5. Discussion

Medicinal plants and phytoconstituents are crucial source of the life saving drugs in modern medicine for majority of the world's population. For instance herbal medicines are used from the ancient time in Ayurvedic system in India and Unani system in Arab countries. The tribal, rural and aboriginal people of our country have been using herb from the prehistoric time in various disorders. Numerous researchers have been continuously working on the identification and characterization of chemo preventive efficacy of diverse range of medicinal plants. Use of anticancer substances present in the foods is regarded as one of the most attractive strategies for cancer control, since certain natural compounds and medicinal plants have been proven to efficiently inhibit tumor development in various organs, including liver (199).

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer. HCC is mainly diagnosed in intermediate- or advanced-stage. Like several other cancers, there is no curative treatment for HCC available at this time. The tyrosine kinsase inhibitor sorafenib is the 1st FDA approved treatment for the advanced stage of HCC. Sorafenib had 9.2-month median overall survival rate and a median time to progression of 5.5 months (16).

Although sorafenib is able to prolong the life to a few months, it is has no impact on patients' quality of life (16). Indeed progression of HCC requires increasingly large doses of sorafenib. Tumor angiogenesis is considered as one of the many processes that contribute to the development of resistance (222-223) leading to progression of the disease. Use of large doses cause increase in adverse effects like lethal hepatotoxicity and anorexia (213). Overall, beneficial effects of sorafenib are compromised in the management of advanced HCC (224-225). Thus, there is a need for researchers to look for new approaches to optimize the beneficial effects of sorafenib by combining it with other agents.

HepG2, a type of hepatatoma cells, maintain some of the morphological characteristics and functionalities of hepatocyte. HepG2 cells are well characterized, readily available, and have been extensively employed in in-vitro cell-based assays to investigate the compound for HCC therapy. To investigate in vivo anticancer activity, HepG2 cells are also implanted in recipient mice (226). Malignant tumors are characterized by aberrant cell invasion, metastasis, and angiogenesis in addition to uncontrolled cell proliferation, apoptosis, and cell cycle (7).

A. aspera, B. diffusa and *E. littorale* are well known ayurvedic medicinal plants used in traditional medicine system for various liver disorders and other cancers (32, 33, 38-43, 46-48). Based on the literature review and with application of network pharmacology principle, interactions were found between various proteins in the key pathways of cancers and these plants phytoconstituents.

Our work began with literature work indicating use of several plants for liver disorders. This work led to identification of following three plants among different plants reviewed: *A. aspera*, *B. diffusa* and *E. littorale*. Consequently a network analysis for understanding interactions was done. In the network analysis, among all the protein studied, p53, VEGF and PI3K had affinity for all the three plants, *A. aspera* (oleanolic acid, chlorgenic acid, kaempferol 3-o- glucoside etc.), *B. diffusa* (boeravinones B & H, quercetin, eupalitin etc.) and *E. littorale* (swertiamarin, betulin, apigenin etc.) phytoconstituents. These proteins are well established for the growth and proliferation of cancer cells.

Following this, five extracts (petroleum ether, ethyl acetate, alcoholic, hydro alcoholic and aqueous) for each of the three plants (roots of *A. aspera*, roots of *B. diffusa* and whole plant parts of *E. littorale)* under investigations and total 15 extracts were screened for cytotoxicity by MTT assay on HepG2 cells.

Among all screened 15 extracts, alcoholic extract of *A. aspera* and *B. diffusa* showed cell viability of only 15% and 12%, respectively at highest concentration (640 µg/mL). While all remaining extracts (petroleum ether, ethyl acetate, hydro alcoholic and aqueous) of these 2 plants showed 39% to 68% and 48% to 72% viability at 640 µg/mL concentration, respectively for *A. aspera* and *B. diffusa*. Alcoholic extract of *E. littorale* showed 39% viability while remaining extracts showed 51% to 77% viability of cells at highest concentration. Calculation of IC_{50} values of the extracts showed that the alcoholic extract had IC_{50} of 192 µg/mL, 141 µg/mL and 373 µg/mL, for *A. aspera, B. diffusa* and *E. littorale*, respectively. Sorafenib showed 22% viability at highest concentration (20 µM) with 6.68 µM IC_{50} value.

