Nutrition and Cancer
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/hnuc20

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Published online: 04 Jun 2014.

To cite this article: Harshita Chaudhary, Prasant Kumar Jena & Sriram Seshadri (2014): In Vivo Evaluation of Eclipta alba Extract as Anticancer and Multidrug Resistance Reversal Agent, Nutrition and Cancer, DOI: 10.1080/01635581.2014.916324

To link to this article: http://dx.doi.org/10.1080/01635581.2014.916324

Nutrition and Cancer, 1–10, 2014

ISSN: 0163-5581 print / 1532-7914 online
DOI: 10.1080/01635581.2014.916324

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In Vivo Evaluation of *Eclipta alba* Extract as Anticancer and Multidrug Resistance Reversal Agent

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The present study investigates the anticancer and multidrug resistance (MDR) reversal potential of hydro-alcoholic *Eclipta alba* extract (EAE) through in vivo experiments. Diethylnitrosamine (DEN) and 2-acetylaminofluorene (AAF) were used for liver cancer induction in animal model, whereas for MDR induction, AAF was used. The level of antioxidant enzymes was studied in serum along with biochemical parameters. Cancer and MDR-induced liver cells have higher levels of reactive oxygen species (ROS) and, in turn, are responsible for the maintenance of the cancer phenotype. Treatment with EAE declines the ROS level and revealed the ROS scavenging properties. Alfa feto protein levels were found to increase significantly in cancer-induced animals confirming induction and progression of liver cancer, EAE treatment was found to bring back the altered levels within normal range indicating the therapeutic effect of plant extract over liver cancer. Zymogram showed the inhibition of MMPs and RT-PCR analysis revealed that the mRNA expression of nuclear factor-*k*B was markedly decreased upon EAE treatment. Further, our results showed that EAE could significantly inhibit mdr1 gene encode P-glycoprotein expression. Our data suggest that EAE is a novel anticancer and potent MDR reversal agent and may be a potential adjunctive agent for tumor chemotherapy.

INTRODUCTION

Hepatocellular carcinoma (HCC) has disappointing results in diagnosis and systemic chemotherapies; therefore the increasing knowledge of the molecular biology of HCC has resulted in innovative targets. The serious problem encountered in cancer chemotherapy, is the multidrug resistance (MDR) developed by many cancer patients to treatment with standard anti-cancer drugs (1). MDR genes (mdr1) encode P-glycoprotein (P-gp), which is responsible for resistance to cancer chemotherapeutic drugs and efflux of xenobiotics of cells. Many of the immunological functions of liver cells are due to high activity of the rel/nuclear factor-*k*B (NF-*k*B) family of transcription factors. These factors are key regulators of genes involved in immunity, wound healing, proliferation, and apoptosis (2). In reference with a pro-oncogenic activity, NF-*k*B promotes expression of several matrix metalloproteinases (MMPs), including MMP-2, -3, and -9, which are key modulators of many biological processes such as angiogenesis, cellular migration, inflammation, and cancer (3). NF-*k*B is generally seen as an antiapoptotic factor because it induces anti-apoptotic genes that block the caspase cascade (4). Therefore, activation of NF-*k*B in cancer cells by chemotherapy or radiation therapy is often related with the acquirement of resistance to apoptosis. NF-*k*B also induces drug resistance through mdr1 expression in cancer cells (5). Although these data raised an admonitory note about the agents that block NF-*k*B and mdr1 gene, they could represent a new and promising strategy in cancer treatment.

Ayurvedic medicine can function as sensitizers, supplementing the effectiveness of cancer chemotherapy (6). Because of high death rate and serious side effects of chemotherapy, many cancer patients seek complementary and alternative methods of treatment. Natural therapies, such as the use of plant-derived products in cancer treatment, may reduce adverse side effects. The plant *Eclipta alba* (Linn.) (Family—*Asteraceae*) has been mentioned in ancient transcripts to be a nervine tonic, antiaggressive effect, hepatoprotective, hair growth promoting, anti-Hepatitis, and antiproliferative (7,8) properties. In addition, it is also reported to possess antinociceptive, antiinflammatory, and bronchodilator activities (9). However in vivo anticancer and mdr study is yet to be explored.

