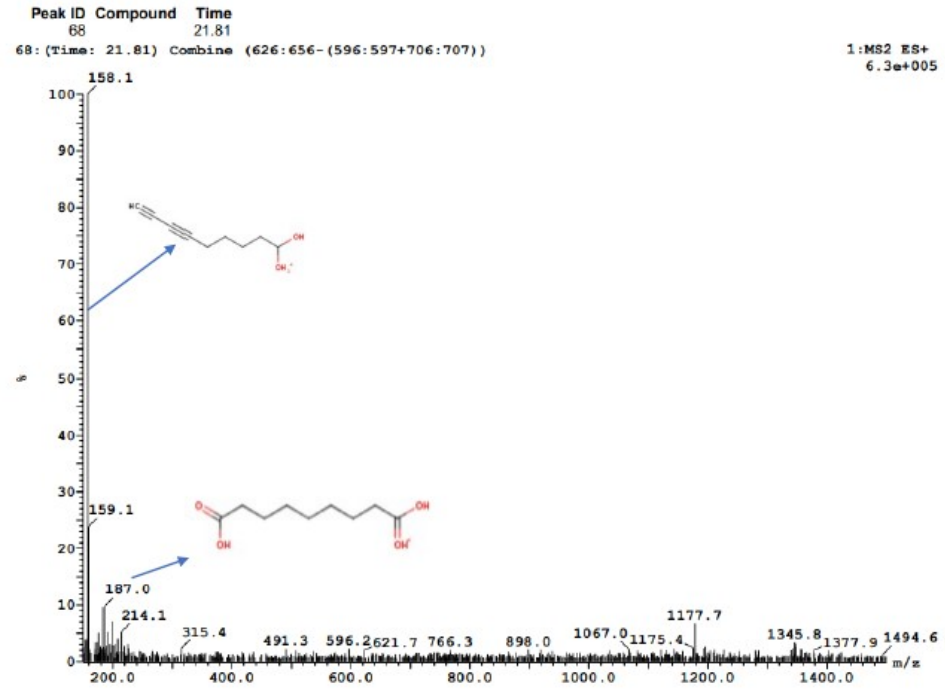
Oleanolic acid



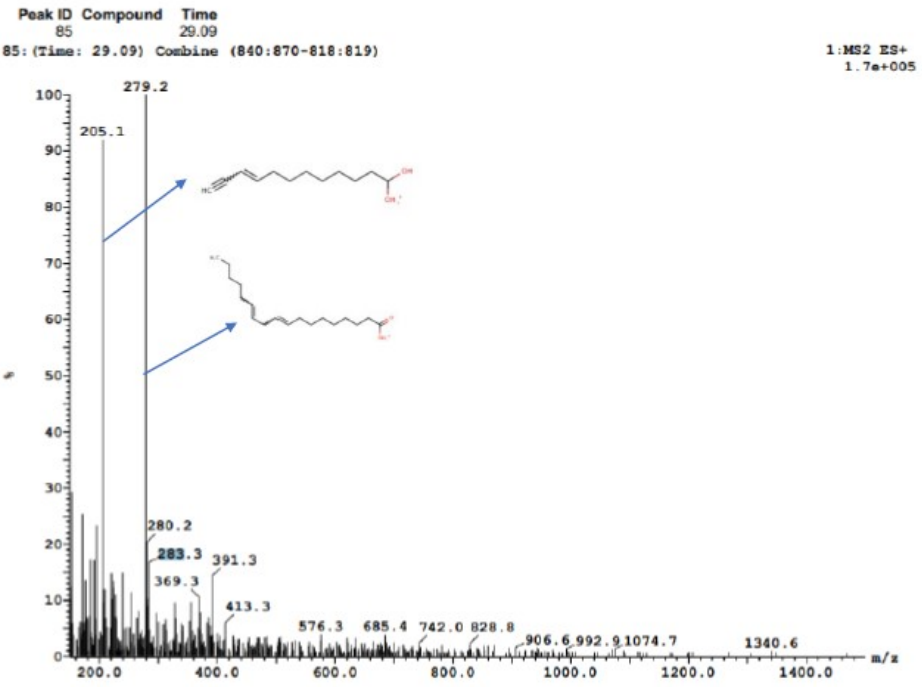
Gentisic acid



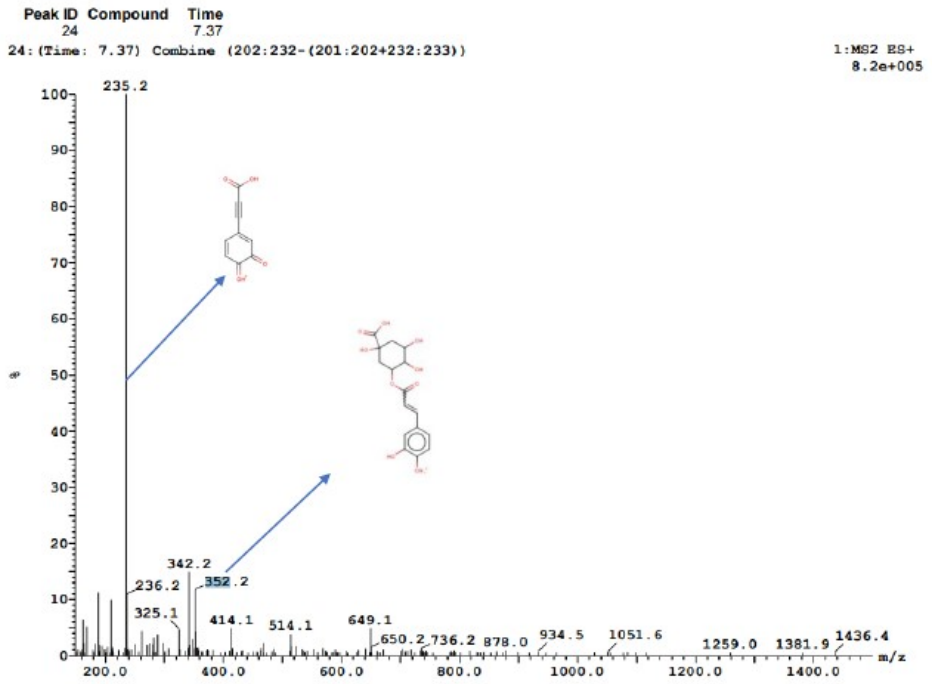
Azelaic acid



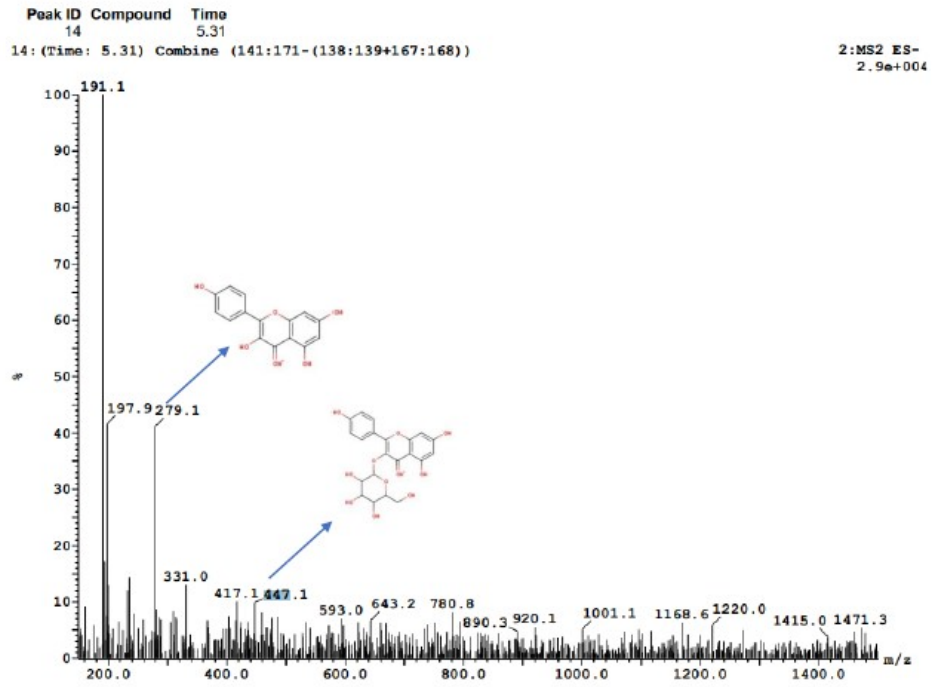
Linolenic acid



Chlorgenic acid



Kaempferol glucoside



4,5 dicaffeoylquinic acid

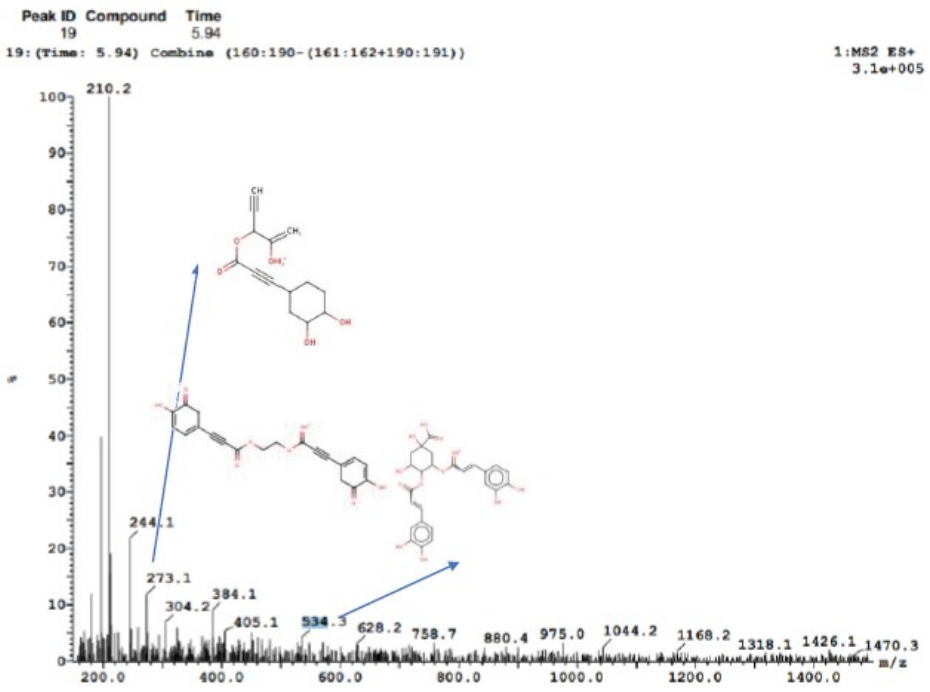


Table 4.11: Compounds identified in alcoholic extract of *B. diffusa* by LCMS

Sr. No.	Phytocompounds	M/Z	Mol Wt.	Rt	Mode	Adduct
1	Protocatechuic acid	152.1	154.1	17.72	ES+	M-2H
2	Quinic acid	191.1	192.1	5.51	ES-	M-H
3	Ferulic acid	227.1	194.1	1.8	ES+	M+CH3OH+H
4	Hydroxy-methylflavone	315.2	282.2	3.11	ES+	M+CH3OH+H
5	Kaempferol	325.1	286.2	9.38	ES+	M+K
6	Epicatechin	329.2	290.2	17.72	ES+	M+K
7	Quercetin	325.2	302.2	12.41	ES+	M+Na
8	Boeravinone B	311.2	312.2	1.2	ES-	M-H
9	Boeravinone E	329.2	328.2	5.46	ES+	M+H
10	Boeravinone A	325.2	326.2	4.29	ES-	M-H
11	Boeravinone G	343.1	342.1	1.45	ES+	M+H
12	Eupalitin	331.1	330.2	4.07	ES+	M+H
13	Isovitexin	433.0	432.3	6.13	ES+	M+H
14	Eupalitin-3-*O*-galactopyranoside	493.1	492.4	3.49	ES+	M+H

Chromatogram of alcoholic extract of *B. diffusa* at positive and negative ion mode is shown in Figure 4.14 and 4.15 respectively. Mass spectrum and structure of phytoconstituents detected by LCMS in alcoholic *B. diffusa* extract is shown in Figure 4.16.

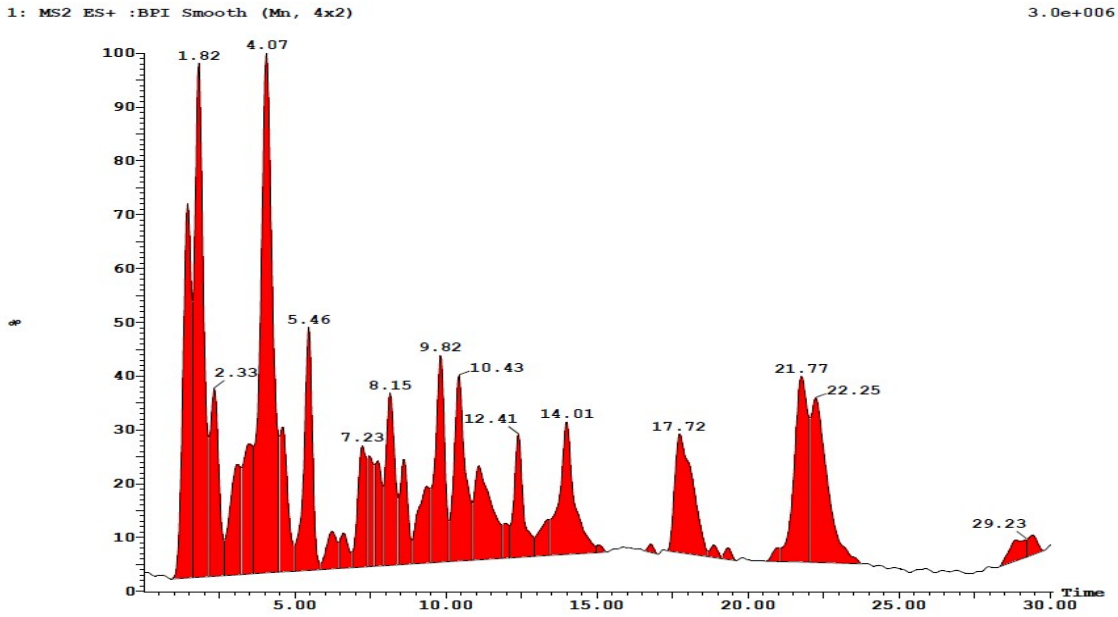


Figure 4.14: LCMS chromatogram of alcoholic extract of *B. diffusa* (ES+ve)

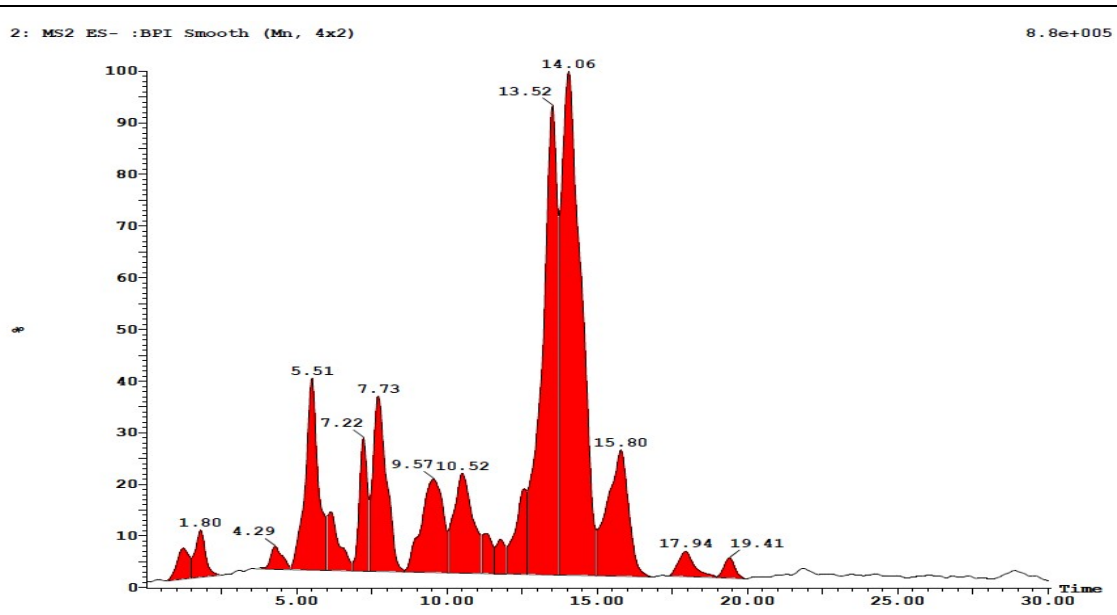
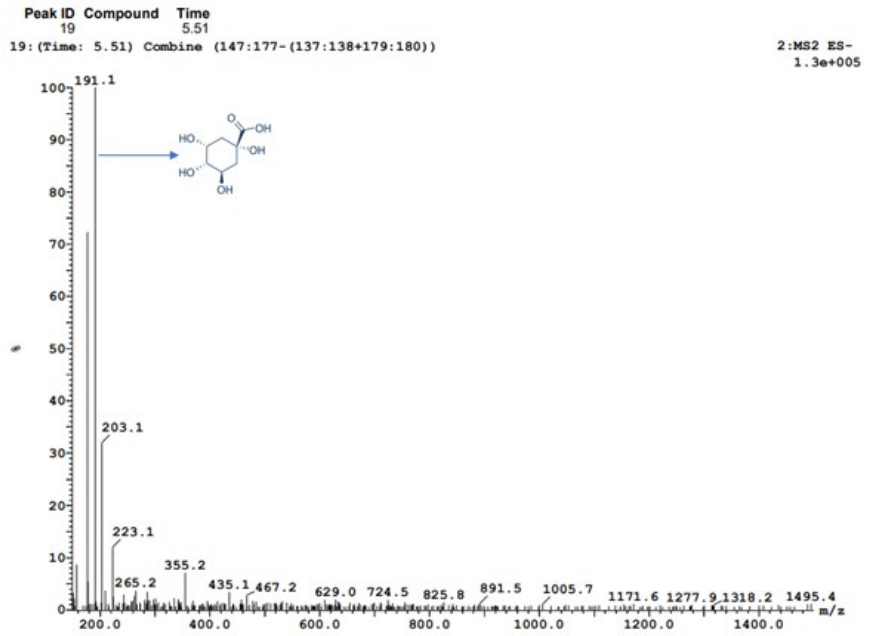


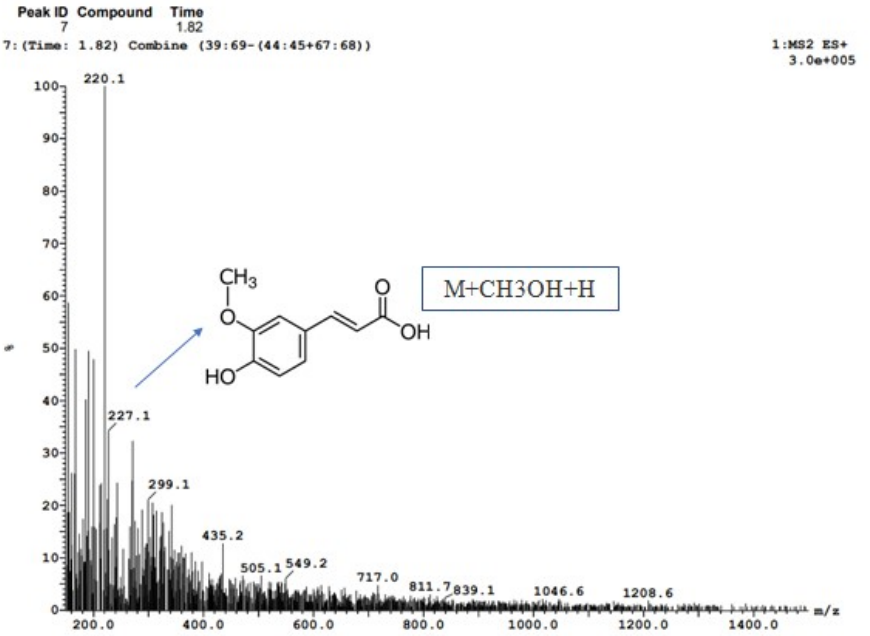
Figure 4.15: LCMS chromatogram of alcoholic extract of *B. diffusa* (ES-ve)

Figure 4.16: Mass spectrum and structure of phytoconstituents detected by LCMS in alcoholic *B. diffusa* extract

