Pharmacological Evaluation of Some Medicinal Plants in Hepatocellular Carcinoma

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1. Introduction

Liver cancer is one of the leading causes of cancer-related mortality worldwide. Hepatocellular carcinomas (HCC), which constitute up 85% to 90% of cases of all liver cancer occurrence. The main causes of HCC are cirrhosis and other non-viral chronic liver diseases, as well as individuals with underlying viral hepatitis-related chronic liver disease, mainly hepatitis B virus (HBV) or hepatitis C virus (HCV) (1, 2). It is the 3rd leading cause of cancer-related death and the 6th most prevalent cancer in the world. The architecture of the liver is altered in cirrhosis, and with time, the liver begins to malfunction. Cirrhosis is a significant risk factor for the malignant development of the liver into HCC. Due to the enormous number of persons with cirrhosis, which is primarily brought on by hepatitis B and C virus infection or exposure to toxins like alcohol or aflatoxin, the incidence of HCC is quickly rising nowadays.

HCC is one of the most prevalent cancers in adults; it affects men more often than women and black people more frequently than white people. HCC is the 5th most frequent cancer in males and the 7th most prevalent cancer in women worldwide and its incidence is increasing at an alarming rate (3). HCC accounts for almost a million deaths annually worldwide.

Men may consume more alcohol, smoke more, and have a higher incidence of cirrhosis than women, which could be the cause of the high prevalence of HCC in men. The function of sex hormones and/or hormone receptors has been hypothesized through studies on animals. In male rats, orchidectomy brings down the carcinogenic effects of chemicals to the same level as in female rats (4).

The vast majority of HCC occurs in Asia and sub-Saharan Africa, in countries where hepatitis B infection is endemic, and many are infected from birth. Due to an increase in hepatitis C virus infections, the incidence of HCC is rising in the United States and other developing nations.

The etiology of HCC is exceedingly complex and involves several different elements. Hepatitis B virus and hepatitis C virus, diabetes, obesity, alcoholic fatty liver disease (AFLD), and nonalcoholic fatty liver disease (NAFLD) are the main risk factors for HCC. Smoking, food pollutants like aflatoxins, familial or genetic factors, and different environmental chemicals that function as carcinogens are additional risk factors that are also known to enhance the occurrence of HCC which are as shown in the figure 1 (5). HCC is brought on by hepatic injury, which includes hepatocyte necrosis, inflammation, and regeneration. Fibrosis, cirrhosis, and ultimately hepatocellular cancer are the stages of this chronic liver condition (6).

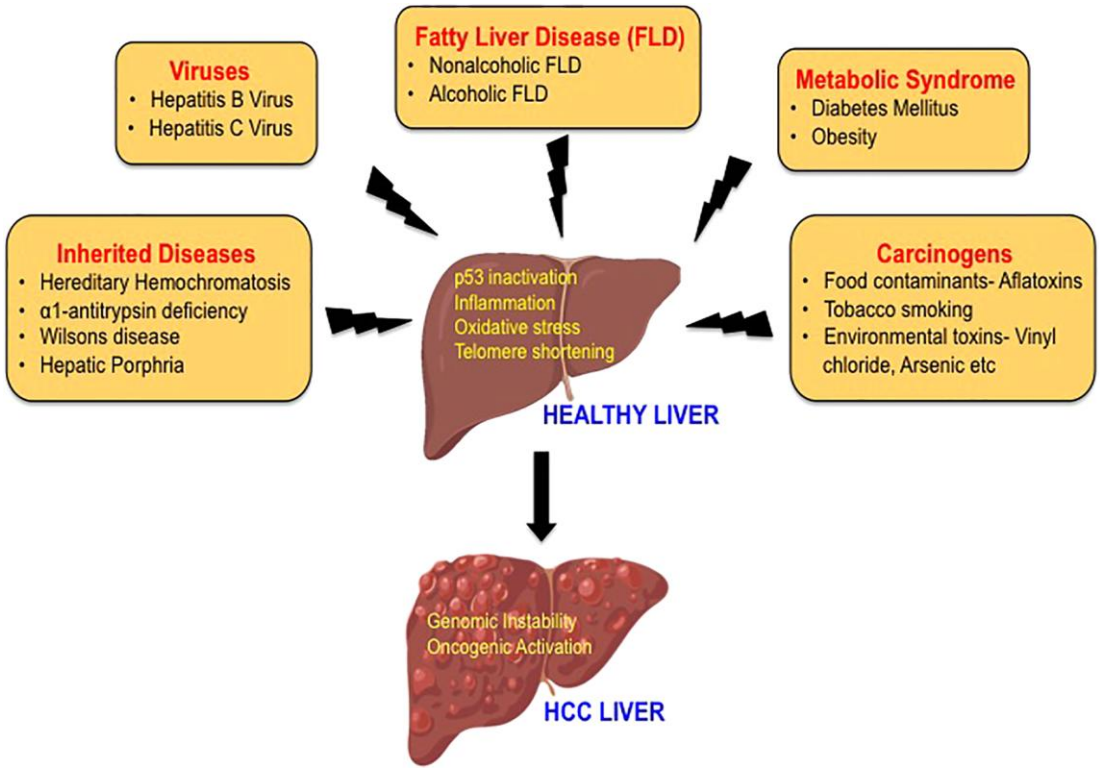

Figure 1: Causative agents of HCC

India is an endemic zone for hepatitis B virus, but there has been no comprehensively analyzed data for HCC in our country, and cancer registries probably do not reflect the accurate incidence. According to GLOBOCAN 2020 data, HCC is the 8th most common cause of cancer-related death in India. In our country, 70%–80% of all HCCs are related to HBV; approximately 15% are related to HCV, and 5% are related to both HBV and HCV. Alcohol alone accounts for approximately 8% of all HCCs. In about 10% of patient no direct etiology is seen. Iron overload and aflatoxin may have a role to play in some geographical areas in India. The underreporting of HCC is possibly because of nonsurveillance of patients with chronic hepatitis and cirrhosis. Most of the HCC cases in the Western countries are diagnosed in early stages because of advanced investigation techniques. However, in India, the majority of the patients are detected in advanced stages, leading to high mortality rates. (7).

1.1 Pathophysiology of hepatocellular carcinoma

Chronic necrosis and inflammation of the liver are important driving forces in the multistep process of hepatocarcinogenesis in the context of underlying risk factors such as HBV and HCV infections, iron overload, aflaotoxin exposure, and the presence of fatty liver disease. It is believed that HCC emerges from normal hepatocytes, through sequential acquisition of essential molecular alterations that empower them with cancer hallmark capabilities. It is a long and cumulative process under the continued clonal selective pressure that certain subclones of cells possessing growth and survival

advantage will dynamically undergo clonal expansion. As a result, the clonal evolution of altered hepatocytes progressively transform into HCC.

HCC primarily arises in a cirrhotic liver, where repeated inflammation and fibrinogenesis predispose the liver to dysplasia and malignant transformation. The structure and function of the liver undergo pathological changes in response to insults and injury, resulting in inflammation, fibrosis, necrosis and cirrhosis. Patients with chronic liver disease have sustained hepatic inflammation, fibrosis, and aberrant hepatocyte regeneration. These abnormalities can cause cirrhosis and favor a series of genetic and epigenetic events that culminate in the formation of dysplastic nodules, which are bonafide preneoplastic lesions. Additional molecular alterations provide dysplastic cells with proliferative, invasive, and survival advantages and complete the transition to full-blown hepatocellular carcinoma (8, 9).

Hepatocellular carcinoma can also arise in patients who have chronic liver disease who do not have established cirrhosis or marked inflammation (e.g., patients with HBV infection). Viral infections with HBV and HCV lead to enhanced hepatocyte turnover as the liver attempts to replace infected cells that have been immunologically attacked (11). Some evidence suggests that HCC develops from hepatic stem cells that proliferate in response to chronic regeneration caused by viral injury (11). The cells in small dysplastic nodules appear to carry markers consistent with stem cells. HBV can also cause HCC in the absence of cirrhosis. HBV integrates its deoxyribonucleic acid into the host genome, leading to genomic instability and chromosomal rearrangements. HCV uses ribonucleic acid to store genetic information and therefore does not integrate into the host genome. HCV-related HCC is found almost exclusively in patients with cirrhosis.

Alterations in numerous signaling pathways occur in cancer, and several specific pathways have been observed to be dysregulated in HCC. Changes in liver tissues induced either by chronic viral infection or by exposure to hepatotoxic agents cause upregulation of components of a number of cellular signaling pathways. The major signaling pathways of HCC are shown in figure 2 (12). The predominant pathways involved in HCC pathogenesis include pathways regulating growth factor signaling such as the insulin like growth factor (IGF), epidermal growth factor (EGF), PDGF, fibroblast growth factor (FGF) and hepatocyte growth factor (HGF/MET); pathways related to cell differentiation such as the WNT, Hedgehog, and Notch pathways; and pathways related to angiogenesis such as the vascular endothelial growth factor (VEGF) and FGF pathways. The major signaling mediators downstream of the receptor tyrosine kinases are the Ras/Raf/MEK/ERK and P13K/AKT/mTOR cascades (13).

In HCC over expression of epidermal growth factor, stimulates tyrosine kinase receptor that in return stimulates cell surface signal transmission to the nucleus. There is also role of MAPK pathway activation acting through the Ras protein in HCC. The Ras protein undergoes activation via phosphorylation, which allows the delivery of signals to the nucleus via downstream components of the pathway, such as ERK1, RAF and MEK. HCC is a highly vascularized tumor with strong angiogenetic activity that is enriched with vascular endothelial growth factor (VEGF) on the surface of tumor cells and which is one of the targeted treatments for HCC.

HCC cell accumulates somatic DNA alterations, including mutations and chromosomal aberrations. Mutations in the TERT promoter are the most frequent genetic alterations, accounting for approximately 60% of cases (14). They can be detected in dysplastic nodules, and the TERT promoter is a recurrent insertion site for the genome of HBV.

Other mutated genes affect the cell cycle (e.g., TP53, accounting for approximately 30% of cases), WNT signaling (CTNNB1 and AXIN1, accounting for approximately 30% and 10% of cases, respectively), or chromatin remodeling (ARID1A and ARID2, accounting for approximately 10% and 5% of cases, respectively).

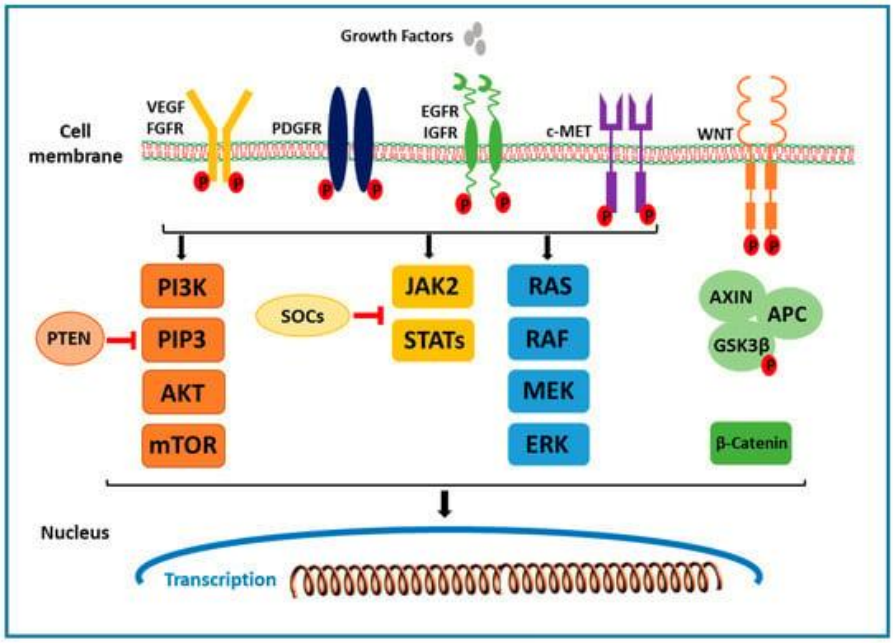

Figure 2: Major signaling pathways involved in HCC

The outstanding histological features of HCC are the resemblance of the tumor cells to normal hepatocytes and of their arrangement to the trabeculae of normal liver. However, the trabeculae are for the most part thicker and reticulin is often scanty or even absent. Between the tumor trabeculae in HCC there is a network of vascular channels lined by endothelium which is positive with

immunostain for CD34, factor VIII related antigen, and other molecules (15). The endothelial lining is a particularly helpful diagnostic feature in the fine needle aspirates.

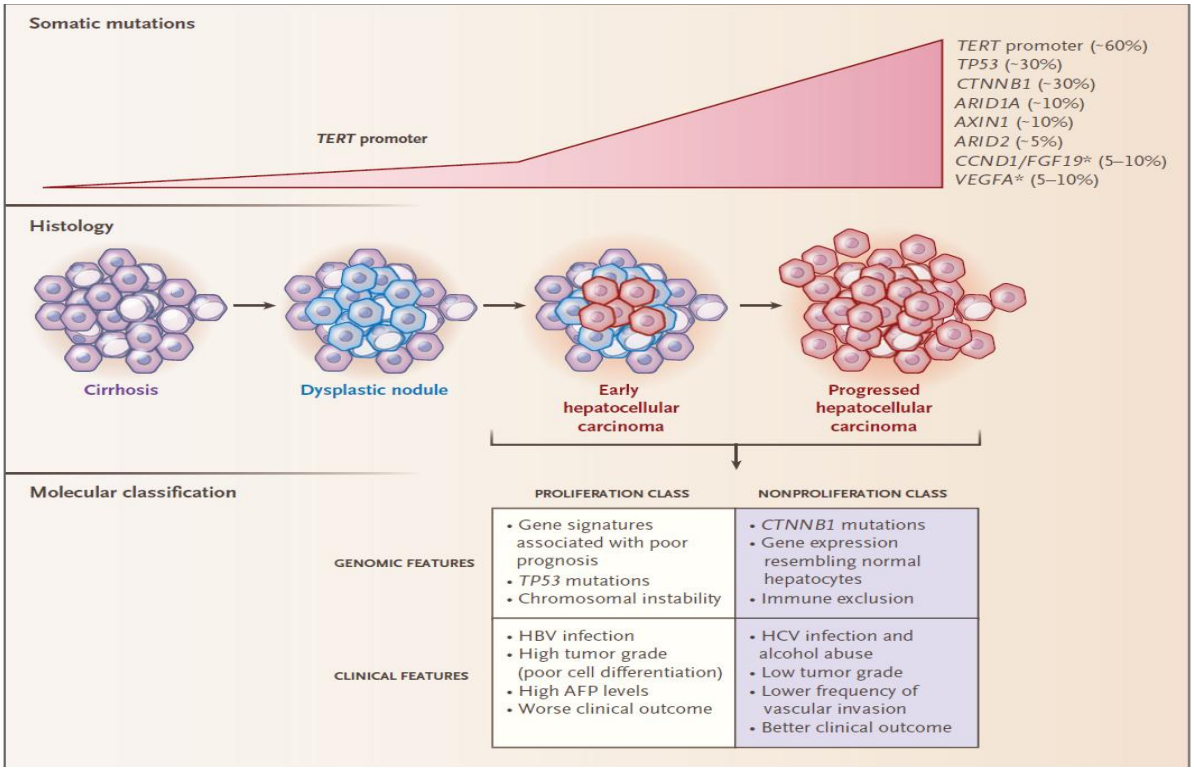

Figure 3: Genetic alteration in hepatocellular carcinoma

HCC is well associated with various metabolic changes including biochemical alterations. Alfa-fetoprotein (AFP) is a glycoprotein in serum that was first recognized as a major marker for HCC. Moreover, HCC development has also been associated with plasma lipid and lipoprotein alterations. These alterations result in cellular dysfunction, reduction in the membrane integrity, fluidity and regulation of cellular processes related to growth and cell survival causing cancer development.

Furthermore, the development and progression of HCC are well associated with the oxidative stress produced by increasing level of reactive oxygen species (ROS) resulting alteration and decrease the antioxidant activity in the tissues. Lipid peroxidation (LPO) is responsible for formation of many toxic products, such as 4-hydroxynonenal and malondialdehyde (MDA) which attack cellular targets, thereby inducing carcinogenicity. Many biochemical and molecular changes leads to free radical metabolites causing the chemical carcinogens induce oxidative stress leading to tumor promotion. The production of cytokines, ROS, and inflammation-mediated events leads to tumor formation. HCC has an anti-apoptotic genes expression and rapid cell proliferation. In HCC increased apoptosis by the down-regulation of the Bcl2 level, the activation of caspase cascade, and the up-regulation of Bax and the p53 level (16) which are shown in figure 3 (17).