Herbal medicinal products are potential sources of microbial contamination, including highly resistant bacteria and heavy metal contamination. Herbal medicines contaminated with bacteria and heavy metals may spread resistant infections, metal toxicities, or even cancers (227). Even ingestion of heavy metals at low levels, can be harmful (228). The U.S. FDA has defined limits for heavy metals (229). Alcoholic extract of *B. diffusa, A. aspera* and *E. littorale* complied for heavy metal and microbial contamination as per the specified limit.

To confirm the safety of the extracts, acute toxicity study was planned as per the OECD guidelines. Alcoholic extract of all the three plants *B. diffusa, A. aspera* and *E. littorale* were found to be safe up to 2000 mg/kg dose and devoid of any toxicity performed by oral toxicity test as per the OECD guideline 423. This is in concurrence with the literature report indicating safety of the extracts (35, 137, 148, 156).

As alcoholic extract of *B. diffusa* and *A. aspera* has potent cytotoxicity (~50% less IC_{50} value), compared to *E. littorale*, both extracts were selected for subsequent work evaluating in vivo anti-tumorigenic activity with two types of animal models. Although alcoholic extract of *E. littorale* showed less cytotoxicity than other two extracts, it was decided to study its vivo activity in animal models, to understand if the activity correlated with its in vitro potency.

First, in-vivo animal model was developed by chemically-induced carcinogenesis in rats. Chemically-induced HCC consisted of two-step protocol. In the first step, diethylnitrosamine was used as initiator of carcinogenesis, which transforms normal hepatocyte to pre-neoplastic or neoplastic cells by genetic alteration (230). In the next step, two different set of experiments, CCl_4 and 2-AAF were used as promoter for the development of HCC.

In both set of animals there was a significant ($p< 0.1$ to 0.001) change in body weight and elevation of liver and spleen index as well as serum ALT and ALP, as compared to vehicle control group was observed. This is consistent with a significant increase in AST, ALT, and ALP enzyme levels in serum as reported in earlier studies after CCl_4 and 2-AAF administration in rats (197).

We followed protocol according to the literature provided by Mohamed et al. (2019) and Mansour et al. (2010). Consistent with their findings, we observed that rats given DEN treatment had altered serum biochemistry, an unformatted architecture, inflammatory cells lining the central vein, necrosis with infiltration of inflammatory cells, and fibrosis formation without apparent hepatocyte damage was observed with larger nuclear sizes in the liver cells. However, liver architecture was devoid of tumor nodules, high hepatocyte cellular damage, and a rise in serum AFP level, which is usually seen in HCC (199, 231, 232). Thus, prolonged exposure might be required for growth of tumors. Wang et. al, reported the limitations like long induction time, repeated dose of carcinogen and low success rate of chemically-carcinogen models (233).

Indeed, Chemically-induced carcinogensis models have limitations such that the sensitivity to such toxic chemicals might differ in animals vs humans. The chemically-induced model is developed in experimental animals in a short time period and high dose levels compared to humans, wherein the exposure might occur over a long time period and at very low doses. This may lead to differences in genetic and phenotypes of the tumors. Additionally, it is known that apart from tumors, chemicals have an effect on healthy cells and tissues; e.g. they may cause oxidative stress, inflammation, or cell death, all of which might change how a tumor behaves and reacts to treatment (113, 234).

Although in our experiments chemical induction caused initial pathology in livers of the animals as discussed above, however significant tumor formation and growth was not seen. In view of above, the data analysis in our study was mainly focused on HepG2 cell bearing xenograft model.

In humans, HCC is more prevalent in males, as compared to females. Inoculation of HepG2 cells in both male and female mice for xenograft development has been well reported in the literature (7, 213). In our experiments, HepG2-induced xenograft model was standardized with 0.5, 1 and 5 million cells per animal in both males and females. However, in our day-to-day experience as well as the literature indicated that male mice showed aggressive behavior which increased the chances of injury during the study (including after the mice are randomly assigned for treatment), which could introduce confounding variables. Therefore, for xenograft studies female mice were used which showed consistent tumor growth without any comfounder such as aggression observed in male animals (235).

In female, 81% of mice, tumor had grown to 300 mm^3 within 2 week of cell inoculation, while in male mice it had reached to just 100 mm^3 in 27% of mice. Thus, females showed high take rates as compared to males.