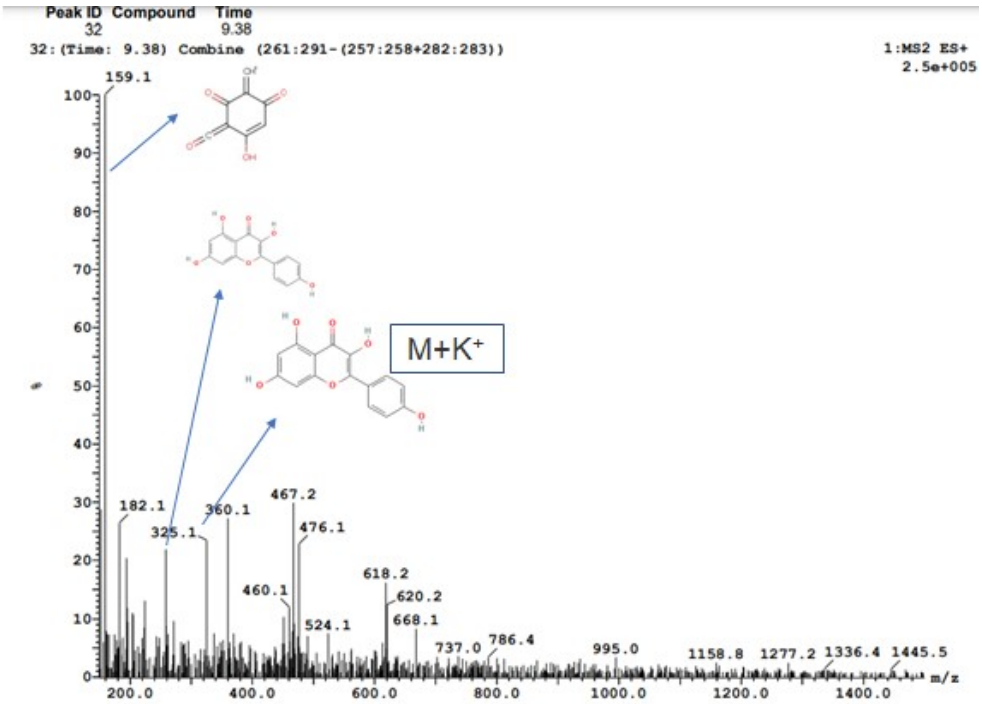
Quinic acid



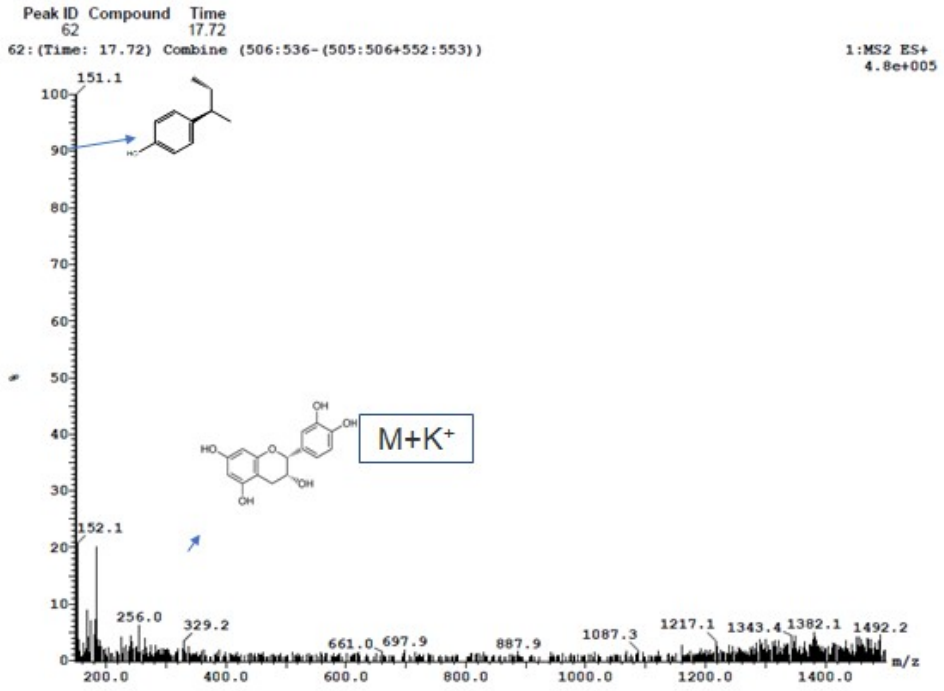
Ferulic acid



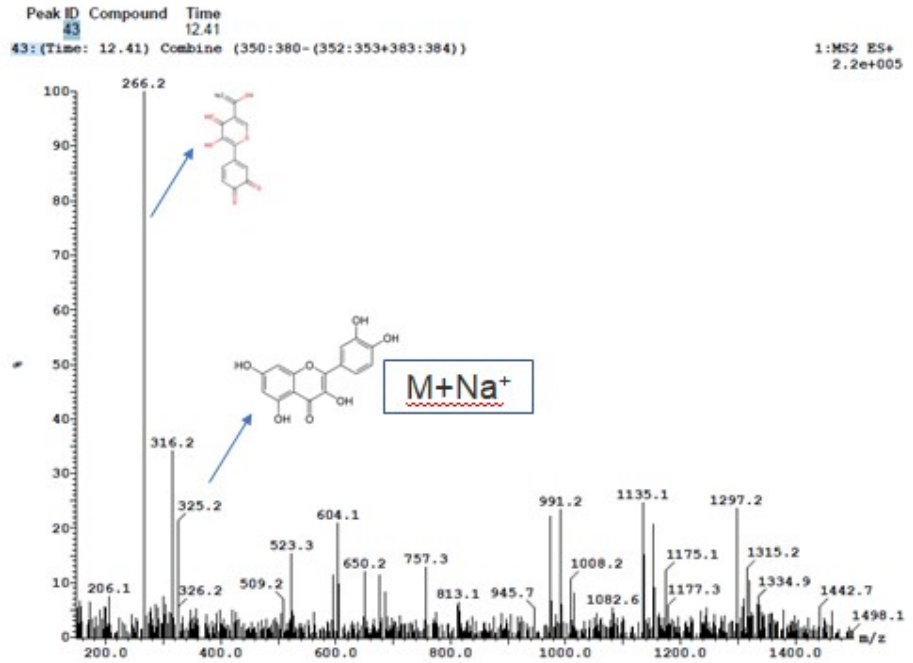
Kaempferol



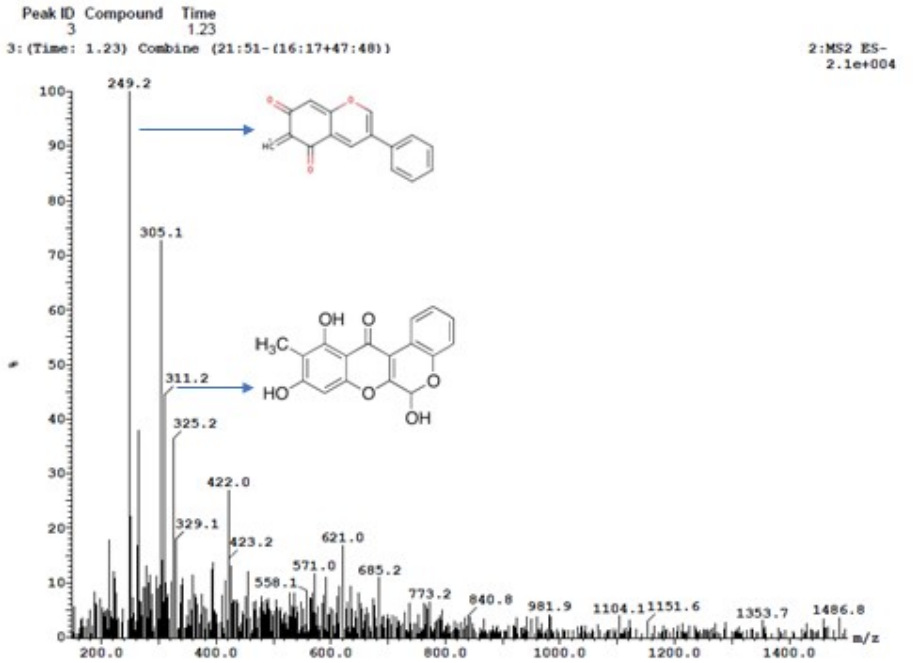
Epicatechin



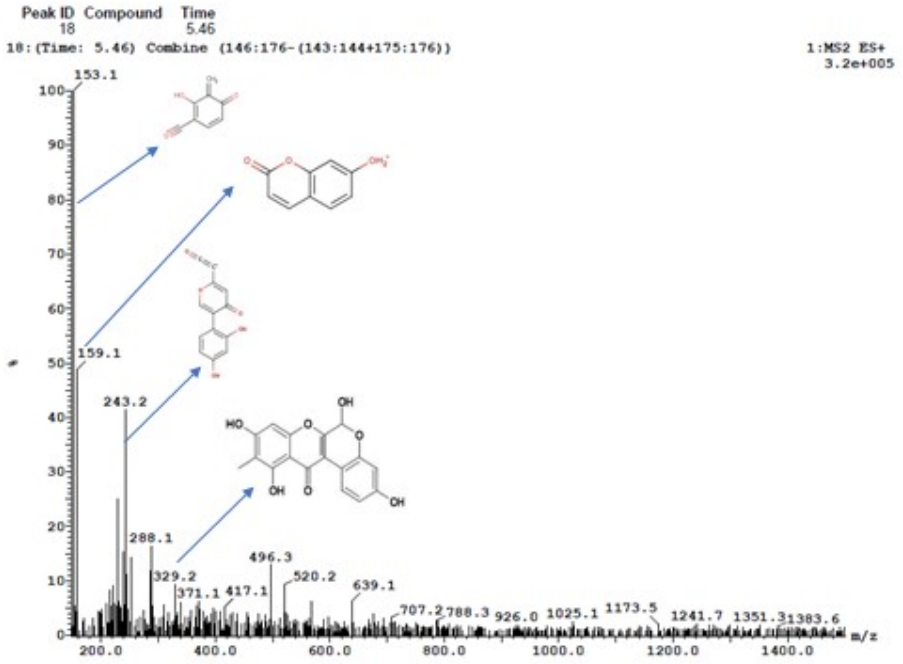
Quercetin



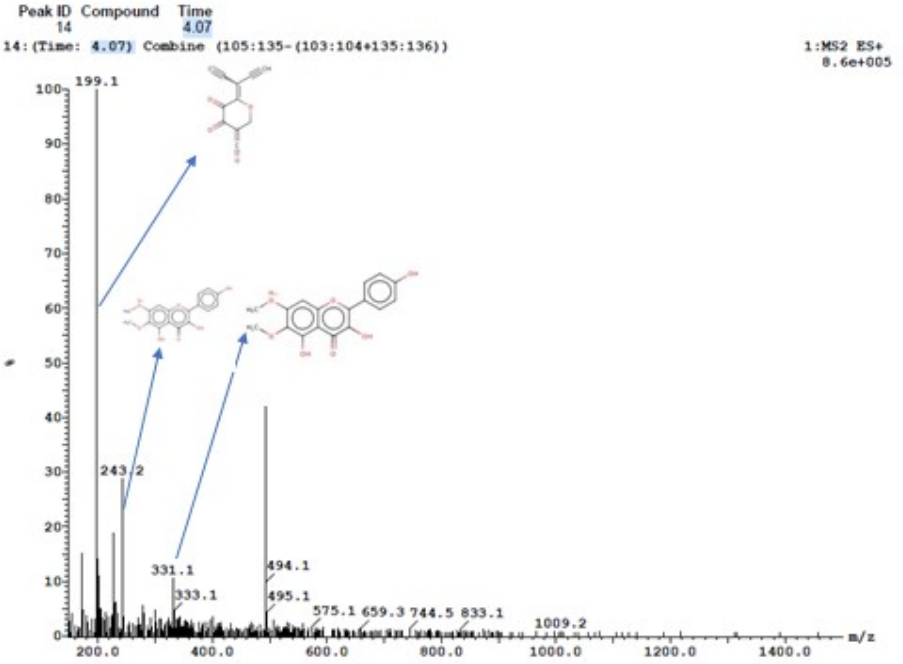
Boeravinone B



Boeravinone E



Eupalitin



4.7 Molecular Docking

Molecular docking for selected phytoconstituents of *A. aspera* and *B. diffusa* extracts was performed. For all three proteins, VEGF, p53, and PI3K molecular docking was performed with Autodock's inbuilt CBDock-2 and Biovia Discovery Studio.

The core proteins PI3K, VEGF, and p53 were used as receptors for molecular docking validation. To determine the binding affinity between the five active ingredients of *A. aspera* and six active ingredients of *B. diffusa* extract with three core proteins, molecular docking was performed. Sorafenib is used as a 1st line treatment option belonging to the TKI class for HCC treatment. It has been observed that sorafenib binds to PI3K, VEGF, and p53.

Compounds with docking (Vina) scores above -10.0 kcal/mol likely have favorable interactions with the protein; scores between -8.0 kcal/mol and -10.0 kcal/mol might have weaker but potentially relevant interactions, and scores below -8.0 kcal/mol suggest a lower predicted binding affinity. The compound docking score is shown in Table 4.12 and 4.13 for *A. aspera* and *B. diffusa,* respectively. Sorafenib showed a docking score of -11.3 kcal/mol which is a high score that indicates that it has favorable interactions with the VEGF protein.

Docking score along with 2D interaction of amino acids and compounds with the target protein are shown in Table 4.14 to 4.16 (VEGF), Table 4.17 to 4.19 (p53), and Table 4.20 to 4.22 (PI3K) for sorafenib, *A. aspera,* and *B. diffusa,* respectively.

Table 4.12: Binding affinities between active components of *A. aspera* and target proteins

Sr. No.	Compound	Docking score (kcal/mol)		
		P53	PI3K	VEGF
1	Oleanolic acid	-9.8	-10.5	-9.7
2	Gentisic acid	-6.2	-6.1	-5.7
3	Linolenic acid	-5.3	-6.0	-6.9
4	Chlorogenic(5-Caffeoylquinic) acid	-7.1	-8.5	-7.2
5	Kaempferol-3-O-glucoside	-9.0	-9.2	-7.8
6	Sorafenib (Standard)	-10.1	-9.5	-11.3

Molecular docking results of the active compound of *A. aspera* are shown in Table 4.12, which highlights that many active components could bind to the core proteins equally or better than the reference standard sorafenib. The PI3K signaling system is crucial for cell division, growth, and survival; as such, PI3K inhibition is a desirable target for anticancer treatment (219).

One of the active ingredients of the extract, oleanolic acid, showed a strong binding affinity of −10.5 kcal/mol docking score for PI3K as compared to sorafenib, which has a binding affinity of −9.5 kcal/mol, while other phytoconstituents, chlorogenic acid and kaempferol-3-O-glucoside, bound to PI3K with docking score values of −8.5 kcal/mol and −9.2 kcal/mol, respectively.

The active ingredients also showed strong binding affinities for p53, with two (oleanolic acid and kaempferol-3-O-glucoside) of them having nearly equal docking scores of that sorafenib.

One phytoconstituent (oleanolic acid) showed good binding affinity with a -9.7 kcal/mol score while that of sorafenib had a -11.3 kcal/mol score for VEGF.

These results suggest that VEGF, p53, and PI3K may be the main targets of *A. aspera* for HCC treatment.

Table 4.13: Binding affinities between active components of *B. diffusa* and target proteins

Sr. No.	Compound	Docking score (kcal/mol)		
		P53	PI3K	VEGF
1	Ferulic acid	-6.6	-6.9	-7.0
2	Boeravinone E/H	-9.4	-8.9	-8.2
3	Eupalitin	-8.8	-8.2	-9.0
4	Epicatechin	-8.6	-8.4	-8.0
5	Quercetin	-9.1	-9.0	-8.0
6	Boeravinone B	-9.3	-9.0	-8.2
7	Sorafenib (Standard)	-10.1	-9.5	-11.3

Molecular docking results of six active compounds of *B. diffusa* are shown in Table 4.13, which highlights that many active components could bind to the core proteins approximately equally of that reference compound sorafenib.

Angiogenesis is crucial to the onset and spread of cancer because tumors need blood vessels to get oxygen and nutrients before they can expand over a certain tumor volume (1-2 mm). The primary regulator of angiogenesis in cancer is VEGF (220).

Five phytoconstituents displayed potentially relevant interaction with VEGF with docking score greater than −8 kcal/mol and sorafenib having strong binding affinity with score of −11.3 kcal/mol.

Three active ingredients of *B. diffusa,* quercetin, boeravinone B, and boeravinone E, with binding scores of -9.0 kcal/mol, -9.0 kcal/mol, and -8.9 kcal/mol, displayed comparative binding affinities as compared to sorafenib with a −9.5 kcal/mol binding score for PI3K.

The active ingredients also showed strong binding affinities for p53, with three of them having stronger docking scores (> -9.0 kcal/mol). These results suggest that PI3K, VEGF, and p53 may be the main targets of *B. diffusa* for HCC treatment.

Table 4.14: Docking of sorafenib on VEGF protein

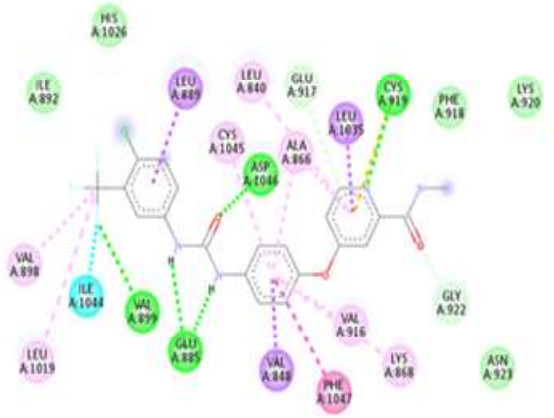
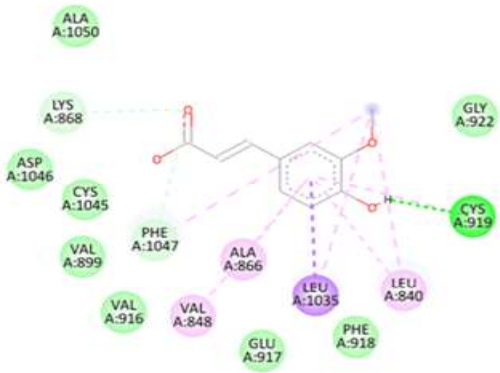
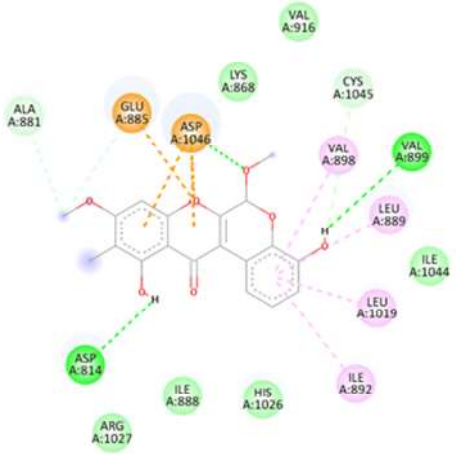
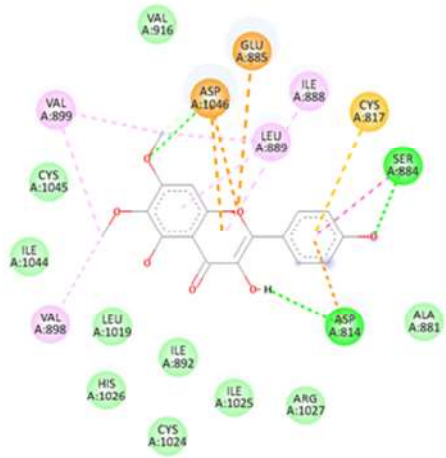
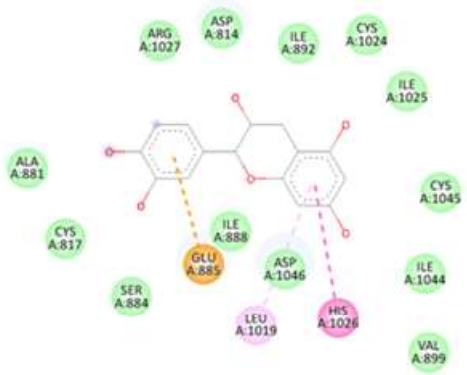
Sr. No.	Standard	CurPocket ID	Vina score	Cavity volume (Å^3)	Center (x, y, z)	Docking size (x, y, z)	2D Interaction
1	Sorafenib	C1	-11.3	1831	-24, -3, -9	27, 27, 27	

Table 4.15: Docking of alcoholic extract of *B. diffusa* on VEGF protein

Sr. No.	Phytoconstituent	CurPocket ID	Vina score	Cavity volume (Å^3)	Center (x, y, z)	Docking size (x, y, z)	2D Interaction
1	Ferulic acid	C1	-7.0	1831	-24, -3, -9	25, 19, 19	
2	Boeravinone H	C1	-8.2	1831	-24, -3, -9	21, 21, 21	