Hypoxia, oxygen (O_2) deprivation, is frequently found in regions of HCC that are devoid of functional blood vessels. Hypoxia can also be induced during palliative HCC treatment such as transcatheter arterial (chemo) embolization (TAE/TACE) with the initial principle to restrict tumor growth through blockade of blood supply. In addition, rapidly proliferating HCC cells quickly consume O_2 therefore deplete O_2 in the tumor microenvironment of HCC. The key molecular mechanism by which cells adapt to hypoxia is through transcription factor, hypoxia inducible factors (HIF). Through hypoxia-inducible factor 1 (HIF-1) which elicits various molecular events, cells are able to overcome low O_2. HIF-1 allows hypoxic cells to survive oxidative stress by generating ATP through glycolysis (18).

The Raf/MEK/ERK pathway is one of the most critical signaling cascades for liver tumorigenesis. Although Raf-activating mutations are relatively rare events in HCC, Raf kinase is activated in a high percentage of HCC tumors. Raf/MEK/ERK pathway can be activated by HBV, HCV infection or mitogenic growth factors and its activation is associated with aggressive tumor behavior (19).

1.2 Classification of HCC

The most accepted clinical classification of HCC has been proposed by the Barcelona Clinic Liver Cancer (BCLC). The BCLC staging system has come to be widely accepted in clinical practice and is also being used for many clinical trials of new drugs to treat HCC.

This clinical classification does stratify patients with HCC into 5 different stages (stage 0 and stages A to D), according to the ECOG Performance Status (PST) and the Child Pugh Classification.

The 5-stage classification categorizes patients into very early HCC (stage 0), early HCC (stage A), intermediate HCC (stage B), advanced HCC (stage C) and end-stage HCC (stage D).

Stage 0 - In this "very early" stage there is a single nodule with size < 2 cm (or carcinoma in situ) without vascular invasion/satellites; portal pressure and bilirubin may be normal or increased.

Patients should undergo curative treatments, such as liver transplantation, or local ablation with percutaneous ethanol injection (PEI) or radiofrequency ablation (RFA).

Stage A – In this early-stage single HCC nodule > 2 cm but < 5 cm, or three nodules < 3 cm; ECOG 0; Child Pugh Class A or B; and (20) absence/presence of associated extra-hepatic diseases.

In the absence of associated diseases, the patients might be candidates to liver transplantation; otherwise, local ablation with PEI or RFA should be considered.

Stage B - Patients in the intermediate stage B show multinodular asymptomatic HCC without an invasive pattern. Liver function may be preserved (Child A), or early decompensation might be seen (Child B). Performance Status is = 0. These patients might receive a survival benefit from transarterial chemoembolization, while other treatments such PEI or RFA should be avoided.

Stage C - These subjects suffer from advanced HCC (N1, M1), that consists of macroscopic vascular invasion (portal vein invasion), extrahepatic spread (lymph nodes and metastasis) or cancer-related symptoms (performance status 1-2). They cannot receive treatments other than first line therapy with sorafenib.

Stage D - Patients with terminal stage (stage D) have cirrhosis (Child C) and PST > 2. Only supportive, symptomatic treatment can be offered.

Patients presenting with very early (stage 0) and early-stage diseases (stage A) represent 20%-30% of patients with HCC. This group, suitable for curative treatments such as resection, liver transplantation, or local ablation with PEI or RFA, have a 5-year survival of 50%-70%. By contrast, patients in intermediate stage B and more advanced stage C, who account for 50%-60% of patients, have a poorer prognosis, presenting a 3-year overall survival of 10%-40%. Finally, symptomatic subjects with end-stage disease (stage D; 10%-20%) have a survival < 3 months.

1.3 Diagnosis and disease marker of HCC

The serum tumour marker alfa-fetoprotein (AFP), radiographic imaging, and liver biopsy are standard diagnostic methods for HCC. For patients with liver cirrhosis, the "gold standard" for HCC screening is the level of AFP in conjunction with abdominal ultrasonography. (21).

Alfa-fetoprotein (AFP)

The human AFP is a 591 amino acid, 70 kD glycoprotein. HCC is regarded as diagnosable by an AFP level of 400–500 ng/ml. The blood marker AFP is frequently used in conjunction with ultrasonography to monitor those who are at high risk for developing HCC (21). At the time of diagnosis, AFP has been demonstrated to correlate with tumour volume and size. According to a study from Thailand, HCC patients with an AFP level of 400 ng/ml or more frequently have larger tumours, bilobar involvement, portal vein thrombosis, and shorter survival times (22-23).

AFP has three major isoforms depends on their affinity for the lectin Lens culinaris agglutinin (AFP-L1, AFP-L2 and AFP-L3. Serum level of AFP, AFP-L3 and Des-gamma-carboxy prothrombin (DCP) are used as parameters to refine the staging system of HCC (24). Serum AFP used alone can be helpful if levels are markedly elevated, which occurs in fewer than half of cases at time of diagnosis. Confirmation by liver biopsy can be performed under circumstances when the diagnosis of HCC remains unclear. Ultrasound imaging is commonly applied in addition to, or in place of, AFP to help detect small hepatic tumors >3 cm.

Des-gamma-carboxy prothrombin (DCP)

Des-gamma-carboxy prothrombin also called PIVKA II (protein induced by vitamin K absence), is a widely used tumor marker in Japan that was as an abnormal form of prothrombin highly specific for HCC

Dickkopf-1 (DKK1)

It is a member of secreted proteins family that play important role in HCC progression by promoting cytoplasmic/nuclear accumulation of beta-catenin in HCC cells via the Wnt/beta-catenin signaling pathway (25).

Golgi protein 73 (GP73)

GP73 is a transmembrane glycoprotein with a molecular weight of 73 kDa. The expression of this protein is significantly increased in liver diseases such as HCC. GP73 is more sensitive and specific for HCC than AFP (25).

Glypican-3 (GPC3)

Significant up regulation of GPC3 mRNA was observed in HCC. It can be detected in 40–53% of HCC patients and 33% of HCC patients seronegative for both AFP and Des-gamma carboxy prothrombin (DCP).

Serum Alpha-1-Fucosidase (AFU)

AFU is a liposomal enzyme that hydrolyzes fucose glycosidic linkages of glycoprotein and glycolipids. It is found in all mammalian cells and its activity increases in the serum of HCC patients (25).

Angiography

Angiography has been used as a diagnostic tool for HCC because of its highly vascular nature; however, the detection of tumors has been disappointing, particularly when less than 2 cm in diameter. At present angiography is more often used to define hepatic anatomy before resection or as guidance for transarterial chemoembolization therapy (25).

Liver biopsy

Liver biopsy offers a safe and effective means to confirm suspicious lesions for HCC. Cytologic and histological samples can be obtained by percutaneous fine-needle aspiration (FNA) and needle core biopsy, respectively, under US or CT guidance. The diagnostic accuracy of liver biopsy is

greater when both FNA and core biopsy techniques are used simultaneously than when either is used alone. The sensitivity and specificity are superior to any other diagnostic test (26).

The diagnosis of HCC poses many challenges which can vary among different regions and centers. AFP and US imaging are most often used every 6 months for surveillance purposes in high-risk individuals.

1.4 HepG2 cell line

HepG2 is human liver carcinoma cells, derived from the liver tissue of a 15-year-old Caucasian male who had a well-differentiated hepatocellular carcinoma. HepG2 is the most widely used human hepatoma cell line in pharmaco-toxicological research. The HepG2 cell line is commonly used in drug metabolism and hepatoxicity studies. HepG2 cells exhibit an epithelial-like morphology with a modal chormosome number of 55 (27).

1.5 Treatment for HCC

Treatment options for HCC are as follows-

1. Multi Kinase Inhibitors (MKI) like sorafenib, regorafenib, lenvatinib, cabozantinib.
2. Vascular endothelial growth factor (VEGF) receptor antibody- bevacizumab
3. Programmed death receptor-1 (PD-1) inhibitor- nivolumab, pembrolizumab, atezolizumab.

According to American Gastroenterological Association (AGA) following are the first line treatment option for HCC. Oral systemic medication include sorafenib (first drug approved by USFDA for HCC) and lenvatinib for the treatment of advanced HCC. In addition to this combination of an anti-angiogenic agent bevacizumab with a checkpoint inhibitor atezolizumab has a small to moderate survival benefit over sorafenib.

For second line treatment oral multi-kinase inhibitors cabozantinib and regorafenib, the IV monoclonal antibody ramucirumab (anti–vascular endothelial growth factor) and the checkpoint inhibitor pembrolizumab (anti-PD1) have recommended for HCC (12).

2. Rationale

Hepatocellular carcinoma is different from many other cancers, in that there is no curative treatment for intermediate- or advanced-stage tumors. Other cancers that have progressed to more advanced stages may respond to adjuvant chemotherapy or radiation. In contrast, for HCC, **neither chemotherapy nor radiation for late-stage disease will reduce mortality**. However, there are effective treatments for early-stage disease. Resection, transplantation, and local ablation of small lesions are potentially curative therapies which reduced mortality. Although, on a population basis,

it remains to be demonstrated that these treatments will reduce mortality, it is hard to imagine that a 90% cure rate, such as is achievable with radiofrequency ablation (RFA) of lesions <2 cm in diameter, 30% long-term cure rate with resection and a 70%-80% cure rate with transplantation which will not translate into a decrease in overall HCC-related mortality, compared to an unscreened group (18).

Anticancer therapy is not without risks and limitations. In HCC first line drug sorafenib is associated with rash, hand-foot syndrome, diarrhea. In addition, other side effects include nausea, vomiting, tiredness, fatigue, hair loss, premature menopause, lowered resistance to infection, bone marrow suppression, neurotoxicity, cardiac dysfunction, decrease in libido, hot flushes, endometrial hyperplasia, and endometrial cancer. Its overall survival in patient is 3-4 month.

As the existing therapy associated with side effects and drawbacks, it becomes the need of an hour to search new cancer chemotherapeutic agents that are effective and non-toxic.
Natural agents are safe and could overcome the resistance produced by the pathogens due to the presence of multiple phytoconstituents that are having different mechanism of action.

Dietary phytochemicals such as curcumin, resveratrol, quercetin, silibinin, N-trans-feruloyl octopamine, lycopene, emodin, caffeine, urolithin A and Phloretin have been found to be useful for the treatment of HCC and other diseases. (27) Recent studies have shown that medicinal herbs and natural agents rich in antioxidants and other safety micronutrients protect against hepatic dysfunction, carcinogenesis, mutagenesis, DNA-damage and LPO. The greatly positive effect of natural antioxidants on membrane stabilizing by mechanisms that include up-regulation of the key apoptotic regulators, modulation of cell cycle arrest and improvement of DNA content by the free radical scavenging, the antimutagenic and antioxidant properties. Thus, it was recommended that the supplementation with edible natural agents may help in safe application of cancer technology in medicine as well as in many other aspects of life (16).
To improve treatment effects, the administration of combination therapies that include synergistic agents such as novel systemic molecular-targeted drugs or traditional herbal medicines has become a new therapeutic approach. The search continues for appropriate drugs that work synergistically with sorafenib to improve its efficacy as well as increase patient survival. An Italian multicenter survey in 2017 published by Berretta et al. concluded that nearly 48.9% of cancer patients ever accepted complementary and alternative medicine. Kristoffersen et al. reported that approximately 33.4% of the surveyed Norwegian cancer patients once accepted traditional and complementary medicine. All this data from the literature encourages the use of natural products as a adjuvant product along with the synthetic compound (33).

Achyrathes aspera (AA) Linn. family; Amaranthaceae is known by different names such as, Chirchita, Apamarga in Hindi. All parts of AA are used in traditional system of medicines such as seeds, roots, and shoots. The tribal, rural and aboriginal people of our country are using this herb from the ancient time in various disorders.

The dried plant is used in abdomen diseases, hemorrhoids, itching, and obesity. The dried root of the plant is used in vomiting, adhmana (tympanites), itching, lymphadenitis, tumor, bhagandara (fistula-in-ano), heart disease, pyrexia, leukoderma, deafness, abdomen diseases, disorders of the liver (28), tooth disease and blood disorders.

AA is used for the management of various diseases such as malaria, dysentery, sinuses, asthma, piles, night blindness, hypertension, and diabetes. The leaf extracts of AA have shown antioxidant, diuretic, antidepressant, hepatoprotective, wound healing, and cancer chemo preventive effects. Other than leaves, roots of AA possess anti-inflammatory and immunomodulatory effects (29).

Few triterpenoid saponins like oleanolic acid have been isolated from the plant. It also contains a water-soluble base, betaine. Various activities such as effect on urinary tract, antibacterial, antifungal, antidiabetic, spasmolytic, antiasthmatic, antiallergic, diuretic and many more are reported in the literature. *Achyrathes aspera* plant is used as anticancer therapy from ancient time by ayurvedic medical practitioners in India. (34).

The species has cooling, pungent, mild astringent, antiperiodic, digestive, purgative, laxative, abortifacient properties. The paste of the root is given to stop bleeding after abortion and to facilitate delivery and stimulate labor pain. The decoction of the leaves is used in early stage of diarrhea and dysentery. The paste of the leaves is externally applied over bites of poisonous insect, wasp, bees, burns and nephrotoxicity. The seeds are used as an emetic, expectorant, brain tonic and are effective in biliousness and bleeding piles. The ash of the plant is said to be effective in cough, chest pain and acidity. It is also observed to be effective in abdominal tumor. (30).

Different marketed formulations like Apamarga Churna, Livol, stone hills etc. containing *A. aspe*ra as a important ingredient which are mainly used for hepatitis, cirrhosis of liver, diuretic and antibacterial. In the xenograft experiment done by Subbarayan et.al. shown *A. aspera* leaf extract has potent activity in pancreatic cancer. A study conducted with root extract has shown good anticancer activity in colon cancer (29) Methanolic extract showed pronounced anticancer activity in skin cancer. The plant extracts showed good anticancer activity in-invitro cells lines- Raji cells. Pharmacological studies have demonstrated that whole-plant possess anti-asthmatic, anticancer, anti-diabetic, antioxidant, nephroprotective, and wound healing properties. Singh et. al. 2021, has studied *A. aspera* methanolic leaf extract in mice which inhibited the proliferation of Dalton's

Lymphoma cells by inducing apoptosis. Cell progression inhibited by a marked reduction in expression of p-PKCα, p-AKT, p-GSK3β, Bcl2 and upregulation in Bax.

Triterpenoid saponins like oleanolic acid have been isolated from the plant. The plant contains different phenolic compounds which are effective in various cancers and having diverse protective pharmacological actions. Important phytoconstituents and their network pharmacology are shown in figure 4

Boerhaavia diffusa **(BD)** Linn. family Nyctaginaceae commonly known as 'Punarnava'. It is a traditional herb (creeping weed) which has been proposed for the treatment of cancer, tumors, jaundice and liver disorders. The roots and the whole plant are used as an Ayurvedic medicine in India and Unani medicine in Arab countries for the treatment of diabetes, jaundice and heart failure (31).

The plant is reported to possess anti-inflammatory, hepatoprotective and diuretic activity. The root is generally used as an infusion to treat internal inflammation. The whole plant extract is hepatoprotective in nature. It is also used for the treatment of diabetes and to treat seminal weakness and high blood pressure.

The plant *B. diffusa* containing phenolic compounds, in particular alkaloids and amino acids have been reported to exhibit strong antioxidant properties. It also contains quinolizidine alkaloids and potassium salts. This plant is also used for liver disorders, asthma, skin diseases, snake bites, inflammation and heart diseases.

