DEN-Induced Hepatocellular Carcinoma in Animal Model

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K. Sivakumari, P. Janani, and S. Rajesh

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K. Sivakumari (✉) · S. Rajesh
Department of Zoology, Presidency College, Chennai, India

P. Janani
Department of Medicine, Stephenson Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

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S. Pathak et al. (eds.), *Handbook of Animal Models and its Uses in Cancer Research*,
https://doi.org/10.1007/978-981-19-3824-5_23

Abstract

Cancer is a major cause of death both in developed and developing countries. Among the various types of cancers, primary liver cancer represents about 4% of all cancers worldwide. Hepatocellular carcinoma (HCC) is a histological type of liver cancer; the fact being that the aspects related to the development of hepatocellular carcinoma and its metastasis are not yet known, and here animal models play an important role in diagnosis, prognosis, and treatment strategies of the disease. Animal models also provide an opportunity to explore new treatment strategies. N-nitroso compounds mainly N-diethylnitrosamine (DEN) is a hepatic carcinogen, which is well known to cause liver necrosis. The current review is based on research and review of works on animal models treated with DEN-induced hepatocarcinogenesis. Despite ongoing debate, animal models could provide valuable information about biotransformation of toxicants and how they worsen the damaging effects on DNA and cell proteins that result in the development of cancer. Today, the emergence of various therapies that target the immune system and the tumor microenvironment emphasizes the importance of the host, conditions of chronic inflammation, and fibrosis. Thus, the use of animal models for anti-HCC drug screening will find our best ability to successfully discover new drugs to combat HCC.

Keywords

Liver cancer · HCC · DEN · Herbal drugs

Introduction

One of the leading causes of cancer-related fatalities worldwide is hepatocellular carcinoma (HCC), a prevalent form of liver illness. People with chronic liver diseases, such as cirrhosis caused by hepatitis B or C infection, severe drinking and diabetes, obesity, and non-alcoholic fatty liver disease are more likely to develop HCC (Balogh et al. 2016; Manimekalai et al. 2016; Rajesh et al. 2016; Hemalatha et al. 2020; Flora Priyadarshini et al. 2020; Rajesh and Sivakumari 2020; Angalammal et al. 2021; Padmavathy et al. 2021). The incidence of HCC is higher in men than in women (2.4:1), with higher incidence in East and South Asia, Central and West Africa, Melanesia, and Micronesia/Polynesia (Ferlay et al. 2010). According to Altekruse et al. (2009), the rate of HCC among Native Americans and Alaskan Indians has increased from 1.6/100,000 to 4.6/100,000 people followed by Blacks, Whites, and Hispanics (Altekruse et al. 2009).

To study the efficacy of drugs against HCC, chemicals are used to induce HCC both in vitro in cell lines and in vivo in animal models. N-nitroso compounds are well-known hepatic carcinogens that cause liver necrosis, especially N-diethylnitrosamine (DEN) (Tricker et al. 1991). N-nitrosamines are known to cause varieties of tumors in several animal models, and are also known to cause

health hazards in humans too (Piot and Sirica 1980; Simonsen and Uirji 1984). These chemicals and their precursors can be present in the environment, in specific workplaces, in foods like meat and dairy products, in tobacco, pharmaceutical and cosmetics items as well as endogenously generated in human body from dietary components (Shank 1975; Bartch and Montesano 1984). Because of this, DEN promotes oxidative stress and cell damage due to increased reactive oxygen species (ROS) generation (Bartsch et al. 1989). By creating free radicals, the cytochrome P450-dependent monoxide system's enzymes increase oxidative stress by producing hydrogen peroxide (H_2O_2) and superoxide anions (Farber and Gerson 1984). The most harmful products of cellular metabolism are reactive oxygen species (ROS), which have a direct impact on cell development, proliferation, and its survival in cancer development. As liver is the primary metabolic biotransformation site for DEN, oxidative stress produced by liver injury may be generated by ROS generation in the liver (Gey 1993). Lipid peroxidation (LPO) is a measure of cell damage caused by ROS (Spiteller 1996). The liver, on the other hand, has a powerful antioxidant system that prevents ROS from causing damage to essential bio-molecules like lipids, proteins, and deoxyribonucleic acid when they are exposed to oxidative stress.

Several research works have been reported the hepatotoxic and carcinogenic effects of DEN (Schmahl et al. 1960; Druckrey et al. 1967; Dhanasekaran et al. 2009; Janani et al. 2009, 2010; Khan et al. 2017; Nithya 2021). In 1963, a study found that giving DEN to rats caused N7 atomic ethylation in nucleic acid guanines in the liver (Magee and Lee 1963), which was a key step toward understanding the chemical mechanisms behind the carcinogenic impact of DEN. A pathway that depends on cytochrome P450 enzymes like CYP2E1 connects the biotransformation of DEN and DMN (dimethylnitrosamine) to alkylating metabolites that result in the production of a DNA adduct (Yang et al. 1990; Verna et al. 1996). CYP2E1 is essential for the bio-activation of nitrosamines, according to studies done on CYP2E1 null mice (Kang et al. 2007). These mice had considerably fewer and smaller tumors, according to observations. After DEN treatment, these mice had a significant drop in tumor size and repetition compared to wild animals. Because of nitrosamine's carcinogenic qualities, it's becoming increasingly popular to utilize these chemicals, particularly DEN, to induce liver tumorigenesis in mice as a test model for human hepatocarcinogenesis (Kang et al. 2007).