3	Eupalitin	C1	-9.0	1831	-24, -3, -9	22, 22, 22	
4	Epicatechin	C1	-8.0	1831	-24, -3, -9	21, 21, 21	

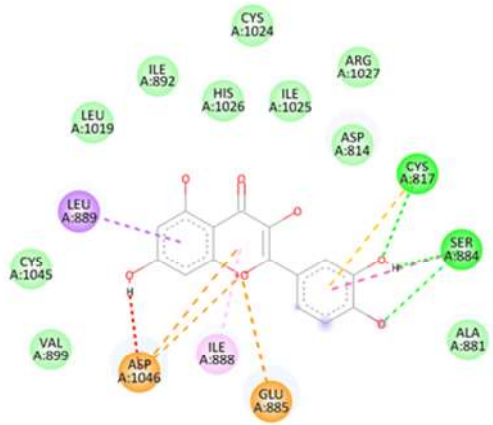
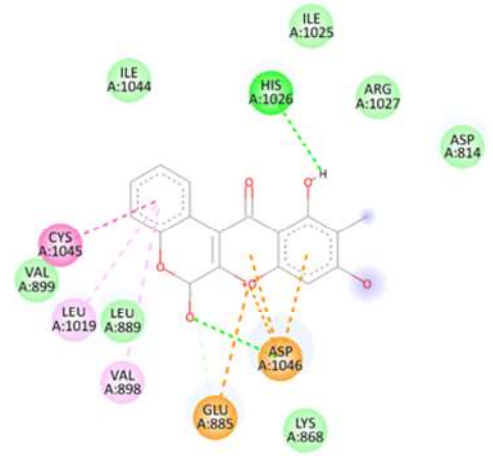
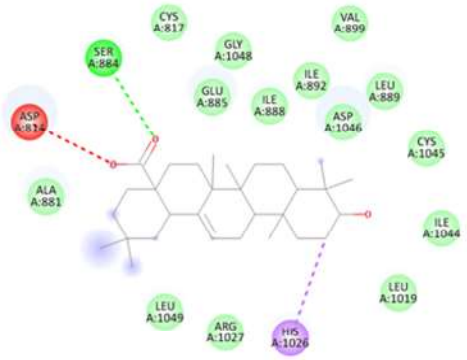
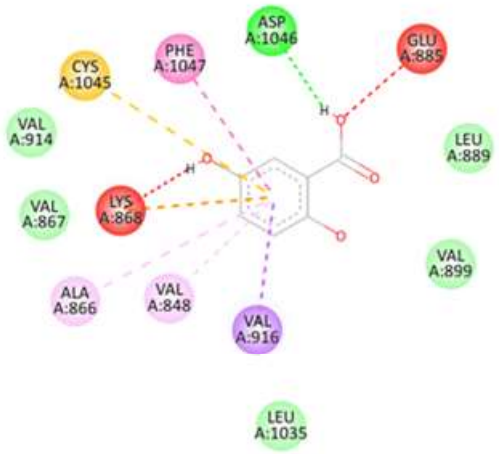
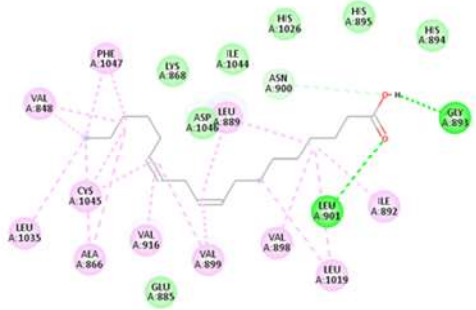
5	Quercetin	C1	-8.0	1831	-24, -3, -9	21, 21, 21	
6	Boeravinone B	C1	-8.2	1831	-24, -3, -9	20, 20, 20	

Table 4.16: Docking of alcoholic extract of *A. aspera* on VEGF protein

Sr. No.	Phytoconstituent	CurPocket ID	Vina score	Cavity volume (Å3)	Center (x, y, z)	Docking size (x, y, z)	2D
1	Oleanolic acid	C1	-9.7	1831	-24, -3, -9	23, 23, 23	

2	Gentisic acid	C1	-5.7	1831	-24, -3, -9	25, 17, 17	
3	Linolenic acid	C1	-6.9	1831	-24, -3, -9	23, 23, 23	

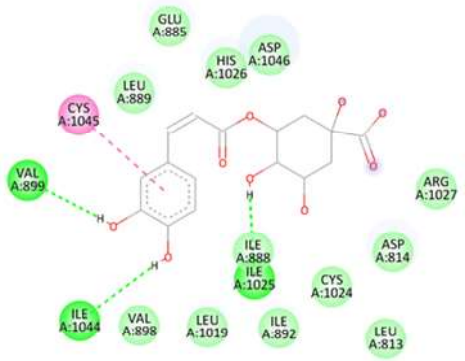
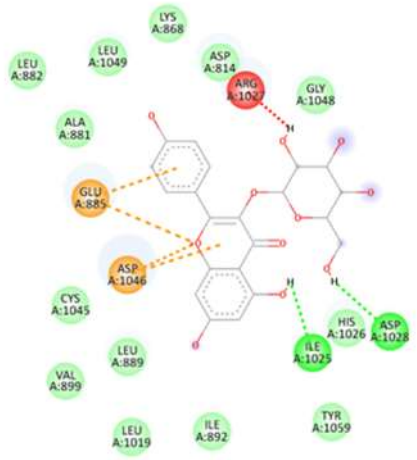
4	Chlorogenic (5-Caffeoylquinic) acid	C1	-7.2	1831	-24, -3, -9	21, 21, 21	
5	Kaempferol-3-O-glucoside	C1	-7.8	1831	-24, -3, -9	22, 22, 22	

Table 4.17: Docking of sorafenib on p53 protein

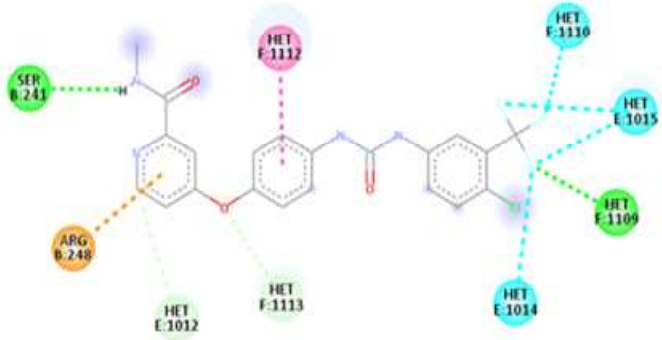
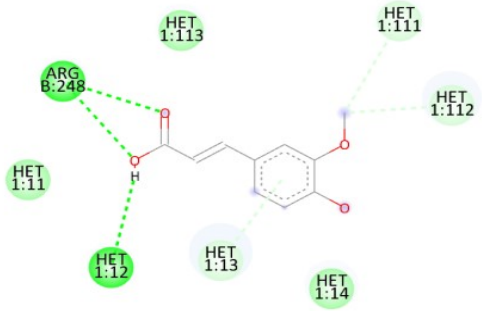
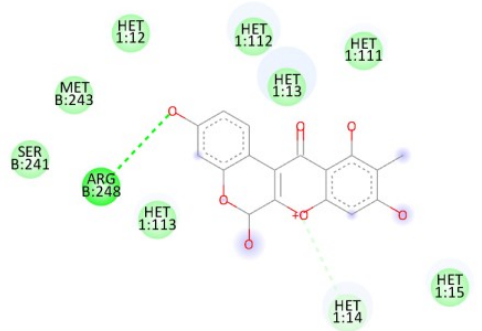
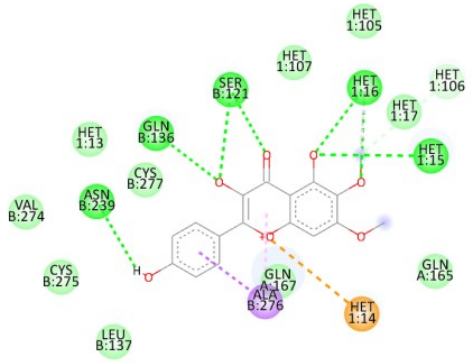
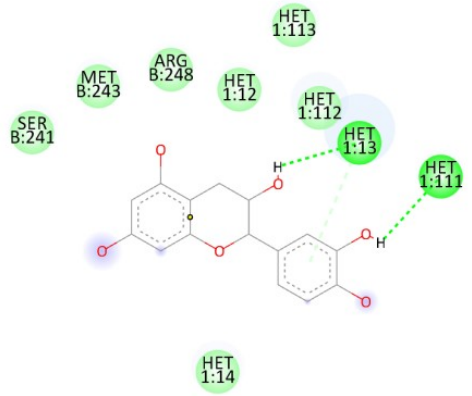
Sr. No.	Standard	CurPocket ID	Vina score	Cavity volume (Å^3)	Center (x, y, z)	Docking size (x, y, z)	2D Interaction
1	Sorafenib	C1	-10.1	2911	62, 32, 76	27, 27, 27	

Table 4.18: Docking of alcoholic extract of *B. diffusa* on p53 protein

Sr. No.	Phytoconstituent	CurPocket ID	Vina score	Cavity volume (Å^3)	Center (x, y, z)	Docking size (x, y, z)	2D Interaction
1	Ferulic acid	C1	-6.6	2911	62, 32, 76	30, 27, 19	
2	Boeravinone H	C1	-9.4	2911	62, 32, 76	30, 27, 21	

3	Eupalitin	**C1**	-8.8	2911	62, 32, 76	30, 22, 22	
4	Epicatechin	**C1**	-8.6	2911	62, 32, 76	30, 27, 21	

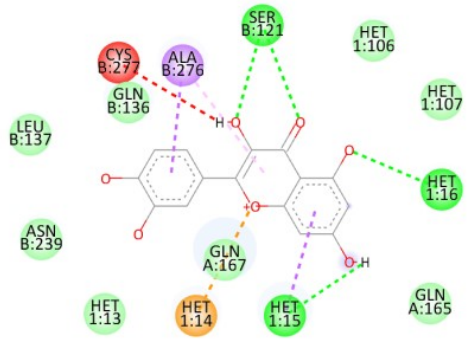
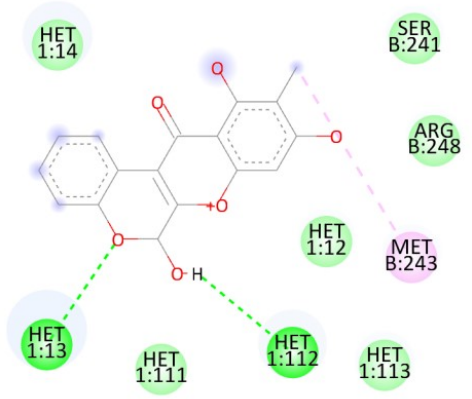
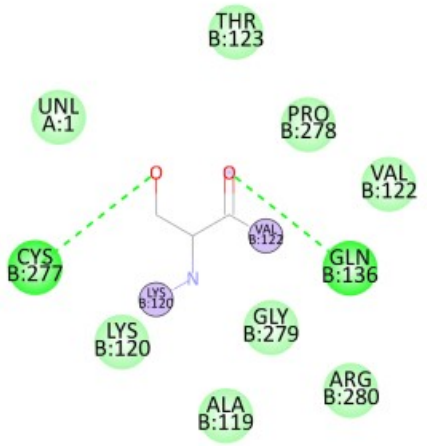
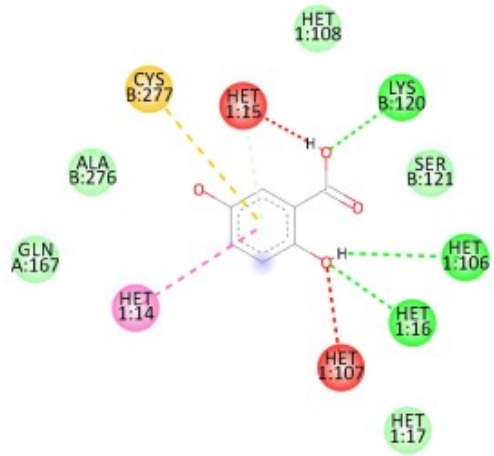
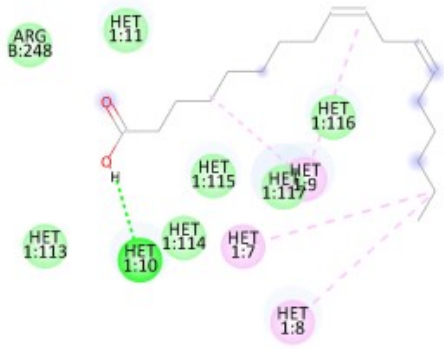
5	Quercetin	C1	-9.1	2911	62, 32, 76	30, 27, 21	
6	Boeravinone B	C1	-9.3	2911	62, 32, 76	30, 27, 20	

Table 4.19: Docking of alcoholic extract of *A. aspera* on p53 protein

Sr. No.	Phytoconstituent	CurPocket ID	Vina score	Cavity volume (Å3)	Center (x, y, z)	Docking size (x, y, z)	2D
1	Oleanolic acid	C1	-9.8	2911	62, 32, 76	30, 23, 23	

2	Gentisic acid	C1	-6.2	2911	62, 32, 76	30, 27, 23	
3	Linolenic acid	C4	-5.3	438	42, 24, 86	23, 23, 23	

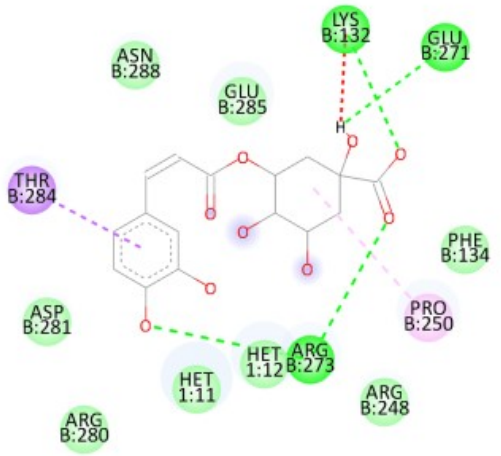
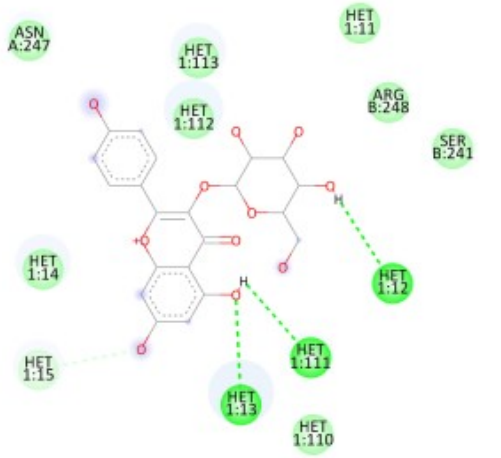
4	Chlorogenic (5-Caffeoylquinic) acid	C5	-7.1	424	55, 25, 89	21, 21, 21	
5	Kaempferol-3-O-glucoside	C1	-9.0	2911	62, 32, 76	30, 22, 22	

Table 4.20: Docking of sorafenib on PI3K protein

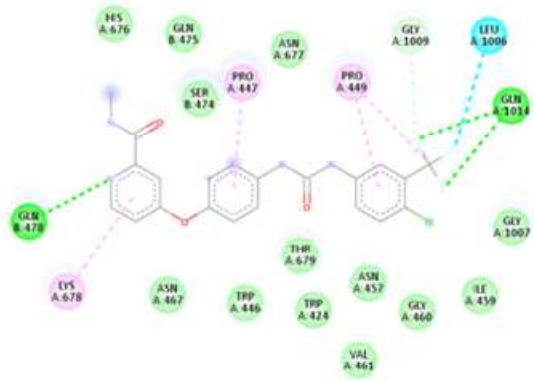
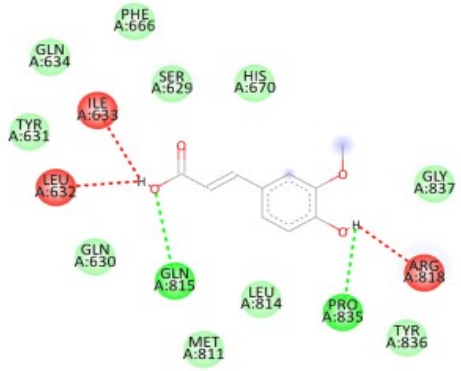
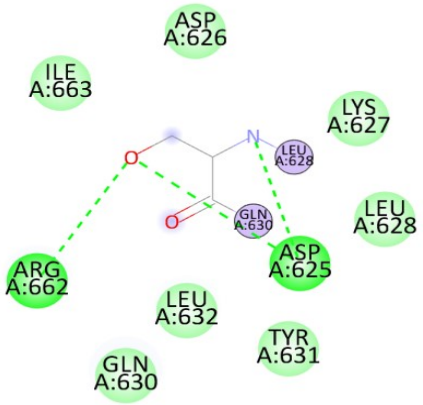
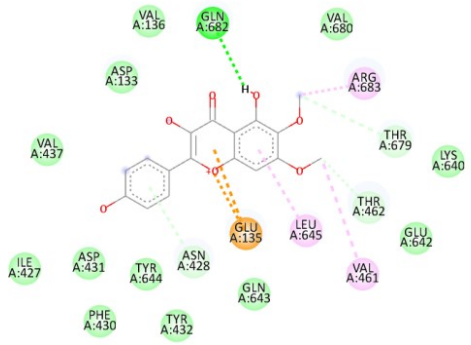
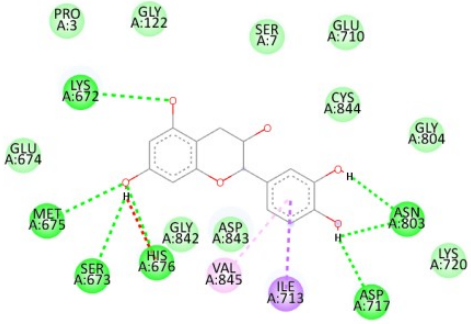
Sr. No.	Standard	CurPocket ID	Vina score	Cavity volume (Å^3)	Center (x, y, z)	Docking size (x, y, z)	2D Interaction
1	Sorafenib	C1	-9.5	36043	7, 133, 42	35, 35, 35	

Table 4.21: Docking of alcoholic extract of *B. diffusa* on PI3K protein

Sr. No.	Phytoconstituent	CurPocket ID	Vina score	Cavity volume (Å^3)	Center (x, y, z)	Docking size (x, y, z)	2D Interaction
1	Ferulic acid	C2	-6.9	18921	21, 134, 24	35, 35, 35	
2	Boeravinone H	C3	-8.9	440	6, 119, 21	21, 21, 30	

3	Eupalitin	C1	-8.2	36043	7, 133, 42	35, 35, 35	
4	Epicatechin	C1	-8.4	36043	7, 133, 42	35, 35, 35	

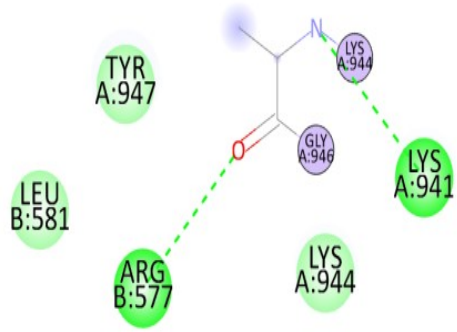
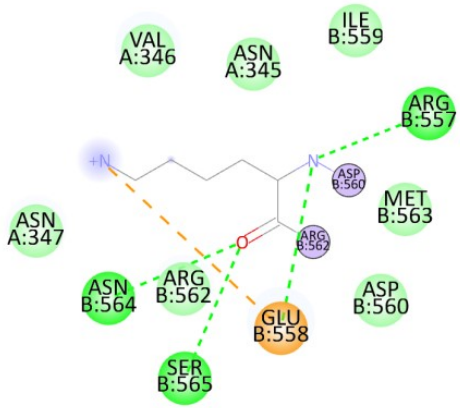
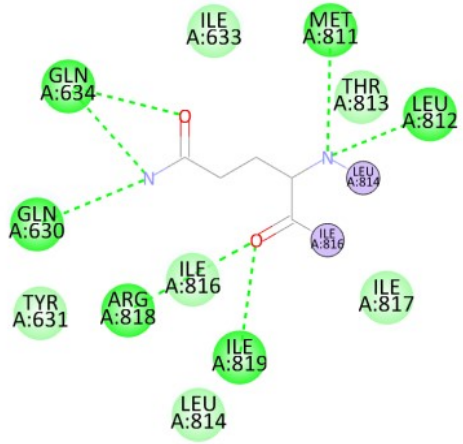
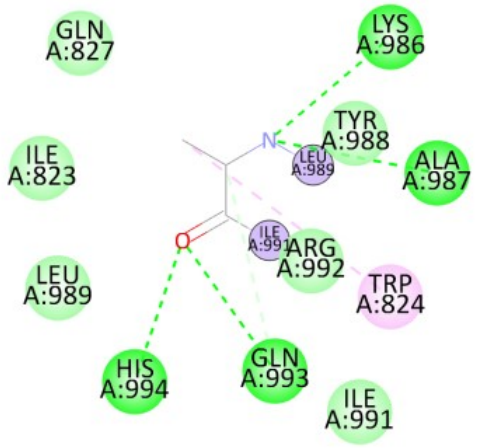
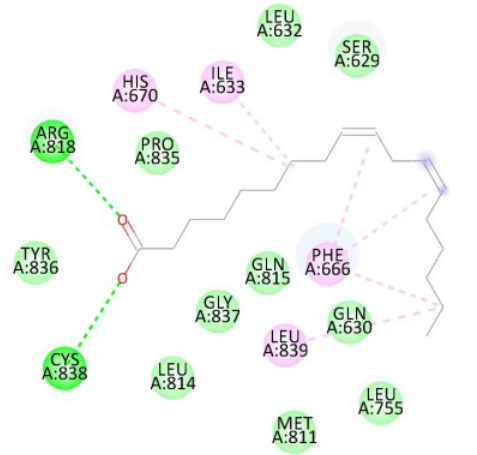
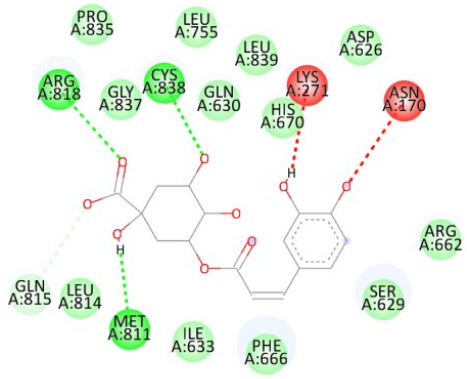
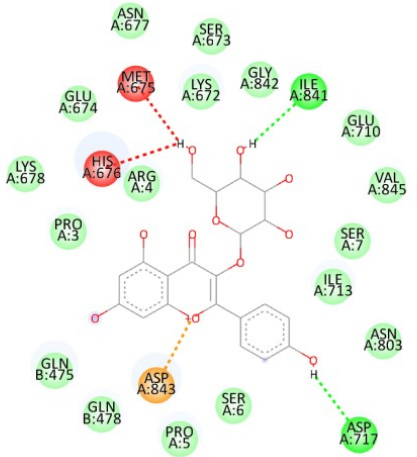
5	Quercetin	C1	-9.0	36043	7, 133, 42	35, 35, 35	
6	Boeravinone B	C1	-9.0	36043	7, 133, 42	35, 35, 35	