In India, a number of tribes use the roots of *Boerhaavia diffusa* to treat liver ailments. The tribal population in South Garhwal used the roots in the treatment of liver enlargement. The roots of 'Punarnava' were given 'in the treatment of jaundice' by the tribal, people of eastern Rajasthan and Gujarat. In the Sagar District of Madhya Pradesh, the roots are also prescribed for liver disorders and kidney ailments (35). Punarnavadyarishta and Punarnavasava are the ayruvedic marketed formulations mainly used for chronic obstructive and advanced stage of jaundice and various liver disorders. The roots of the plant are used in poly-herbal formulae and are mainly given for gastric and liver cancers in Sri Lanka (36). Whole plant is used in cervical cancer (37). Ethanolic extract is used in human cervical cancer cell. Methanolic leaf extract is effective against MCF-7 cell line. Ethanolic extract of leaves has been evaluated against Dalton's ascitic lymphoma (DAL) (38).

Roots of the plant were found to have antiproliferative effects on Hela cancer cells acting through apoptosis pathway by triggering caspase 3/9 (39). Methanol extract of the whole plant demonstrated anti-proliferative effects in MCF-7 cells with an arrest in gap1 phase in the cell cycle; indicating

potential anti-oestrogenic activity of the plant against human breast cancer cells. Administration of an aqueous methanol (3:7) extract of the whole plant was found to be effective in hindering the formation of B16F10 melanoma induced lung metastases in mice by inhibiting the expression of matrix metalloproteinases 2/9 which are associated with cell invasion and angiogenesis. The treated mice had showed much lower lung collagen hydroxyproline content indicating a reduced fibrosis and a smooth alveolar function. A reduction in the number of lung tumour nodules that are metastatic colonies of melanoma, correlated with the findings. This plant is also used for liver disorders, asthma, skin diseases, snake bites, inflammation and heart diseases.

Different phytoconstituent from flavonoid, phenolic, rotenoids are found in the plant having anticancer activity by targeting different pathways like apoptosis, PI3K-Akt, and antioxidant activity which is shown in network digram (figure 5) of *B.diffusa*.

Enicostemma littorale **(EL)** family- Gentianaceae also called as Chota chirayata in Hindi, Mamejovo in Gujarati, and Vellarugu in Tamil. It has been used traditionally for many diseases. The plant found throughout India, is traditionally used in the treatment of rheumatism, ulcer, hypoglycemia, and insect poisoning. It is found to be active against hyperglycemia, inflammation, and tumor.

The plant is used in folk medicine for the treatment of diabetes mellitus, rheumatism, peptic ulcers, hernia, swelling, itching and insect poisoning. It inhibited carrageen-induced edema and its anti-inflammatory activity is comparable to that of hydrocortisone. Ethnomedical studies of North Gujarat revealed the use of hot aqueous extract of *E. littorale* by the tribal inhabitants for the treatment of diabetes, fever, stomach pain, dyspepsia and malaria. The root extract showed antimalarial activity both *in vitro* and *in vivo*. This herb is also known for its hypoglycemic, antioxidant and hypolipidemic potential in newly diagnosed non-insulin dependent diabetes mellitus (NIDDM) patients (32).

Enicostemma littorale modulate effect on the expression pattern of apoptotic, cell proliferative, inflammatory and angiogenic markers during 7, 12-dimethylbenz (a) anthracene induced hamster buccal pouch carcinogenesis. (40). Alcohol extract of whole plant are hepatoprotective in CCl_4-induced hepatic damage in rats. Anticancer activity of this plant has been evaluated for Dalton's ascetic lymphoma. Swertiamarin is an important constituent of the extract which exerts anticancer activity on human cervical cancer by targeting MEK-ERK pathway. Swertiamarin has antioxidant and hepatoprotective activity against D-GalN induced hepatotoxicity in rats. Methanolic extract of E. littorale was effective in Ehrlich's Ascitic Carcinoma (EAC) in mice which increased the mean survival time from 18.5 to 31 days.

All the three plants or their important active phytoconstituents act on different cancer pathways. *A. aspera* and *B. diffusa* are shown in figure 4 and 5 respectively. Standard treatment also targets some of these pathways which encourage to think the plants use in HCC. AA reduced the expression of p-AKT, Bcl2 and upregulated the Bax. Swertiamarin is an important constituent of the EL extract which exerts anticancer activity on human cervical cancer by targeting MEK-ERK pathway. The roots of BD are used in polyherbal formulae for different cancers (36).

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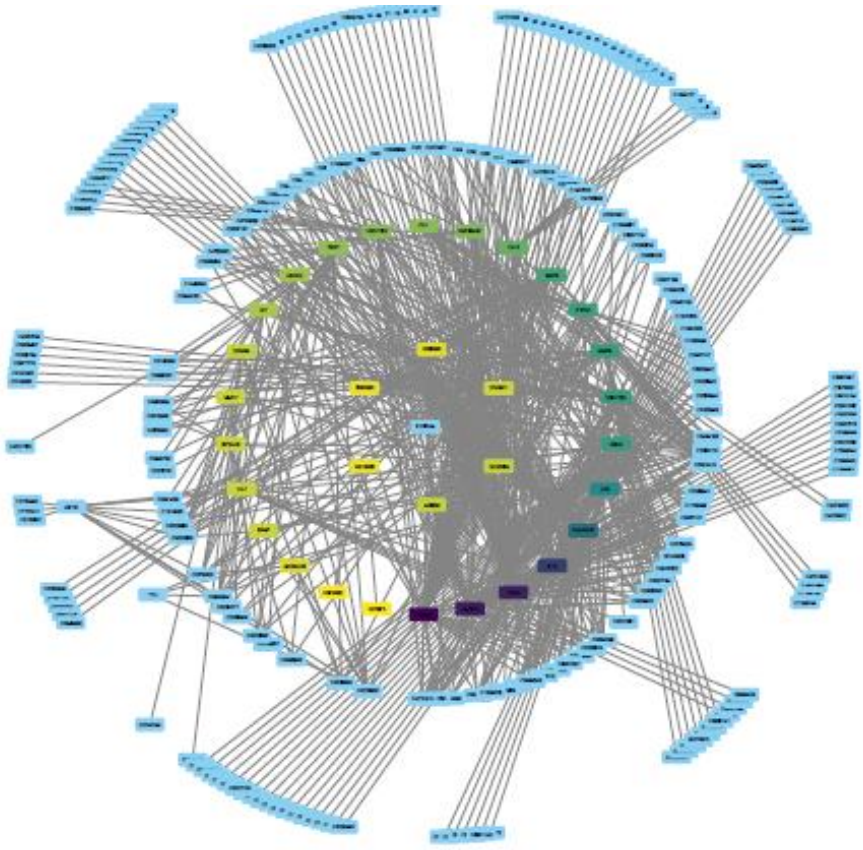


Figure 4: Network pharmacology diagram for *B. diffusa*

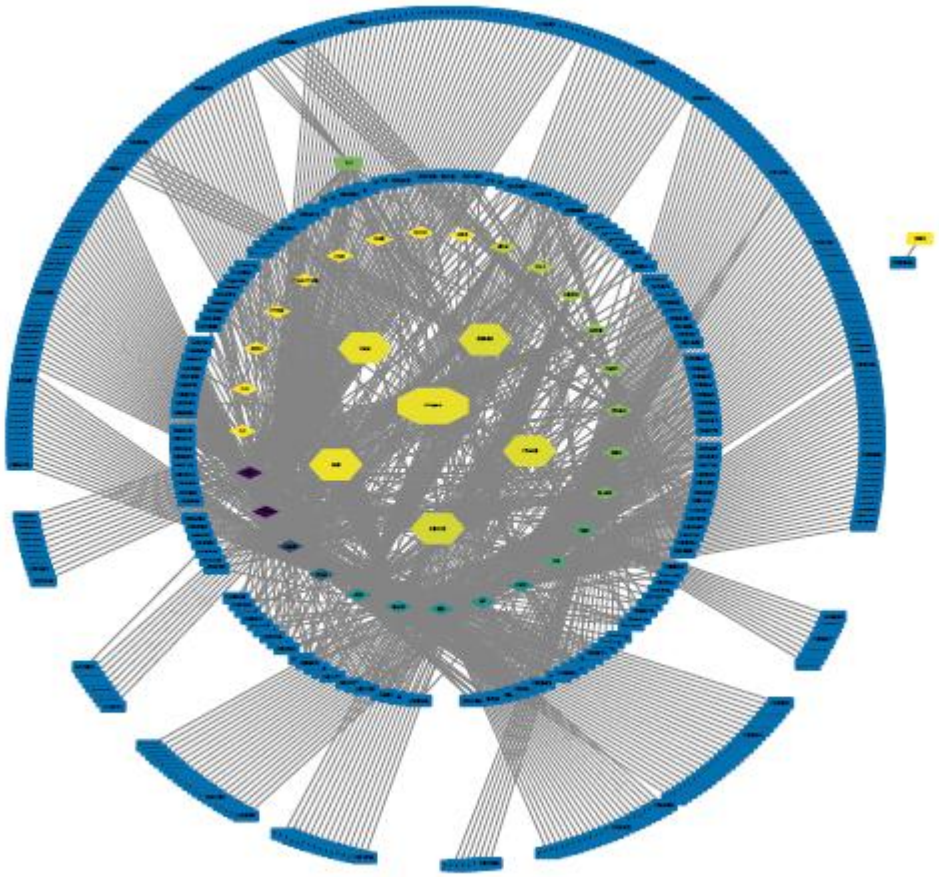


Figure 5: Network pharmacology diagram for *A. aspera*

3. Aim/ Objectives:

Title: Pharmacological Evaluation of Some Medicinal Plants in Hepatocellular Carcinoma

Objectives of the planned study are as follows-

1. Extraction of plant material and preliminary phytochemical screening
2. To evaluate the cytotoxic potential of various extracts (aqueous, alcoholic, hydroalcoholic, ethyl acetate and petroleum ether) of *A. aspera*, *B. diffusa* and *E. littorale* plants on HepG2 cells
3. To evaluate the effect of alcoholic extracts of *A. aspera* and *B. diffusa* on HepG2 cell migration
4. To analyze the chemical components of *A. aspera* and *B. diffusa* extract by LC-MS method
5. Development and standardization of different animal models of HCC
6. To evaluate activity of plant extracts by in-vivo xenograft model
7. To evaluate efficacy of plant extracts in combination with reference standard
8. To assess biomarker for the HCC by immunohistochemistry and western blot method
9. To evaluate safety of the plant extracts

4. Material and methods

4.1 Reagents

Diethylnitrosamine (DEN), 2-Acetylaminofluorene (2-AAF), (3-4, 5- dimethylthiazol-2, 5 biphenyl tetrazolium bromide) propidium iodide (MTT), 3, 3′-Diaminobenzidine (DAB) tablet, paraformaldehyde (PFA) and citrate buffer were procured from Sigma Aldrich.

Dimethyl sulphoxide (DMSO), Eagle's minimum essential medium (EMEM), fetal bovine serum (FBS), penicillin streptomycin antibiotic solution, trypsin- EDTA, HEPES buffer, haematoxylin, eosin was purchased from Himedia.

Tris-buffered saline (TBS), Tween 20, ECL, RIPA buffer, BCA kit, protease/phosphatase inhibitor, bovine serum albumin (BSA) was obtained from Bio-Rad. Carbon tetrachloride (CCl_4), xylene, 2-propanol, Tween 80 were obtained from SDFCL. Matrigel was purchased from Corning India while ABC kit was purchased from Vector laboratory. Hydrogen peroxide (H_2O_2), Triton X 100, methanol, hydrochloric acid (Hcl), sodium azide, magnesium chloride, and sodium hydroxide were purchased from Merck, India.

Antibody against Ki67 was obtained from Abcam, while antibody against CD31, ERK, p-ERK, ß-actin, and c-caspase were obtained from Cell Signaling Technology.

Sorafenib and doxorubicin were received from Sun Pharma Industries Limited. Sorafenib tablet (Soranib™) was purchased from Maheshwari medical agency, Ahmedabad.

All other reagents used for the experiment were of analytical grade.

4.2 Collection of plant materials

Plant materials *A. Aspera* (roots), *B. Diffusa* (roots) and *E. littorale* (whole plant) was collected from Dr Hitarthini N. Chudasama (Vinayak Ayurved Clinic, Waghodia road, Vadodara) for preparation of different extracts.

4.3 Herbarium preparation and plant identification from botanist

Herbarium of all the three plants was prepared and submitted to BARO herbarium in charge, Department of Botany, Faculty of Science, The Mahraja Sayajirao University of Baroda for identification purpose. Plant was authenticated by Dr P Nagar, Associate Professor, Department of Botany, Faculty of Science, The MS University of Baroda with specimen no. K000357272 (*A. aspera*) K000438312 (*E. littorale*) and K001138105 (*B. diffusa*).

4.4 Extraction of plant material

The plant material was shade dried, coarsely powdered and defatted with petroleum ether for the preparation of different extracts. Coarse powder (50 gm) of plant material was packed in Soxhlet apparatus and extracted; the extraction was continued until the colour of the solvent in the siphon tube became colorless. All the three plants were extracted with different solvents viz. petroleum ether, ethyl acetate, alcohol and distilled water separately using a Soxhlet apparatus. All the extracts were concentrated at 40˚C under reduced pressure at 40°C with rotatory evaporator. Hydroalcoholic (70:30) extracts of all the three-plants were purchased from Amine Biotech Ltd. Vadodara. Alcoholic extracts of all the three-plants were procured from Amsar Ltd. Indore

4.5 Phytochemical screening

All the plant extracts were assessed for the existence of the phytochemical analysis by using the standard laboratory methods, to detect the presence of different secondary metabolites such as alkaloids, flavonoids, saponins, tannins, steroid glycosides, phenols, reducing sugars, protein, anthraquinones, quinones, fat and fixed oil. The solution of extract was prepared and assessed for various phytochemical tests (41-42).

4.6 Phytochemical analysis by LC-MS

Mass spectrometry was conducted at high-resolution mass spectrometer Waters XEVO-TQD #QCA1301. Powder of 1 mg from extract was sonicated for 45 minutes with methanol, and then centrifugated at 13 000 rpm for 10 minutes. An aliquot of the supernatant was filtered through 0.22 μm filter membrane. The mobile phase consisted of phase A (5mM Ammonium Acetate) and phase B (Acetonitrile). The flow rate of mobile phase was 1.5 mL/min, and the gradient elution program was set as follows: 0 to 1 minutes, 95% B; 1 to 10 minutes, 70% B; 10 to 16 minutes, 40% B; 16 to 32 minutes, 20% B; 32 to 40 minutes, 90% B. The injection volume was 2 μl. The temperatures of the column oven and auto-sampler were maintained at 35°C and room temperature, respectively (43).

4.7 Cell culture:

The hepatic liver cancer cell line, HepG2 was cultured in EMEM medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/mL), and penicillin (100 U/mL). All cells were fostered at 37°C in a humidified atmosphere containing 5% CO2. The cell line was obtained from *In-vitro* Biology Department, Sun Pharma Advanced Research Company Ltd (SPARCL). MTT assay was deciding factor for further screening of the extract as shown in the figure 6.

