

Introduction

1. Introduction

Cancer is a disease characterized by the uncontrolled growth and spread of abnormal cells in the body. The human body is composed of trillions of cells. Over a hundred different types of cancer can result from abnormal cell proliferation of any kind of cell. Malignant or benign tumors are the two types of tumors that can arise. A benign tumor stays in its original position and does not invade neighbouring healthy tissue or spread to other sections of the body. A malignant tumor, on the other hand, has the capacity to affect neighbouring normal tissue and metastasis, or spread throughout the body through the lymphatic or circulatory systems. It is appropriate to refer to tumors that are malignant solely as cancers.

In developed countries, cancer ranks no. 1 in terms of cause of death, whereas in underdeveloped nations, it ranks second. Primary liver cancer, which makes up around 4% of newly diagnosed cases and is a leading cause of cancer-related death, is the most prevalent disease diagnosed worldwide (1).

The type of cancer that starts in the liver is called primary liver cancer. Of the various types of primary liver cancer, hepatocellular carcinoma (HCC) is the most common. Intrahepatic cholangiocarcinoma, a bile duct cancer, is the second most prevalent kind of primary liver cancer in adults.

HCC accounts for 85 to 90 percent of instances of liver cancer. The primary causes of HCC include individuals with underlying viral hepatitis-related chronic liver disease, particularly hepatitis B virus (HBV) or hepatitis C virus (HCV), as well as cirrhosis and other non-viral chronic liver disorders (2, 3). HCC is the sixth most commonly diagnosed type of cancer globally and ranks third in terms of cancer-related death. The risk of HCC increases with the number of people with cirrhosis. Cirrhosis is primarily caused by the infection with the hepatitis B and C viruses or by exposure to toxins such as aflatoxin or alcohol. Because prolonged cirrhosis alters the architecture of the liver, it is a substantial risk factor.

The most prevalent kind of cancer in adults is HCC, which affects more men than women. Compared to white individuals, black people are more likely to have HCC. Globally, HCC ranks 5th in incidence among males and 7th in women.

1.1 Epidemiology

The incidence of HCC varies geographically throughout the world. In Asia and sub-Saharan Africa, where the virus is common and many people are infected from birth, the majority of

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hepatitis B infections are reported. A rise in hepatitis C virus infections is contributing to an increase in the incidence of HCC in the United States and other emerging nations.

The alarming global increase in incidence of HCC results in one million deaths from the disease each year (4). Because men are more prone than women to smoke, drink, and develop cirrhosis, it is possible that men will experience a higher incidence of HCC (5). Furthermore, the male predominance of HCC suggested a role for testosterone and androgen receptor signaling. Studies conducted on humans have shown a strong association between raised testosterone levels and an increased risk of HCC (6).

1.2 HCC therapeutics

HCC treatments can be divided into three categories: curative treatments, locoregional treatments, and systemic chemotherapy.

1.2.1 Curative treatments

For early stage HCC, the only first line curative options are surgical resection (tumor removal) and liver transplantation (7). Surgical resection is a preferred curative treatment option reserved for patients with single nodules and adequate hepatic function. It has good long-term survival rates for intermediate or advanced HCC in the absence of portal hypertension, a condition that independently predicts poor prognosis. This treatment option may become important as the incidence of HCC increases while the availability of sufficient livers for transplant diminishes (8).

Liver transplant is another curative option and is recommended in patients with HCC who have decompensated cirrhosis. To qualify for liver transplant, patients must have tumors that fall within the Milan criteria (i.e., a solitary tumor up to 5 cm in size or 3 tumors up to 3 cm in size). The recurrence-free survival rates were greater than 90% in liver transplants (9).

The majority of patients are diagnosed with advanced stages of the disease. So such patients diagnosed with advanced stages of HCC are no longer eligible for surgical resection or liver transplant.