Table 4.22: Docking of alcoholic extract of *A. aspera* on PI3K protein

Sr. No.	Phytoconstituent	CurPocket ID	Vina score	Cavity volume ($Å^3$)	Center (x, y, z)	Docking size (x, y, z)	2D
1	Oleanolic acid	C1	-10.5	36043	7, 133, 42	35, 35, 35	

2	Gentisic acid	C2	-6.1	18921	21, 134, 24	35, 35, 35	
3	Linolenic acid	C2	-6.0	18921	21, 134, 24	35, 35, 35	

4	Chlorogenic (5-Caffeoylquinic) acid	C2	-8.5	18921	21, 134, 24	35, 35, 35	
5	Kaempferol-3-O-glucoside	C1	-9.2	36043	7, 133, 42	35, 35, 35	

4.8 Acute toxicity study as per OECD guideline 423

After dosing, each animal was examined separately for at least 30 minutes, then periodically for the next 24 hours, receiving extra care during the first 4 hours and every day after that, for a total of 14 days. Every rat was checked at least twice a day in order to note any behavioral abnormalities or signs of illness.

There was no change in physical parameters such as body temperature, skin, or eye color during the study duration. Body temperature of animals was measured periodically with the help of a rectal probe. The body temperature was normal in all the animals across the group. Animals had shown normal exploratory activity and non irritability behavior post-administration of extract which confirms that a single dose of extract does not have any deleterious effects on the CNS system. Observations for the limit test were displayed in Table 4.23, 4.24 and 4.25 for alcoholic extracts of *A. aspera*, *B. diffusa*, and *E. littorale,* respectively.

Animals treated with all three extracts showed normal behavioral observations and did not show any visible toxicity at a 2000 mg/kg oral dose. Thus, the extracts were found to be safe untill 2000 mg/kg body weight.

Table 4.23: Observations for the limit test for alcoholic extract of *A. aspera* in rats

Observation	**0 hour**	**4 hours**	**24 hours**	**168 hours (7^{th} day)**	**336 hours (14^{th} day)**
Body weight (gm)	239 ± 6.4	238 ± 6.2	252 ± 8.1	264 ± 10.8	269 ± 11.0
Body temperature (^{0}C)	37.1 ± 0.8	37.4 ± 0.2	37.6 ± 0.6	37.5 ± 0.4	37.5 ± 0.2
Skin color	N	N	N	N	N
Eye color	N	N	N	N	N
Alertness - exploratory activity	N	N	N	N	N
Irritability	Nil	Nil	Nil	Nil	Nil
Sensory response-touch response	N	N	N	N	N

N: Normal

Table 4.24: Observations for the limit test for alcoholic extract of *B. diffusia* in rats

Observation	0 hour	4 hour	24 hour	168 hour (7^{th} day)	336 hour (14^{th} day)
Body weight (gm)	231 ± 20.4	232 ± 20.6	244 ± 19.3	263 ± 19.1	272 ± 18.0
Body temperature (^{0}C)	37.6 ± 0.2	37.6 ± 0.3	38.0 ± 0.5	37.7 ± 0.2	37.6 ± 0.5
Skin color	N	N	N	N	N
Eye color	N	N	N	N	N
Alertness - exploratory activity	N	N	N	N	N
Irritability	Nil	Nil	Nil	Nil	Nil
Sensory response-touch response	N	N	N	N	N

N: Normal

Table 4.25: Observations for the limit test for alcoholic extract of *E. littorale* in rats

Observation	0 hour	4 hour	24 hour	168 hour (7^{th} day)	336 hour (14^{th} day)
Body weight (gm)	222 ± 7.4	222 ± 7.1	233 ± 11.5	253 ± 13.9	259 ± 15.5
Body temperature (^{0}C)	37.4 ± 0.4	37.6 ± 0.4	37.5 ± 0.3	37.5 ± 0.1	37.6 ± 0.3
Skin color	N	N	N	N	N
Eye color	N	N	N	N	N
Alertness - exploratory activity	N	N	N	N	N
Irritability	Nil	Nil	Nil	Nil	Nil
Sensory response-touch response	N	N	N	N	N

N: Normal

4.9 DEN and 2-AAF induced HCC in rats

In this experimental model, HCC was induced in rats by DEN as the initiator and 2-AAF as the promoter of hepatocarcinogensis. Rats in group 1 (control group) received a single intraperitoneal (i.p.) injection of normal saline, and from the 2nd week onward saline was given by p.o. route for 2 weeks. In group 2, rats were injected with a single intraperitoneal (i.p.) dose of DEN (200 mg/kg) for initiating hepatocarcinogenesis. After two weeks, liver cancer development was promoted with daily dose of 2-AAF (30 mg/kg, p.o.) for the next two weeks.

As presented in Table 4.26, liver weight, liver index, and spleen index of DEN and 2-AAF-treated group rats were significantly ($p < 0.1$ to 0.001) increased as compared to control group rats. As compared to the control (normal) group rat (27.9%), a statistically significant reduction in body weight was observed in the DEN and 2-AAF treated group (8.1%), as shown in Table 4.26 and Figure 4.17. On day 28 (at the end of the study), serum ALT ($p < 0.1$), ALP ($p < 0.001$) and total bilirubin ($p < 0.001$) levels were noticeably higher than in the animals under control (normal). While there was no significant change in AST and creatinine level in the DEN and 2-AAF-treated group of rats compared with control (normal) animals (Table 4.27). The mean serum levels of all the enzymes were within the normal range for the control group.

The hepatic sections from the control (normal) group animals showed normal liver architecture. There were no visible anatomical deformities or any tumor nodules seen on livers isolated from the rats in any group except infiltration of inflammatory cells and single cell necrosis, which indicate that damage to the liver has initiated but is devoid of any cancer development in DEN and 2-AAF group rats (Figure 4.18).

Table 4.26: Data of percentage change in body and liver weight, liver and spleen index on day 28

Treatment	% change in body weight	Liver weight (gm)	Liver index	Spleen weight (gm)	Spleen index
Control	27.9 ± 4.3	10.2 ± 0.8	3.8 ± 0.3	0.6 ± 0.1	0.2 ± 0.04
DEN (200 mg/kg, i.p.) and 2-AAF (30 mg/kg, p.o)	8.1 ± 5.5***	18.0 ± 2.9*	7.8 ± 1.6***	0.7 ± 0.1	0.31 ± 0.07***

Remark: 2-AAF was administered daily for 2 weeks from 2nd week of DEN administration. Data are articulated as mean ± SD (n=6). Statistical analysis was carried out using paired t test for multiple comparisons. *p < 0.1 and ***p < 0.001 as compared to vehicle treated (control) group.

Table 4.27: Data of liver function parameters on day 28

Treatment	ALT (U/L)	AST (U/L)	ALP (U/L)	Creatinine (mg/dL)	Total bilirubin (mg/dL)
Normal range	**13-56**	**34-109**	**95-611**	**0.2-0.7**	**0.2-0.7**
Control	58.7 ± 10.1	156.5 ± 49.9	542.8 ± 46.2	0.7 ± 0.1	0.2 ± 0.1
DEN (200 mg/kg, i.p.) and 2-AAF (30 mg/kg, p.o)	88.9 ± 20.1*	168.6 ± 44.7	868.9 ±170.6***	0.6 ± 0.1	0.7 ± 0.7***

Remark: 2-AAF was administered daily for 2 weeks from 2nd week of DEN administration Data are articulated as mean ± SD (n=6). Statistical analysis was carried out using paired t test for multiple comparisons. *p < 0.1, ***p < 0.001 as compared to vehicle treated (control) group.

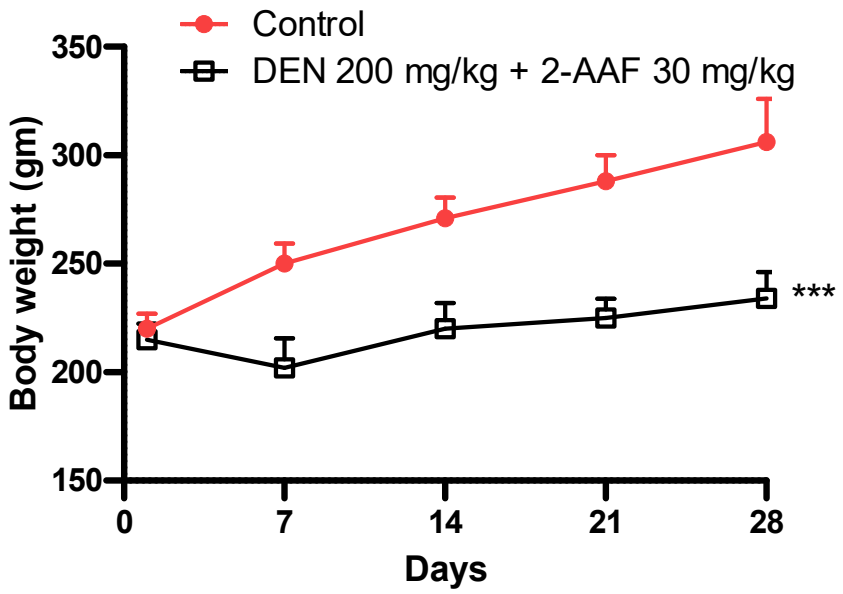


Figure 4.17: Body weight in DEN and 2-AAF induced HCC model

Remark: 2-AAF (30 mg/kg, p.o.) was administered daily for 2 weeks from 2nd week of DEN (200 mg/kg, i.p.) administration. Data are articulated as mean ± SD. Statistical analysis was carried out using paired t test for multiple comparisons. ***$p < 0.001$ as compared to vehicle treated (control) group.

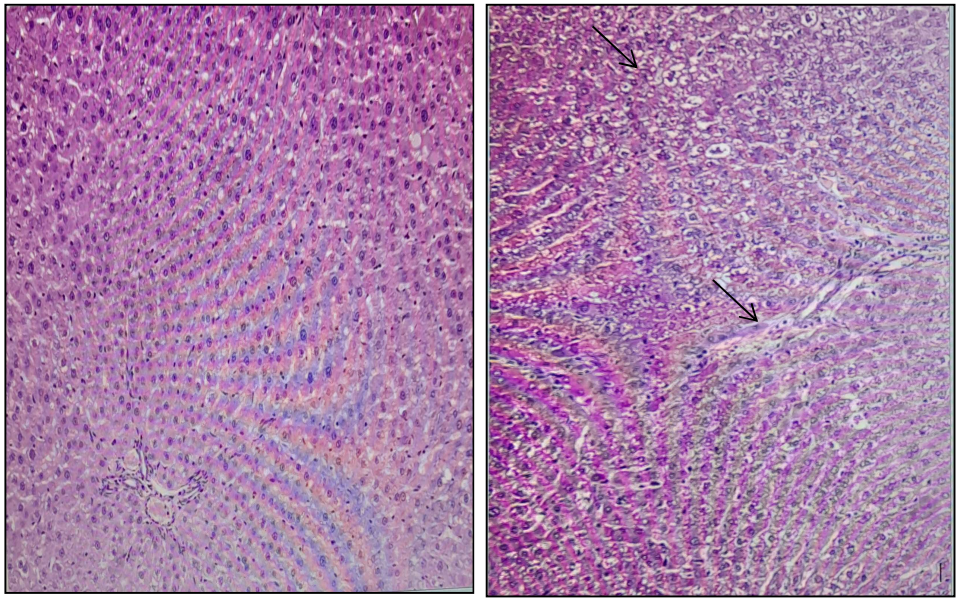


Figure 4.18: Photomicrographs of liver sections of normal and DEN with 2-AAF administered rats

Representative histological pictures of liver tissue sections stained with (A–B) hematoxylin-eosin (H&E). Liver samples were obtained at the termination of the experiment in rats. Rats received saline, control group (A), DEN (single dose, i.p.) and 2-AAF p.o., once daily for 2 week, post 2 week of DEN administration, disease control group (B) captured at 20X objective, 50 µm bar. ↑Indicate necrosis

4.10 DEN and CCl_4 induced HCC in rats

This protocol contains two-stage application of chemicals to the liver for the initiation and promotion of hepatocellular tumors. In this animal model, DEN was used to initiate and CCl_4 to promote rat carcinogenesis. Rats in group 1 (control group) received a single intraperitoneal (i.p.) injection of normal saline and 2 weeks later received corn oil with saline 1:1 (2 ml/kg, i.g.) for 3 weeks. Rats in group 2 were injected with a single dose of DEN (200 mg/kg, i.p.), 2 weeks later received a single dose of CCl_4 (2 mL/kg, i.g.) by gavage, while in group 3, rats were injected with DEN (200 mg/kg, i.p.), and 2 week later received weekly CCl_4 (2 mL/kg, i.g) for 3 weeks.

Post-24 hr (day 14) of DEN injection rats in both groups 2 and 3 have found with slow movement, listlessness, which was continuous till 2-3 days. Rats in group 2 (13.8%) and 3 (4.7%) showed a substantial decrease in body weight in comparison to the rats in the control group (23.9%). Data shown in Table 4.28 and Figure 4.19. Subsequently, the liver and spleen

index was significantly ($p < 0.001$) higher in both groups 2 (6.4% and 0.28%) and 3 (7.5% and 0.29%) respectively, as compared to the control group (3.4% and 0.19%). Data are shown in Table 4.28.

Liver function was monitored by measuring serum liver enzymes (ALT, AST, ALP, total protein, creatinine and total bilirubin) on days 14, 28, and 35. The mean serum levels of all the enzymes were within the normal range for the control (vehicle-treated) group. Mean serum levels of ALT, AST, ALP, and creatinine were significantly ($p < 0.001$) higher in the model groups 2 and 3 as compared to the control group. There was no significant difference in serum enzyme levels of total bilirubin level between groups 2 and 3 as compared to the control group (Table 4.29). In serum total protein significant difference was found between groups 3 as compared to the control group.

The liver collected from the rats in all groups showed no obvious anatomical abnormalities or tumor nodules. The control group's liver histology was normal, with fewer inflammatory cell infiltrations and hepatocytes with rounded nuclei. In group 2, rats showed single-cell necrosis and high infiltration of inflammatory cells, while in group 3, showed more damage with single cell necrosis and formation of fibrosis (Figure 4.20). There was not any visible tumor or severe damage observed in groups 2 and 3 animals. Though damage was initiated, to reach HCC development requires more exposure of carcinogens.

In both the animal models, an increase in serum markers was found, which indicates that the process of tumor generation through inflammation, necrosis, and fibrosis was initiated. But to generate the HCC, it will require long duration and continuous exposure of the carcinogen. The serum AFP levels (Table 4.29) were nearly the same in all three groups, which also confirms that the HCC was not developed with given carcinogen.

Table 4.28: Data of percentage change in body weight, liver and spleen index

Group	Treatment	% Change in body weight	Liver weight (gm)	Liver index	Spleen weight (gm)	Spleen index
1	Control	23.9 ± 3.6	11.8 ± 1.2	3.4 ± 0.6	0.60 ± 0.1	0.19 ± 0.03
2	DEN (200 mg/kg, i.p.) and CCl_4 (2 mL/kg, p.o.) x single dose	13.8 ± 3.6*	16.9 ± 2.8**	6.4 ± 1.0***	0.75 ± 0.2	0.28 ± 0.05***
3	DEN (200 mg/kg, i.p.) and CCl4 (2 mL/kg, p.o.) x three dose	4.7 ± 8.9***	19.7 ± 1.9***	7.5 ± 0.6***	0.77 ± 0.1	0.29 ± 0.05***

Remark: CCl_4 was administered on 3rd and 3rd, 4th, 5th week in 2nd and 3rd group, respectively post DEN administration Data presented as mean ± SD (n=6). The statistical analysis of the study was done with one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. *p < 0.1, **p < 0.01 and ***p < 0.001 as compared to vehicle treated (control) group.

Table 4.29: Data of liver function parameters

Blood biochemistry	Normal range	Day	Control	DEN (200 mg/kg, i.p.) and CCl_4 (2 mL/kg, p.o.) x single dose	DEN (200 mg/kg, i.p.) and CCl_4 (2 mL/kg, p.o.) x three dose
ALT (U/L)	13-56	14	64.2 ± 8.4	69.7 ± 9.9	76.5 ± 8.2
		28	67.8 ± 11.1	92.5 ± 7.0	94.7 ± 10.8
		35	68.9 ± 12.4	193.3 ± 35.5***	240.2 ± 34.3***
AST (U/L)	34-109	14	139.8 ± 26.3	147.5 ± 49.4	137.8 ± 26.2
		28	140.3 ± 30.1	174.2 ± 54.7	172.0 ± 19.2
		35	138.2 ± 34.5	239.8 ± 25.3***	255.8 ± 30.4***
ALP (U/L)	95-611	14	567.2 ± 71.0	539.7 ± 41.7	542.7 ± 47.7
		28	483.0 ± 79.0	670.0 ± 96.9	678.4 ± 65.1
		35	488.1 ± 122.4	816.3 ± 61.9***	877.8 ± 122.1***
Creatinine (mg/dL)	0.2-0.7	14	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.0
		28	0.8 ± 0.1	0.6 ± 0.0	0.5 ± 0.0
		35	0.5 ± 0.1	0.8 ± 0.1***	0.8 ± 0.1***
Total bilirubin (mg/dL)	0.2-0.7	14	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1
		28	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0
		35	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.0

Total protein (gm/dL)	**6.3-7.3**	14	7.2 ± 0.3	6.6 ± 0.2	6.7 ± 0.3
		28	8.1 ± 0.9	6.8 ± 0.4	7.1 ± 1.1
		35	7.3 ± 0.3	6.6 ± 0.5*	6.6 ± 0.6*
Alpha feto protein (ng/dL)	**0-10**	14	2.27 ± 0.4	2.32 ± 0.3	2.28 ± 0.2
		28	2.71 ± 0.3	2.70 ± 0.3	2.80 ± 0.3
		35	2.73 ± 0.3	2.72 ± 0.3	2.97 ± 0.2

Remark: CCl_4 was administered on 3rd and 3rd, 4th, 5th week in 2nd and 3rd group respectively post DEN administration. Data presented as mean ± SD (n=6). The statistical analysis of the study was done with two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. *$p < 0.1$ and ***$p < 0.001$ as compared to vehicle treated (control) group.