Inoculation of 5 million cells led to aggressive tumor growth and mice reached the human end point (1500 mm^3 tumor volume) within 5 weeks of the inoculation. One millions cells caused steady tumor growth pattern throughout the study, which was ideal for requirement of screening of anticancer activity. With 0.5 million cells, the tumor growth was initially slow for 5 weeks of cell inoculation, however following this the tumor growth caught up aggressively in the subsequent one week, thus leading to tumor size almost similar to that of 1 million cells bearing mice.

Initial efficacy studies were conducted in HepG2 cells with all the three extracts using tumor bearing mice inoculated with 0.5, 1 and 5 million cells (see discussion on HepG2 model standardization above). The study showed tumor growth inhibition as compared to control mice across the groups inoculated with 3 cell concentrations. Among the three extracts, *B. diffusa* and *A. aspera* extract showed statistically significant ($p < 0.001$) tumor growth inhibition activity with % TGI of 40% to 46% and 32% to 46%, respectively, across the three cell concentrations at 500 mg/kg dose level. The alcoholic extract of *E. littorale* showed non-significant antitumor activity as compared to vehicle control group, except with 5 million cell concentrations bearing mice with % TGI of 24% to 31% at 500 mg/kg dose across the 3 cell concentrations.

Thus, in line with in-vitro cytotoxic activity, *E. littorale* extract showed less efficacy across the three inoculated cell concentrations in xenograft model, compared to alcoholic extract of *A. aspera* and *B. diffusa*. Hence, further experiments were performed with alcoholic extract of *A. aspera* and *B. diffusa*.

Further selection of these two extracts was done for in vitro and in vivo investigations. To explore the anticancer potential invasion, migration and apoptosis of both the extracts using in vitro experiments were planned with sorafenib as a standard drug.

Cancer patients typically have poor prognoses due to cancer cell migration and metastases (193). In scratch motility assay, *A. aspera* and *B. diffusa* extract inhibited the migration, invasion of cells in scratch area and healed the wound of scratch by only 35% and 45%, respectively as compared to untreated cells, while sorafenib healed the wound area to 29%. ***A. aspera* treatment showed good activity in scratch motility assay. Its activity was similar to that of sorafenib with no significant difference seen in percentage wound healing activity of sorafenib and *A. aspera*** treated cells. On the other hand, sorafenib activity was significantly ($p<0.1$) better than that of *B. diffusa* treated cells.

Cancer cells had high proliferating capacity. In clonogenic cell survival assay cells proliferation capacity was measured by its ability to form cell colonies. The ability of cells to form cell colonies was used to test the proliferative capability of the cells in the clonogenic cell survival assay. In this experiment, treatment of extracts for 2 weeks reduced the colony formation ability of cells by 74%, 68% and 16%, respectively with treatment of *A. aspera*, *B. diffusa* extract and sorafenib. **In all the treatment groups, cells were remarkably hampered for the survival and proliferation.**

Both, the alcoholic extract of *B. diffusa* and *A. aspera* induced early-and late stage apoptosis in Annexin V-FITC and propidium iodide (PI)-induced apoptosis assay. **Exposure of *A. aspera* extract damaged (46% mortality of cells and 21% cells in early apoptosis stage) significantly ($p<0.1$ to 0.01) to the cancer cells, as compared to sorafenib (37% dead cells and 17% cells in early apoptosis stage) treated cells.**

Treatment of alcoholic extract of *B. diffusa* showed similar activity as that of sorafenib. Thus, both the treatments had 16% and 17% cells, respectively in early apoptosis stage.

To understand the mechanism basis of efficacy observed, the alcoholic extract of *A. aspera* and *B. diffusa* were subjected to LC-MS. This led to identification 12 and 14 number of molecules, respectively *in A. aspera* and *B. diffusa* extract

In docking studies, interaction of active constituents with p53 was studied. The active phytoconstituents of the *B. diffusa* boeravinone B and H, and quercetin and *A. aspera* oleanolic acid and kaempferol 3-o glucoside showed a docking score of > -9.0 kcal/mol, as compared to sorafenib which showed a docking score of -10.1 kcal/mol. Thus, it indicates potentially relevant interaction with p53.

