Abstract

Aim: To evaluate in vitro and in vivo anticancer activity of *A. aspera*, *B. diffusa* and *E. littorale* in hepatocellular carcinoma (HCC)

Introduction: HCC is one of the most common forms of liver cancer, accounting for 85% to 90% of cases, which is the leading cause of cancer-related death globally. Currently available drugs like sorafenib and regorafenib slightly increase the overall survival rate of HCC. However, these medications have a number of limitations, such as severe side effects and drug resistance. Hence, there is an urgent need for alternative or adjuvant therapy to currently available treatment options. In Ayurvedic pharmacotherapy *A. aspera, B. diffusa* and *E. littorale* are well known medicinal plants used in traditional medicine system for various liver disorders and other cancers. Numbers of Indian tribes are using these plants as a remedy for liver disorders. Additionally anticancer activity of the selected plants was studied in breast, oral, lung, and cervical cancer. In our initial study in network pharmacology analysis, interactions were found between plant phytoconstituents and various proteins of key pathways of cancer. Thus, employing HepG2 cell lines in in-vitro and tumor model, the current work is set out to ascertain the anticancer properties of selected plant extract.

Material and Methods: Initially phytochemical screening of the plant extracts was perfomed following which in-vitro anticancer activity of plant extracts undertaken. LCMS analysis was performed and interaction of phytoconstituents with relevant proteins involved in HCC was done using Network pharmacology analysis and molecular docking. For cytotoxic activity extracts were evaluated in HepG2 cells using MTT. Next, scratch motility, colony formation assay and Annexin V/propidium iodide apoptosis assay was done by FACS. Sorafenib was used as reference standard drug for both in vitro and in vivo experiments.

For assessment of in vivo activity of the extracts, chemically-induced HCC in rats and HepG2 cell line-bearing tumor models were standardized. Two chemically-induced models were developed. In the first model, DEN and 2-AAF were given to rats, and different parameters such as animal body weight, serum enzyme levels of ALT, AST, ALP, creatinine, total bilirubin, and histopathology of the liver were performed.

In the second chemically-induced model, DEN and CCL_4 were administered to rats, and different parameters mentioned in the first model along with serum AFP analysis were performed.

Then the HepG2 tumor cell-bearing mice model was standardized with three concentrations of HepG2 cells in male and female mice. In our next studies, anticancer activity of extracts alone and in combination with the standard drug sorafenib was performed. Mice were monitored for body weight, tumor volume, and any clinical symptoms. Post completion of the 21-day dosing schedule, blood was collected, and serum ALT, AST, ALP, total bilirubin, and creatinine were analyzed for assessment of hepato-renal toxicity. Tumor volume-related parameters like percentage tumor growth inhibition (% TGI), % T/C, and percentage change in tumor volume (on day 21 as compared to day 1) were calculated. Tumors were dissected from mice, weighted, and appropriately stored for CD31 and Ki67 analysis by immunohistochemistry and VEGF, p-ERK, and t-ERK by western blot technique. Moreover, plant extracts were evaluated for an acute toxicity study (limit test) as per the OECD guidelines.

Results: Selected plant extracts showed a variety of phytoconstituents identified by proximate tests, and compounds were confirmed by LCMS analysis. In Network analysis, identified phytoconstituents showed affinity for proteins such as p53, VEGF, and PI3K. These proteins are known to play important role in growth and proliferation of cancer cells. In network pharmacology analysis it was found that these are the common targets of all three plants.

In cytotoxicity assay, the alcoholic extracts of three plants showed promising anticancer activity against the HepG2 cells as compared to petroleum ether, ethyl acetate, hydroalcoholic, and aqueous extracts. All the three plant alcoholic extracts complied with heavy metal and microbial contamination as per the specified limit by the regulatory agencies. Further alcoholic extract was found safe in acute toxicity study.

Two chemically induced animal models were developed as per the literature. In both the animal models, an increase in body weight, liver and spleen index, and elevation of serum ALT, AST, ALP, total bilirubin, and creatinine levels were observed as compared to control (vehicle-treated) group rats. In histochemical analysis, necrosis with infiltration of inflammatory cells, fibrosis formation without apparent hepatocyte damage was observed, but no significant nodule formation occurred. Hence, based on the chemically-induced HCC model experiment, it was concluded that for the development of HCC, prolonged exposure to chemicals was required.

In vivo studies the HepG2 cell-bearing mice model was standardized with 0.5, 1, and 5 million HepG2 cells per animal in male and female mice. The female mice had shown good

tumor development as compared to male mice. The cell inoculation with 5 million cells led to aggressive tumor growth, and 1 million cells showed less aggressive growth, which is the optimum requirement for screening of anticancer activity. All three extracts showed tumor growth inhibition/anticancer activity as compared to the disease-control mice across the 3 cell concentrations. Based on this study, two extracts with better activity were selected for further study. In a new set of experiments, anticancer activity was performed with extract, sorafenib, and their combination in tumor-bearing mice. On the basis of tumor volume and percentage tumor growth inhibition, extract alone and in combination with sorafenib showed good anticancer activity without affecting animal body weight or serum enzyme level.

Serum enzyme level (indicative for hepatotoxicity), VEGF and p-ERK expression level, Ki67 (proliferation marker), and CD31 (indicative of mean vascular density of blood vessels) were significantly decreased by the extracts. These effects were further intensified with the combination treatment approach using sorafenib as a standard drug Additionally, the extracts showed reduction of hepatotoxicity induced by sorafenib, in combination treatment. 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.

Conclusion: The alcoholic extract of *A. aspera* and *B. diffusa* suppressed the cancer-proliferating (Ki67) cells, angiogenesis (VEGF and CD31), and enhanced apoptosis in HepG2 cells and showed anti-cancer activity. Compared to extracts and sorafenib monotherapy, combined treatment yielded a better therapeutic outcome. As a result, the extract may be utilized in combination or as an adjuvant therapy with the existing standard-of-care for hepatocellular carcinoma, albeit at a reduced dose.