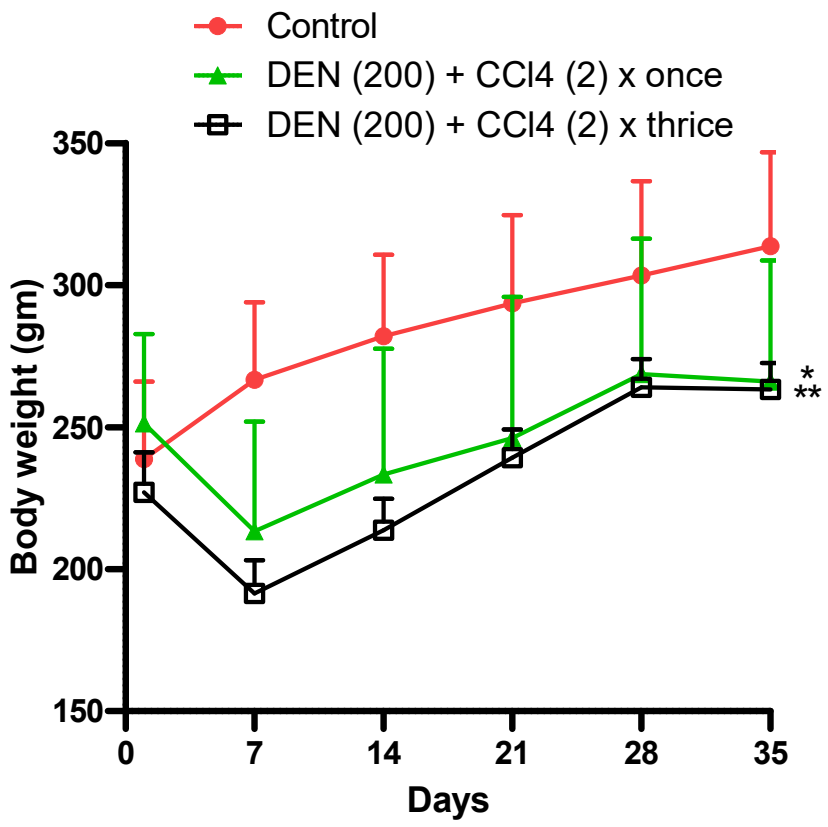


Figure 4.19: Body weight in DEN and CCl_4 induced HCC model

Remark: CCl_4 (2 mL/kg, i.g.) was administered on 3rd and 3rd, 4th, 5th week in group 2 and 3 respectively post DEN (200 mg/kg, i.p.) administration. Data are articulated as mean ± SD (n=6). The statistical analysis of the study was done with one-way ANOVA followed by Bonferroni's post hoc test for multiple comparison. *p < 0.1, **p < 0.01 as compared to vehicle treated (control) group.

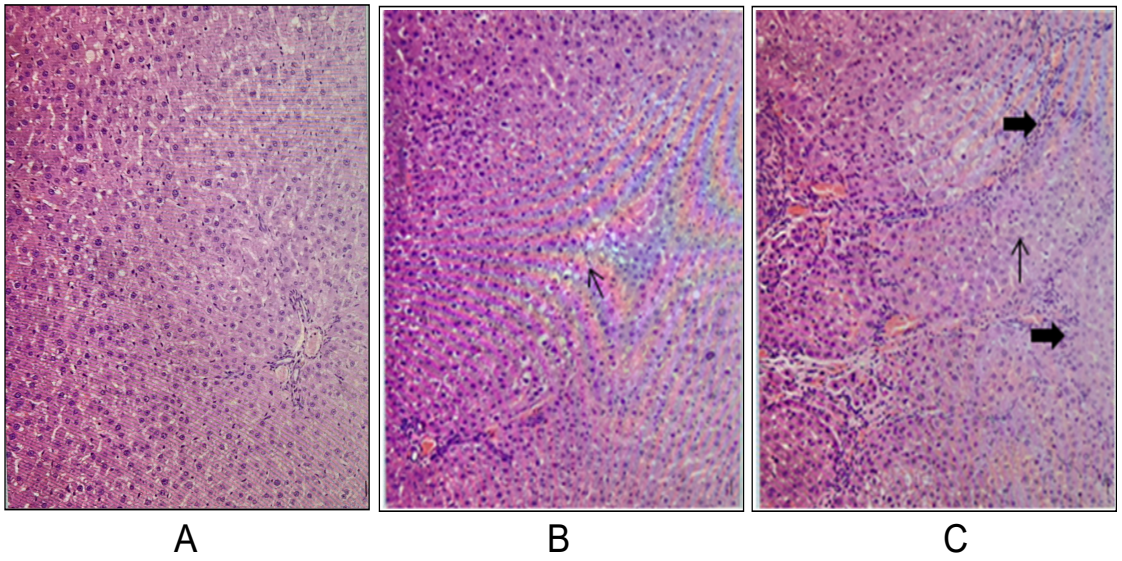


Figure 4.20: Photomicrographs of liver sections of normal, DEN with CCl_4 (single dose) and DEN with CCl_4 (three dose) administered in rats

Representative histological pictures of liver tissue sections stained with (A–C) hematoxylin-eosin (H&E). Liver samples were obtained at the termination of the experiments in rats that received saline, control group (A), DEN (200 mg/kg, i.p. single dose) and CCl_4 (2 ml/kg, i.g.) single dose post 2 week of DEN administration, disease control group (B) and DEN (200 mg/kg, i.p. single dose) and CCl_4 (2 ml/kg, i.g.) once weekly for 3 week post 2 week of DEN administration, disease control group (C), post 2 week of DEN captured at 20X objective, 50 µm bar, ↑indicate necrosis, ➡indicate fibrosis formation

4.11 Subcutaneous xenograft model

4.11.1 Growth pattern of HepG2 cells in male and female mice

In mice, a palpable tumor began to appear within two weeks of cell inoculation. Within 14 days, the palpable tumor growth in female mice reached a volume of around 300 mm^3. In contrast, the tumor volume in male mice reached around 100 mm^3 in 14 days (Figure 4.21). After two weeks of cell inoculation, the tumor growth pattern in female mice was faster than in male mice (almost doubling). On day 14, the average tumor take rate in female mice was 81.3%, but in male mice it was 26.7%. Data is shown in Figure 4.21 and Table 4.30. In female mice the tumor growth rate was dependent on the cell concentration. The tumor take rate in female was found to be 71%, 76%, and 91% for 0.5, 1.0 and 5.0 million cells per mouse, while in male mice it was 20% to 40% for three cell concentration.

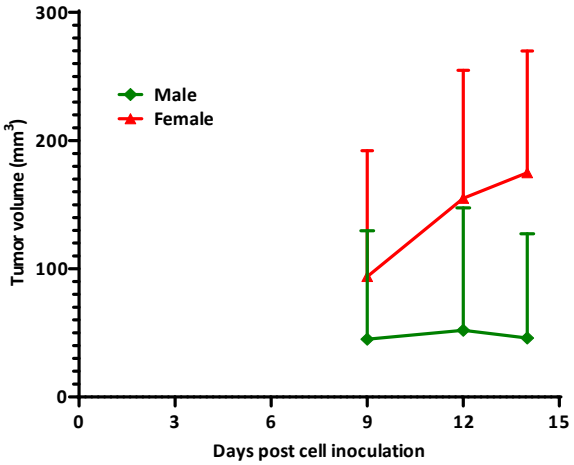


Figure 4.21: HepG2 cell growth pattern in male and female mice

Table 4.30: Growth rate of tumors in male and female mice

HepG2 cell concentration	Female mice	Male mice
0.5 million	71.4%	20.0%
1 million	76.2%	20.0%
5 million	90.9%	40.0%
Average of all cell concentration	**81.3%**	**26.7%**

4.11.2 Standardization of xenograft model with different cell number

It was found that the number of inoculated cells directly correlated with the growth pattern of HepG2 cells. On day nine, following cell inoculation, the palpable tumor growth began. In contrast to 0.5 and 1 million cells/animal, the mice injected with 5 million cells per mouse exhibited aggressive tumor growth and reached human endpoint one week early (day 36) as compared to the mice inoculated with 0.5 and 1 million cells (day 42). The.0.5 million cell-bearing mice showed slow tumor growth as compared to 1 million cell mice till the 5th week of cell inoculation, but thereafter the cells grew aggressively, and within 1 week their growth pattern was similar to that of 1 million cells. Data on tumor growth pattern is as shown in

Figure 4.22. Since one million cells per animal showed less aggressive growth than the 5 million cells (the optimum growth), further efficacy study was done using 1 million cells.

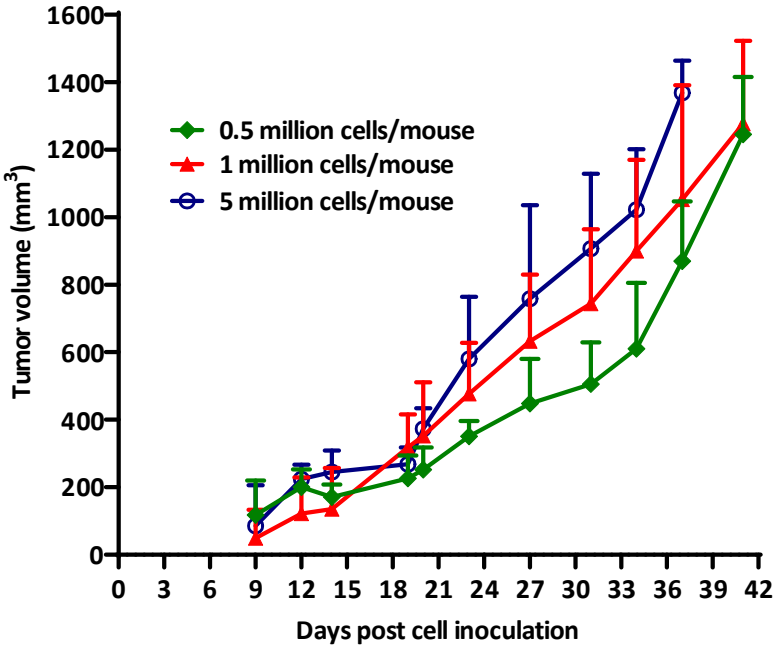


Figure 4.22: Growth pattern of HepG2 cells inoculation with different cell number per mouse

4.11.3 Efficacy study of extracts in xenograft model with different cell number

Over the course of the 21-day investigation, the mice in the vehicle (disease control) group displayed substantial tumor growth at each of the three cell concentrations (0.5, 1 and 5 million cells per animal). The alcoholic extract of *A. aspera* and *B. diffusa* at 500 mg/kg dose demonstrated statistically significant ($p<0.001$) anticancer activity in comparison to the vehicle group in all three cell concentrations (Figure 4.23, 4.24, 4.25). Also, sorafenib (dose 30 mg/kg), a standard drug, has shown statistically significant ($p<0.001$) anticancer activity. At 0.5 and 1.0 million cell concentration, the alcoholic extract of *E. littorale at 500* mg/kg dose exhibited non-significant activity when compared to the vehicle-treated group. Only at a concentration of 5 million cells the extract exhibited statistically ($p < 0.01$) significant effect. At lower dose 150 mg/kg, *A. aspera* (except at 1 million cells) and *E. littorale* does not

shown any activity. So there data was excluded in the thesis. Data of %TGI of *B. diffusa* at 150 mg/kg dose is shown in Table 4.31.

The body weight of mice was not affected by treatment in any group. The subsequent experiment was designed with one million cells in female mice due to the aggressive tumor growth with five million cells and the optimal tumor growth with one million cells. In HepG2 xenografts, alcoholic extracts of *A aspera* and *B. diffusa* have shown good tumor-reducing ability. Data of percentage tumor growth inhibition (% TGI) is shown in Table 4.31.

Therefore, a single dose of each extract as well as a combination with sorafenib was used in the next experiment to evaluate the activity of the extract and sorafenib as alone and in combination treatment.

Table 4.31: Percentage tumor growth inhibition on day 21

Treatment	Dose (mg/Kg)	% Tumor Growth Inhibition (% TGI)		
		0.5 million cells/mouse	1 million cells/mouse	5 million cells/mouse
Alcoholic extract of *A. aspera*	150	-	28.0	-
Alcoholic extract of *B. diffusia*	150	11.6	26.3	21.5
Alcoholic extract of *E. littorale*	150	-	-	-
Alcoholic extract of *A. aspera*	500	45.5	45.6	40.1
Alcoholic extract of *B. diffusa*	500	44.5	45.7	32.2
Alcoholic extract of *E. littorale*	500	30.8	24.1	28.5
Sorafenib	30	55.6	54.7	46.6

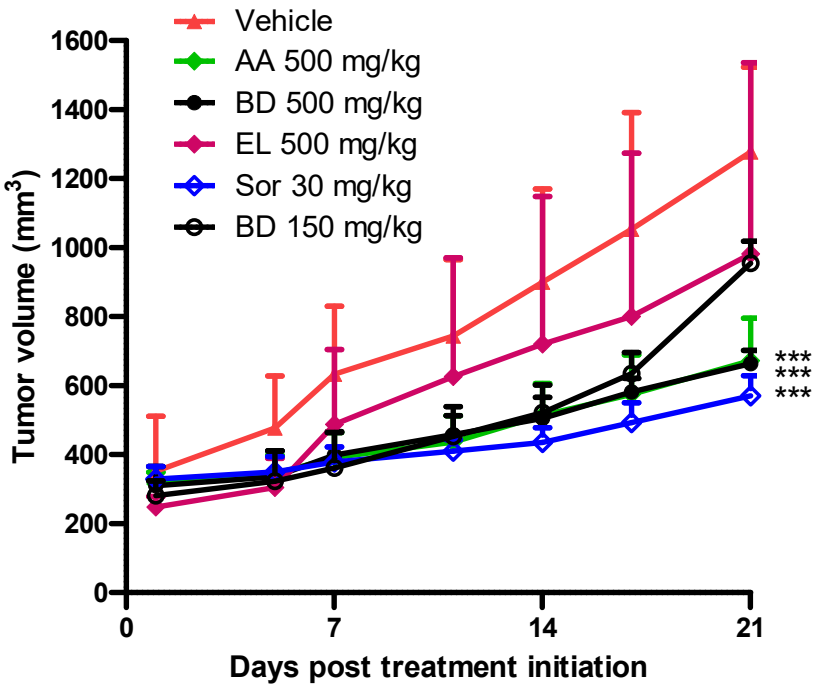


Figure 4.23: Efficacy of extracts in female mice (0.5 million cells/animal)

Mice with HepG2 tumors were given with vehicle, *A. aspera* (AA), 500 mg/kg/day, *B. diffusa* (BD) 150 and 500 mg/kg/day, *E. littorale* (EL) 500 mg/kg/day, and sorafenib (Sor) 30 mg/kg/day. All treatment was given by p.o. for 21 days. Data presented as mean ± SD. Statistical analysis was carried out using two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. *$p < 0.1$ ***$p < 0.001$ as compared to vehicle treated group.

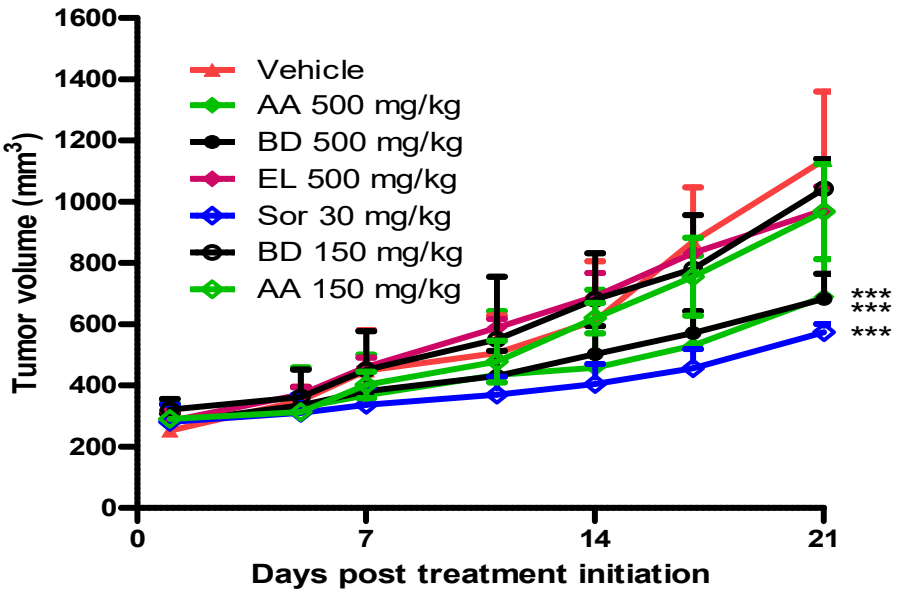


Figure 4.24: Efficacy of extracts in female mice (1 million cells/animal)

Mice with HepG2 tumors were given with vehicle, *A. aspera* (AA) 150 and 500 mg/kg/day, *B. diffusa* (BD) 150 and 500 mg/kg/day, *E. littorale* (EL) 500 mg/kg/day, and sorafenib (Sor) 30 mg/kg/day. All treatment was given by p.o. for 21 days. Data presented as mean ± SD. Statistical analysis was carried out using two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. ***$p < 0.001$ as compared to vehicle treated group.

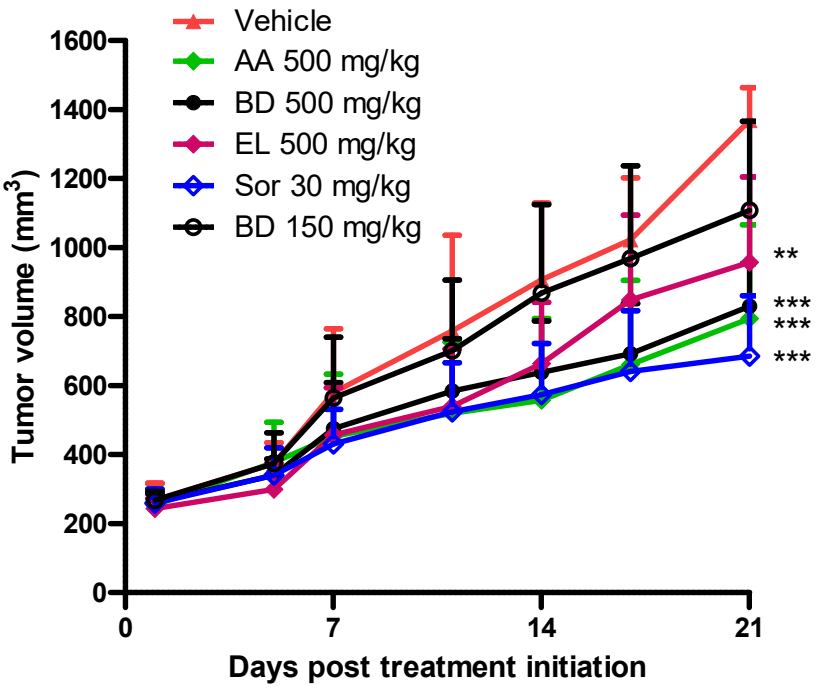


Figure 4.25: Efficacy of extracts in female mice (5 million cells/animal)

Mice with HepG2 tumors were given with vehicle, *A. aspera* (AA) 500 mg/kg/day, *B. diffusa* (BD) 150 and 500 mg/kg/day, *E. littorale* (EL) 500 mg/kg/day, and sorafenib (Sor) 30 mg/kg/day. All treatment was given by p.o. for 21 days. Data presented as mean ± SD. Statistical analysis was carried out using two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons.**$p < 0.01$, ***$p < 0.001$ as compared to vehicle treated group.

4.11.4 Evaluation of efficacy of extracts alone and in combination with sorafenib in tumor bearing mice

Subcutaneous injection of HepG2 cells into the right flank of mice resulted in the development of tumors. After injecting HepG2 cells, visible tumors appeared within two weeks. When the tumor volume reached 150–250 mm^3, the mice were randomly assigned to treatment groups based on the size of the tumor.

The treatment was given once daily orally for 21 days. Tumor growth curves (Figure 4.26 and 4.27) were established, and the tumor volume was monitored at least twice a week. When the course of treatment is over, there was no discernible difference in the mean body weight between the mice in the treatment and vehicle groups, confirming that the different extracts and sorafenib had no effect on the body weights of HepG2 cell tumor-bearing mice (Table 4.32).

Table 4.32: Body weight Data

Treatment	Dose (mg/Kg)	Initial (Day 1) body weight (gm)	Final (Day 21) body weight (gm)
Vehicle	-	18 ± 1.0	24 ± 1.5
Alcoholic extract of *A. aspera*	500	18 ± 1.4	25 ± 1.6
Alcoholic extract of *B. diffusa*	500	19 ± 1.3	26 ± 1.2
Sorafenib	30	19 ± 1.4	24 ± 2.0
Sorafenib	15	18 ± 1.8	24 ± 1.4
Alcoholic extract of *A. aspera* & Sorafenib	500 + 30	19 ± 1.2	23 ± 1.2
Alcoholic extract of *A. aspera* & Sorafenib	500 + 15	18 ± 1.0	24 ± 1.2
Alcoholic extract of *B. diffusia* & Sorafenib	500 + 30	18 ± 1.2	24 ± 0.8
Alcoholic extract of *B. diffusia* & Sorafenib	500 + 15	19 ± 1.2	25 ± 1.0

The data is presented as mean ± S.D. for n =6.