1.2.2 Locoregional treatments

Locoregional treatments are minimally invasive, image-guided treatments for liver cancer. Percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), microwave ablation

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(MWA), cryoablation, and transarterial chemoembolization (TACE) are the major modalities utilized to control the growth of HCC lesions. This therapy falls under the locoregional treatments.

PEI is one of the oldest image-guided modalities and involved direct injection of ethanol into the lesion, which induces tumor necrosis. Initially, PEI was considered a first-line therapy for small HCC lesions. But after the introduction of RFA, it was preferred over PEI due to its adverse effects, like postprocedural pain, and typically requires multiple sessions to achieve a complete treatment effect and has a high recurrence rate (10).

In RFA, a probe is inserted percutaneously or laparoscopically under ultrasound guidance, and high-frequency alternating current is applied to the tumor. The radiofrequency waves generate thermal energy, leading to tumor necrosis.

MWA is beneficial for tumors smaller than 3 cm. Under ultrasound guidance, a tiny needle is injected into the tumor during MWA. By using a microwave, the temperature is raised, causing necrosis and the destruction of the liver tumor. One possible substitute for RFA is MWA (11).

Cryoablation has become less prevalent as more advanced ablative techniques have emerged, while it is still helpful in some circumstances. Laparoscopy is required in order to apply liquid nitrogen or argon gas and a cryoprobe directly to the HCC lesion. Freezing causes tissue damage that cannot be reversed. Usually, two or three cycles are completed in a single session, and real-time tumor removal is tracked using intraoperative ultrasound. Cryoablation is a treatment modality that can be utilized alone or in conjunction with other therapies.

TACE has emerged as one of the most popular HCC treatment techniques, with applications ranging from primary therapy to tumor downstaging and bridging to the transplantation method (12). TACE involves percutaneous hepatic artery catheterization, which targets the feeding branches of the tumor that supply nutrients and aid in tumor shrinking (13).

TACE is associated with significant post-treatment complications seen in up to 90% of patients. For this, the patient needs to be hospitalized for observation and symptom management. During TACE, there is a temporary elevation of bilirubin and aminotransferases, as well as ileus, pain in the right upper quadrant, and nausea (14).

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Transarterial radioembolization (TARE) is a newer treatment modality for HCC and involves selective catheterization of hepatic arteries with infusion of radioactive material into the feeding artery to induce tumor necrosis.

Irreversible electroporation (IRE) is a unique treatment that utilizes electrical pulses to create pores in the cellular membrane, leading to apoptosis and cell death. With this approach, there is minimal damage to surrounding parenchyma and vascular structures (15).

1.2.3 Systemic chemotherapy

Systemic chemotherapy is reserved for patients with advanced HCC or Barcelona clinic liver cancer (BCLC) stage C or D, and includes traditional agents such as doxorubicin, gemcitabine, and oxaliplatin. The first oral chemotherapeutic agent proven to have survival benefit in HCC is sorafenib (Nexavar, Bayer), an oral multikinase inhibitor, which is approved by the US Food and Drug Administration (FDA) as a first-line therapy for advanced HCC (16).

1.3 Medicinal plants with anticancer activity

Medicinal plants are a gift from nature that helps people live healthy, disease-free lives. It is essential to maintaining our health. Plants and plant-derived products have long been utilized as a source of medicine. The World Health Organization (WHO) reports that traditional plant-based medicines represent the primary source of healthcare for over 80% of the world's population, the majority of whom live in developing and impoverished nations (17). As medicinal plants are a significant source of medicinally valuable biodynamic chemicals (18), having low cost with fewer side effects, demand for them has been raised in global trade for an alternative or adjuvant to allopathic treatments for a variety of diseases.

India is among the nations with the most diverse medical and cultural traditions, and the use of medicinal herbs is still highly appreciated as part of an ancient practice. The Rigveda, which was composed between 4500 and 1600 BC, contains the first record of the use of herbs as medicine (19). Western civilization caused our traditional natural healing method to vanish during the British era. It is now resurfacing as a result of awareness of its significance in treating illnesses without causing negative side effects. A growing body of research indicates that the demand for medicinal plants is rising due to the worldwide trend towards an enhanced "quality of life." (20). Hence, the medicinal plant use has extensively increased, both directly in the traditional medicinal system and indirectly for the preparation of pharmaceutical aid (21).