Plants and their derivatives have long been recognized as efficient and versatile chemopreventive treating agents for various malignancies. Medicinal plants have been utilized for treating and preventing several diseases, as well as for the promotion of good health, since antiquity. Anticancer therapy has progressed significantly, as a result of medicinal plant-derived drug research, which has resulted in considerable advancements in anticancer therapies. India is known as the "Medicinal Garden of the World" because of the vast quantity of medicinal plants nature has bestowed upon us. In the armory of modern medicine, the drugs manufactured from phytocompounds or medicinal plants have been investigated for their efficacy against specific diseases, so that they would be valuable therapeutic agent in modern medicine.

Effects of Herbal Medicine Against DEN-Induced HCC

Curcumin

Chuang et al. (2000a) looked into the effect of curcumin on DEN-HCC mice model. Curcumin was demonstrated to be a potential inhibitor of DEN-induced hepatocarcinogenesis in C3H/HeN mice. p21 (ras) levels, nuclear antigen (PCNA) expression, and CDC2 protein levels increased significantly in DEN-treated mice's hepatic tissues, but curcumin decreased the levels of all these biological indicators (Chuang et al. 2000a). Curcumin might also significantly inhibit liver inflammation induced by DEN and hyperplasia in rat HCC model, according to Chuang et al. (2000b). The oncogenic p21 (ras), p53 proteins, PCNA, cyclin E, factor NF-, and p34 (cdc2) proteins were likewise suppressed by curcumin, but not Cdk2, c-Jun, and c-Fos, as revealed by immunoblotting studies (Chuang et al. 2000b). Curcumin's antioxidant, anti-inflammatory, and apoptotic potential in HCC models in vitro as well as in vivo, as well as its role in multiple molecular signaling mechanisms, have all been well documented (Table 1). The potential challenges, viz., the bioavailability, drug delivery, pharmacokinetics of curcumin in HCC, and the lacunae in its clinical studies have also been reviewed (Darvesh et al. 2012).

Terminalia arjuna

In male Wistar albino rats, the antioxidant potential of *Terminalia arjuna* bark ethanolic extract (EETA) against DEN-induced liver cancer was investigated by Sivalokanathan et al. (2006). Induction of liver cancer by DEN (200 mg/kg) was followed after 2 weeks by Phenobarbital (PB) for cancer promotion up to 14 weeks. Then, the HCC bearing rats were fed with EETA extract (400 mg/kg) and the samples of serum, liver and kidneys were collected for biochemical analysis. LPO levels such as H_2O_2, ascorbate, and $FeSO_4$ were estimated in serum, liver, and kidney of control and DEN treated rats. Likewise, non-enzymatic antioxidants like Vitamin C (Vit-C) and Vitamin E and enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Vit-E) were also assessed. With DEN therapy, LPO levels increased significantly, but enzymatic and non-enzymatic antioxidant levels declined. In DEN-treated rats, EETA administration at a dose of 400 mg/kg dramatically improved these changed enzyme levels. As a result, EETA's protective impact was linked to DEN-induced LPO inhibition and antioxidant enzyme levels being maintained (Sivalokanathan et al. 2006).

Annona squamosa

In DEN-induced Swiss albino mice, Raj et al. (2009) investigated the hepatoprotective effects of custard apple (*Annona squamosa*). Total protein, GOT, GPT, ACP, ALP, AFP, Total bilirubin and Direct bilirubin in serum and liver along

Table 1 Effects of herbal products against DEN-induced HCC

Herbal products	Animal model	Selected for the study ages/weight	Treatment duration	Observations	References
Curcumin (*Curcuma longa*)	C3H/HeN mice	5 weeks	48 weeks	Curcumin reversed the increased levels of p21 (ras), PCNA and CDC2 proteins to normal values in hepatic tissues.	Chuang et al. (2000a)
Curcumin (*Curcuma longa*)	Male Wistar rat	4 weeks	42 days	Curcumin strongly inhibited DEN-mediated the increased expression of oncogenic p21(ras) and p53 proteins in liver tissues of rats. Curcumin selectively reduced the expression of proliferating cell nuclear antigen (PCNA), cyclin E and p34 (cdc2), but not Cdk2 or cyclin D1. Curcumin also inhibited the DEN-induced increase of transcriptional factor NF-kappa B. However, Curcumin failed to affect DEN-induced c-Jun and c-Fos expression	Chuang et al. (2000b)
Terminalia arjuna	Male Wistar albino rat	4 weeks	28 days	EETA significantly decreased LPO levels and maintained enzymic and non-enzymic antioxidants, thus exhibiting its protective effect	Sivalokanathan et al. (2006)
Annona squamosa	Swiss albino mice	30–40 g	30 days	The levels of GOT, GPT, ALP, Total and Direct Bilirubin (both in serum and tissue), ACP, AFP (only in serum) decreased in DEN-induced plus *Annona squamosa* extract groups. Total proteins increased in DEN-induced plus *Annona squamosa* extract groups. Histopathology also confirmed the hepatoprotective effect of *Annona squamosa*	Raj et al. (2009)
Tinospora cordifolia	Male Wistar albino rats	120–150 g	20 weeks	Treatment of ECD in both preventive and curative DEN-induced animals increased the level of antioxidants (SOD, CAT) and detoxification	Dhanasekaran et al. (2009)