The wild-type TP53 protein plays important roles in apoptosis after DNA damage and in cell cycle regulation (236). TP53 mutation is the most common mutation in HCC, and is known to affects the progression and prognosis of HCC. Alterations in TP53 are correlated with serum alpha-fetoprotein (AFP) levels, tumour stage, vascular invasion, tumour differentiation and shorter overall survival (OS) (237). Literature review revealed that p53 expression is involved in the ruling of cell apoptosis during chemotherapeutic drugs treatment (193). Both the extract might produce the apoptosis of cells by interacting with p53 protein. Singh et. al has reported the role of *A. aspera* extract in apoptosis by regulating PKCα signaling pathway and mitochondrial cascade in Dalton's Lymphoma cells (35). In another study, punarnavine from *B. diffusa* has been reported to inhibit NF-kappaB signaling, causing B16F-10 melanoma cells to undergo apoptosis (238).

Next, in the new set of experiments combination studies of the extracts with two dose levels of sorafenib were undertaken in mice xenograft model. The recommended human dose of sorafenib is 400 mg twice daily which is equivalent to 30 mg/kg/day dose in mice. Hence, in combination study, sorafenib was used at 30 mg/kg and at one level low dose of 15 mg/kg in view of its good efficacy without any anticipated toxicity. The extracts were used at 500 mg/kg dose levels.

The extracts were well tolerated during the 21-day dosing period. There was no discernible difference in the average body weight across all treatment groups. On the basis of tumor volume, tumor growth inhibition rate was calculated on day 21. Sorafenib monotherapy caused the TGI of 36.1% and 55.1% at 15 mg/kg and 30 mg/kg doses, respectively. *A. aspera* extract administered alone had TGI of 46.8%, which was increased to 60.6% and 73.4% in combination with sorafenib doses of 15 mg/kg and 30 mg/kg, respectively. Treatments with *B. diffusa* extract administered alone produced the TGI of 38.3% which was augmented to 49.9% and 66.1%, respectively with combination of sorafenib at 15 mg/kg and 30 mg/kg dose.

The second important parameter, T/C ratio was calculated from the tumor weight. As per the NCI criteria T/C ratio of less than 0.42 is considered as the active response of the treatment (221). Both the extracts and sorafenib monotherapy had the T/C ratio greater than 0.42 indicating significant activity as per the NCI criteria.

Combination of *A. aspera* with sorafenib showed the synergistic activity. The T/C ratio was found to be 0.39, 0.24 with combination of *A. aspera* and sorafenib doses of 15 and 30 mg/kg. While monotherapy with *A. aspera* and sorafenib showed 0.51, 0.48 and 0.54 T/C ratios respectively at 500, 15 and 30 mg/kg dose.

***B. diffusa* monotherapy had 0.58 as T/C ratio which was reduced to 0.34 and 0.42 respectively with combination of sorafenib (15 and 30 mg/kg).**

Calculations of tumor volumes also demonstrated that the combination of sorafenib and A. aspera treatment was effective in preventing the tumors from growing. Thus, at **the end of the study (i.e. day 21), the percentage change in tumor volume (as compared to day 1) for *A. aspera* and sorafenib (15 mg/kg and 30 mg/kg) was well below 100% in 2 and 3 out of the 6 mice, respectively.** While only 1/6 mice had below 100% change in tumor volume in sorafenib 30 mg/kg alone treated group.

The *B. diffusa* extract and sorafenib (30 mg/kg) combination caused tumor growth below 200% as compared to its day 1 tumor while in monotherapy of *B. diffusa* extract and

sorafenib (30 mg/kg) where the percentage change in tumor growth was up to 800% and 350%, respectively. This indicated the synergistic activity of the extract to inhibit the tumor growth when administered with sorafenib.

Sorafenib has been reported to induce hepatotoxicity (188). In sorafenib (30 mg/kg) treated group serum ALT level was significantly ($p<0.01$) increased as compared to the vehicle control group. While no significant difference was observed between AST, ALP, total bilirubin and creatinine level between the groups.