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The three primary traditional medical systems practiced here are Ayurveda, Unani, and Siddha. Ayurveda includes references to using plants to treat a variety of cancers. In Ayurveda, the tumor known as "Arbuda" or "Gulma" and liver cancer are referred to as "Yakrut Udara" and "Yakrit Gulma." The traditional Indian medical system (Ayurveda) and ethnomedical practices use a variety of medicinal herbs and their preparations for liver diseases (22).

Natural products found in dietary fruits, vegetables, and spices inhibit cancer development and stimulate mechanisms related to disease prevention by activating antitumor, antiproliferative, anti-inflammatory, and antioxidant systems. As an anti-cancer therapy, the properties of natural products to selectively regulate enzyme activities leading to carcinogenesis are very important. Certain classes of natural products, such as alkaloids, phenols, fatty acids, quinones, terpenoids, flavonoids, etc. are considered to be selective inhibitors of tumor-cell invasion and migration, induce apoptosis, and suppress angiogenesis and proliferation of cancer cells.

These natural compounds originating from plants provide many benefits and a variety of targets and molecular pathways; however, their low solubility and poor bioavailability limit their usefulness for clinical use. Thus, the development of novel therapies with high efficacy and minimal side effects is imperative for the prevention and treatment of liver cancer.

Numerous studies have demonstrated the significant potential benefit of using natural substances in conjunction with chemotherapy to treat liver cancer.

Combination therapy has the potential to both lessen adverse effects and boost the effectiveness of the treatment. Certain natural compounds have the potential to increase susceptibility to conventional cytotoxic therapy, strengthen the medication's effect, reverse drug resistance, heighten the combined effect of both delivered medicines, or particularly target tumor cells with cytotoxic effects. Hence, the present study is planned to investigate the effect of plant extracts in hepatocellular carcinoma (23, 24).

1.4 Rationale

HCC 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.**

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However, there are effective treatments for early-stage disease. Resection, transplantation, and local ablation of small lesions are potentially curative therapies that reduce 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, a 30% long-term cure rate with resection and a 70%-80% cure rate with transplantation. Still, it does not translate into a decrease in overall HCC-related mortality due to diagnosis of disease in advanced stages (25).

The last decade has witnessed substantial advances in screening, diagnosis, staging, and treatment of HBV, HCV, and HCC. These developments have improved outcomes at the patient level (26). However, whether they have translated to a reduction in liver disease burden at a population level is less clear. HCC prognosis remains poor even after the receipt of potentially curative treatments. Thus, the population-level benefit of improved HCC management on survival requires ongoing evaluation.

Anticancer therapy is not without risks and limitations. In HCC, the first line drug sorafenib is associated with rash, hand-foot syndrome, and 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 patients is 3-4 months.

As the existing therapy is associated with side effects and drawbacks, it becomes the need of an hour to search for new cancer chemotherapeutic agents that are effective and non-toxic. HCC is typically caused by liver damaged-cirrhosis. Thus, hepatoprotective drugs are effective in HCC.

Natural agents are safe and could overcome the resistance produced by the pathogens due to the presence of multiple phytoconstituents that have different mechanisms 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 (77).

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

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stabilizing by mechanisms that include up-regulation of the key apoptotic regulators, modulation of cell cycle arrest and improvement of DNA content by free radical scavenging, the ant mutagenic 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 (28).

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 (29). All this data from the literature encourages the use of natural products as adjuvant products along with the synthetic compound.

1.4.1 *Achyrathes aspera* Linn. Family, Amaranthaceae is known by different names, such as Chirchita, and Apamarga in Hindi. All the parts of the plant such as seeds, roots, and shoots are used in traditional systems of medicine. The tribal, rural and aboriginal people of our country have been using this herb since ancient times for various disorders.