(continued)

Table 1 (continued)

Herbal products	Animal model	Selected for the study ages/weight	Treatment duration	Observations	References
				enzymes (GSH, GPx), and decreased serum transaminase level and hepatic marker enzymes (SGOT, SGPT, LDH) to near normal. Histopathological and nodular incidence also confirmed that ECD remarkably reduced tumor incidence and reversed damaged hepatocytes to normal	
Bacoside A (*Bacopa monniera*)	Male albino rats	160–180 g	7 days	The liver weight, lipid peroxidation (LPO), and activity of serum marker enzymes (aspartate transaminases, alanine transaminases, lactate dehydrogenase, alkaline phosphatase, and gamma-glutamyl transpeptidase) were near normal in bacoside A-pretreated rats. Activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutatione-S-transferase, and reduced glutathione) in liver also significantly elevated in bacoside A-pretreated rats. It is concluded that pretreatment of bacoside A prevents the elevation of LPO and activity of serum marker enzymes and maintains the antioxidant system and thus protects the rats from DEN-induced hepatotoxicity	Janani et al. (2009, 2010)
Saffron	Rat	4 weeks	22 weeks	Saffron significantly reduced the DEN-induced increase in the number and the incidence of hepatic dyschromatic nodules. Saffron also decreased the number and the area of placental glutathione S-transferase-positive foci in livers of DEN-treated rats. Furthermore, saffron counteracted DEN-induced oxidative stress in rats	Amin et al. (2011)

				as assessed by restoration of superoxide dismutase, catalase, and glutathione-S-transferase levels and diminishing of myeloperoxidase activity, malondialdehyde and protein carbonyl formation in liver. The results of immunohistochemical staining of rat liver showed that saffron inhibited the DEN-mediated elevations in numbers of cells positive for Ki-67, cyclooxygenase 2, inducible nitric oxide synthase, nuclear factor-kappa B p-65, and phosphorylated tumor necrosis factor receptor. Saffron also blocked the depletion in the number of cells positive for TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) and M30 CytoDeath in liver tissues of DEN-treated rats. In vitro experiments carried out using HepG2 cells also confirmed these findings and showed inhibition of nuclear factor-kappa B activation, increased cleavage of caspase-3, as well as DNA damage and cell cycle arrest upon saffron treatment	
Luteolin	Male Wistar albino rats	130–150 g	16 weeks	Administration of LVME as 200 mg/kg b.w. (to NDEA treated rats) significantly (i) reduced the preneoplastic lesions alleviated lipid peroxidation through scavenging free radicals, (ii) enhanced antioxidant status and reverted liver/disease marker enzymes (ALP, ALT, AST) plausibly by modulating xenobiotics metabolizing enzymes (XMEs) and by exhibiting antiproliferative and cytoprotective effects	Balamurugan and Karthikeyan (2012)

(continued)

Table 1 (continued)

Herbal products	Animal model	Selected for the study ages/weight	Treatment duration	Observations	References
Leucas aspera	Wistar albino rat	150–180 g	6 weeks	A significant lowering of the activity of ALP indicated the inhibition of pre-cancerous transformation in the liver on hydro-ethanolic and aqueous extract treatment in DEN + CCL4 animals, indicating the chemo-preventive efficacy of both the extracts in decreasing cell proliferation and hepatic nodulogenesis	Gupta et al. (2015)
Graptopetalum paraguayense	Male Wistar albino rats	150–180 g	84 days	*Graptopetalum paraguayense* enhanced PTEN expression and decreased AKT phosphorylation at Ser473 in a concentration-dependent manner in HCC cells. Moreover combination of GP or HH-F3 and sorafenib synergistically inhibited the proliferation of Huh7 cells. The treatment of a rat model with diethylnitrosamine (DEN)-induced liver cancer with extracts of GP and HH-F3 decreased hepatic collagen contents and inhibited tumor growth	Hsu et al. (2015)
Oldenlandia diffusa	Male Sprague-Dawley rats	180–200 g	60 days	The survival in *Oldenlandia diffusa* treated groups was shown to have a greater therapeutic effect than the control group. 28 days after drug treatment. *Oldenlandia diffusa* treated groups resulted in a significant reduction in tumor number, size, ^{18}F-FDG uptake, and serum levels such as alanine transaminase, aspartate transaminase, and alkaline phosphate compared to the control group. Also, proliferated cells in tumor sites by *Oldenlandia diffusa* were reduced compared to the control group. Furthermore, several rats in *Oldenlandia diffusa* treated group	