Administration of extracts was found safe. The body weight, liver, and renal function (by evaluating the levels of ALT, AST, ALP, Cr, and total bilirubin) did not show any appreciable change. **Indeed, combination treatment did not show increase in ALT. Thus, combination assuages the increase of ALT stimulated by sorafenib.**

Further, to determine the molecular mechanism of anti-cancer activity of extracts, immunohistochemistry and western blot experiments were performed. The Ki-67 protein which is frequently employed as a marker of human tumor cell growth was determined by immunohistochemistry. The proliferation of tumor cells with a high likelihood of metastasis is closely associated with the expression of Ki-67 (239, 240). The proliferation index of Ki67 was determined to be 50% in the vehicle control group. In treatment group Ki67 was determined to be 39%, 36%, 35% and 19%, respectively in *B. diffusa*, *A. aspera*, sorafenib 15 mg/kg and 30 mg/kg treated group.

Combination treatment showed synergistic activity. Thus, Ki67 expression was reduced to 21% and 10% in *A. aspera* with sorafenib (15 mg/kg and 30 mg/kg), and 23% and 15% in *B. diffusa* with sorafenib (15 mg/kg and 30 mg/kg) treated mice.

VEGF is an essential growth factor and signaling molecule in angiogenesis and vasculogenesis. On the other hand, endothelial cells have a high expression of CD31, which is frequently used to monitor the density of vessels in malignant tissue (241). **When *A. aspera* and *B. diffusa* were combined with sorafenib, the VEGF expression was considerably ($p<0.001$) lower than in the group that received vehicle treatment. Microvessel density (MVD) in the tumors measured in terms of %CD31 positive cells was reduced significantly ($p<0.1$ to 0.001) in combination group as compared to vehicle treated group. VEGF and CD31 are well established markers of angiogenesis. These mediators are crucial for the growth and spread of HCC tumors since the HCC tumors are**

extremely vascular in nature. Hence, inhibition of these mediators is an effective strategy in treatment of HCC (242). In our network pharmacology analysis and docking study important phytoconstituents identified by LCMS had a strong VEGF binding affinity.

Phytoconstituents of *B. diffusa* like boeravinone B & H, eupalitin, quercetin, and epicatechin had docking (Vinna) score of -8 to -9 kcal/mol compared to sorafenib which had a docking score of -11.3 kcal/mol. Oleanolic acid of *A. aspera* had a docking score of -9.7 kcal/mol. **This high docking score of these compounds showed high affinity for VEGF, which might be responsible for its anticancer activity by inhibiting angiogenesis in tumor.**

Boeravinone B & H and quercetin of *B. diffusa* had a docking score of -9 kcal/mol for PI3K. **Oleanolic acid and kaempferol 3-o-glucoside of *A. aspera* had a docking score of -10.5 kcal/mol and -9.2 kcal/mol, respectively for PI3K which was comparable to the standard drug sorafenib which had -9.5 kcal/mol score.**

Several constituents of both the extracts demonstrated strong affinity for PI3K which is one of the main therapeutic targets for HCC. Elevated tumor microvessel density, invasive capacity of cancer cells, and elevated PI3K expression are all correlated with the advancement of tumors (243). Both, the extracts and sorafenib monotherapy decreased ERK phosphorylation in western blot experiment and the combination of treatments boosted the inhibitory effect more prominently as compared to single agent treatment. This data is noteworthy since p-ERK is a key marker of sorafenib sensitivity in HCC. Sorafenib mainly targets Ras/RAF/ERK pathway (193). Suppression of ERK phosphorylation is the primary anticancer mechanism of sorafenib (244).

Finally, angiogenesis is another essential mechanism for HCC growth. As per the literature sorafenib down-regulates VEGF pathway and is known to inhibit the angiogenesis. Both the extracts inhibited angiogenesis and their activity was further increased in combination treatment by downregulation of VEGF and CD31 expression. In docking study, both the extract components showed binding affinity for VEGF receptor. Therefore, we hypothesized that the combination of *A. aspera* and *B. diffusa* extract with sorafenib may show better therapeutic efficacy than sorafenib alone. This hypothesis was confirmed both in in-vivo and docking results.

In order to improve the anticancer efficacy and circumvent the resistance that sorafenib often develops within six months of treatment (245), a combination strategy was found in the literature through due diligence. In a double-blind phase II trial, sorafenib with doxorubicin exhibited longer overall and progression-free survival as compared to doxorubicin monotherapy (246). Another study found that the lipid-lowering drug fulvastatin, when combined with sorafenib, substantially inhibited the growth of cancers associated with HCC in vivo experiment (247).