The dried plant is used in abdomen diseases, hemorrhoids, itching, and obesity. The dried root of the plant is used in vomiting, itching, lymphadenitis, tumor, bhagandara (fistula-in-ano), heart disease, pyrexia, leukoderma, deafness, abdomen diseases, disorders of the liver (30), tooth disease, and blood disorders.

Achyrathes aspera is used for the management of various diseases such as malaria, dysentery, sinuses, asthma, piles, night blindness, hypertension, and diabetes.

The leaf extracts of the plant have shown antioxidant, diuretic, antidepressant, hepatoprotective, wound healing, and cancer chemo preventive effects. Other than leaves, roots of *A. aspera* possess anti-inflammatory and immunomodulatory effects (31).

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

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many more are reported in the literature. *Achyrathes aspera* plant is used as anticancer therapy from ancient times by ayurvedic medical practitioners in India. (32).

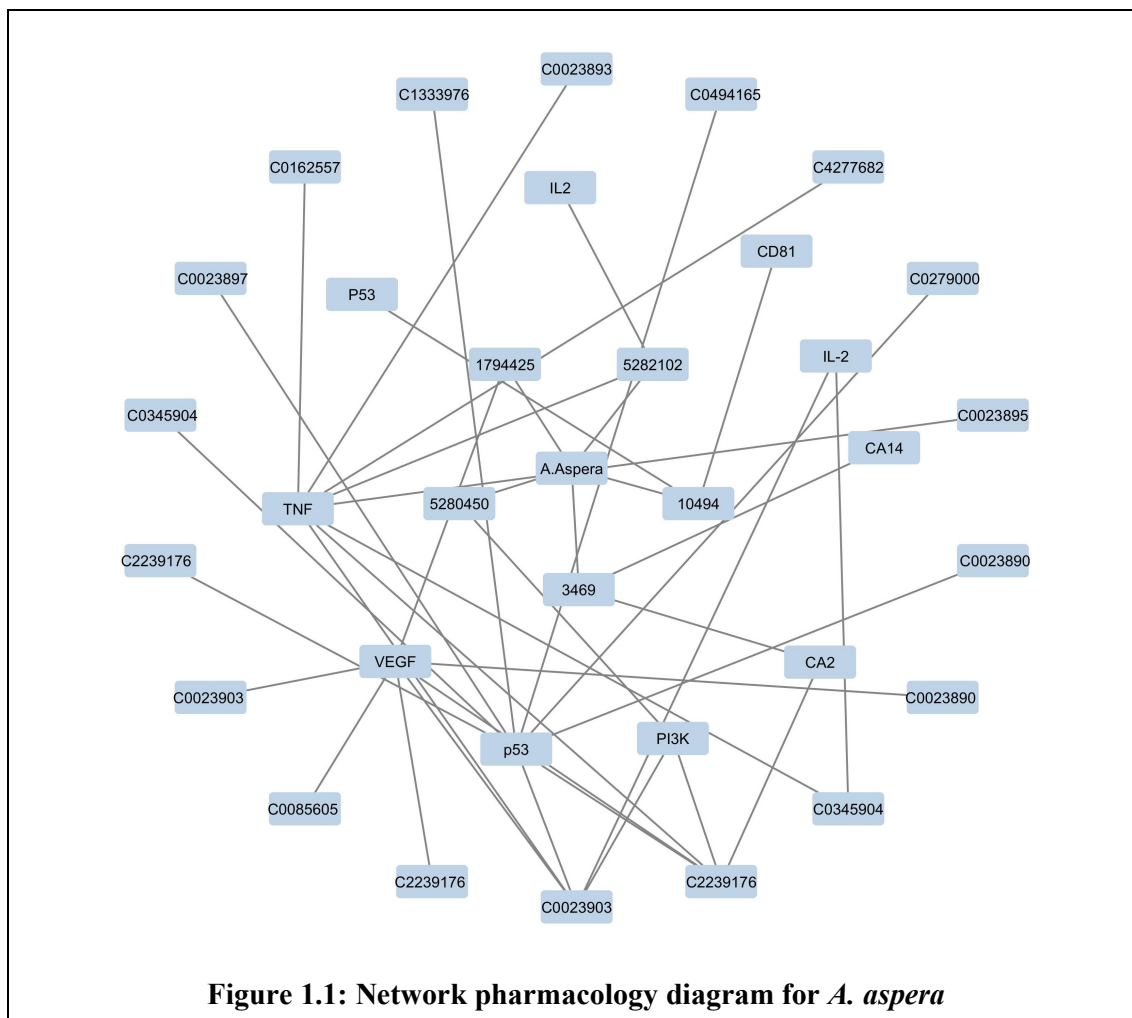
The species has cooling, pungent, mild astringent, antiperiodic, digestive, purgative, laxative, and abortifacient properties. The paste of the root is given to stop bleeding after abortion and to facilitate delivery and stimulate labor pain. The decoction of the leaves is used in the early stages of diarrhea and dysentery. The paste of the leaves is externally applied over bites of poisonous insects, wasps, bees, and burns. 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 (33).