				survived over 60 days and liver morphology of these rats showed the difference between tumor mass and normal tissue	
Celastrol (*Tripterygium wilfordii*)	Male Sprague-Dawley rats	130–150 g	20 weeks	Celastrol significantly decreased the mortality rate, the number of tumor nodules and the index of liver in the Celastrol groups compared with DEN-treated group. Moreover, Celastrol obviously improved the hepatic pathological lesions and decreased the elevated levels of ALT, AST, ALP and AFP. Meanwhile, Celastrol suppressed the expression of the protein MDM2, activated the intrinsic mitochondrial apoptosis pathway induced by p53, inhibited anti-apoptotic Bcl-2 and Bcl-xl, induced the pro-apoptotic Bax, cytochrome C, PARP and caspases	Chang et al. (2016)
Tetilla dactyloidea	Male Sprague-Dawley rats	4 weeks	14 weeks	Oral administration of crude methanolic extract of *Tetilla dactyloidea* at a dose of 400 mg/kg body weight to DEN treated rats restored the nodule incidence, body weight, liver marker enzymes, enzymatic and non-enzymatic antioxidant, Phase I metabolizing and liver macromolecular damaging enzymes and immuno-histopathological changes were to near normal levels compared to control. The biochemical results were consistent with histopathological observations suggesting marked hepatoprotective effect of Crude Methanolic Extract of *Tetilla dactyloidea* in a dose-dependent manner	Gowri Shankar et al. (2017)
Phoenix dactylifera	Aalbino male rats	5–6 weeks (or) 100–120 g	10 weeks	Histological features of HCC in rats treated with extract of ajwa dates (ADE) showed partial to complete reversal of normal liver architecture.	Khan et al. (2017)

(continued)

Table 1 (continued)

Herbal products	Animal model	Selected for the study ages/weight	Treatment duration	Observations	References
				Antioxidant enzymes such as superoxide dismutase (SOD), glutathione reductase (GR), glutatione peroxidase (GPx) and catalase (CAT) increased, while the liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels and lipid peroxidation significantly decreased than that of DEN treated groups. Pro-inflammatory cytokines such as interleukin (IL)-1α, IL-1β, GM-CSF increased in the serum of DEN treated rats, while the anti-tumor cytokines (IL-2, IL-12) increased in ADE treated groups. In addition, Alpha-Feto Protein (AFP) and IL-6 gene expression levels were up-regulated in DEN treated groups, while they were significantly down-regulated in ADE treated groups	
Garcinia mangostana	Wistar rats	–	16 weeks	Significant increase in serum AFP, CEA, hepatic hydroxylproline, and total tissue protein levels in HCC group versus the negative control group. In contrast, the groups with HCC subjected to either high or low dose of GME elicited significant reduction of AFP, CEA, hepatic hydroxylproline, and increase in total protein in serum compared to the untreated HCC rats. Interestingly, treatment with *Garcinia mangostana* extract elicited marked improvement in the liver histological feature and down-regulation of tumor necrosis factor-alpha levels in HCC groups. *Garcinia*	Priya et al. (2018)

				mangostana extract possess chemopreventive benefits by reducing the tumor promoting growth factor levels in HCC-induced group	
Wedelia calendulacea	Male Swiss albino Wistar rats	150–200 g	22 weeks	19-α-Hydroxyurs-12(13)-ene-28 oic acid-3-O-β-D-glucopyranoside (HEG) in *Wedelia calendulacea*, confirmed the reduction of growth and deoxyribonucleic acid synthesis of both cell lines. DEN successfully induced HCC in all group, which was significantly altered by the HEG in a dose-dependent manner. The decreased level of pro-inflammatory cytokines and altered membrane-bound enzyme activity were also observed. HEG inhibited the phase I, II and antioxidant enzymes at the effective dose-dependent manner, which is considered as the precursor of the HCC. The alteration of phase I, II and antioxidant enzymes confirmed the inhibition of inflammatory reaction and oxidative stress, which directly or indirectly inhibited the NF-kB expression. Collectively, we can conclude that the HEG inhibited the growth of hepatocellular carcinoma via attenuating the NF-kB pathway	Verma et al. (2018)
Cynanchum auriculatum	Male Sprague-Dawley rats	150–180 g	20 weeks	Baishouwu extract pre-treatment successfully attenuated liver injury induced by DEN, as shown by decreased levels of serum biochemical indicators (AST, ALT, ALP, TP, and T-BIL). Administration of Baishouwu extract inhibited the fibrosis-related index in serum and live tissue, respectively from inflammation stage to HCC stage after DEN treatment. It significantly reduced	Ding et al. (2019)

(continued)

Table 1 (continued)

Herbal products	Animal model	Selected for the study ages/weight	Treatment duration	Observations	References
				the incidence and multiplicity of DEN-induced HCC development in a dose-dependent manner. Macroscopic and microscopic features suggested that pre-treatment with Baishouwu extract for 20 weeks was effective in inhibiting DEN-induced inflammation, liver fibrosis, and HCC. Furthermore, TLR4 overexpression induced by DEN was decreased by Baishouwu extract, leading to the markedly down-regulated levels of MyD88, TRAF6, NF-κB p65, TGF-β1 and α-SMA in hepatitis, cirrhosis, and hepatocarcinoma	
Echinacoside	Male C57BL/6 J mice	6–8 weeks	8 weeks	Echinacoside (ECH) attenuated diethylnitrosamine (DEN)-induced HCC in mice, and exerted anti-proliferative and pro-apoptotic functions on HepG2 cell line. ECH exposure in HepG2 cells dose-dependently reduced phosphorylation of AKT (p-AKT) and enhanced the expression of p21 (a cell cycle inhibitor) and Bax (a proapoptotic protein). Furthermore, ECH significantly suppressed insulin-like growth factor-1-induced p-AKT and cell proliferation. These data indicated that phosphoinositide 3-kinase (PI3K)/AKT signalling was involved in the anti-HCC activity of ECH. Gene set enrichment analysis results revealed a positive correlation between the PI3K pathway and triggering receptors expressed on myeloid cells 2 (TREM2) expression in HCC tissues. ECH	Ye et al. (2019)