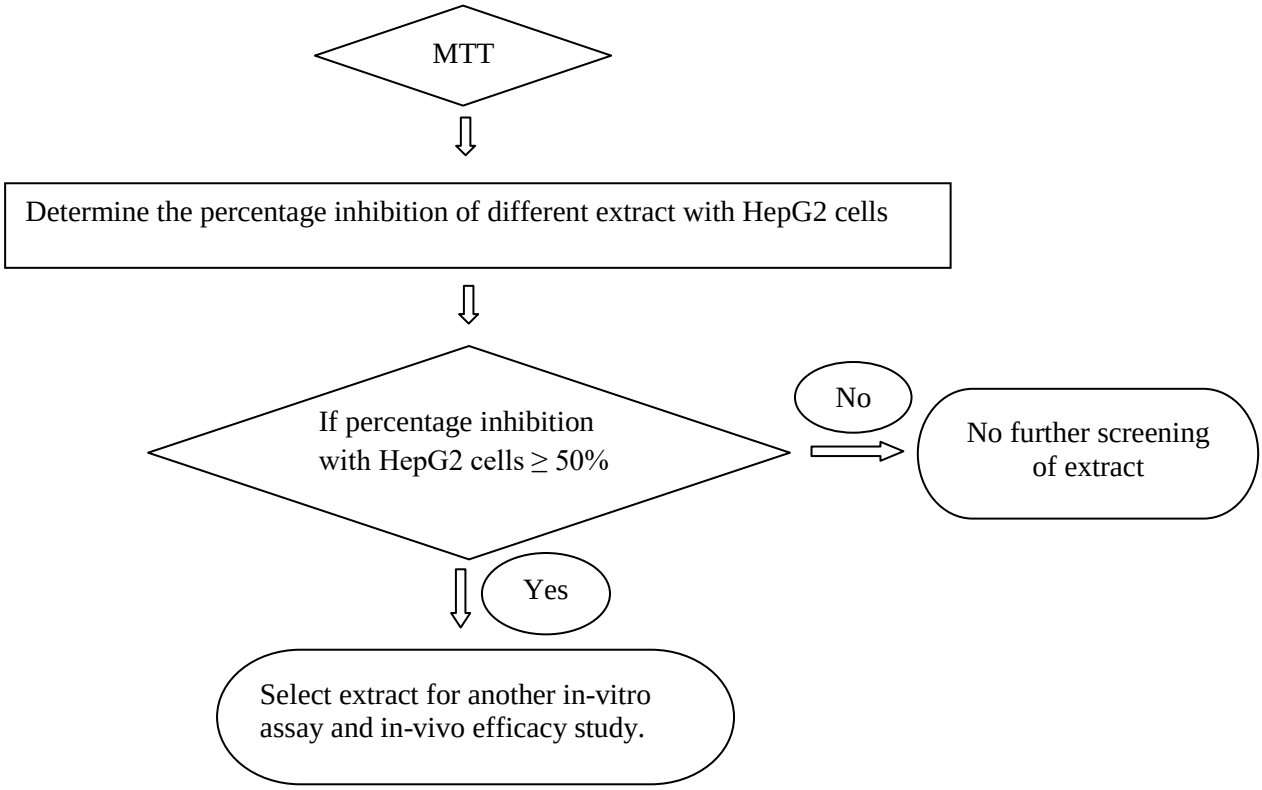


Figure 6: Flow chart for selection of extract for *in-vitro* (cell migration) and *in-vivo* (animal model) assay based on MTT assay.

4.8 *In-vitro* Assay

4.8.1 MTT Cell viability assay

Principle:

Cell viability is determined by measuring cellular metabolic activity using the MTT test. This colorimetric assay relies on the transformation of purple formazan crystals into a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) by metabolically active cells as shown in the figure. The MTT is converted to formazan by the NAD(P)H-dependent oxidoreductase enzymes found in the live cells as shown in figure 7 (44). The insoluble formazan crystals are dissolved using a solubilization solution and the resulting-colored solution is quantified by measuring absorbance at 570 nanometers using a spectrophotometer. The darker the solution, the greater the number of viable, metabolically active cells.

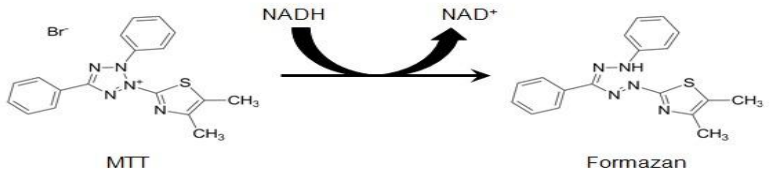


Figure 7: Mechanism for conversion of MTT to formazon

Procedure:

MTT Cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazo-lium bromide (MTT) assay. This colorimetric assay measures the reduction of yellow tetrazolium salt MTT to an insoluble, coloured (dark purple) formazan product by mitochondrial succinate dehydrogenase. Briefly, 5×10^3 of HepG2 cells were seeded into 96-well plates for 24 h, followed by incubation with various doses of extract and reference standard for indicated time. After adding 0.5 mg/ml/well of MTT solution, the cells were incubated for another 4 h. Then the supernatant was removed, and the formazan crystals were dissolved in 100 µL/well DMSO. The absorbance at 570 nm of each sample was measured using multilabel plate reader. The proportion of viability was calculated from equation.

Percentage cell viability = [1-(ODt/ODc) x 100%]

Where ODt is the optical density of wells treated with the investigated sample and ODc is the mean optical density of untreated cells. Triplicate wells were evaluated for each investigation. The concentration essential to cause fatal effects in 50% of intact cells (IC_{50}) was assessed from graph plots of the dose response curve for each concentration using Graph Pad Prism software (29,45).

4.8.2 Assay of cell motility (Scratch Motility Assay):

Principle:

The scratch-wound assay measures the basic cell migration parameters such as speed, persistence, and polarity. Cells are grown to confluence and a thin "wound" introduced by scratching with a pipette tip. Cells at the wound edge polarize and migrate into the wound space.

Procedure:

To confirm the migration capability of the HepG2-treated cells, the scratch motility assay was carried out. HepG2 cells (3.0×10^5 cells/well) were seeded in a 12-well plate and grown till confluent. The cell monolayer was scratched with a sterile pipette tip vertically and washed thrice with PBS and incubated with media containing extracts (Concentration: IC_{50} obtained in MTT assay on HepG2 cell line at 48 hours). The cells in the denuded area were photographed with camera attached to inverted microscope and counted. The experiment was performed in triplicates (29, 46). The area covered by the cells was calculated by Image J software.

The percentage wound closure was determined by following formula

Wound closure (%) = ((W0-Wt)/W0)x 100

Where, W0 = Wound area at 0 h, Wt = Wound area after completion of incubation

4.9 IAEC approval

The experimental protocol for conducting animal studies was approved by the Institutional Ethical Committee of Sun Pharma Advanced Research Committee Ltd., IAEC No. IAEC/908. All experimental procedures were conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests and Climate Change, Government of India. Male and Female athymic nude mice (18-24 g), age of 6-8 weeks was used in the xenograft study. In each cage 2-3 mice were housed after tumor induction. Male Sprague-Dawley rats weighing 180 to 200 g were housed in ventilated plastic cages. The animals were acclimatized for one week before grouping and beginning of the study. Cages were maintained under constant temperature (18-26°C), humidity (30%-70%) and lighting conditions (12 h light and 12 h dark). Animals received reverse osmosis (RO) water and Harlan rodent diet standard normal chow diet *ad libitum*.

4.10 Evaluation of the extract in in-vivo animal model

4.10.1 Evaluation of the extracts for safety by acute toxicity study

Acute toxicity test was performed as per OECD guideline 423 (47). All experiments and protocols described in the study were approved by the Institutional Animal Ethical Committee (IAEC) of Sun Pharma Advanced Research Company Limited. The study was performed using healthy young adult female rats, nulliparous, non-pregnant, and weighing 200-250 g. Female rats were selected because literature surveys of conventional LD_{50} tests show that usually there is little difference in sensitivity between sexes, but in those cases where differences are observed, females are generally slightly more sensitive. The animals were randomly selected and kept in their cages for 1 week before dosing to allow for acclimatization to the laboratory conditions. The animals were housed individually in clean polypropylene cages. Room temperature and humidity were maintained at 22°C (±3°C) and 55-65%, respectively. Artificial lighting was provided, the sequence being 12 hrs light, 12 hrs dark.

The animals were fed with commercially available standard pellet chow and unlimited supply of filtered drinking water.

Procedure for limit test

Animals were fasted overnight and alcoholic extracts of *B. diffusa*, *A. aspera* and *E. littorale* was orally administered in a single dose. The dose volume of extracts given was 2 ml/kg body weight. Following the period of fasting, the fasted body weight of each animal was determined, and the dose was calculated according to the body weight. Extracts were administered orally at a dose of 2000 mg/kg and after administration food was withheld for further 3-4 hrs. If no mortality was

observed in the animals, then the extract was administered in another set of three animals to confirm the same. If test substance-related mortality is produced, further testing at the next lower level may need to be carried out.

4.10.2 Development and Standardization of Animal Model for HCC

4.10.2.1 Chemically induced HCC in rats

a) Diethyl nitrosamine (DEN) and 2- acetyl amino furorene (2-AAF) induced HCC in rats

Male Wistar rats (200–250 g) were used for the experiment. The animals were divided randomly on the basis of body weight into two groups, n=6 animals in each group. The first group (control) was vehicle treated group.

The second group was given a single intraperitoneal injection of N-nitrosodiethylamine (DEN) at a dose of 200 mg/kg, i.p. After 2 week of DEN administration 2- acetyl amino furorene (2AAF) was given at 30 mg/kg, p.o. daily for 2 week. 2 AAF formulation was prepared in 0.5% carboxy methyl cellulose (CMC). Required quantity of 2-AAF was weighed and transferred to mortar then it was triturated with 0.5% CMC with the help of pestle. The 2-AAF was administered at 2 mL/kg dose volume (48-49).

The baseline rat body weight was measured on day one before DEN administration and every other week throughout the experimental period for all rats in each group. The weight gain was calculated and expressed as a percentage with following formula:

% weight gain = (Final weight - Initial weight)/Initial weight x100

Twenty-four hour of the last dose of 2- AAF, animal's blood was collected from the retro-orbital plexus under the isoflurane anesthesia and all animals were euthanized by a slow CO_2 inhalation. Blood was kept at room temperature for 30 minutes and then centrifuged at 3000 rpm at 24°C for 10 minutes; serum was separated and kept in -70°C freeze until analysis. The liver and spleen was quickly isolated, washed with saline, blotted dry on filter paper, and weighed. Percentage change in body weight as compared to day 1, liver and spleen index was calculated.

Rat liver and spleen index were calculated as follows:
Liver index = liver wet weight (g) / body weight (g) × 100%;
Spleen index = spleen wet weight (g) / body weight (g) × 100%.

Serum biochemical analysis

Serum was analysed for serum aspartate transaminase (AST), serum alanine transaminase (ALT), alkaline phosphatase (ALP), creatinine, and total bilirubin by Randox Daytona chemistry analyzer.

Histopathology of liver

At the end of the study, liver from each rat was be excised after dissection. A small piece from liver of each rat of all groups was fixed in 10% neutral buffered formalin for 24 h. Then, all specimens were cleared and embedded in paraffin and then transferred to 70% alcohol. A paraffin block of liver tissues were prepared and 5 micron sections taken on slides for staining. The obtained tissue sections were stained by hematoxylin and eosin (H&E) stain. The examination of section was performed under the microscope for the presence of cancerous cells and to confirm the development of the cancer.

b) Diethyl nitrosamine (DEN) and CCl_4 induced HCC

Male Wistar rats (200-250 g) were used for the experiment. The animals were divided randomly into two groups, 6 animals in each group. The first group (control) received vehicles used for preparation of DEN. The second group was injected with DEN (200 mg/kg i.p.) to initiate hepatocarcinogenesis. Two week later animals in group 2 were given a single dose of CCl_4 (2 mL/kg i.g) by gavage as 1:1 dilution in corn oil to stimulate liver cells proliferation and regeneration according to previously published protocols (50). In group 3 two more dose of CCl_4 (2 mL/kg i.g) was given at the interval of one week each of post 1^{st} CCl_4 administration (51-52).

Blood samples were taken by retoro-orbital plexuses periodically on day 14 (2^{nd} week post DEN injection) day 28 and day 35 under isoflurane anesthesia into non-heparinized eppendorf tubes. Blood was kept at room temperature for 30 minute and then centrifuged at 3000 rpm at 24°C for 10 minute; serum was separated and kept in -70°C freeze until analysis. At the end of treatment protocol, after 5 weeks Animals were euthanized by a slow CO_2 inhalation and the liver and spleen was quickly isolated, washed with saline, blotted dry on filter paper, and weighed. Percentage change in body weight as compared to day 1, liver and spleen index was calculated.

Serum biochemical and tumour markers estimation

Serum was analysed for serum aspartate transaminase (AST), serum alanine transaminase (ALT), alkaline phosphatase (ALP), total protein, total bilirubin, and creatinine by Randox Daytona chemistry analyzer.

Histopathology of liver

At the end of the study, liver from each rat was excised after dissection. A small piece from liver of each rat of all groups was fixed in 10% neutral buffered formalin for 24 h. Then, all specimens were cleared and embedded in paraffin and then transferred to 70% alcohol. A paraffin block of liver tissues were prepared and 5 micron sections taken on slides for staining. The obtained tissue sections were stained by hematoxylin and eosin (H&E) stain. The examination of section was performed under the microscope for the presence of cancerous cells and to confirm the development of the cancer.

4.10.2.2 Xenograft tumor models in nude mice

The hepatic liver cancer cell line, $HepG_2$ was cultured in EMEM medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/mL), and penicillin (100 U/mL). All cells were fostered at 37°C in a humidified atmosphere containing 5% CO_2. The cell line was obtained from In-vitro Biology Department, SPARCL.

Human hepatocellular carcinoma HepG2 cells were inoculated into immunodeficient nude mice to establish the animal model of HCC.

Male and female nude mice, 6-8 week-old, were supplied by Laboratory Animal Resources department SPARCL These animals were housed under controlled conditions (temperature 22 ± 2◦C, relative humidity 50 ± 10%) with a natural light-dark cycle and acclimatized for at least one week before the experiment was carried out.

a) Standardization of xenograft model with different cell concentration

The HepG2 cell at concentration of 5×10^7 cells/mL was collected in sterile falcon tube. Cell suspension was further diluted with media to get final cell concentration of 1×10^7 and 5×10^6 cells/mL. Each concentration of cells was mixed at 1:1 volume with ice cold BD-Matrigel (Corning, India) and injected subcutaneously with a volume of 0.2 ml on the right rear flank of nude mouse. So the final inoculated cell concentration was 5, 1, and 0.5 million cells per mouse (53-55).

b) Standardization with male vs. female mice species for xenograft model development

To compare the gender difference in growth pattern of the cells, HepG2 cells were inoculated with same concentration 5, 1, and 0.5 million cells per mouse in both male and female species.

Tumor volume measurement

Once tumor were palpable, the long diameter (a), short diameter (b), of tumor was measured in mm^3 with the vernier caliper and tumor volume was calculated with formula-

Tumor volume = (a x b^2)/2

Then the relative tumor volume (RTV) and the percentage tumor growth inhibition (% TGI) was calculated with following formula.

RTV =V_t/V_0

Where, V_0 is the volume measured after the first drug administration, day 1; V_t is the volume measured at concerned day of tumor measurement.

% TGI = (1-RTV of test or reference compound group/ RTV of vehicle control group) x 100

(56).

Dose selection

Sorafenib tablet (Soranib™) was purchased from Cipla Ltd. The recommended human dose of sorafenib is 400 mg twice daily; 30 mg/kg/day of sorafenib was given orally in order to have similar exposure to mice. Sorafenib was given orally which is clinical route of drug administration. Sorafenib formulation was prepared by triturating tablet with 0.4% Tween 80 and 0.5% CMC to form uniform suspension for in vivo experiment (57-60).

A) Evaluation of efficacy of extracts alone in xenograft model

Fasting of mice

Mice were fasted 2-3 hr before the dosing and food was placed back after 1-2 hr of dosing. Water was available ad libitum throughout the study.

Study design

Animals were randomized based on the tumor volume when tumors reached a volume of 200-300 mm^3 and treatment was initiated. All the formulations were prepared freshly in 0.4% Tween 80 (w/v) and 0.5% CMC. Required quantity of extract and sorafenib was weighed and transferred in mortar and triturated with Tween 80 (0.4% w/v of formulation) and 0.5% CMC was added slowly with trituration to get the uniform suspension.

The formulation was administered with 24 gauge stainless steel feeding cannula. Dose volume was calculated on the basis of body weight and was administered at 10 mL/kg dose volume.

Mice were randomized into following different groups-

Group I: Vehicle control

Group II: Alcoholic extract of A aspera (500 mg/kg, p.o.)

Group III: Alcoholic extract of B diffusa (500 mg/kg, p.o.)

Group IV: Alcoholic extract of E littorale (500 mg/kg, p.o.)

Group V: Sorafenib (30 mg/kg, p.o.)

Group VI: Alcoholic extract of B. diffusa (150 mg/kg, p.o.)

Group VII: Alcoholic extract of A. aspera (150 mg/kg, p.o.)

All vehicle control mice received 0.4% Tween 80 in 0.5% CMC. Treatment was given for 21 days. Mice tumor dimensions were measured twice weekly with the vernier caliper and body weights were recorded on alternate days starting with the first day of treatment. Mice were observed for body weight change, any clinical sign and tumor volume during the experimental period. Mice having tumor volume greater than the 10% of their body weight were humanely euthanized.