Different marketed formulations like Apamarga Churna, Livol, stone hills etc. contains *A. aspera* as an important ingredient and are mainly used for hepatitis, cirrhosis of the liver, diuretics 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 (32) Methanolic extract showed pronounced anticancer activity in skin cancer. The plant extract showed good anticancer activity in the Raji cell line. Pharmacological studies have demonstrated that whole-plant possess anti-asthmatic, anticancer, anti-diabetic, antioxidant, nephroprotective, and wound healing properties (34). Singh et. al. (2021), have studied *A. aspera* methanolic leaf extract in mice which inhibited the proliferation of Dalton's Lymphoma cells by inducing apoptosis (35). Cell progression is inhibited by a marked reduction in expression of p-PKC α , p-AKT, p-GSK3 β , Bcl2 and upregulation in Bax. *A. aspera* had shown hepatoprotective activity by alleviating oxidative stress in a chemically (NDEA and CCl₄)-induced HCC model (36).

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.

Based on the literature different phytoconstituents of *A. aspera* were screened for target network analysis and based on interaction relationship for liver cancer compound-target network was constructed. The nodes represented compounds or targets, and edges represented interaction relationships. The *A. aspera* network contains 34 nodes and 40 edges for liver cancer and their network pharmacology is shown in Figure 1.1. The analysis identifies significant interactions between various proteins and compounds, including IL2, CD81, CA2 and 14, **p53**, **PI3K**, TNF, and **VEGF**.

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1.4.2 *Boerhaavia diffusa* Linn. family Nyctaginaceae commonly known as ‘Punarnava’. It is a traditional herb (creeping weed) which has been proposed for the treatment of cancer, 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 (37).

The plant is reported to possess anti-inflammatory, hepatoprotective and diuretic activity. The root is generally used as an infusion to treat 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, alkaloids and amino acids which have been reported to exhibit strong antioxidant properties. It also contains quinolizidine alkaloids