A xenograft efficacy study using a similar methodology to our experiment was conducted and recently published in January 2024 by Tan et al. For the purpose of adjuvant therapy, the researcher demonstrated that ellagic acid potentiates the efficacy of sorafenib at lower doses by triggering apoptosis through inhibition of the MAPK and Akt/mTOR signaling pathways (245). Furthermore, a number of studies have demonstrated the synergy between sorafenib and several bioactive components found in plant extracts, including, resveratrol in grapes, peanuts, and red wine (248) and corosolic acid in *Actinidia chinensis* (249). Literature review showed that roots of *A. aspera* are rich in saponins which might be responsible for its cytotoxic effect (244, 250).

The roots of *B. diffusa* contains alkaloids, rotenoids, flavonoids, amino acids, lignans, ß-sitosterols and tetracosanoic, esacosanoic, stearic and ursolic acids (211). The roots of *B. diffusa* are used in traditional Sri Lankan medicine in poly-herbal formulae for gastric and liver cancers (39).

Literature review suggested that ferulic acid, a phytoconstituents of *B. diffusa,* reduced the cell viability by inducing apoptosis and suppressing metastasis by reversing the epithelial-mesenchymal transition in breast cancer (251). Rutin, a quercetin, aids in reversing multidrug resistance in breast cancer by inhibiting P-gp and BCRP pumps (252). Boeravinone B causes decreased cell survival and apoptosis in colon cancer by internalizing ErbB2 and EGFR receptors (253). Phytochemical screening and LC-MS analysis has shown the presence of saponin, alkaloid, phenolic compound as a phytoconstituent which might be responsible for the anticancer activity of the extract.

As a phytoconstituent of *A. aspera*, chlorogenic acid is widely found in coffee and some fruits like apples, pears, and berries. It works by disrupting the NF-κB/EMT signaling pathway, which prevents breast cancer cells from proliferating, causing apoptosis, and migration (254).

When it comes to evaluating and assessing potential novel anticancer drug candidates, xenograft models are invaluable. In the present study, alcoholic extract of *A. aspera*, *B.*

diffusa and the standard drug sorafenib alone showed a significant reduction in tumor growth compared to control group in HepG2 xenograft model. Furthermore, the tumor growth inhibition was enhanced when each extract combined separately with a low dose of sorafenib. The combination did not exacerbate the adverse effects of sorafenib, rather it tended to reduce it. These results show that combined treatment of each extract with low dose of sorafenib might offer a potential adjuvant therapy for HCC with less adverse effect.

Lastly, the safety and strong anti-tumor, anti-proliferative impact of the sorafenib with extracts combination imply that both the extracts may be investigated further as a possible adjuvant to support sorafenib therapy in HCC.

Though, both, *A. aspera and B. diffusa* extract showed significant early and late stage apoptosis activity as compared to untreated cells, *A. aspera* extract (21%) had significant early stage apoptosis as compared to sorafenib (17%) while *B. diffusa* (16%) had shown no significant activity as compared to sorafenib (Sor). Also, in different parameters of anticancer activity in animal model, *A. aspera* extract (AA) had shown non significant but better activity than that of *B. diffusa* (BD) extract. For inhibition of cancer metastasis by wound healing ability of AA (34%)> BD (45%), % TGI for AA (51%)> BD (45%), T/C ratio AA (51%)> BD (58%), lower T/C ratio was better, Ki67 proliferation index AA(36%)> BD (39%).

In combination group at lower dose of sorafenib (15 mg/kg) with *A. aspera* had better activity with percentage T/C of 39.0 (42.0 with BD and Sor), %TGI of 68.3 (60.0 with BD and Sor), Ki67 proliferation index 10% (15% with BD and Sor), MVD for CD31 1.9% ((2.2% with BD and Sor) than that of sorafenib (15 mg/kg) with combination of *B. diffusa* extract group. Thus, the *A. aspera* extract had non-significant but better activity than that of *B. diffusa* extract.

This work shows that the synergistic use of extracts and sorafenib might help the patients of HCC with the targeted therapy that targets various tumor-specific signaling pathways. Such combination may help to improve the drug resistance and extend the survival with better quality of life.