B) Evaluation of efficacy of extracts alone and combination with sorafenib in xenograft model

The HepG2 cell at concentration of 1×10^7 cells/mL was collected in sterile falcon tube. Cells were mixed at 1:1 volume with ice cold BD-Matrigel (Corning, India) and injected subcutaneously with a volume of 0.2 ml on the right rear flank to get 1 million cells per mouse.

Mice tumor dimension was measured twice weekly with the vernier caliper and body weights were recorded on alternate day starting with the first day of treatment. Mice were observed for body weight change, any clinical sign and tumor volume during the experimental period. Mice having tumor volume greater that the 10% of their body weight were humanely euthanized. Treatments producing >20% lethality and/or 20% net body weight loss were considered ‘‘toxic’’.

Study design

Animals were randomized based on the tumor volume. Treatment was initiated when tumors reached a volume of 150-250 mm^3. All the formulations were prepared freshly in 0.4% Tween 80 (w/v) in 0.5% carboxymethyl sodium. Required quantity of test and reference item was weighed and transferred in mortar and triturated with Tween 80 (0.4% w/v of formulation) and 0.5% carboxy methyl cellulose was added slowly with trituration to get the uniform suspension.

The formulation was administered with 24 gauge stainless steel feeding cannula. Mice dose was calculated on the basis of body weight and dosed at 10 mL/kg dose volume.

Mice (n=6 for each group) were randomized into following different groups-

Group I: Vehicle control

Group II: Alcoholic extract of *A. aspera* (500 mg/kg, p.o.)

Group III: Alcoholic extract of *B. diffusa* (500 mg/kg, p.o.)

Group IV: Sorafenib (30 mg/kg, p.o.)

Group V: Combination of alcoholic extract of *A. aspera* (500 mg/kg, p.o.) & Sorafenib (30 mg/kg, p.o.)

Group VI: Combination of alcoholic extract of *B. diffusia* (500 mg/kg, p.o.) & Sorafenib (30 mg/kg, p.o.)

Group VII: Sorafenib (15 mg/kg, p.o.)

Group VIII: Combination of alcoholic extract of *B. diffusia* (500 mg/kg, p.o.) & Sorafenib (15 mg/kg, p.o.)

Group IX: Combination of alcoholic extract of *A. aspera* (500 mg/kg, p.o.) & Sorafenib (15 mg/kg, p.o.)

All vehicle control mice received 0.4% Tween 80 in 0.5% CMC. Treatment was given upto 21 days. All control mice received 0.4% Tween 80 in 0.5% CMC.

After 2 hr of the last dose administration, blood was collected from the retro-orbital sinus under the isoflurane anesthesia. Blood was kept at room temperature for 30 minute and then centrifuged at 3000 rpm at 24°C for 10 minute, serum was separated and kept in -70°C freeze until analysis. Mice were euthanized by a slow CO_2 inhalation and tumors were isolated. One part of tumor was fixed by 4% paraformaldehyde for IHC and histopathology while other part of tumor was snap frozen for biomarker analysis by western blot.

Analysis of biochemical factors

To evaluate liver and kidney functions, serum was separated from blood via centrifugation at 3000 rpm for 10 min, and the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, and creatinine (Cr) were determined by using an automated biochemical analyzer.

Western Blot Assay

Western blot assay was conducted to detect expression levels of p-ERK, total-ERK, cleaved caspase-3, and β-actin. Total proteins of the tumor tissues were extracted using a total protein assay kit. After the determination of protein contents via bicinchoninic acid assay (BCA assay), these protein samples (40–50 μg) were separated by 10% SDS-PAGE and electrotransferred to nitrocellulose membrane. The membrane was washed in Tris-buffered saline containing 0.1% Tween 20 (TBST), blocked with TBST for 2 h at room temperature, and incubated with following specific antibodies: caspase-3, p-ERK, total-ERK and β-actin overnight at 4°C. The membrane was washed three times in TBST, followed by incubation with the appropriate horseradish peroxidase-

(HRP) linked secondary antibodies for 1 h at room temperature. The specific proteins on the blots were developed using enhanced chemiluminescence and visualized as bands on ChemiDoc® imaging system (59-61).

Immunohistochemistry

The immunohistochemical analysis was processed according to standard procedures. Formalin-fixed paraffin-embedded tumor sections were dewaxed in xylene and hydrated in gradient alcohol. Then the slides were kept with citrate buffer at 97˚ C for antigen retrieval. Then, 3% H_2O_2 was added to the sections to remove endogenous peroxidase. Immunostained sections were preincubated with normal serum and then incubated with specific primary antibodies against Ki-67 for overnight at 4˚ C The sections were incubated for 2 hr with the horseradish peroxidase streptavidin biotinylated secondary antibody (Vector Laboratories) followed by diaminobenzidine (Sigma). For the negative controls, the primary antibody was replaced with normal serum. Staining intensities were determined by measurement of the integrated optical density (IOD) with light microscopy using a computer-based Image System. The results are expressed as the mean value of at least three randomly chosen field from each slides (n=3) in each group. The results were expressed as the mean number of vessels ± standard error of the mean. For Ki67, percentage positivity was determine from no of cells and positive cells for no. of Ki67 nuclear staining with the help of QuPath software version 0.3.2 (62).

4.10.2.3 Statistical analysis

Data were expressed as mean ± standard deviation (SD). The GraphPad Prism 5.0 software (Graph Pad Software Inc., San Diego, CA, USA) was used for the statistical analysis. All data were analyzed using one or two-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison post hoc test. p value of <0.05 was considered as an indication for statistical significance. The graph pad prism software was used to analyze the data.

5. Results

5.1 Extraction of plant material

The air-dried plant part 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 soxhlet apparatus. All the extracts were concentrated under reduced pressure and controlled temperature (40–50^{0}C) in a rotary evaporator. The extracts were stored in air-tight containers for further experimental purposes. Organoleptic evaluation refers to evaluation of the extracts by color, odor, consistency and percentage yield.

B. diffusa extracts have characteristics odor with 5 to 7% w/w yield. Preliminary photochemical screening of *B. diffusa* confers the content of flavonoid, glycosides, alkaloid and phenolic compounds which are found as per the reported literature (63).. The extract complies for heavy metal and microbial count as per the USFDA guideline (64-65).

A. aspera extracts also has characteristic odor with yield range from 3 to 7% w/w. Preliminary photochemical screening of *A. aspera* showed presence of flavonoid, glycosides, alkaloid, phenolic compounds, and saponins which are matched as mentioned in the literature paper (28). Alcoholic extract of the plant contains 7.36% w/w of saponin (reference CoA of the extract). The alcoholic extract complies with USFDA guideline for heavy metal and microbial count (64-65).

Alcoholic extract of *E. littorale* has the highest yield of 12%. All other extracts of the *E. littorale* has yield from 4 to 7 % w/w with extracts showed the presence of flavonoid, glycosides, steroid, alkaloid, phenolic compounds, and saponins by phytochemical screening which are as per the literature (66). Also the extract complies for heavy metal and microbial count as per the USFDA guideline (64-65).

The organoleptic characters and preliminary phytochemical screening of the extracts are summarized in Table 1 to 3 and Table 4 to 6 respectively.

Table 1: Organoleptic properties of *B. diffusa* extracts

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

Table 2: Organoleptic properties of A. aspera extracts

Specification	Petroleum ether	Ethyl acetate	Alcoholic	Hydroalcoholic	Aqueous
Color	Green	Dark green	Pale yellow	Brown	Black
Consistency	Semisolid-liquid	Semisolid	Semisolid	Lyophilized powder	Semisolid
Odor	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
Yield (% w/w)	3.0	5	6.5	6.2	7.0

Table 3: Organoleptic properties of *E. littorale* extracts

Specification	Petroleum ether	Ethyl acetate	Alcoholic	Hydroalcoholic	Aqueous
Color	Green	Blackish green	Dark green	Brown	Blackish green
Consistency	Semisolid	Semisolid	Semisolid	Lyophilized powder	Semisolid
Odor	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
Yield (% w/w)	4	8	12.0	7.0	6.8

Table 4: Preliminary phytochemical screening of *B. diffusa* root extract

Class	Test/ Reagents	Petroleum ether	Ethyl acetate	Alcoholic	Hydroalcoholic	Aqueous	Presence of phytoconstituents as per literature
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	-	-	+	+	-	Punarnavoside, β-sitosterol-β-D-glucoside
	Liebermann's Test	-	-	+	+	-	
Saponins	Foam Test	-	+	-	+	-	-
Flavonoids	Alkaline Reagent Test	-	+	+	+	+	Quercetin, kaempferol
Alkaloids	Dragendorff's Test	-	+	+	+	+	Punarnavine
	Mayer's Test	-	+	+	+	+	
	Wagner's Test	-	+	+	+	+	
Tannins and phenolic compounds	Ferric chloride Test	+	+	+	+	+	Boeravinones G, H

+ present, - absent

Table 5: Preliminary phytochemical screening of *A. aspera* root extract

Class	Test/ Reagents	Petroleum ether	Ethyl acetate	Alcoholic	Hydroalcoholic	Aqueous	Presence of phytoconstituents as per literature
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	-	-	+	+	+	Oleanolic acid, ursolic acid
Flavonoids	Alkaline Reagent Test	-	+	+	+	+	-
Alkaloids	Dragendorff's Test	-	-	+	+	+	-
	Mayer's Test	-	-	+	+	+	
	Wagner's Test	-	-	+	+	+	
Tannins and phenolic compounds	Ferric chloride Test	-	-	+	+	+	*p*-coumaric acid, vanillic acid, ferulic acid

+ present, - absent

Table 6: Preliminary phytochemical screening of *E. littorale* whole plant extract

Class	Test/ Reagents	Petroleum ether	Ethyl acetate	Alcoholic	Hydroalcoholic	Aqueous	Presence of phytoconstituents as per literature
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	-	-	+	+	+	Swertiamarin
	Liebermann's Test	-	-	+	+	+	
Saponins	Foam Test	-	+	+	+	+	Betulin
Flavonoids	Alkaline Reagent Test	-	+	+	+	+	Genkwanin, apigenin, swertisin, isovitexin, saponarin, quercetin
Alkaloids	Dragendorff's Test	-	+	+	+	+	-
	Mayer's Test	-	+	+	+	+	
	Wagner's Test	-	+	+	+	-	
Tannins and phenolic compounds	Ferric chloride Test	+	+	+	+	+	Vanillic acid, syringic acid, p-coumaric acid, ferulic acid

+ present, - absent

5.2 Phytochemical analysis by LC-MS

The screening of alcoholic extract of *A. aspera* and *B. diffusa* by LC-MS leads to identification of 12 (table 7) and 14 (table 8) phytoconstituent respectively. Each listed chemical component contains the following information: compound name, retention time, ionization mode, adducts ions, and fragments ions m/z.

Table 7: Compounds identified in alcoholic extract of *A. aspera* by LC-MS method

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	534.32	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	5.40	ES+	M+H
12	Oleonolic acid	458.1	456.7	6.52	ES+	M+H

Table 8: Compounds identified in alcoholic extract of *B. diffusa* by LC-MS method.

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

5.3 MTT (cytotoxicity) assay

MTT assay is the colorimetric assay which measures the reduction of MTT (yellow) to an insoluble colored (dark purple) formazan product by mitochondrial succinate dehydrogenase.

All the plant extracts were assessed for cytotoxicity on HepG2 cells by *in-vitro* experiments. The HepG2 cells were exposed for 48 hr to varying concentrations (5 to 640 µg/mL) of different plant extracts, sorafenib (0.156 to 20 µM) and doxorubicin (0.156 to 20 µM) for 48 hr and the cytotoxicity was measured by the MTT assay.

The alcoholic and aqueous extract of all the three plants, *B. diffusa, A. aspera and E. littorale* was prepared in water and remaining extracts (hydroalcohol, ethyl acetate and petroleum ether) were prepared in DMSO. Sorafenib and doxorubicin were also prepared in DMSO. Sorafenib and doxorubicin were used as positive controls in the assay. All the extracts and reference standards showed dose dependent cytotoxicity on HepG2 cell line.

Alcoholic extract of *A. aspera* showed statistically significant cytotoxicity as compared to ethyl acetate and petroleum ether extracts (P<0.001). Though alcoholic extract cytotoxicity was not significant compared to aqueous and hydroalcoholic extract but the cells percentage viability at 640 µg/mL (highest concentration) was lower (20%) than both the extract (63 and 50% for aqueous and hydroalcoholic extract). The IC_{50} value of alcoholic extract was found to be 192 µg/mL.

The alcoholic extract *B. diffusa* leads to less formazan crystal formation measured by MTT assay (Cytotoxicity) with IC_{50} value of 141 µg/mL compared to its aqueous, ethyl acetate (P<0.1) and petroleum ether (P<0.01) extract. The cytotoxicity was not significant with hydroalcoholic extract with 48% cells were viable at 640 µg/mL concentration compared to 12% with alcoholic extract.

The alcoholic extracts of *E. littorale* showed highest cytotoxicity with IC_{50} value of >370 µg/mL compared to its other extract. Sorafenib and doxorubicin showed dose dependent cytotoxicity with IC_{50} value 6.68 µM and 1.41 µM respectively. As the alcoholic extracts of the three plants showed highest activity compared to other (aqueous, hydroalcohol, ethyl acetate and petroleum ether) extracts, alcoholic extracts were selected for further screening. The cytotoxicity profiles of different extracts and reference standards with percentage viability are shown in Figures 8–11. The IC_{50} values are shown in Table 9.

Table 9: IC_{50} value of extract and reference standards

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
Doxorubicin	1.41 ± 0.7 µM

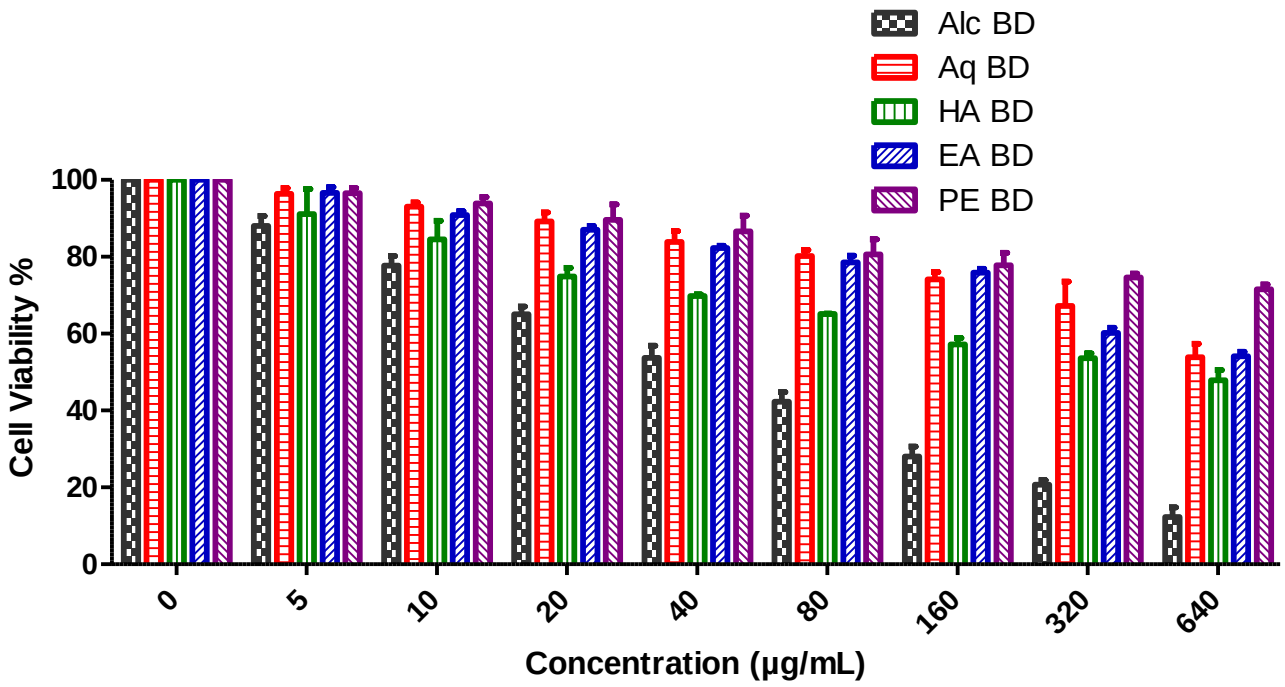


Figure 8: Percentage cell viability of *B. diffusa* extracts in MTT assay

HepG2 cell lines were treated with *B. diffusa* extracts at the indicated concentrations and cell viability was determined by MTT assays. All values mean ± SD (n = 3-5). Alc-alcoholic extract, Aq-aqueous extract, HA-hydroalcoholic extract, EA-ethyl acetate extract, PE-petroleum extract