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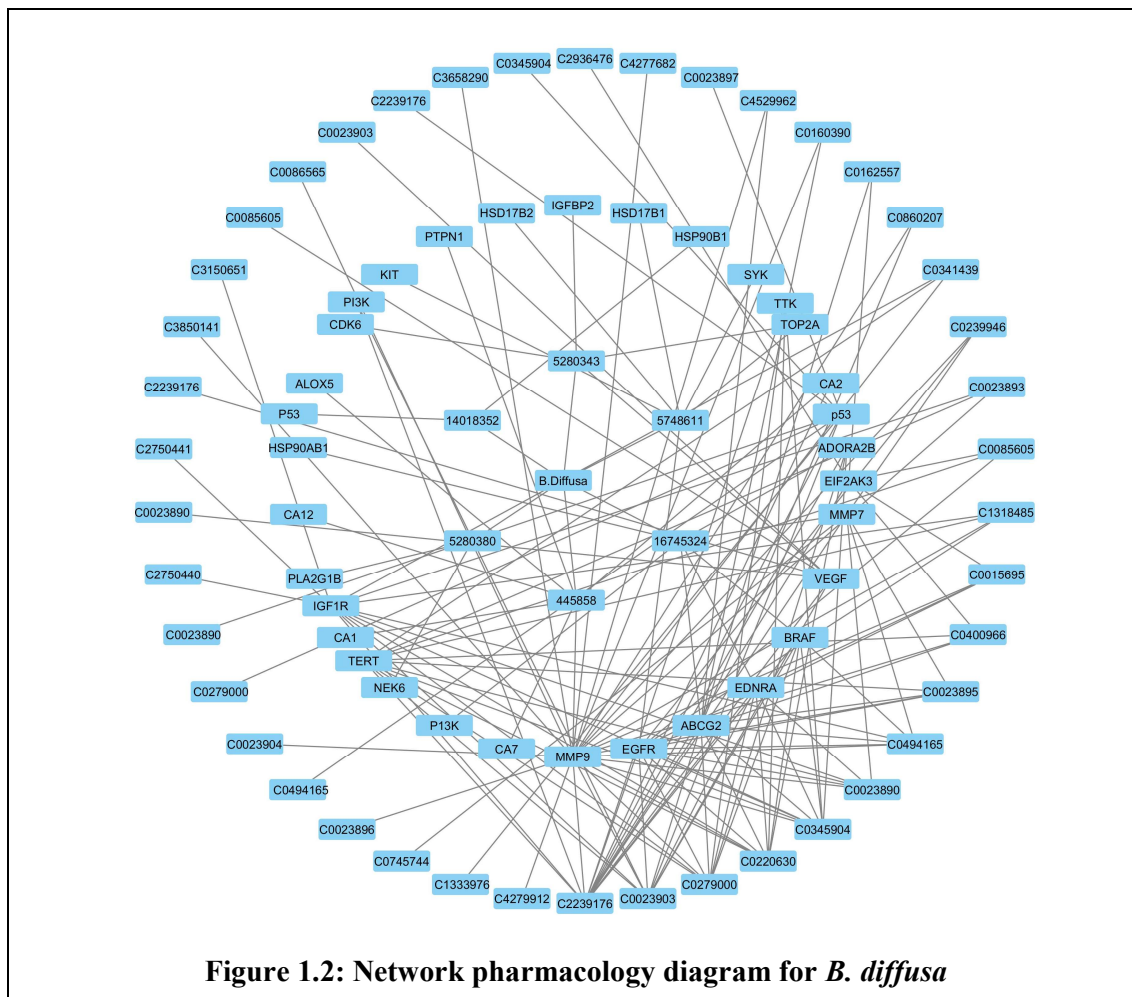
and potassium salts. This plant is also used for liver disorders, asthma, skin diseases, snake bites, inflammation and heart diseases.

In India, a number of tribes use the roots of *B. 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 (38). Punarnavadyarishta and Punarnavasava are the ayurvedic 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 (39). Whole plant's ethanolic extract is used in human cervical cancer (40). Methanolic leaf extract is effective against MCF-7 cell line. Ethanolic extract of leaves has been evaluated against Dalton's ascitic lymphoma (DAL) (41).

Roots of the plant were found to have antiproliferative effects on Hela cancer cells acting through apoptosis pathway by triggering caspase 3/9 (42). 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 (43). 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 (44). This plant is also used for liver disorders, asthma, skin diseases, snake bites, inflammation and heart diseases. Flavonoid, phenolic compounds and rotenoids in *B. diffusa* showed anticancer activity by targeting apoptosis pathways and, PI3K-Akt pathway

A network pharmacology analysis was performed for *B. diffusa* phytoconstituents. On the basis of interaction relationship with liver cancer pathways, it showed 82 nodes and 158 edges. The network pharmacology diagram of *B. diffusa* for liver cancer is shown in Figure 1.2. The analysis identifies significant interactions between various proteins and compounds, including KIT, PTPN1, HSD1782, HSP, TTK, CDK6, ALOX5, **p53**, TERT, **PI3K**, EGFR, BRAF, **VEGF**, MMP7, and SYK.