				exposure significantly decreased TREM2 protein levels in HepG2 cells and DEN-induced HCC. Furthermore, ECH-mediated proliferation inhibition and AKT signalling inactivation were notably attenuated by TREM2 over-expression	
Ginger	Male Wistar albino rats	180–210 g	22 weeks	Ginger restored the activities of superoxide dismutase, catalase, GST and glutathione. Immunohistochemical bleaching in rat livers showed that ginger prevented the increase in cell-positive numbers for Ki-67, cyclooxygenase-2 and nuclear factor kappa B p65. Ginger also inhibited the number of positive cells in DEN/2-AAF-treated rats for TUNEL, M30 and caspase-3 liver tissues	Hamza et al. (2021)
Cow ark with *Allium sativum*	Wistar rats	–	28 days	Cow ark has the potential to accelerate Reactive Oxygen Species production, and allow to increasing membrane permeability (MP) and efficient discharge of Cytochrome c in HCC cancer cells while, no remarkable change was recorded in control hepatocytes	Nithya (2021)

with histological investigations of the liver were carried out. GOT, GPT, ALP, Total and Direct Bilirubin (both in blood and tissue), ACP, and AFP (only in serum) levels increased in DEN-treated groups, while all values reduced in the DEN and *Annona squamosa*-treated groups. Total protein levels were lower in DEN-treated mice and higher in DEN and *Annona squamosa*-treated mice. *Annona squamosa*'s hepatoprotective activity was further validated by histopathological examinations (Raj et al. 2009).

Tinospora cordifolia

With a diterpenoid (5R, 10R)-4R, 8R-dihydroxy-2S, 3R: 15, 16-diepoxycleroda-13 (16), 17, 12S: 18,1S-dilactone (ECD), eluted from *Tinospora cordifolia*, Dhanasekaran et al. (2009) assessed the chemopreventive inhibitory efficacy against DEN that produced HCC in mice. Antioxidant activity (SOD, CAT) and detoxifying enzymes (GSH, GPx) decreased in DEN-treated animals, but hepatic signaling activity increased (SGOT, SGPT, LDH). The treatment of ECD resulted in an increase in antioxidants and detoxification enzymes, as well as a drop in blood transaminases and hepatic indicators to normal levels in both treatment groups. ECD effectively reduced tumor incidence according to histopathological and nodular incidence (Dhanasekaran et al. 2009).

Tinospora cordifolia ECD also helped to avoid additional damage. In a solid tumor model, it proved efficient in inhibiting tumor growth. This work indicated ECD's chemopreventive potential in DEN-induced hepatocarcinogenesis, which was attributable to ECD's antioxidant and detoxifying mechanisms. Reduced serum transaminase secretion preserved membrane function, which is also attributable to ECD's protective impact. ECD's chemopreventive activities were also validated by biochemical and histological tests. ECD performs a dual effect by suppressing carcinogen metabolic activity and increasing carcinogen detoxification, according to Dhanasekaran et al. (2009).

Bacoside A

Janani et al. (2009) reported that bacoside A (BA), a substance derived from Bacopa monniera Linn., protects rats from DEN-induced liver damage. The activity of serum marker enzymes, viz., AST, ALT, LDH, ALP, and GGT rose significantly, as did liver weight, lipid peroxidation (LPO), and liver weight in rats treated with DEN, while the markers enzymes were on par with the levels of the above parameters in rats treated with BA. Antioxidant enzyme activity, viz., SOD, CAT, GSH-Px, GR, GSTs and reduced GSH in the liver was diminished in rats treated with DEN. The findings imply that pre-treating BA decreases LPO and serum marker enzyme activity while maintaining antioxidant activity, protecting rats from DEN-induced hepatotoxicity (Janani et al. 2009).

Likewise, the impact of BA on the activity and expression of MMP-2 and MMP-9 during HCC was examined by Janani et al. (2010). Co-treatment with BA considerably reduced the activity of MMP-2 and MMP-9, which increased during HCC, according to the results of a gelatin zymography investigation. Immunoblot analysis demonstrated a decrease in the expression of MMP-2 and MMP-9 in BA co-treated rats, than that of DEN-induced HCC rats. By suppressing MMP-2 and MMP-9 activity as well as expression, BA inhibits the metastasis of DEN-induced HCC (Janani et al. 2010).

Saffron

Saffron was found to be a strong medication against HCC in another investigation by Amin et al. (2011). The number and incidence of hepatic dyschromatic nodules caused by DEN were considerably reduced by saffron. In the livers of rats treated with DEN, saffron decreased the intensity and distribution of placental GST positive foci. It also protected rats against the oxidative stress caused by DEN by restoring the levels of SOD, CAT, and GST. Similarly, MPO activity, MDA activity, and COb formation were inhibited in liver. According to immunohistochemical labelling of rat liver, Saffron lowers the amount of cells positive for Ki-67, COG 2, inducible NOS, NF-kB, p-65, and phosphorylated TNF receptors in rats treated with DEN. In the liver tissues of mice treated with DEN, saffron decreased the number of cells positive for TUNEL and M30 Cyto-Death (Amin et al. 2011).