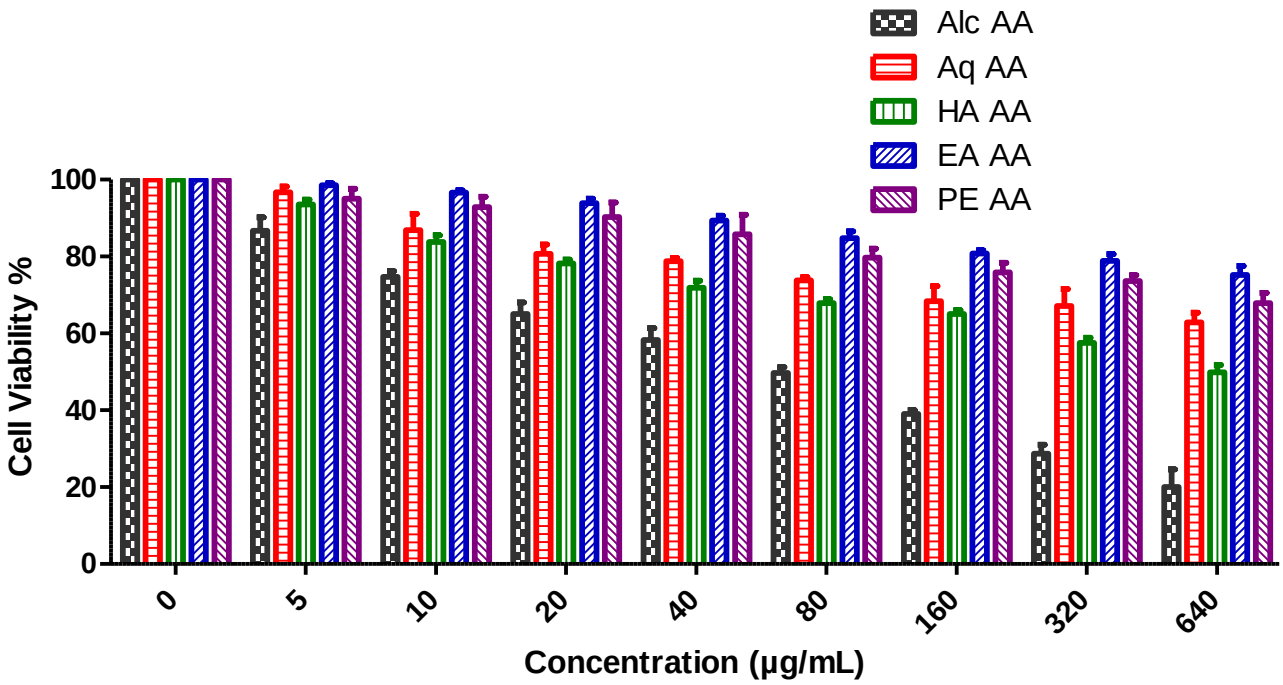


Figure 9: Percentage cell viability of *A. aspera* extracts in MTT assay

HepG2 cell lines were treated with *A. aspera* extracts at the indicated concentrations and cell viability was determined by MTT assays. All values mean ± SD (n = 3-5). Alc-alcoholic extract, Aq-aqueous extract, HA-hydroalcoholic extract, EA-ethyl acetate extract, PE-petroleum extract

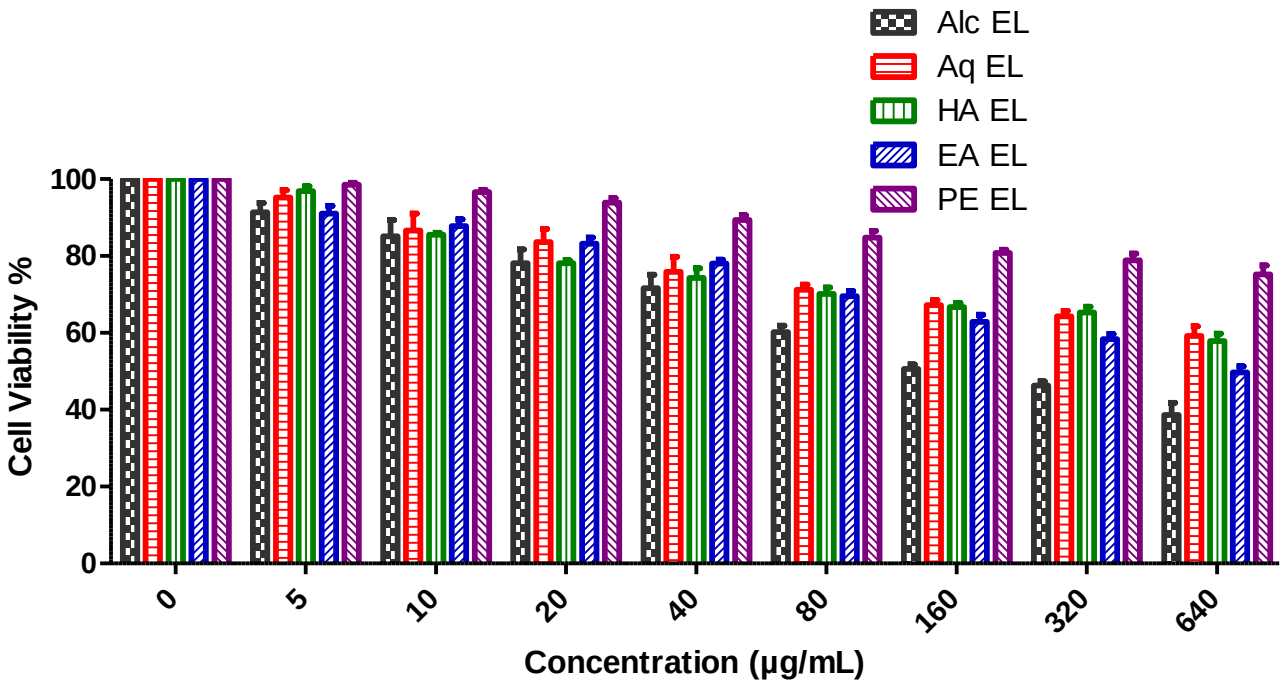


Figure 10: Percentage cell viability of *E. littorale* extracts in MTT assay

HepG2 cell lines were treated with *E. littorale* extracts at the indicated concentrations and cell viability was determined by MTT assays. All values mean ± SD (n = 3-5). Alc-alcoholic extract, Aq-aqueous extract, HA-hydroalcoholic extract, EA-ethyl acetate extract, PE-petroleum extract

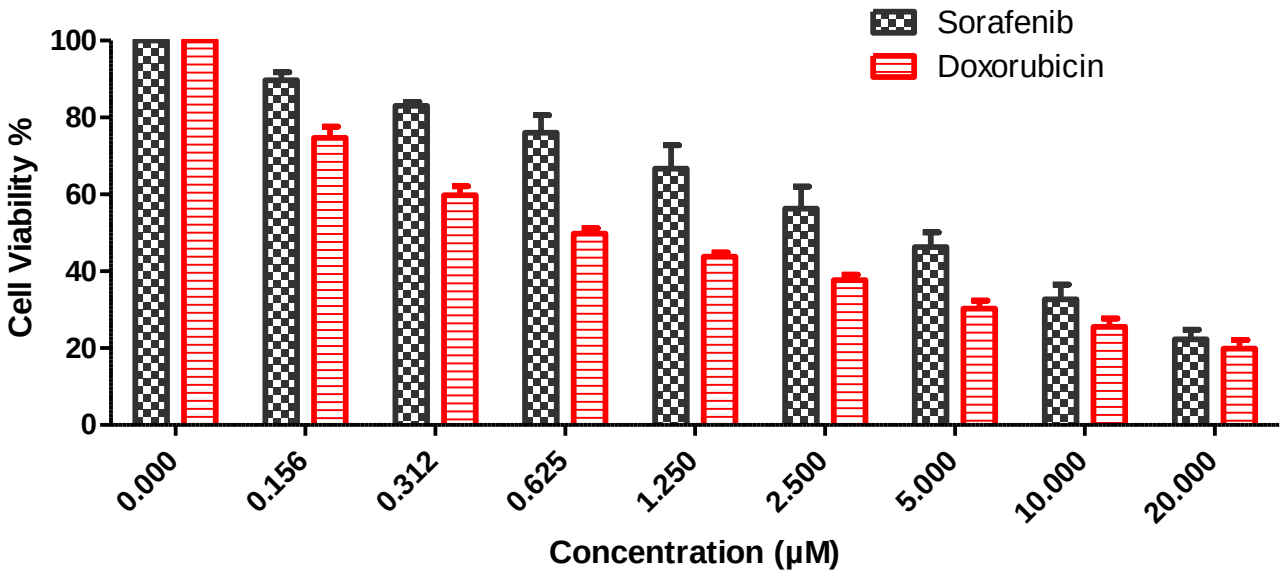


Figure 11: Percentage cell viability of reference standards in MTT assay

HepG2 cell lines were treated with sorafenib and doxorubicin at the indicated concentrations and cell viability was determined by MTT assays. All values mean ± SD (n = 3-5).

5.4 Scratch motility assay

Alcoholic extracts of *B. diffusa* and *A. aspera* were assessed for scratch motility assay on HepG2 cells by *in-vitro* experiments. The HepG2 cells were exposed to IC_{50} value of the extracts and sorafenib and the percentage area of cells moved were measured. Both the extract and sorafenib inhibited the migration of the cells as compared to control group.

5.5 Acute toxicity study as per OECD guideline 423

Animals were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hrs, with special attention given during the first 4 hrs, and daily thereafter, for a total of 14 days. All the rats were observed at least twice daily with the purpose of recording any symptoms of ill-health or behavioral changes.

There was no change in physical parameters such as body temperature, skin and eye color during the study duration. An animal's body temperature was measured periodically with the help of rectal probe. The body temperature was normal in all the groups animals. Animals have shown normal exploratory activity and non irritability behavior post administration of extract confirms that single dose of extract does not have any deleterious effects on CNS system. Observations for the limit test were displayed in Table 10, 11, and 12 for alcoholic extract of *A. Aspera*, *B. diffusa*, and *E. littorale* respectively.

Animals treated with all the three extracts showed normal observations and does not show any visible toxicity at 2000 mg/kg oral dose. The LD_{50} was found to be higher than 2000 mg/kg body weight.

Table 10: 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 11: 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 12: 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

5.6 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 were injected with a single intraperitoneal (i.p.) dose of

DEN (200 mg/kg body weight) for initiating hepatocarcinogenesis. After two weeks, liver cancer development was promoted with daily dose of 2-AAF (30 mg/kg, p.o.) for next two weeks.

As presented in Table 14, liver weight, liver index and spleen index of control group rats were significantly increased as compared to normal rats. As compared to normal rat group (27.9%) statistically significant reduction in body weight was observed in DEN and 2-AAF treated group (8.1%), data shown in table 14 and figure 12. Serum ALT, ALP and total bilibrubin were enhanced significantly while no change in AST and creatinine level in DEN-treated rats compared with normal animals at the end of the study (table 16).

The hepatic sections from the normal 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 necrosis which indicate that damage to liver has initiated but devoid of cancer development in DEN and 2-AAF group (figure 13).

Table 13: Data of mean body weight of rats

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28
Control	220 ± 7.0	250 ± 9.3	271 ± 9.5	288 ± 12.0	306 ± 19.9
DEN (200 mg/kg, i.p.) and 2-AAF (30 mg/kg, p.o)	215 ± 7.4	202 ± 13.6***	220 ± 11.9***	225 ± 8.8***	234 ± 12.2***

Remark: 2-AAF was administered daily for 2 week 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 < 0.001 as compared to vehicle treated (control) group.

Table 14: Data of percentage change in body weight, liver and spleen index at the end of the study

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 < 0.001 as compared to vehicle treated (control) group.

Table 15: Data of mean ± SD of liver function parameter 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.

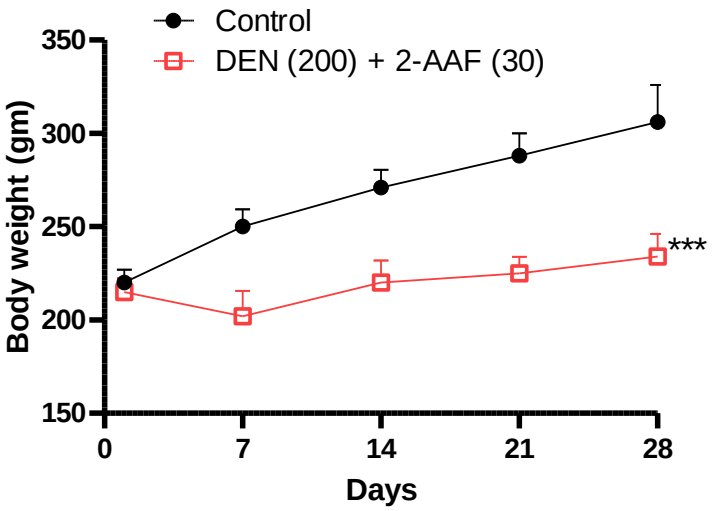
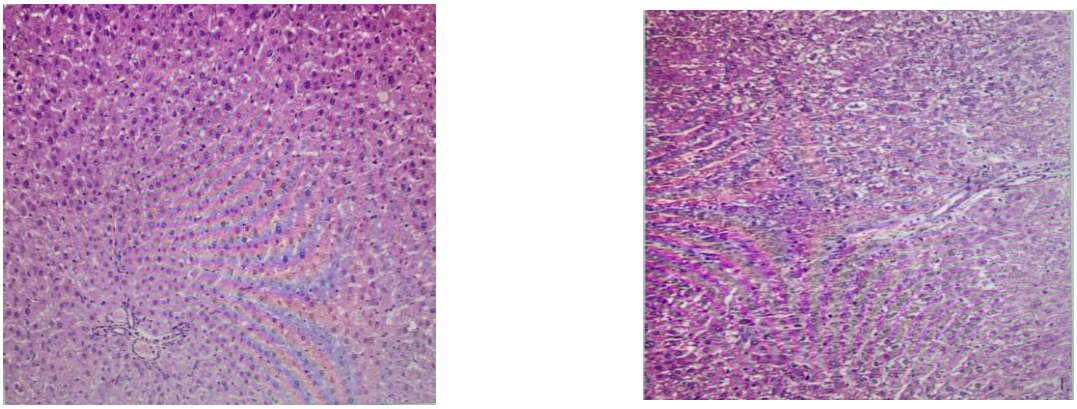


Figure 12: Body weight in DEN and 2-AAF induced HCC model

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.



A B

Representative histological pictures of liver tissue sections stained with (A–B) hematoxylin-eosin (H&E). Liver samples were obtained at the termination of the experiments in rats that 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, disease control group (B) captured at 20X objective, 50 µm bar.

Figure 13: Photomicrographs of liver sections of normal and DEN/2-AAF administered rat

5.7 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. 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 and 2 week later received weekly CCl_4 (2 mL/kg i.g) for 3 week.

Post 24 hr DEN injection rats in both the group have found with slow movement, listlessness which was continuous till 2-3 days. As compared to control group (23.9%) significant reduction in body weight was observed in both group 2 (13.8) and 3 (4.7%). Data shown in table 16 and figure 14. Subsequent the liver and spleen index was significantly higher in both group 2 (6.4% and 0.28%) and 3 (7.5% and 0.29%) respectively as compared to control group (table 16).

Liver function was monitored by measuring serum liver enzymes (ALT, AST, ALP, total protein, creatinine and total bilirubin) on day 14, 28 and 35. Mean serum level of all the enzymes were within the normal range for the control group. Mean serum levels of ALT, AST, ALP and creatinine were significantly higher in the model group 2 and 3 as compared to the control group. There was not much difference in serum enzyme levels of total protein and bilirubin between group 2, group 3 compared to the control group (table 17 and 18).