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1.4.3 *Enicostemma littorale* family- Gentianaceae also called as Chota chirayata in Hindi, Mamejovo in Gujarati, and Vellarugu in Tamil. It has been used traditionally for many diseases. This plant, which grows all over India, has long been utilized for treating rheumatism, ulcers, hypoglycemia, and insect poisoning. It has been discovered to be effective against tumors, inflammation, and hyperglycemia (45).

In traditional medicine, the plant is used to treat a variety of conditions, including rheumatism, peptic ulcers, diabetes mellitus, hernias, swelling, itching, and insect poisoning. It inhibited carrageen-induced edema and its anti-inflammatory activity is comparable to that of hydrocortisone. Studies conducted ethnomedically in North Gujarat have shown that *E. littorale* hot aqueous extract is used by the tribal inhabitants for the treatment of diabetes, fever, stomach pain, dyspepsia and malaria. In vivo and in vitro antimalarial activity has been shown by the root extract. This herb is also known for its hypoglycemic, antioxidant and

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hypolipidemic potential in newly diagnosed non-insulin dependent diabetes mellitus (NIDDM) patients (46).

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. (47). Alcohol extract of whole plant are hepatoprotective in CCl₄-induced hepatic damage in rats. Anticancer activity of this plant has been evaluated for Dalton's ascetic lymphoma (48). 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 (49).

Network pharmacology analysis with *E. littorale* phytoconstituents for liver cancer showed 69 nodes and 89 edges. Interaction for phytoconstituents and proteins in network pharmacology for liver cancer is shown in Figure 1.3. The analysis identifies significant interactions between various proteins and compounds, including CDK9, ADK, **P53**, **VEGF**, CA2, **PI3K**, MMP9, ESR1, FLT3 and ALOX5.

1.5 Aim/ Objectives:

Title: Pharmacological Evaluation of Some Medicinal Plants in Hepatocellular Carcinoma

Objectives of the planned study are as follows:

1. To perform network pharmacology of *A. aspera*, *B. diffusa*, and *E. littorale* for target identification
2. To collect, authenticate and herbarium preparation of *A. aspera* (roots), *B. diffusa* (roots), and *E. littorale* (whole plant parts)
3. Extraction of plant material and preliminary phytochemical screening
4. To evaluate the cytotoxic potential of various extracts (petroleum ether, ethyl acetate, alcoholic, hydro-alcoholic and aqueous) of *A. aspera* (roots), *B. diffusa* (roots) and *E. littorale* (whole plant parts) on HepG2 cells
5. To evaluate the effect of alcoholic extracts of *A. aspera*, and *B. diffusa* on HepG2 cell migration ability
6. To evaluate the impact of alcoholic extracts of *A. aspera* and *B. diffusa* on HepG2 cells colony formation ability
7. To evaluate the effect of alcoholic extracts of *A. aspera* and *B. diffusa* on HepG2 cells apoptosis activity
8. To analyze the chemical components of *A. aspera* and *B. diffusa* extract by LCMS method
9. To perform molecular docking for phytoconstituents of *A. aspera* and *B. diffusa*
10. To evaluate the safety of alcoholic extract of *A. aspera*, *B. diffusa* and *E. littorale*
11. Development and standardization of different animal models of HCC
12. To evaluate the activity of plant extracts by in-vivo xenograft model
 - Standardization of animal models for different cell concentrations
 - Standardization of animal model for cell growth pattern in male and female mice
13. To evaluate efficacy of plant extracts in combination with a reference standard