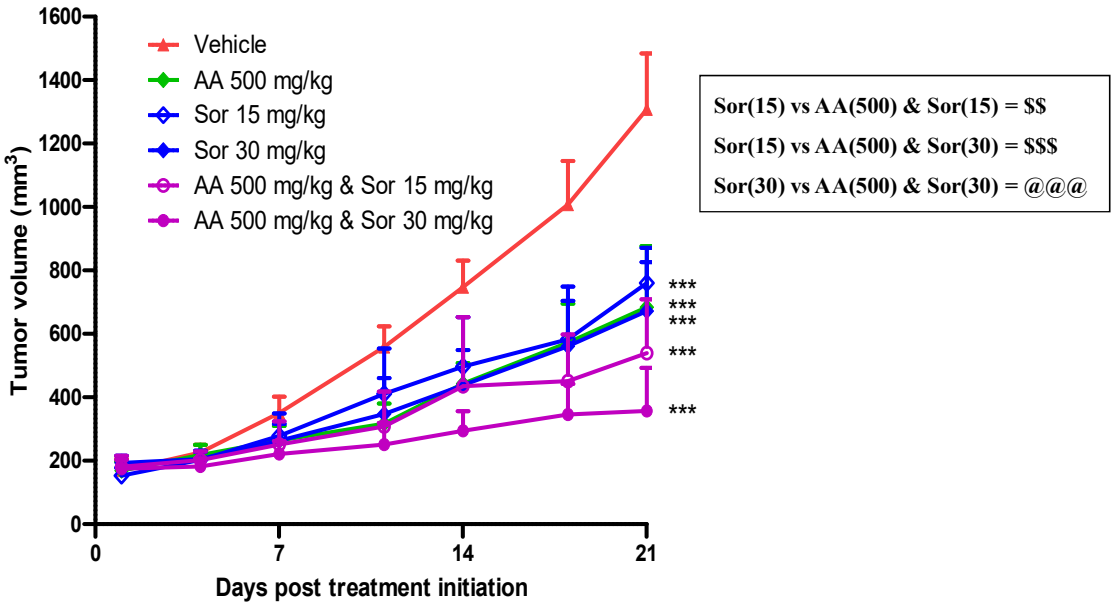


Figure 4.26: Efficacy of *A. aspera* in HepG2 CDX model

Mice with HepG2 tumors were given vehicle, *A. aspera* (AA) 500 mg/kg/day, sorafenib (Sor) 15 and 30 mg/kg/day, and combination of extract with sorafenib. All the treatment was given by p.o. for 21 days. Data presented as mean ± SD. Statistical analysis was carried out using two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. ***$p < 0.001$ as compared to vehicle treated group. $^{\$\$}p < 0.01$ and $^{\$\$\$}p < 0.001$ as compared to Sor (15 mg/kg) treated group. $^{@@@}p < 0.001$ as compared to Sor (30 mg/kg) treated group.

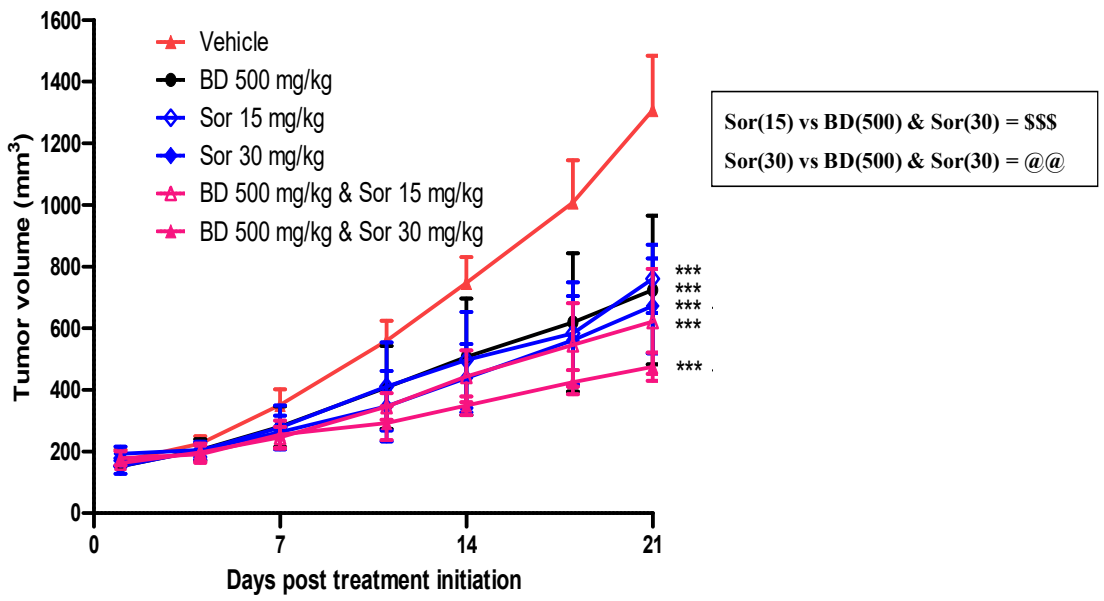


Figure 4.27: Efficacy of *B. diffusa* in HepG2 CDX model

Mice with HepG2 tumors were given with vehicle, *B. diffusa* (BD) 500 mg/kg/day, sorafenib (Sor) 15 and 30 mg/kg/day and combination of *B. diffusa* 500 mg/kg/day with sorafenib 15 and 30 mg/kg/day. All the treatment was given by p.o. for 21 days. Data presented as mean ± SD. Statistical analysis was carried out using two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. ***$p < 0.001$ as compared to vehicle treated group. $^{\$\$\$}p < 0.001$ as compared to Sor (15 mg/kg) treated group. $^{@@}p < 0.01$ as compared to Sor (30 mg/kg) treated group.

On day 21, the percentage tumor growth inhibition (% TGI) rate of sorafenib at 15 mg/kg and 30 mg/kg was 47.5% and 58.6%, respectively. %TGI for the *A. aspera* extract alone treated group was 51.3%; %TGI with the combination *A. aspera* with sorafenib at dose levels of 15 mg/kg and 30 mg/kg was 68.3% and 84.2%, respectively. The %TGI with *B. diffusa* extract monotherapy was 44.9% which was enhanced to 60.0% and 74.2% when extract was combinated with sorafenib at 15 and 30 mg/kg doses, respectively (Table 4.33).

Table 4.33: Data of percentage tumor growth inhibition on day 21

Treatment	Dose (mg/Kg)	% TGI
		Day 21
Alcoholic extract of *A. aspera*	500	51.3
Alcoholic extract of *B. diffusa*	500	44.9
Sorafenib	15	47.5
Sorafenib	30	58.6
Alcoholic extract of *A. aspera* & Sorafenib	500 + 15	68.3
Alcoholic extract of *A. aspera* & Sorafenib	500 + 30	84.2
Alcoholic extract of *B. diffusia* & Sorafenib	500 + 15	60.0
Alcoholic extract of *B. diffusia* & Sorafenib	500 + 30	74.2

Further, the efficacy of all treatments was assessed by calculating the T/C ratio. For calculating the T/C ratio, the final mean weight of the tumors in the drug-treated arm (T) and the arm in the control group (C) was used. A value of less than 0.42 was accepted to be an active response, according to the NCI standards of the Drug Evaluation Branch of the Division of Cancer Treatment (221).

Alone treatment with extracts and sorafenib had the T/C ratio greater than 0.42. Only the combination groups were found to be more active with T/C ratios of 0.39, and 0.24 with *A. aspera* and sorafenib at doses of 15 and 30 mg/kg, respectively, and the second combination of *B. diffusa* and sorafenib at doses of 15 and 30 mg/kg with a 0.42 and 0.34 T/C ratio (Table 4.34).

Table 4.34: Data of T/C ratio with tumor weight

Treatment	Dose (mg/Kg)	T/C
Vehicle	-	100.0
Alcoholic extract of *A. aspera*	500	0.51
Alcoholic extract of *B. diffusa*	500	0.58
Sorafenib	30	0.48
Sorafenib	15	0.54
Alcoholic extract of *A. aspera* & Sorafenib	500 + 30	**0.24**
Alcoholic extract of *A. aspera* & Sorafenib	500 + 15	**0.39**
Alcoholic extract of *B. diffusia* & Sorafenib	500 + 30	**0.34**
Alcoholic extract of *B. diffusia* & Sorafenib	500 + 15	**0.42**

Data are expressed as mean ± S.D. n=6.

During the experiment period, animals within all groups displayed normal behavior while eating, drinking, and excreting. On dissection of mice, there was no evidence of any visible vital organ damage.

It has been noted in the literature that sorafenib can cause hepatotoxicity (214). To evaluate the potential hepatotoxicity due the course of treatment, clinical examinations of HCC patients' ALT and AST levels are conducted on a regular basis. The mice serum levels of ALT and AST were assessed at the end of the experiment. Compared to the mice in the vehicle (control) group, the level of ALT was considerably ($p<0.01$) increased following sorafenib treatment at a dose of 30 mg/kg, which is equivalent to the clinical dose. When comparing the ALT level of the mice treated with extract alone or combined with sorafenib to the vehicle control group, there was no appreciable difference (Figure 4.28).

The levels of ALT, AST, creatinine, bilirubin, and ALP did not significantly alter in the combination group (extracts + sorafenib), indicating minimal hepato-renal damage (Figure 4.28 to 4.32). Combinations of extracts also result in a drop in ALT levels, which were previously elevated as a result of therapy with sorafenib at a dose of 30 mg/kg. This indicates that both the extracts have no obvious damage to the liver, or kidney function.

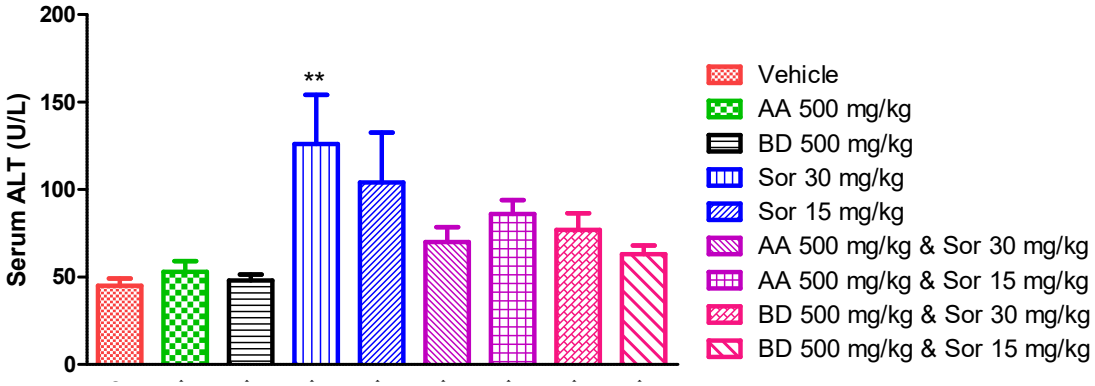


Figure 4.28: Effect of *A. aspera*, *B. diffusa* and sorafenib on serum ALT level

Mice with HepG2 tumors were given with vehicle, *A. aspera* (AA), *B. diffusa* (BD) extracts, sorafenib (Sor) and their combinations by oral route for 21 days. Serum was analysed on day 21. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. **p < 0.01 as compared to vehicle treated group.

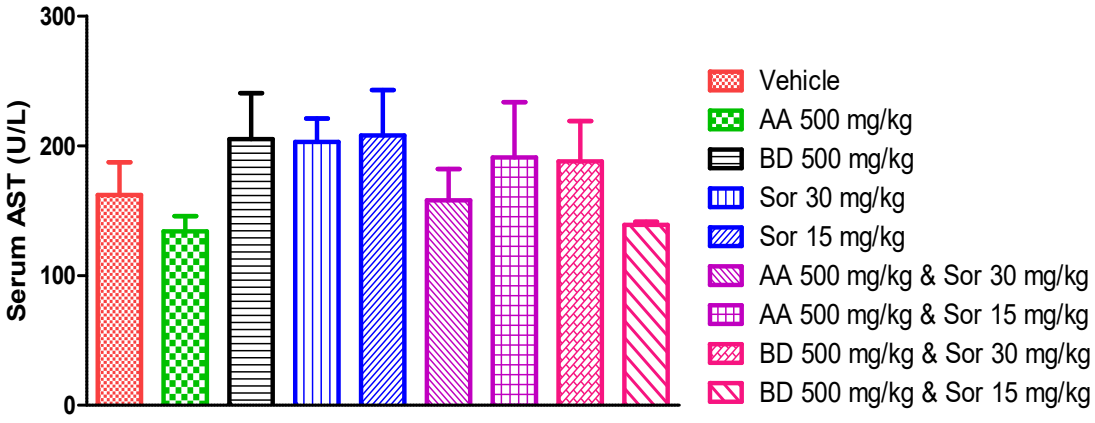


Figure 4.29: Effect of *A. aspera*, *B. diffusa* and sorafenib on serum AST level

Mice with HepG2 tumors were given with vehicle, *A. aspera* (AA), *B. diffusa* (BD) extracts, sorafenib (Sor) and their combinations by oral route for 21 days. Serum was analysed on day 21. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. All groups are non significant as compared to vehicle treated group.

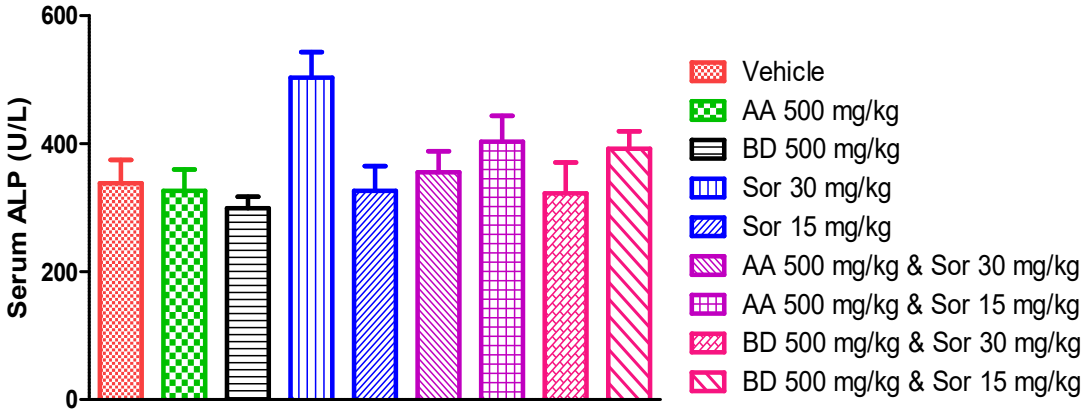


Figure 4.30: Effect of *A. aspera*, *B. diffusa* and sorafenib on serum ALP level

Mice with HepG2 tumors were given with vehicle, *A. aspera* (AA), *B. diffusa* (BD) extracts, sorafenib (Sor) and their combinations by oral route for 21 days. Serum was analysed on day 21. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. All groups are non significant as compared to vehicle treated group.

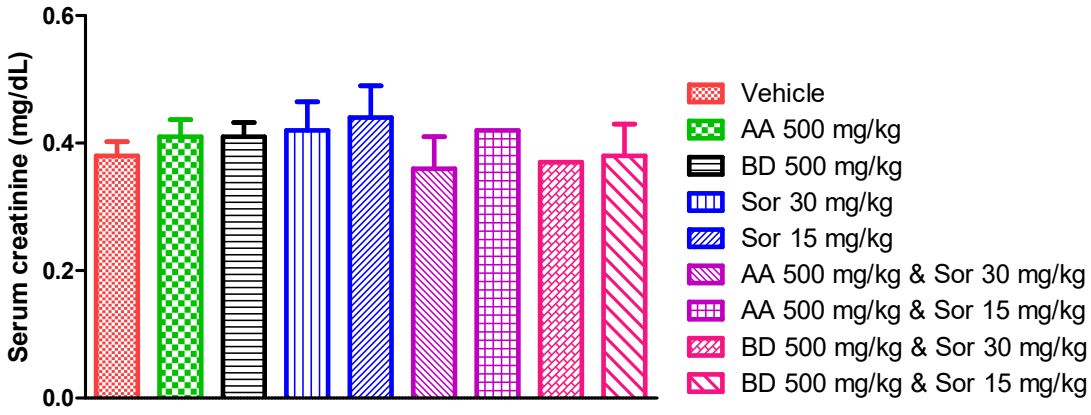


Figure 4.31: Effect of *A. aspera*, *B. diffusa* and sorafenib on serum creatinine level

Mice with HepG2 tumors were given with vehicle, *A. aspera* (AA), *B. diffusa* (BD) extracts, sorafenib (Sor) and their combinations by oral route for 21 days. Serum was analysed on day 21. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. All groups are non significant as compared to vehicle treated group.

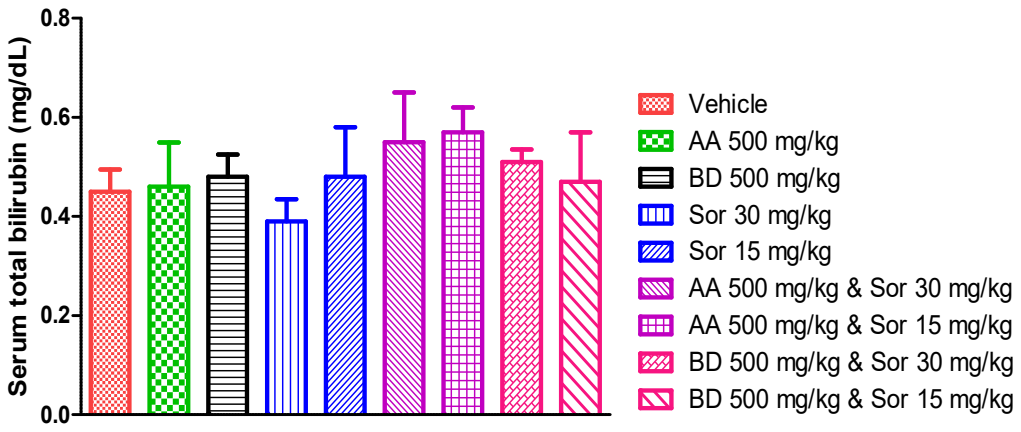


Figure 4.32: Effect of *A. aspera*, *B. diffusa* and sorafenib on total bilirubin level

Mice with HepG2 tumors were given with vehicle, *A. aspera* (AA), *B. diffusa* (BD) extracts, sorafenib (Sor) and their combinations by oral route for 21 days. Serum was analysed on day 21. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. All groups are non significant as compared to vehicle treated group.

The sorafenib and extracts treatments considerably reduced ($p < 0.001$) the tumor weight and volume in mice compared to those in the vehicle control group. The anticancer activity was further increased when they were administered in combination. The data for tumor weight on day 21 is presented in Figure 4.33.

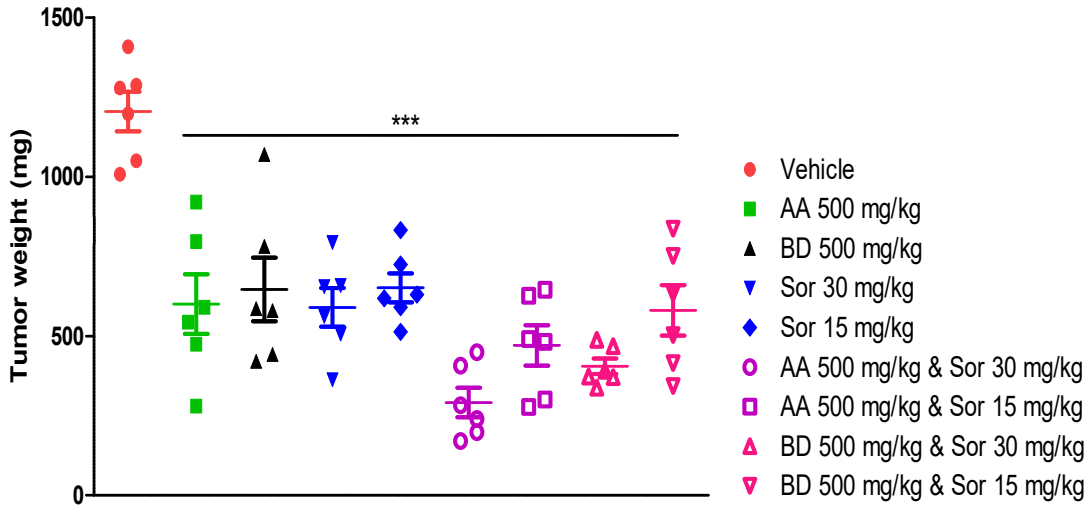


Figure 4.33: Effect of *A. aspera*, *B. diffusa* and sorafenib on tumor weight

Mice with HepG2 tumors were given with vehicle, *A. aspera* (AA), *B. diffusa* (BD) extracts, sorafenib (Sor), and their combinations by oral route for 21 days. Tumors were dissected, and their weight was taken on day 21. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. ***$p < 0.001$ as compared to the vehicle-treated group.