There were no visible anatomical deformities or any tumor nodules seen on livers isolated from the rats in any group. The control group showed normal liver histology with hepatocytes having rounded nucleus and fewer infiltrations of inflammatory cells. In group 2 rats shown necrosis and high infiltration of inflammatory cells while in group 3 showed more damage with necrosis, formation of fibrosis and initiation of tumor nodule in one animal (figure 15). There was no any visible tumor or severe damage. Though damage initiated but to reach it to HCC development requires more exposure of carcinogen.

In both the animal models there was increase in serum marker 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 chemicals. The serum AFP levels was nearly same in both the groups which also confirms that the HCC was not generated.

Table 16: Data of percentage change in body weight, liver and spleen index

Treatment	% change in body weight	Liver Weight (gm)	Liver index	Spleen Weight (gm)	Spleen index
Control	23.9 ± 3.6	11.8 ± 1.2	3.4 ± 0.6	0.60 ± 0.1	0.19 ± 0.03
DEN (200 mg/kg, i.p.) and CCl_4 (2 mL/kg, p.o.)	13.8 ± 3.6	16.9 ± 2.8	6.4 ± 1.0***	0.75 ± 0.2	0.28 ± 0.05***
DEN (200 mg/kg, i.p.) and CCl_4 (2 mL/kg, p.o.)	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). 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 (control) group.

Table 17: Data of liver function parameter

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

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). 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 (control) group.

Table 18: Data of liver function parameter

Treatment	Total protein (gm/dL)			Total billirubin (mg/dL)			Creatinine (mg/dL)		
	Day 14	Day 28	Day 35	Day 14	Day 28	Day 35	Day 14	Day 28	Day 35
Normal range	6.3-7.3			0.2-0.7			0.2-0.7		
Control	7.2 ± 0.3	8.1 ± 0.9	7.3 ± 0.3	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.5 ± 0.1
DEN (200 mg/kg, i.p.) and CCl_4 (2 mL/kg, p.o.)	6.6 ± 0.2	6.8 ± 0.4	6.6 ± 0.5	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.8 ± 0.1***
DEN (200 mg/kg, i.p.) and CCl_4 (2 mL/kg, p.o.)	6.7 ± 0.3	7.1 ± 1.1	6.6 ± 0.6	0.5 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.8 ± 0.1***

Remark: CCl_4 was administered on 3^{rd} and 3^{rd}, 4^{th}, 5^{th} week in 2^{nd} and 3^{rd} group respectively post DEN administration

Data presented as mean ± SD (n=6). 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 (control) group.

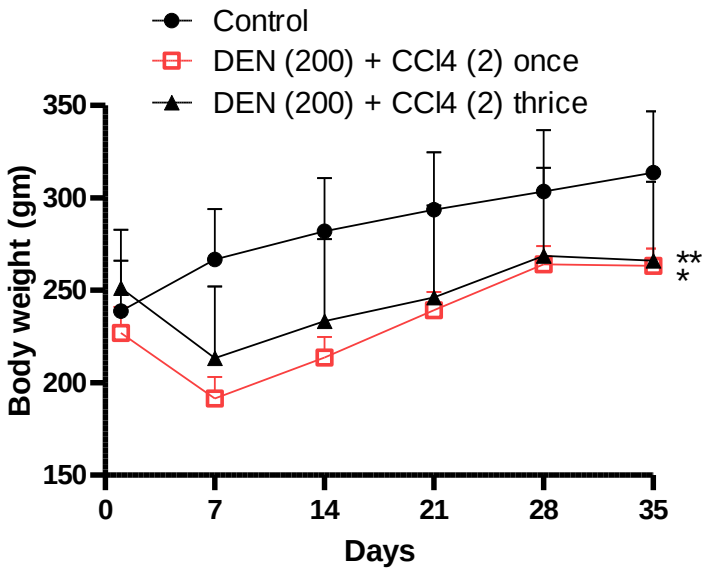
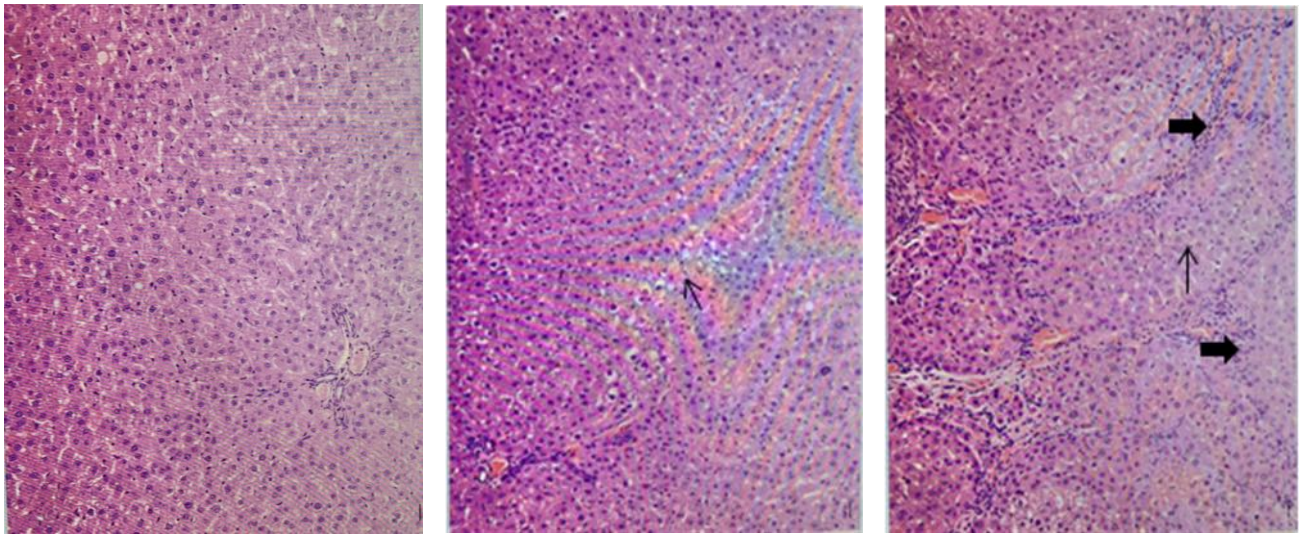


Figure 14: Body weight in DEN and CCl4 induced HCC model

Data are articulated as mean ± SD (n=6). Statistical analysis was carried out using paired t test for multiple comparisons. *p < 0.1, **p < 0.01 as compared to vehicle treated (control) group.



A B C

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 (single dose, i.p.) and CCl_4 p.o. single dose disease control group (B) and DEN (single dose, i.p.) and CCl_4 p.o. once weekly for 3 week disease control group (C), post 2 week of DEN captured at 20X objective, 50 µm bar, ↑indicate necrosis, ➡indicate fibrosis formation

Figure 15: Photomicrographs of liver sections of normal, DEN/CCl_4 (single dose) and DEN/CCl_4 (three dose) administered to rat

5.8 Subcutaneous Xenograft Model

a) Growth pattern of HepG2 cells in male vs. female mice

Initiation of palpable tumor was established in mice within 2 week of cell inoculation. In female mice palpable tumor growth was initiated and tumor volume was grown upto ~300 mm^3 within 14 day while in male mice tumor volume was reached to ~100 mm^3 in 14 days. So the tumor growth pattern was faster in female mice as compared to male mice at 2 week of cell inoculation. The tumor take rate in female mice was 81.3 % as compared to male mice (26.7%) on day 14.

b) Standardization of xenograft model with different cell concentration

The growth pattern of HepG2 cells was found to be cell concentration dependent. The mice injected with 5 million cells showed aggressive tumor growth and reached human end point one week early as compared to 1 and 0.5 million cells/ animal. Optimum growth was observed with 1 million cells so further efficacy study was performed with 1 million cells.

c) Standardization of efficacy of extracts in xenograft model with different cell concentration:

In all the three cell concentrations (0.5, 1, and 5 million cells/mL) alcoholic extract of *A. aspera* and *B. diffusa* showed statistically significant antitumor activity as compared to vehicle group (figure 16-18). Sorafenib was used as a standard. Alcoholic extract of *E. littorale* showed no significant activity as compared to vehicle group at 1 and 5 million cells concentration. The extract showed statistically significant activity at 0.5 million cell concentration. Percentage tumor growth inhibition (% TGI) for alcoholic extract of *A. aspera* was in the range of 41% to 49% while that for alcoholic extract of *B. diffusa* was 39% to 47% at 500 mg/kg dose level. Percentage TGI for alcoholic extract of *E. littorale* was 26% to 35% at 500 mg/kg dose. Treatment in all group does not impact on body weight. Due to aggressive tumor growth with 5 million cells and optimum tumor growth with 1 million cells next experiment was planned with 1 million cells in female nude mice. Alcoholic extract of *A. aspera* and *B. diffusa* showed good tumor reducing activity in HepG2 xenograft. So next experiment was planned with single dose of both the extract along with combination of sorafenib.

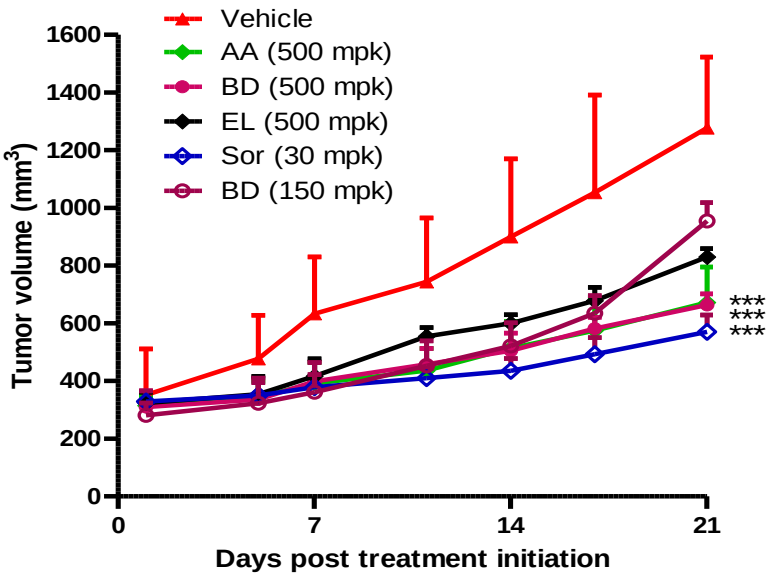


Figure 16: Efficacy of extracts in female mice (0.5 million cells/animal)

HepG2 tumor bearing mice were administered with vehicle, *A. aspera* (150 and 500 mg/kg/day, p.o.), *B. diffusa* (150 and 500 mg/kg/day, p.o.), *E. littorale* (500 mg/kg/day, p.o.) and Sorafenib (30 mg/kg/day, 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.

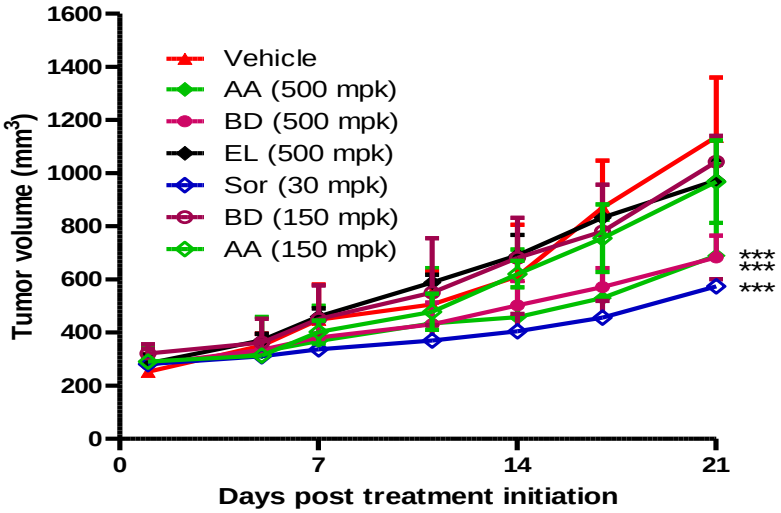


Figure 17: Efficacy of extracts in female mice (1 million cells/animal)

HepG2 tumor bearing mice were administered with vehicle, *A. aspera* (500 mg/kg/day, p.o.), *B. diffusa* (150 and 500 mg/kg/day, p.o.), *E. littorale* (500 mg/kg/day, p.o.) and Sorafenib (30 mg/kg/day, 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.

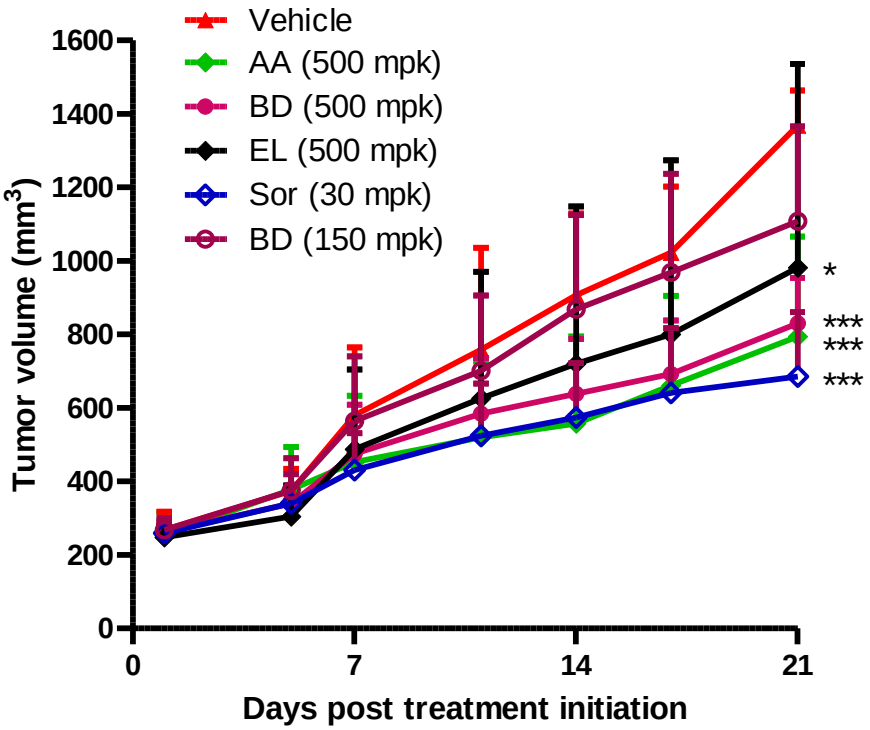


Figure 18: Efficacy of extracts in female mice (5 million cells/animal)

HepG2 tumor bearing mice were administered with vehicle, *A. aspera* (500 mg/kg/day, p.o.), *B. diffusa* (150 and 500 mg/kg/day, p.o.), *E. littorale* (500 mg/kg/day, p.o.) and Sorafenib (30 mg/kg/day, 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.

D) Evaluation of efficacy of extracts alone in xenograft model

Xenograft tumors were developed by implanting HepG2 cells subcutaneously into right flank of mice. Within 2 week of HepG2 cell injection palpable tumours were established. The mice were randomized into different groups based on their tumor volume when tumor volume reached to 150-250 mm^3.

The treatment was given once daily for 21 days. The tumor volume was measured atleast twice in a week and the growth curves of the tumors were established. No significant change in average body weight was found in mice that received treatment compared with control mice at the end of the experiment which indicate that the sorafenib and extracts had no effect on body weights of the hepatocellular carcinoma-bearing nude mice.

The tumor volume and weight was significantly inhibited by both the sorafenib and extracts treatments alone and in combined application as compared with the vehicle control group. Data are shown in Figure 19 and 20.

Tumor growth inhibitory rate of sorafenib at 15 mg/kg and 30 mg/kg was 36.1% and 55.1% respectively on day 21. Alone treatment of *A. aspera* extract has TGI of 46.8% while with combination of sorafenib at 15 mg/kg and 30 mg/kg dose levels produced TGI of 60.6% and 73.4% respectively. Treatments with *B. diffusa* extract lead to the TGI of 38.3%. *B. diffusa* extract with combination of sorafenib at 15 mg/kg and 30 mg/kg has a TGI of 49.9% and 66.%1 respectively.

Sorafenib has been reported to induce hepatotoxicity. Clinically, the secretion of ALT and AST of HCC patients are routinely examined to avoid potential hepatotoxicity. ALT and AST levels in the serum of HepG2 xenograft mice were examined at the endpoint of the experiment. The level of ALT was augmented after sorafenib as compared the control group. The alone extract and combination treatment group has no significant difference in ALT as compared to control group (figure 21 and 22).