Luteolin

Balamurugan and Karthikeyan (2012) investigated the effectiveness of luteolin in Wister albino rats with DEN-induced HCC. The researchers looked at non-enzymatic antioxidant enzymes including AST, ALP, LDH, and c-GT, as well as enzymatic antioxidants like SOD, CAT, GSH, and GPx, along with histopathological alterations. In the DEN-treated groups, tissue-damaging enzymes were higher, while enzymatic antioxidants were lower. The DEN-treated rats developed severe lesions and cirrhosis. The levels of tissue-damaging enzymes and enzymatic antioxidants recovered in DEN-treated rats after treatment with luteolin, which almost entirely healed the damaged lesions in the liver induced by DEN. In albino rats, luteolin functions as a potential anti-HCC agent (Balamurugan and Karthikeyan 2012).

Leucas aspera

In Wister rats, Gupta et al. (2015) investigated the chemoprotective efficacy of *Leucas aspera* against DEN-induced and CCL4-stimulated hepato-carcinogenesis.

Except control, all other groups got a single dose of CCl4 (2 ml/kg i.p.) 2 weeks after the commencement of the test protocol to enhance liver cell proliferation and regeneration. The extent of protection was measured once the treatment period was completed by analyzing blood antioxidant indicators. To validate the effect of toxicants on the liver and to assess the chemoprotective potential of *Leucas aspera* extracts, biochemical parameters of the liver were measured. In addition to an increase in GGT levels, which indicated hepatic carcinogenesis, DEN administration in animals resulted in an increase in ALP activity, which could be attributable to changes in enzyme production, as in other examples of hepatotoxicity. The extracts normalized serum GGT levels and lowered serum AST and ALT levels, indicating a hepatoprotective action and suppression of carcinogenesis. In rats treated with aqueous and hydro-ethanolic extracts of DEN + CCL4, a significant reduction in ALP activity indicated suppression of pre-cancerous alterations in the liver. As a result, both extracts of *Leucas aspera* were found to be efficient in suppressing cell proliferation and hepatic nodulogenesis (Gupta et al. 2015).

Graptopetalum paraguayense

In HCC cells, the release of *Graptopetalum paraguayense* (GP) suppressed the expression of many oncoproteins, including AURKA, AURKB, and FLJ10540, according to Hsu et al. (2015). When the fractions eluted from the extracts were tested for their effects on onco-protein exposure in HCC cells, it was discovered that the HH-F3 fraction enriched with active components had cytotoxic effects and inhibited onco-protein expression. Studies on apoptosis showed that HH-F3 caused HCC cells to undergo apoptosis by increasing the energy loss from the mitochondrial membrane and the production of active oxygen species. In a concentration-dependent manner, HH-F3 improved PTEN expression and reduced AKT phosphorylation at Ser473 in HCC cells. The combination of GP or HH-F3 and sorafenib also suppressed Huh7 cell proliferation. Treatment with GP and HH-F3 reduced hepatic collagen levels and prevented tumor growth in DEN-treated mice. The results clearly depicted the protection of liver by GP and HH-F3 extracts liver, and that they could be used to treat HCC (Hsu et al. 2015).

Oldenlandia diffusa

Demonstrated *Oldenlandia diffusa*'s therapeutic potential in vitro and in vivo. *Oldenlandia diffusa* enhanced apoptosis and anti-proliferation activities while reducing the ability of HCC cells to migrate. In in vivo experiments, *Oldenlandia diffusa*, when given twice daily for 28 days following confirmation of the HCC model utilizing two images – [^{18}F]-fluoro-2-deoxy-D-glucose (^{18}F-FDG) imaging, showed a higher survival rate in *Oldenlandia diffusa* treated group than in the control group. Tumor counts, size, tumor cell proliferation, ^{18}F-FDG uptake, and serum enzyme levels such as ALT, AST, and ALP were all considerably less in the *Oldenlandia*

diffusa treated group than control group after 28 days of therapy. Furthermore, several *Oldenlandia diffusa*-treated rats lived for more than 60 days, and their liver morphology revealed variations between tumor mass and normal tissue compared to control rats.

Celastrol

Chang et al. (2016) investigated the anti-tumorogenic activity of Celastrol, an active component in *Tripterygium wilfordii*, in DEN-induced HCC in Sprague-Dawley rats. For 16 weeks, DEN (10 mg/kg) was given intragastrically 6 days a week. Hematoxylin-Eosin (HE) staining was used to determine the number of nodules developed and hepatic pathological abnormalities. Similarly, Elisa kits were used to determine serum ALT, AST, ALP, and AFP levels, as well as p53 protein levels, MDM 2, Bax, Bcl-2, Bcl-xl, cytochrome C, Caspase-3, Caspase-9, and PARP levels. In comparison to rats treated with DEN, Celastrol dramatically decreased liver index, tumor nodule count, and mortality in rats treated with Celastrol. Celastrol also lowered elevated levels of ALT, AST, ALP, and AFP and appeared to improve liver pathological abnormalities. Celastrol, on the other hand, inhibited anti-apoptotic Bcl-2 and Bcl-xl and activated pro-apoptotic Bax, cytochrome C, PARP, and caspases by repressing MDM2 protein expression, activating the p53-induced intrinsic mitochondrial apoptotic pathway (Chang et al. 2016).