In percentage change in tumor volume on day 21, all animals (n = 6) in the vehicle, *B. diffusa*, *A. aspera* extract, and sorafenib at 15 mg/kg group showed tumor growth of more than 100% while one animal out of 6 in sorafenib (30 mg/kg) and 2 mice in combination of sorafenib at 15 mg/kg with *A. aspera* extract showed less than 100% change in tumor growth. The combination group of *A. aspera* extract with sorafenib at 30 mg/kg was more effective. In this group, 3 animals had less than 100% tumor growth. Data is presented in Figure 4.34.

In *B. diffusa* extract and sorafenib (30 mg/kg) treated groups, percentage tumor growth was found up to 800% and 350%, respectively. The combination group showed a tremendous effect, and the percentage tumor growth was below 200%. Data is presented in Figure 4.35.

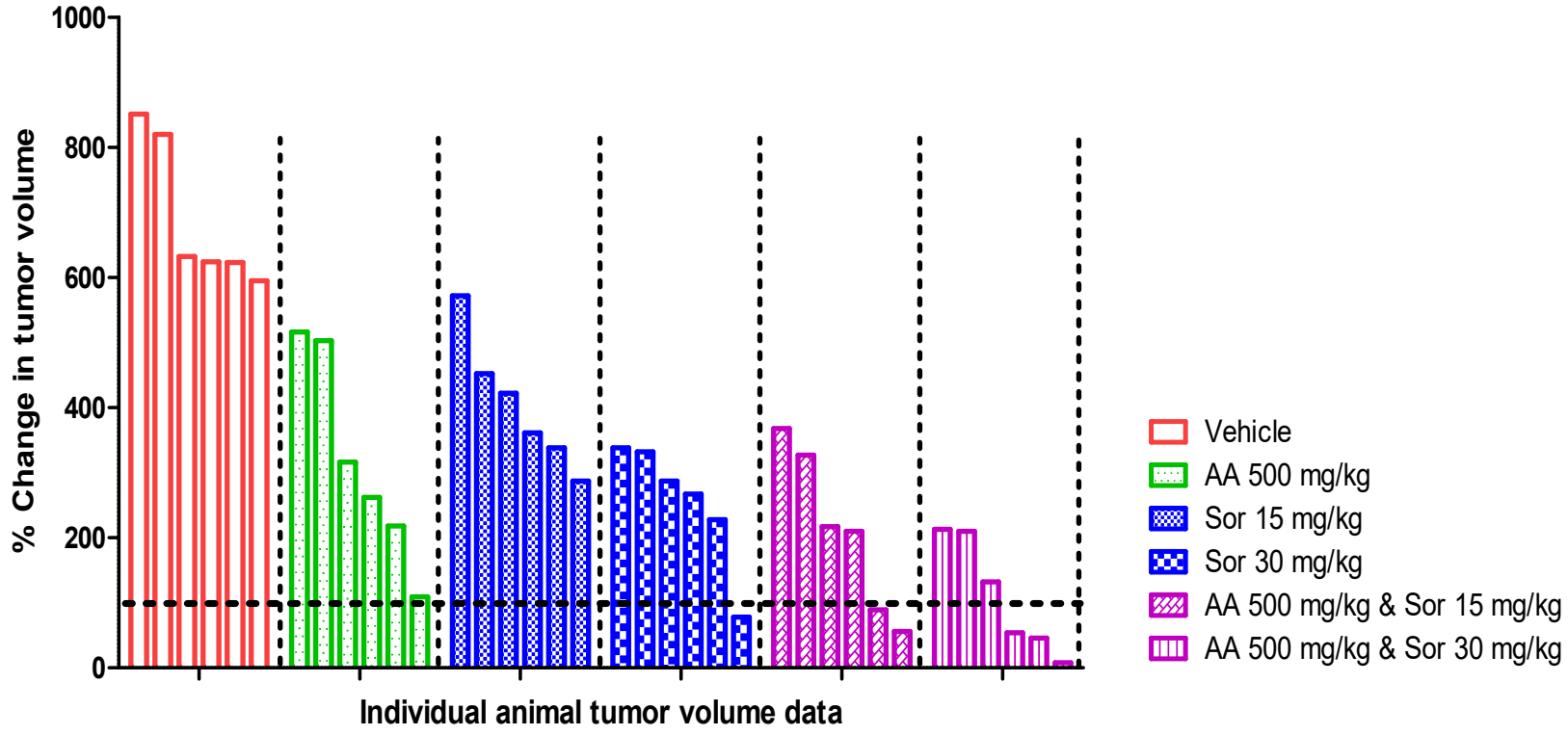


Figure 4.34: Effect of *A. aspera* and sorafenib on percentage change in tumor volume on day 21

Mice with HepG2 tumors were given with vehicle, *A. aspera* (AA) extracts, sorafenib (Sor) and their combinations by oral route for 21 days. Percentage change in tumors volume was calculated from day 21 and day 1 tumor volume. n=6

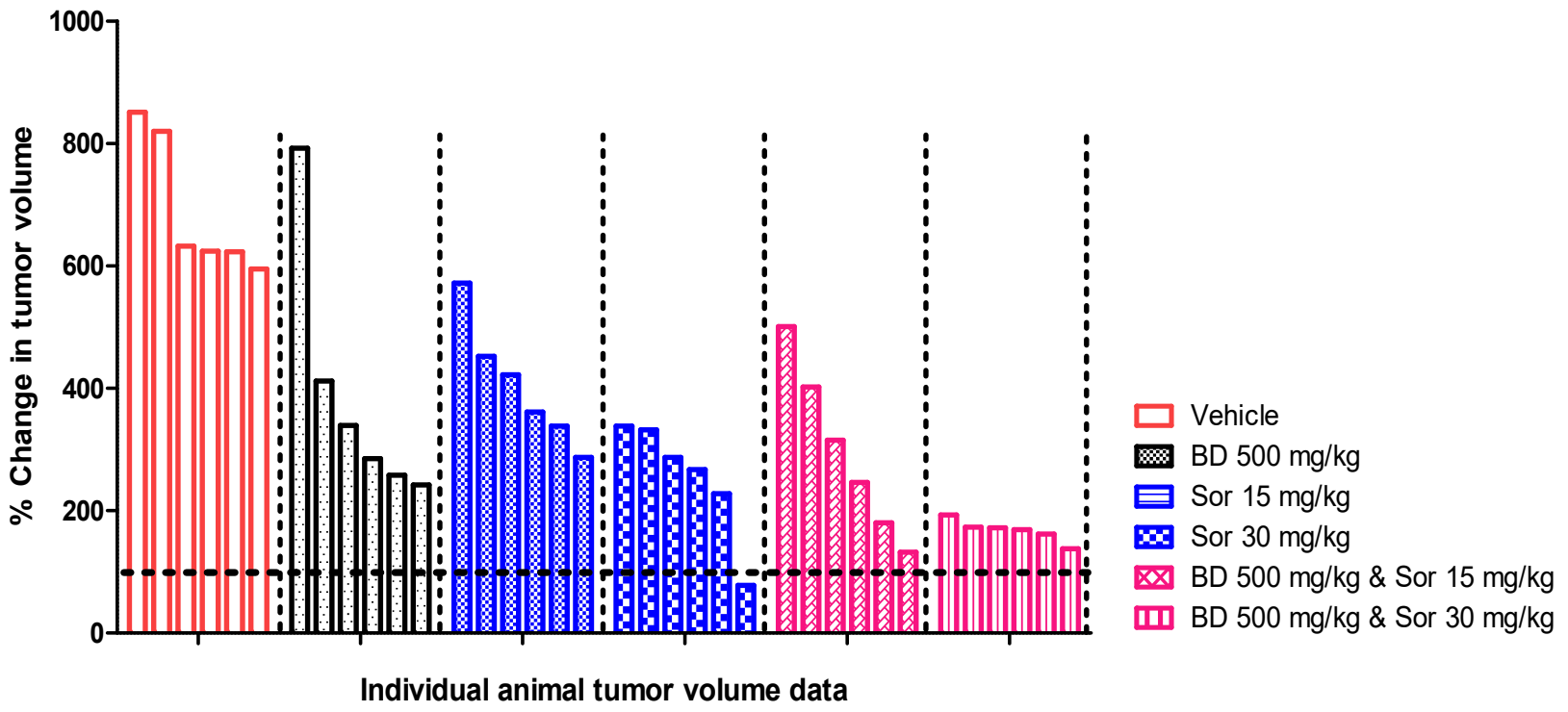


Figure 4.35: Effect of *B. diffusa* and sorafenib on percentage change in tumor volume on day 21

Mice with HepG2 tumors were given vehicle, *B. diffusa* (BD) extract, sorafenib (Sor) and their combinations by oral route for 21 days. Percentage change in tumors volume was calculated from day 21 and day 1 tumor volume. n=6

4.11.4.1 Effect of extracts, sorafenib and their combination on VEGF and p-ERK protein expression analyzed by western blot

To correlate the mechanism basis for the anti-tumor effect of extract, VEGF and p-ERK protein were evaluated using western blot technique. *A. aspera* and sorafenib significantly ($p< 0.1$ to 0.01) reduced the expression of VEGF. Combination of *A. aspera* with sorafenib leads to further reduction ($p<0.001$) of VEGF expression as compared to the vehicle-treated group (Figure 4.36 and 4.37).

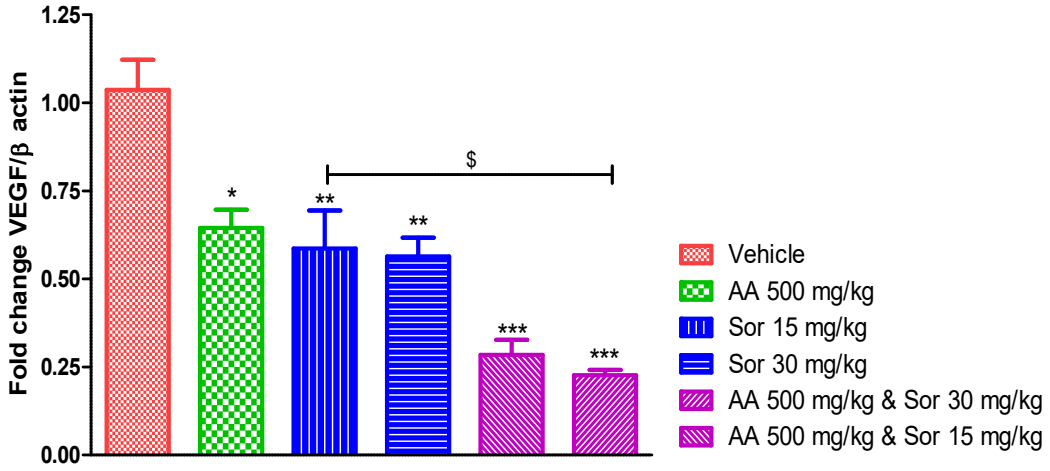


Figure 4.36: Effect of *A. aspera,* sorafenib and their combination on fold change of VEGF expression

Mice with HepG2 tumors were administered with vehicle, *A. aspera* (AA) extract, sorafenib (Sor), and their combinations by oral route for 21 days. Tumors were dissected, and expression of VEGF was determined by western blot. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. *$p < 0.1$, **$p < 0.01$ and ***$p < 0.001$ as compared to the vehicle-treated group. $^{\$}p < 0.1$ as compared to Sor (15 mg/kg) treated group.

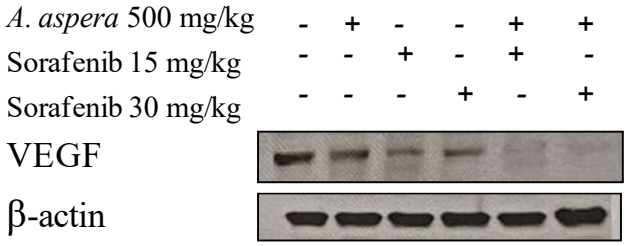


Figure 4.37: Effect of *A. aspera,* sorafenib and their combination on VEGF expression

A. aspera monotherapy slightly reduced the unregulated p-ERK, while sorafenib at 30 mg/kg significantly ($p < 0.1$) reduced the p-ERK expression. Combination of *A. aspera* extract with 15 and 30 mg/kg doses of sorafenib further significantly ($p < 0.01$ and 0.001) reduced the p-ERK levels as compared to the vehicle group (Figure 4.38 and 4.39).

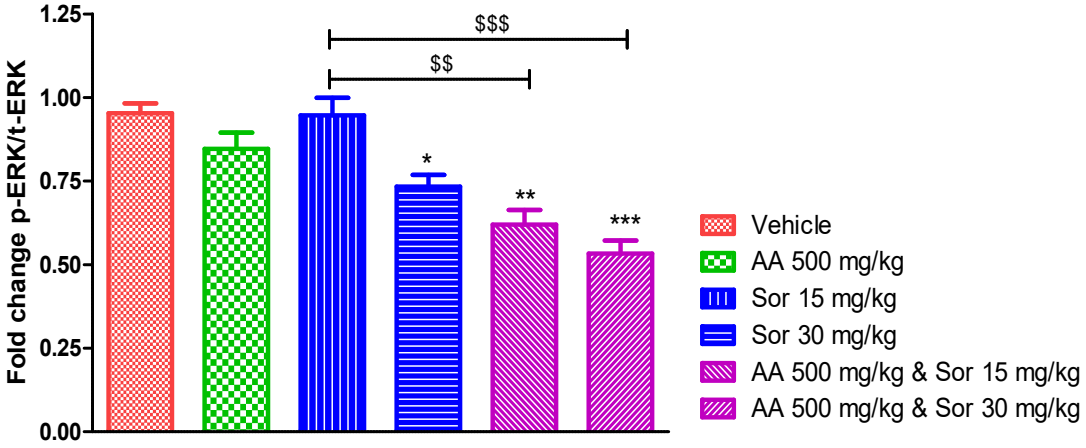


Figure 4.38: Effect of *A. aspera*, sorafenib and their combination on fold change in p-ERK/t-ERK ratio

Mice with HepG2 tumors were administered with vehicle, *A. aspera* (AA), *B. diffusa* (BD) extracts, sorafenib (Sor) and their combinations by oral route for 21 days. Tumors were dissected and expression of p-ERK and t-ERK was determined by western blot. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. *$p < 0.1$, **$p < 0.01$, and ***$p < 0.001$ as compared to the vehicle-treated group. $^{\$\$}p < 0.01$ as compared to Sor (15 mg/kg) treated group.

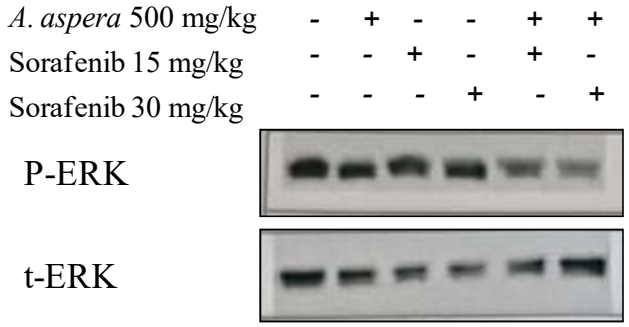


Figure 4.39: Effect of *A. aspera,* sorafenib and their combination on p-ERK expression

Though sorafenib significantly (p< 0.1 & 0.01) reduced the expression of VEGF, alone treatment of *B. diffusa* was unable to reduce the level of VEGF significantly as compared to the vehicle treatment group. But the combination of both enhanced the activity and significantly reduced (p<0.001) the VEGF expression as compared to vehicle-treated mice (Figure 4.40 and 4.41).

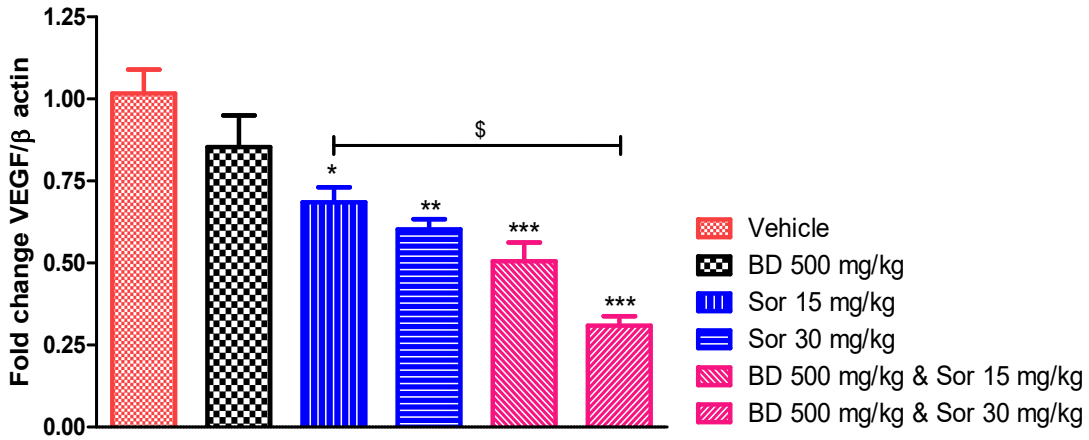


Figure 4.40: Effect of *B.diffusa* extract and sorafenib on fold change in VEGF

Mice with HepG2 tumors were administered with vehicle, *B. diffusa* (BD) extracts, sorafenib (Sor) and their combinations by oral route for 21 days. Tumors were dissected and expression of VEGF was determined by western blot. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. *p < 0.1, **p < 0.01 and ***p < 0.001 as compared to the vehicle-treated group. $^{\$}$p < 0.1 as compared to Sor (15 mg/kg) treated group.

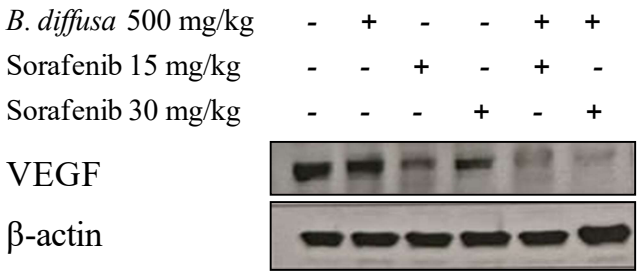


Figure 4.41: *B.diffusa,* sorafenib and their combination effect on VEGF expression

Sorafenib at 30 mg/kg significantly ($p< 0.1$) reduced the upregulated p-ERK level. Further combination of it with *B. diffusa* extract significantly ($p< 0.001$) enhanced the activity and downregulated the p-ERK level. Alone treatment of *A. aspera* and sorafenib at 15 mg/kg was unable to significantly reduce the upregulated p-ERK level (Figure 4.42 and 4.43).

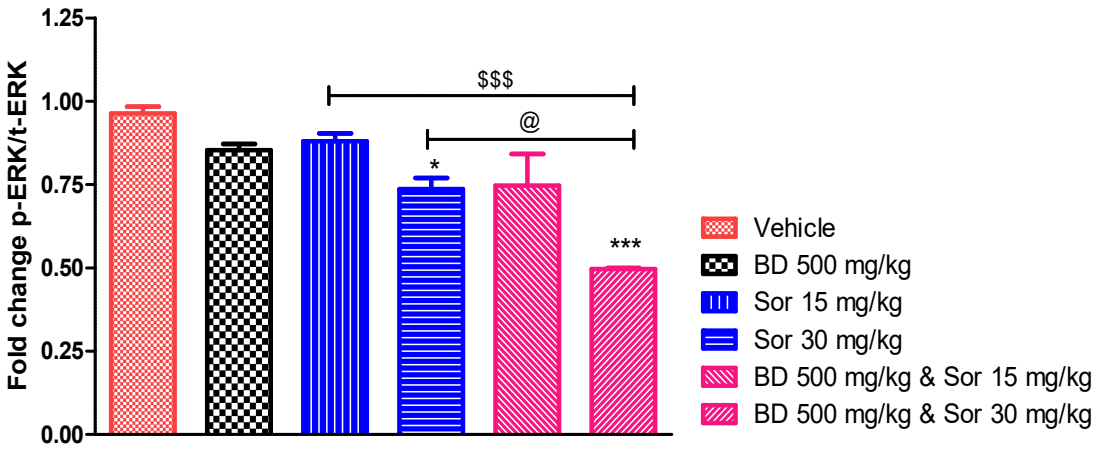


Figure 4.42: Effect of *B.diffusa* extract and sorafenib on fold change of p-ERK/t-ERK ratio

Mice with HepG2 tumors were administered with vehicle, *B. diffusa* (BD) extracts, sorafenib (Sor) and their combinations by oral route for 21 days. Tumors were dissected and expression of p-ERK and t-ERK was determined by western blot. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. *$p < 0.1$, **$p < 0.01$ and ***$p < 0.001$ as compared to the vehicle-treated group. $^{\$\$\$}p < 0.001$ as compared to Sor (15 mg/kg) treated group. $^{@}p < 0.1$ as compared to Sor (30 mg/kg) treated group

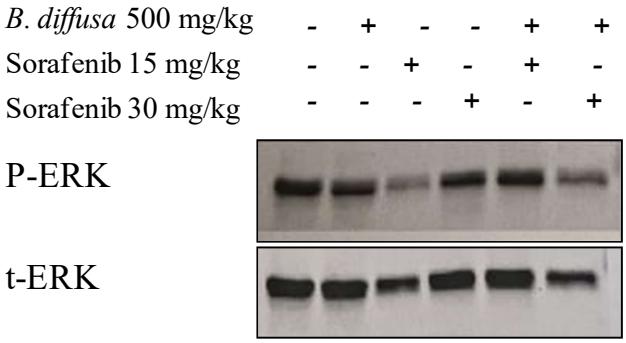


Figure 4.43: Effect of *B. diffusa*, sorafenib and their combination on p-ERK expression

4.11.4.2 Effect of extracts, sorafenib and their combination on proliferation and angiogenesis markers

To correlate the mechanism, we evaluated tumor sections using immunohistochemistry for proliferation (Ki-67) and angiogenesis (CD31) marker.