The levels of aspartate aminotransferase, creatinine, or alkaline phosphatase were not changed significantly between the indicating that the combination of extracts and sorafenib had no significant hepato-renal toxicity. Also combinations of extract lead to decrease in the level of ALT which was increased due to sorafenib treatment.

Animals in each group behaved normally during the experiment. After the experiment, nude mice of each group were dissected, and vital organs were observed visually. No visual organ damage was observed.

The percentage positivity of Ki67 cells were significantly reduced in both the extract and sorafenib as compared to control group by Immunohistochemistry assay.

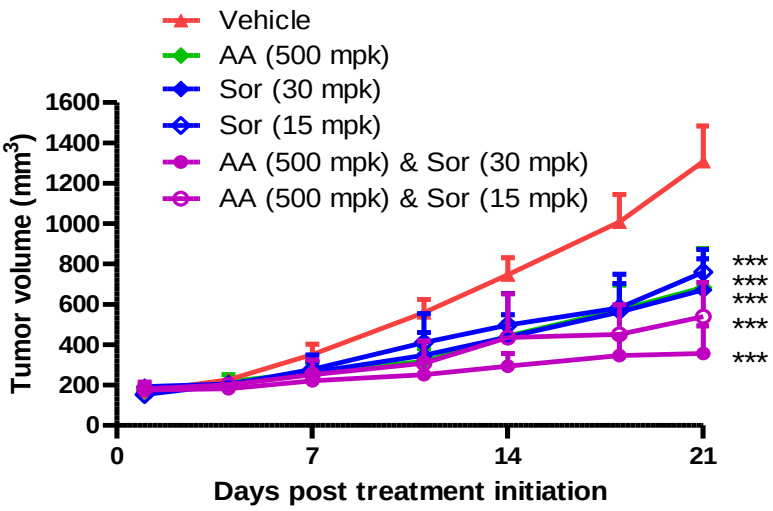


Figure 19: Efficacy of *A. aspera* in HepG2 CDX

HepG2 tumor bearing mice were administered with vehicle, *A. aspera* (500 mg/kg/day, p.o.), Sorafenib (15 and 30 mg/kg/day, p.o.) and combination of *A. aspera* (500 mg/kg/day, p.o.) with Sorafenib (15 and 30 mg/kg/day, 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.

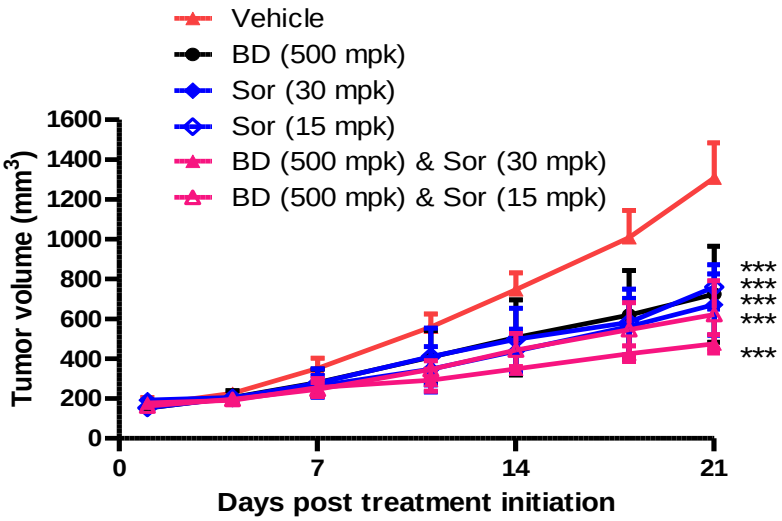


Figure 20: Efficacy of *B. diffusa* in HepG2 CDX

HepG2 tumor bearing mice were administered with vehicle, *B. diffusa* (500 mg/kg/day, p.o.), Sorafenib (15 and 30 mg/kg/day, p.o.) and combination of *B. diffusa* (500 mg/kg/day, p.o.) with Sorafenib (15 and 30 mg/kg/day, 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.

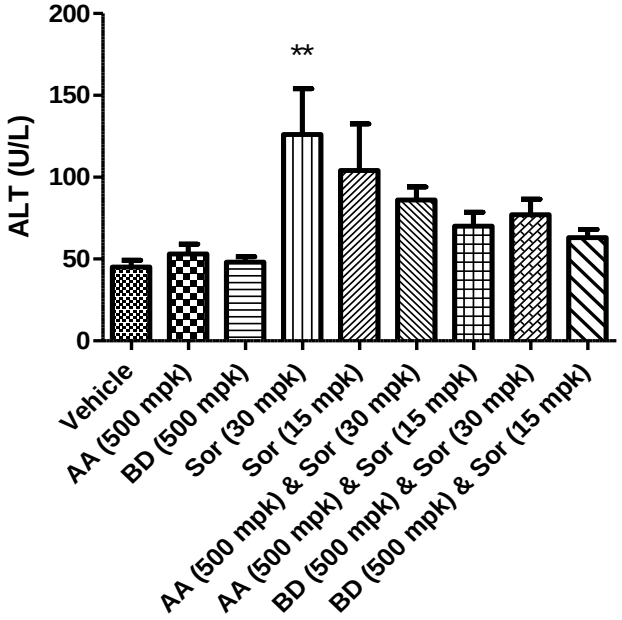


Figure 21: Serum levels of ALT on day 21

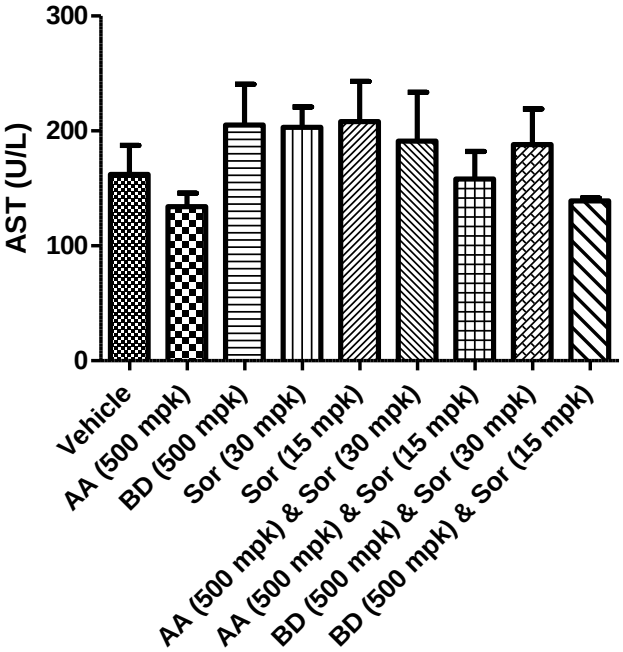


Figure 22: Serum levels of AST on day 21

6. Discussion

Medicinal plants and phytoconstituents are crucial source of the life saving drugs for majority of the world's population. They are used from the ancient system in Ayurvedic medicine in India and Unani medicine in Arab countries. The tribal, rural and aboriginal people of our country are using this herb from the prehistoric time in various disorders. Herbs are integral part of our life. Numerous researchers have been continuously working on the identification and characterization of chemopreventive efficacy of diverse range of medicinal plants. Anticancer substances present in foods are regarded as one of the most attractive strategies for cancer control, since certain natural compounds and medicinal plants have efficiently inhibited tumor development in various organs, including liver (50)

Hepatocellular carcinoma is the most common type of primary liver cancer. HCC is mainly diagnosed in intermediate- or advanced-stage. At this stage there is no curative treatment. In this advanced stage sorafenib is 1st FDA approved treatment option for HCC. Sorafenib is the kinase inhibitor has 9.2 month median overall survival rate and a median time to progression of 5.5 months (67).

Though sorafenib is able to prolong the life to a certain extent it is unable to improve the patients' quality of life. The disease progression and development of resistance lead to large doses of sorafenib which diminished the beneficial effects of sorafenib in the management of HCC patients (68-69).

Alcoholic extract of all the three plants *B. diffusa, A. aspera* and *E. littorale* has showed good dose dependent cytotoxicity on HepG2 cell line. All the three extract were found safe and devoid of any toxicity performed by oral toxicity test as per the OECD guideline 423.

The alchoholic extract of *B. diffusa* and *A. aspera* showed better cytotoxicity as compared to alcoholic extract of *E. littorale* with IC_{50} value of 141 µg/mL and 192 µg/mL respectively on HepG2 cells. Also both the extract and sorafenib inhibited the migration of the cells as compared to control group in scratch motility assay.

To analyze the translation of in-vitro activity into in-vivo system two types of animal models; chemically induced HCC and xenograft model were developed.

Chemically induced HCC consist of two step protocol in which diethyl nirosamine is used as the initial step of the multistage carcinogenesis which transforms normal hepatocytes to preneoplastic or neoplastic cells by genetic alteration (70). In 2 different set of experiment CCl_4 and 2-AAF were used as promoter for development of HCC.

In both the animal models significant change in body weight and elevation of serum ALT, AST, and ALP levels were observed as compared to control group. A significant increase in AST,

ALT, and ALP enzyme levels in serum has also been reported after inducing hepatocellular tumors by administering CCl_4 in rats (49).

In our model cancer development might be initiated. But still it was not able to generate the carcinogesis. In histochemical analysis necrosis, fibrosis formation was observed but no significant nodule formation was occurred. So from the result of the experiment it was concluded that for development of HCC prolong exposure of chemicals was required.

Then the xenograft model was developed in athymic nude mice with HepG2 cell line. The model was standardized with 0.5, 1 and 5 million HepG2 cells per animal in male and female mice. In our experiment female mice had shown good tumor development as compared to male mice. The cell inoculation with 5 million cells lead to aggressive tumor growth and 1 millions cells showed optimum tumor growth with good anticancer activity. All the three extracts shown tumor growth inhibition as compared to control mice across the 3 cell concentrations. Among the three extracts, *B.diffusa* and *A.aspera* showed statistically significant tumor growth inhibition activity.

Sorafenib is the first-line treatment for advanced HCC, approved by the FDA. Despite the positive effect of sorafenib on the survival of patients with unresectable HCC, a minimal response rate with less than 3 months of additional survival was reported (67).

In the new set of experiment combination approach of the extract with sorafenib at two dose levels was assessed. The recommended human dose of sorafenib is 400 mg twice daily which is equivalent to 30 mg/kg/day dose in mice. To avoid the toxicity of sorafenib with expecting good efficacy, combination study of soarfenib and extracts was performed at 30 mg/kg and one lower dose of sorafenib at 15 mg/kg.

On the basis of tumor volume, tumor growth inhibiton rate was calculated on day 21. *A. aspera* extract has TGI of 46.8% which was enhanced to 60.6% and 73.4% with combination of sorafenib at 15 mg/kg and 30 mg/kg with respectively. Treatments with *B. diffusa* extract produced the TGI of 38.3% which was augmented to 49.9% and 66.1% respectively compared to sorafenib at 15 mg/kg and 30 mg/kg dose. Sorafenib at 15 mg/kg and 30 mg/kg has 36.1% and 55.1% TGI respectively. Both the extract and sorafenib reduced the tumor proliferating (Ki67) cells by immunohistochemistry assay.

No significant change in average body weight was found between all the treatment group suggesting that the extract does not impact on body weight of the mice. Sorafenib has been reported to induce hepatotoxicity. In our experiment serum ALT level of sorafenib at 30 mg/kg was increased compared to control group. Increase in serum ALT level of sorafenib 30 mg/kg group was significantly higher as compared to control group. There was not much difference

between AST, total bilirubin and creatinine level between the groups. The combination assuage the secretion of ALT stimulated by sorafenib.

Literature review shown that roots of *A. Aspera* are rich in saponins which might be responsible for its cytotoxic effect (29, 71, 72).

The root of *B. diffusa* contains alkaloids, rotenoids, flavonoids, amino acids, lignans, ß-sitosterols and tetracosanoic, esacosanoic, stearic and ursolic acids (63). The root of Boerhavia diffusa is used in traditional Sri Lankan medicine in poly-herbal formulae for gastric and liver cancers (36).

Phytochmical screening and LC-MS analysis has shown presence of saponin, alkaloid, phenolic compound as a phytoconstituent which might be responsible for the anticancer activity of the extract.

Xenograft models play an important role in the screening and evaluation of candidates for new anticancer agents. Alcoholic extract of *A.aspera, B. diffusa* and sorafenib alone showed a significant reduction in tumor growth compared to control group in HepG2 xenograft model. Furthermore, the tumor growth inhibition effect was enhanced when each extract combined combined separately with lower dose of sorafenib. These results show that combined treatment of each extract with lower dose of sorafenib might offer a potential adjuvant therapy for HCC with less adverse effect.

7. **Conclusion**

In conclusion, the alcoholic extract of *A. aspera* and *B. diffusa* has good anticancer activity in HepG2 xenograft. Further it provides valuable information for combination of extract with Sorafenib. Both the extracts inhibit the cancer proliferating (Ki67) cells and help to reduce the potential toxicity of sorafenib (heptotoxicity). Thus, the extract has potential to be used as adjuvant therapy with the lower dose of current standard of care in hepatocellular carcinoma.

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9. Pending works:

Sr. No.	Activity
1	Biomarker estimation by Western Blot method

10. Participatation in conferences (poster/ oral presentation)/paper/courses

1. Attended National Online Workshop on "**Generating the Highest Level of Evidence through Systematic Review and Meta-analysis:Best Alternative for Hospital Based Projects During the Current Pandemic Situation**" Organized by The Department of Pharmacy Practice NIPER, Guwahati on 28th & 29th August 2020

2. Attended International webinar on **Coming Closer to Nature for Impactful Publication (An Authors Worshop)** by NIPER, Ahmedabad on 22nd September 2020.

3. Attended Prof Ambikanandan Misra memorable International e conference on **Recent Advances and Trends Novel Drug Delivery Systems** organized by MSU Pharmacy Alumni Association (MPA) & Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda 24-25 September 2021

4. Attended GUJCOST sponsored International e conference on **Recent Trends in Molecular Pharmacology** by LJ institute of pharmacy, LJ University, Ahmedabad on 11-12 March 2022

5. Attended GUJCOST sponsored one day National Virtual Seminar on **Current Trends and Challenges: Regulations and Standardization of Herbals** organized by Anand pharmacy college, Anand on 26th March 2022

6. Attended US FDA annual conference on **Regulatory Education for Industry (REdI)** by US FDA on 6-7 June 2022

7. Attended US FDA worksop on Office of Study Integrity and Survillance Workshop 2022 on **CDER Inspection of Good Laboratory Practice, Animal Rule, and Bioavailability/Bioequivelance Study Sites** by US FDA on 19-20 July 2022

8. Oral presentation entitled "***B. diffusa*** **as an antitumor agent against hepatocellular carcinoma"** presented at "National Conference on Trends in Natural Products in Immunotherapeutics" held at RPCP, CHARUSAT, Changa on 3rd & 4th March, 2023" **authors** Zanwar Sachin, Patel Kirti

9. Oral presentation entitled "**Smart drug delivery in cancer treatment"** presented at "National Seminar on Recent Advances in 'Smart' Drug Delivery Systems" held at School of Pharmaceutical Sciences, Chettinad Academy of Research and Education, Chettinad Health City, Tamil Nadu on 5th April, 2023" **authors** Zanwar Sachin, Patel Kirti

10. Book chapter entitled "**Chemically induced animal models as tool in cancer research**" in a book Recent Advances in Pharmaceutical Sciences, Volume 7, page no. 379 to 401, by Innovative Academic Sciences Pvt. Ltd. Bhopal (ISBN No. 978-81-952065-9-9) Sachin B. Zanwar, Kirti V. Patel, Aarti S. Zanwar, Dhanya B. Sen, Rajesh A. Maheshwari, Ashim Kumar Sen

11. Attended one day Indo-US Seminar on **Drivers of Future Pharmaceuticals** organized by NIPER-Ahmedabad on 9th July 2023

12. Attended one day National conference on **Intelligent Automation in Pharmaceutical Industry** organized by IQAC Kamla Nehru College of Pharmacy Butibori Nagpur and APTI (MS) on 15 July 2023.