Tetilla dactyloidea

Gowri Shankar et al. (2017) investigated the zoochemical status, antioxidant capability, and anti-cancer efficacy of *Tetilla dactyloidea* crude methanol extract (CMETD) in Sprague Dawley (SD) rats treated with DEN. Nodule formation, body mass, hepatic marker enzymes, enzymatic and non-enzymatic antioxidants, Phase-I metabolizing and hepatic macromolecular enzymes, and immuno-histopathological alterations were evaluated in the DEN and DEN + CMETD treated groups. Following oral administration of 400 mg/kg body weight of CMETD, all parameters in the DEN-treated groups were restored to normal levels. The recovered biochemical levels were in accordance with histological findings, indicating that CMETD has a dose-dependent hepatoprotective effect. Six chemicals were detected in CMETD after GCMS screening. In DEN-induced HCC, the results demonstrated that CMETD reduced liver damage, protected the antioxidant immune system, and exhibited anti-cancer activities (Gowri Shankar et al. 2017).

Ajwa Dates, *Phoenix dactylifera*

Khan et al. (2017) conducted another study in Wister rats, this time looking at the anti-cancer properties of Ajwa dates (*Phoenix dactylifera* L.), where HCC was

caused by DEN administration. The liver architecture of the DEN-treated rats was reversed from partial to complete, while the liver architecture of the AD-treated rats was reversed from partial to complete. Antioxidant enzymes like SOD, GR, GPx, and CAT elevated, whereas liver enzymes like ALT, AST, and ALP, as well as LPO, declined in AD treated rats compared to DEN. Antitumor cytokines like IL-2 and IL-12 were found to be elevated in DEN-treated groups' serum, while pro-inflammatory cytokines like IL-1 and GM-CSF increased in AD-treated groups' serum. Furthermore, gene levels of Alpha-Feto Protein (AFP) and IL-6 were up-regulated in DEN-treated groups, but down-regulated in AD-treated groups. AD extract helped to restore normalcy to a damaged liver that had been treated with DEN. Following AD treatment, antioxidant enzymes, liver enzymes, cytokine balance, and gene expression all restored to normal, proving that AD enhances the function of liver and protects it against HCC (Khan et al. 2017).

Garcinia mangostana

In a rat animal model of DEN-induced HCC, Priya et al. (2018) revealed a protective mechanism of *G. mangostana* fruit extract (GME). In rats treated with DEN, the levels of HCC indicators such as AFP, CEA, TNF-α, hepatic hydroxyproline and total protein were determined by ELISA. Immunohistochemistry was used to detect the expression of vascular endothelial growth factor in liver tissue. Serum AFP, CEA, hepatic hydroxyproline and total protein levels were significantly higher in the DEN-treated rats than that of the control group. Treatment with GME at low or high dosages resulted in significant decline in AFP, CEA, hepatic hydroxyproline, and an elevation rise in total blood protein levels in the DEN-treated rats. Interestingly, treatment with GME resulted in significant improvements in the histological architecture of the liver and down-regulated tumor necrosis factor alpha levels. GME thus exhibited its chemopreventive potential against DEN-induced HCC by reducing the expression of tumor promoting growth factor (Priya et al. 2018).

Wedelia calendulacea

In Wistar rats and HepG-2 and HuH-7 cell lines, Verma et al. (2018) investigated the hepatoprotective potential of 19--Hydroxyurs-12 (13)-hypertensive method of 28 oic acid-3-O-D-glucopyranoside (HEG) eluted from *Wedelia calendulacea* against DEN-induced oxidative stress, hyperproliferation, inflammation, and apoptotic tissue damage. To cause liver damage, single dose of DEN (200 mg/kg) and two doses of phenobarbitol were given. This was followed by a 22-week HEG treatment. Hepatic nodules were confirmed by macroscopic examination, and serum and hepatic samples were subjected to additional biochemical and histological analyses. Inflammatory cytokines such as TNF-α, IL-6, IL-1, and NF-kB were also evaluated, as were hepatic and non-hepatic Phase I and II antioxidant enzymes (NF-kB). To examine the changes that have occurred in the liver of both DEN and

HEG treated rats, histopathological changes were identified. HCC induced by DEN in all groups was significantly altered in a dose-dependent manner by HEG. Likewise, tumor growth and DNA synthesis was reduced by HEG in both cell lines. Pro-inflammatory cytokines were found to be decreased and membrane-bound enzyme activity was altered by HEG. HEG inhibited phase I, II and antioxidant enzymes in an active dose-dependent way, and proved HEG as a precursor in combating HCC. According to alterations in phase I, II, and antioxidant enzymes, HEG suppressed inflammatory responses and oxidative stress that either explicitly or implicitly decreased NF-kB expression. HEG inhibited the growth of HCC by inhibiting the NF-kB pathway (Verma et al. 2018).