Ki67 is a proliferating cell-associated nuclear antigen marker that is found to increase tremendously in cancer. In the vehicle group, the proliferation index of Ki67 was found to be 50% and it was reduced significantly to 36%, 35%, and 19%, respectively, in the *A. aspera*, sorafenib 15 mg/kg, and 30 mg/kg treated groups. Ki-67 was significantly ($p<0.001$) abridged by combined therapy of *A. aspera* and sorafenib at 15 mg/kg, and 30 mg/kg to 21% and 10%. In the *B. diffusa*-treated group, the Ki67 index was reduced from 39% to 23% and 15%, respectively, in combination with extract with sorafenib at 15 mg/kg and 30 mg/kg treated groups (Figure 4.44 and 4.45).

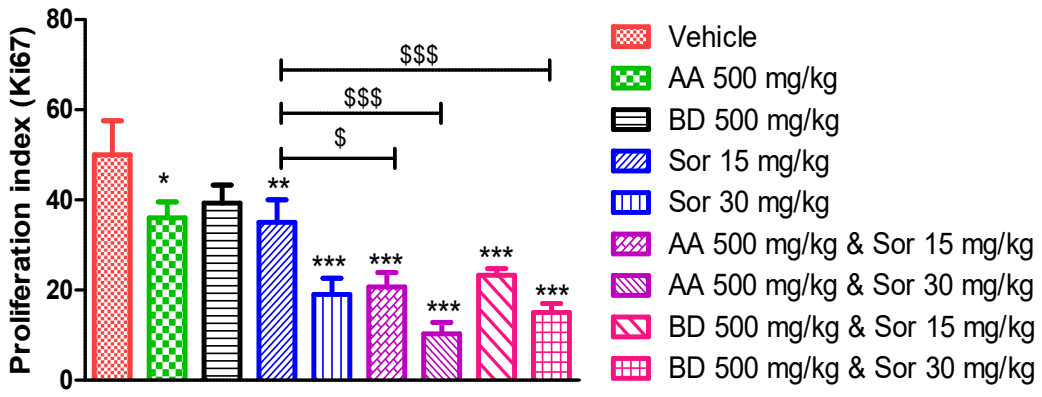


Figure 4.44: Proliferation index of Ki67 by immunohistochemistry assay

Mice with HepG2 tumors were administered with vehicle, *A. aspera* (AA), *B. diffusa* (BD) extracts, sorafenib (Sor), and their combinations by oral route for 21 days. Tumors were dissected and the proliferation index (Ki67) was determined by immunohistochemistry. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. *$p < 0.1$, **$p < 0.01$ and ***$p < 0.001$ as compared to the vehicle-treated group. $^{\$}p < 0.1$, $^{\$\$}p < 0.01$ and $^{\$\$\$}p < 0.001$ as compared to Sor (15 mg/kg) treated group.

Anticancer activity of alcoholic *A. aspera* and *B. diffusa* extract is summarized in Figure 6-48.

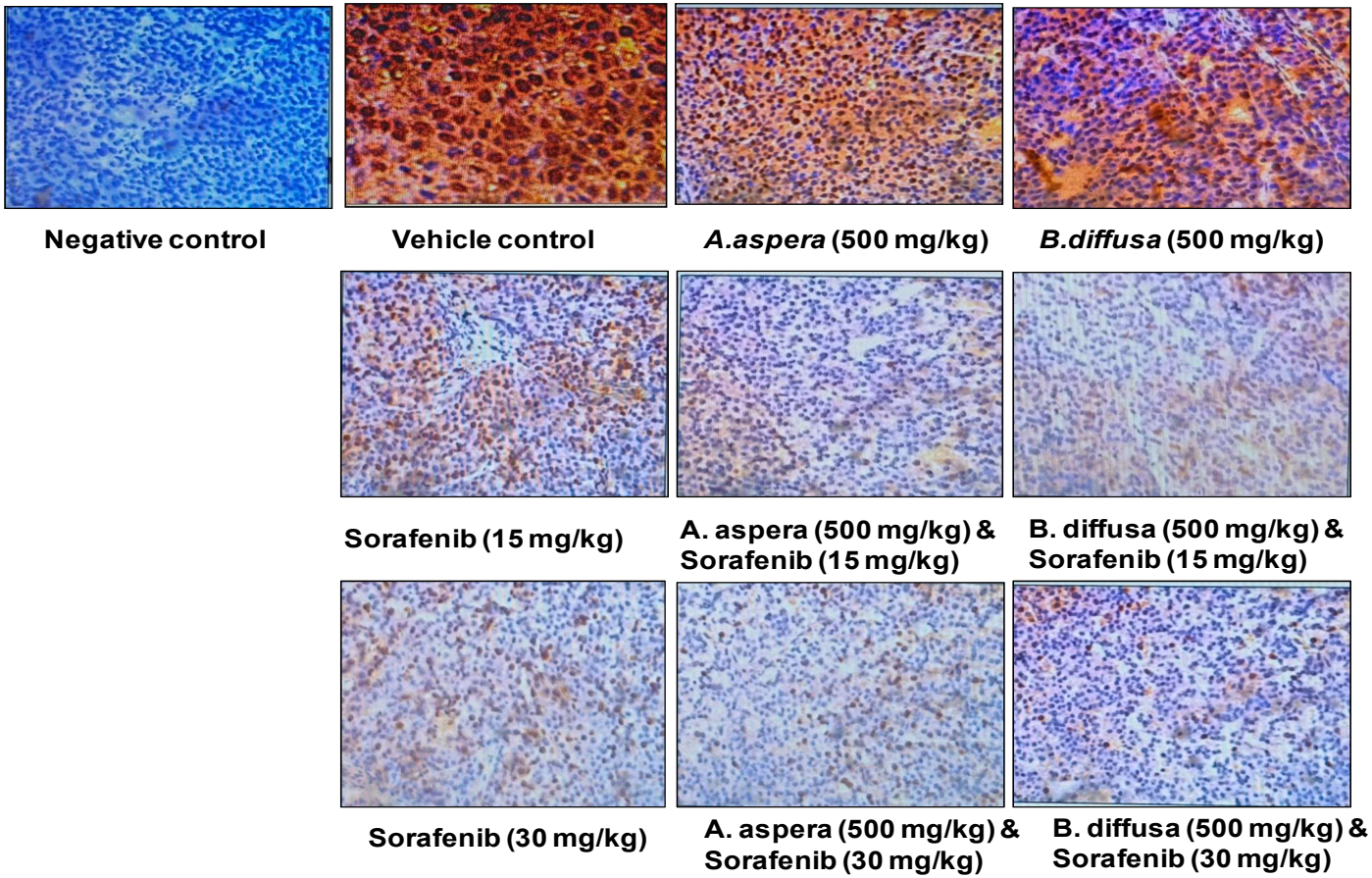


Figure 4.45: Representative images of Ki67 staining by Immunohistochemistry assay

Images are captured at 40X objective, 100 µm scale bar

The angiogenesis marker CD31 was identified by immunohistochemistry to reveal microvessel density (MVD), which reflects % of CD31-positive cells. CD31-positive blood vessels in tumors treated by *A. aspera* and *B. diffusa* extract with a combination of sorafenib at both 15 and 30 mg/kg doses were significantly decreased as compared to the vehicle control group. The control group had the highest MVD, followed by *B. diffusa*, *A. aspera*, then sorafenib at 15 and 30 mg/kg, and then the combination groups (Figure 4.46 and 4.47).

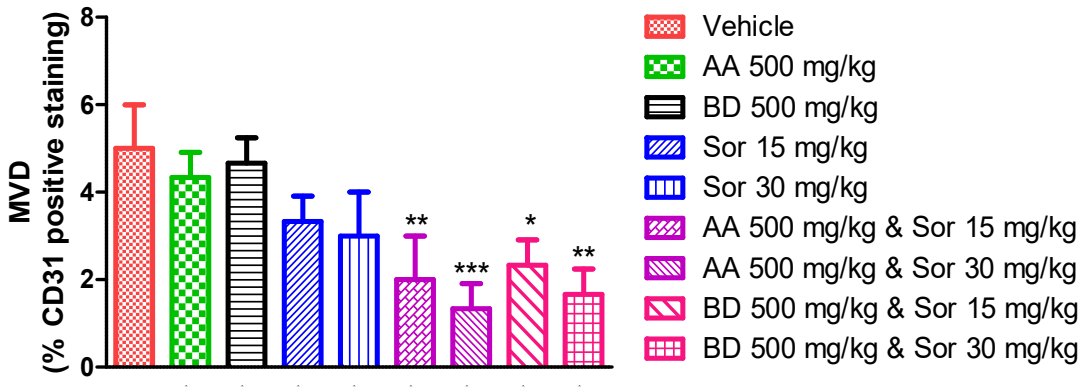


Figure 4.46: Mean Vascular density (MVD) of CD31 by immunohistochemistry assay

Mice with HepG2 tumors were administered with vehicle, *A. aspera* (AA), *B. diffusa* (BD) extracts, sorafenib (Sor), and their combinations by oral route for 21 days. Tumors were dissected and CD31 was determined by immunohistochemistry. Data presented as mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. *$p < 0.1$, **$p < 0.01$ and ***$p < 0.001$ as compared to vehicle treated group.

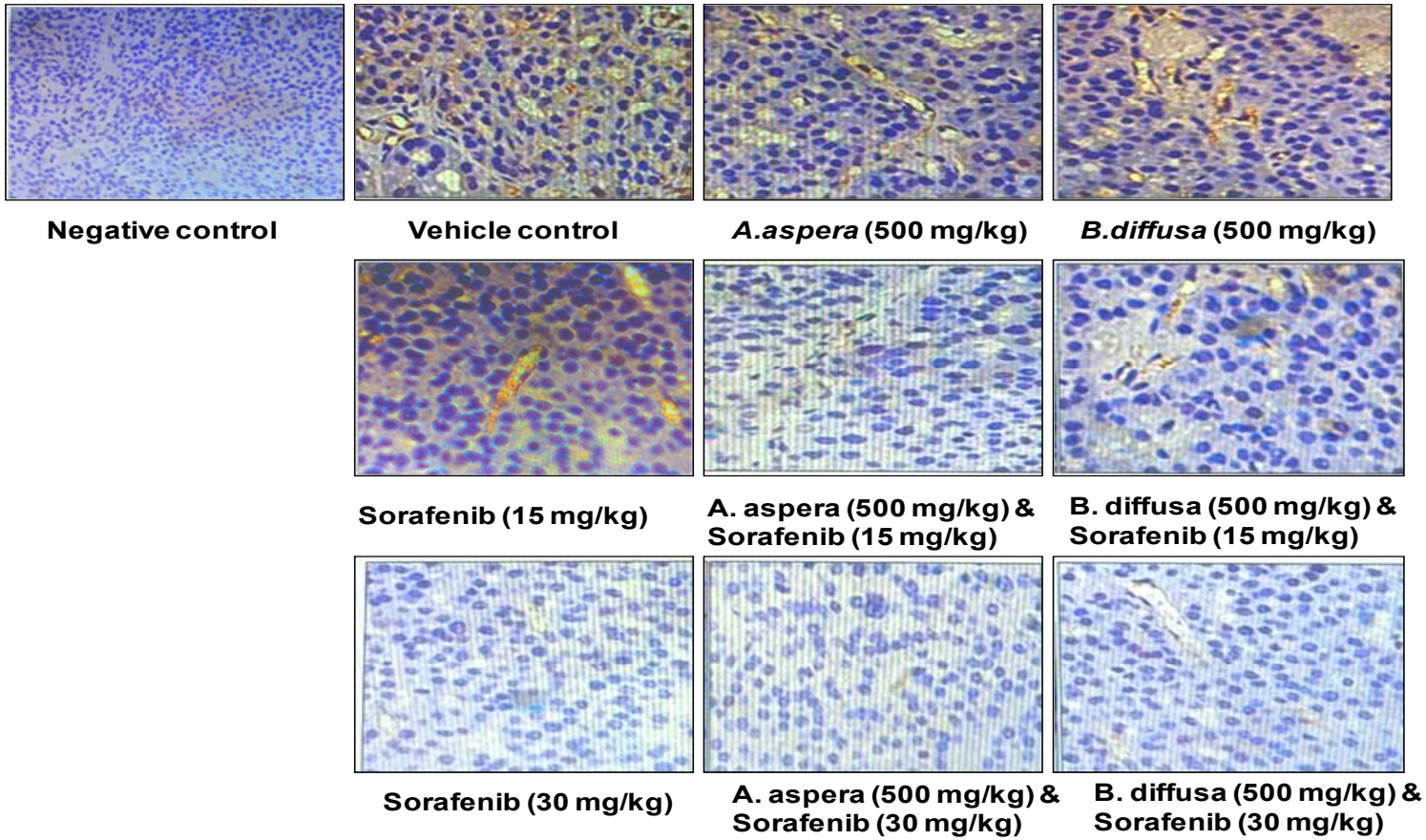


Figure 4.47: Representative images of MVD of CD31 by Immunohistochemistry assay

Images are captured at 40X objective, 100 µm scale bar

Thus the combined treatment showed superior activity compared to extracts and sorafenib alone treatment in constraining cell proliferation (Ki67) and mean vascular density of HepG2 xenograft tumors.

Anticancer activity of alcoholic *A. aspera* and *B. diffusa* extract is summarized in Figure 6.48. All the work performed in the thesis is summarized in graphical abstract in Figure 4.49.

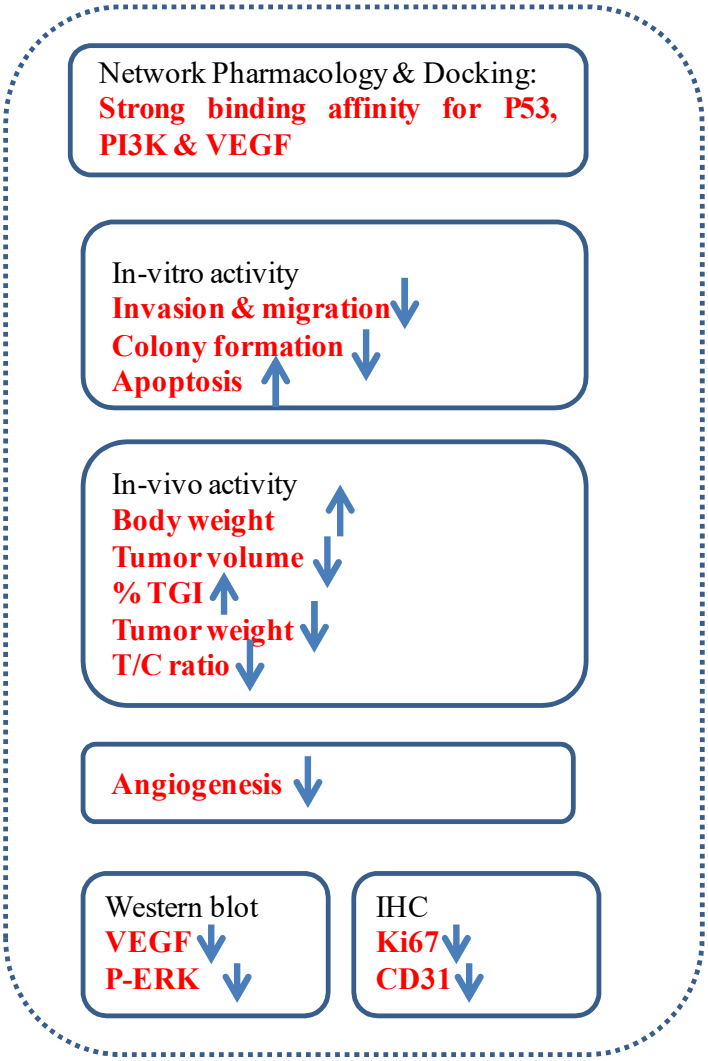


Figure 4.48: Schematic representation of anti-cancer activity of alcoholic *A. aspera* and *B. diffusa* extract

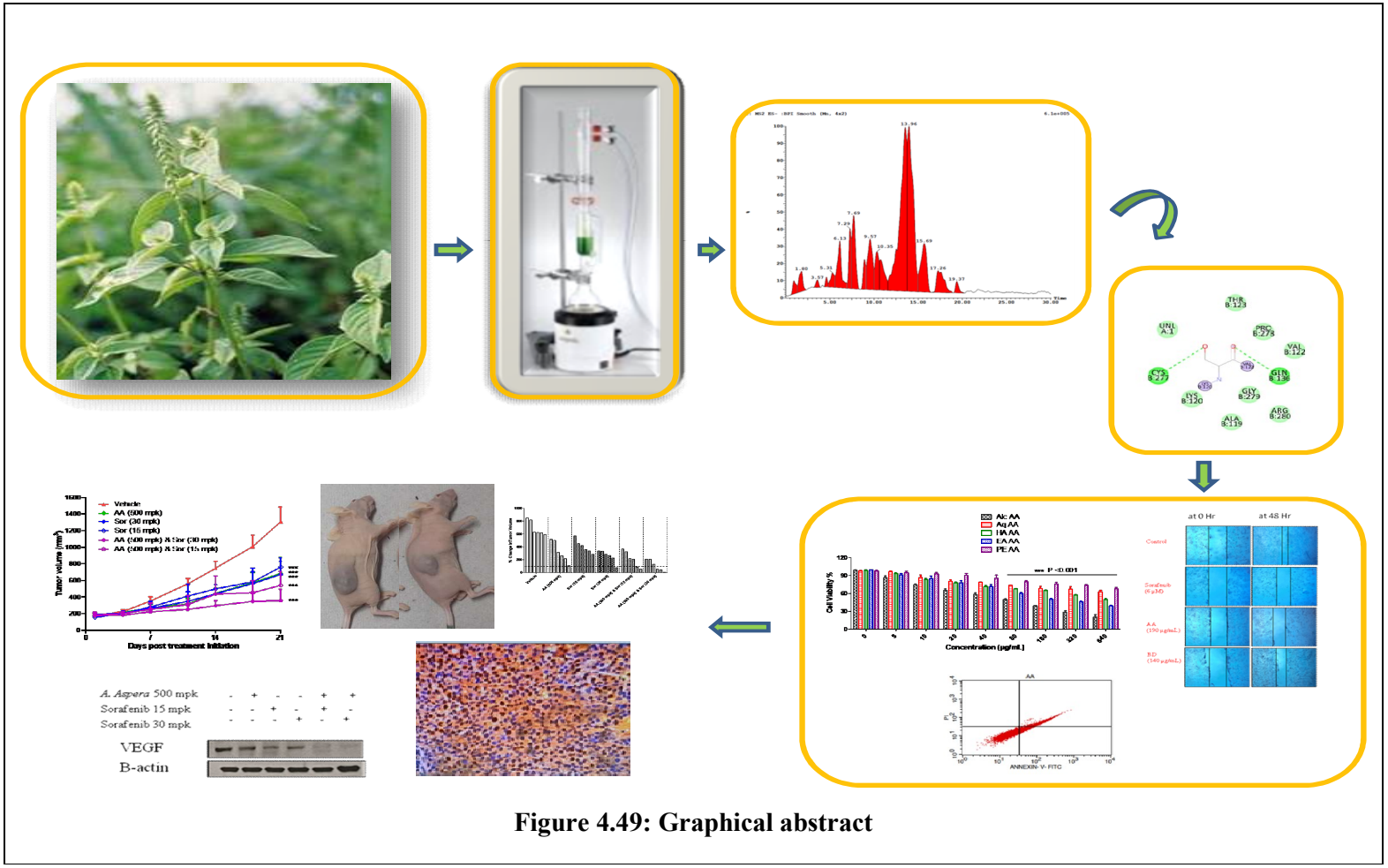


Figure 4.49: Graphical abstract