Cynanchum auriculatum (Baishouwu)

Ding et al. (2019) investigated the effect of Baishouwu extract (BE) on DEN-induced HCC as well as the potential mechanisms involved in its treatment. Animals were treated simultaneously with BE, which was administered daily by oral gavage for 20 weeks to investigate its preventive benefits, and multistep hepatocarcinogenesis was commenced by injecting DEN. To assess the effect of BE on hepatic carcinogenesis, a serum sample was taken at a predetermined time and organ samples were taken from each group. BE co-treatment significantly reduced the liver damage caused by DEN in rats, as seen by lower levels of serum biochemical markers (AST, ALT, ALP, TP, and T-BIL). From the inflammatory phase until the HCC stage, BE decreased the fibrosis-related index in blood and tissues, which was produced by DEN. In a dose-dependent way, BE dramatically reduced the incidence and frequency of DEN-induced HCC development. Pre-treatment with BE for 20 weeks appeared to be successful in avoiding inflammation produced by DEN, liver fibrosis, and HCC, according to macroscopic and microscopic findings. Furthermore, BE reduced TLR4 over DEN expression, resulting in considerable downregulation of MyD88, TRAF6, NF-B, p65, TGF-1, and -SMA in hepatitis, cirrhosis, and HCC (Ding et al. 2019).

Echinacoside

A phenylethanoid glycoside called as echinacoside (ECH) is obtained from the Chinese herb Cistax'nches salsa. Ye et al. (2019) explored the impact of ECH on HCC as well as the mechanisms involved. ECH decreased DEN-induced HCC in mice and had anti-proliferative and pro-apoptotic effects in the HepG-2 cell line, according to the findings. ECH suppressed AKT (p-AKT) phosphorylation and increased p21 and Bax expression in HepG-2 cells in a dose-dependent manner. ECH reduced p-AKT and cell proliferation generated by insulin-like growth factor-1, demonstrating that PI3K/AKT signaling was involved in ECH's anti-HCC effect. It was shown that the activation of receptors expressed in myeloid cells 2 (TREM2) expression in HCC tissues was positively linked with the PI3K pathway. TREM2

protein levels in HepG-2 cells and DEN-induced HCC in mice were both dramatically reduced after exposure to ECH. Overexpression of TREM2 also substantially inhibited ECH-mediated proliferation inhibition and AKT signaling inactivation. Eventually, the ECH suppressed tumor growth by inhibiting TREM2 expression and PI3K/AKT signaling (Ye et al. 2019).

Ginger

In Wister rats, Hamza et al. (2021) studied the mechanisms of ginger rhizome extracts against DEN-induced HCC. At dosages of 75, 150, and 300 mg/kg/day, ginger was found to have chemopreventive potential in the liver damage caused by DEN and 2-acetylaminofluorene in rats (2-AAF). After 22 weeks of cancer induction, ginger decreased the quantity of placental GST in the liver of the DEN/2-AAF treated groups, as well as the number and incidence of hepatic dyschromatic nodules and positive focal regions. In addition, ginger reduced the levels of myeloperoxidase, malondialdehyde, and protein carbonyl in the liver via inhibiting oxidative stress with DEN. The restoration of SOD, CAT, GST, and glutathione was used to determine this. Ginger decreased the proliferation of Ki-67 cell counts, cyclooxygenase-2 (COX-2) and NF-B p65 in rat liver, as depicted by immunohistochemical staining. In mice treated with TUNEL DEN/2-AAF, M30, and caspase-3 liver tissue, ginger lowered the number of cancer cells. Ginger has a significant chemopreventive potential against liver cancer, as per this study, by slowing cell growth and increasing apoptosis. Ginger protects the rat liver from cancer by lowering oxidative damage and inflammation (Hamza et al. 2021).

Cow Ark with *Allium sativum*

In a study by Nithya (2021), the ability of Cow Ark to control and regulate cancer activity in Wister rats induced with DEN + 2AAF. The weight loss in rats showed that HCC was caused by DEN + 2AAF in the experimental group. On the other hand, Cow ark significantly reduced MMP in mitochondria of HCC hepatocytes in a time-phased way. *Allium sativum* extracts independently showed an insignificant effect on MMP of liver mitochondria, isolated from the control group. Likewise, production of Hydrogen peroxide in the liver mitochondria of the HCC cells was found to be enhanced in rats induced by DEN + 2AAF, whereas the H_2O_2 activity in plant extract did not have significant effects in control group treated only with plant extract. Significant increase in H_2O_2 production was observed in rats treated with Cow Ark and plant extract, due to their synergistic effect. Thus the enhancing potential of Cow ark and plant extract was established by the radical scavenging into the antioxidant activity. In addition, the synergistic action of Cow Ark and plant extract treated mitochondria increased cytochrome c release by cleaving the mitochondrial membrane integrity in liver mitochondria from DEN + 2AAF treated rats, but no such effect was seen in the control group (Nithya 2021).

Conclusion

Based on previous findings, it could be concluded that animals can be considered to be the exact models for drug testing against HCC. Today, the emergence of new therapies that target the immune system and the tumor microenvironment emphasizes the importance of the host, conditions of chronic inflammation, and fibrosis. Hence, use of animal models for a wide range of cancer research will help us to discover new drugs to combat HCC.

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