The present study was designed to test the hypothesis that hydroalcoholic *Eclipta alba* extract (EAE) may confer reversal against cancer and MDR.

MATERIAL AND METHOD

Plant Material

Eclipta alba leaves were obtained from LVG (Ayurvedic product supplier; Ahmedabad, India) and was authenticated by Dr. Vasant A. Patel, Department of Botany, Smt. S.M.P. Science College, Hemachandracharya North Gujarat University,

Submitted 13 July 2013; accepted in final form 10 March 2014.
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Gujarat, India. Specimen sample of *Eclipta alba* has been submitted at the Institute of Science, Nirma University, Ahmedabad, Gujarat, India with the voucher no. ISNU/EA/CN-120421/01.

Preparation of Hydro-Alcoholic Extract of Plants

Dried leaves were grinded in 50% ethanol in ratio 1:3. The obtained hydroalcoholic extracts were stirred overnight at 50°C, followed by filtration under sterile conditions. The filtrate was vaccum dried at 50°C to remove the solvent completely, weighed, and reconstituted in double distilled water to form a final concentration of 50 mg/ml. The yield of the EAE was 11.6% (w/w) and was stored at −20°C in 1 ml aliquots until further use.

Cancer and MDR Induction

Adult healthy male Wistar rats weighing 250–350 g were procured from Zydus Research Centre, Ahmedabad, India, compelling to the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines. The Guidance for Care and Use of Animals for Scientific Research (10) was strictly followed (IAEC Protocol No. IS/BT/PHD10-11/2001). The animals were acclimatized for 5 days prior to the experiments. The animals were monitored every day for their body weight, food, and water intake and any visible symptoms, during the whole experiment, hepatocarcinogenesis was induced with xenobiotics (DEN and 2-AAF). DEN (Sigma Chemical Co., St. Louis, MO, USA) was given i.p once in concentration of 200 mg/kg body weight. AAF was given orally in the concentration of 20 mg/kg body weight 5 days a week, for 3 weeks as an enhancer to enhance the effect of DEN in the induction of liver cancer after that EAE treatment was started orally. For MDR induction, animals were treated with 2-AAF (20 mg/kg body weight) daily for 5 days and after that EAE treatment started. The protection with EAE seemed to be dose-dependent, hence in the present investigation EAE at 250 and 500 mg/kg body weight was used for anticancer studies and EAE at 500 mg/kg body weight was used for MDR reversal studies.

Autopsy Schedule

The animals were divided into 4 groups, control group (vehicle-treated), DEN + AAF treated group, and EAE treated groups (250 and 500 mg/kg body weight) for liver cancer study. Autopsy of animals was performed after induction and treatment. Four animals from each group were sacrificed after every 20 days from the day when extract dose started till the treatment schedule continued (60 days). For the MDR study, animals were divided into 3 groups: control group (vehicle treated), AAF treated group, and EAE treated group (500 mg/kg body weight) Animals were sacrificed on 3 days, 7 days, and 14 days after treatment with EAE. Blood and liver were used for the assay of the following parameters.

Histopathological Study

Liver from all the study groups was cancer-induced and treated animals were removed at the time of autopsy. The tissues were cleared of the visible fats and were fixed immediately in Bouin's fluid for 24 hours, dehydrated in ethanol, cleared in benzene, infiltrated and embedded in paraffin wax. Five-μm thick sections was cut and stained with Harris hematoxylin and eosin. The histopathological tissues sections were viewed and digitally photographed using a Cat-Cam 3.0 MP Trinocular microscope with an attached digital 3XM picture camera (Catalyst Biotech, Mumbai, India).

Serum Profiling

Serum was separated from the blood collected at the time of autopsy. The serum was used for the biochemical estimation of aspartate aminotransferase (AST), alanine aminotransferase (ALT) assay, bilirubin, high-density lipoprotein (HDL) cholesterol, and gamma-glutamyl-transferase (GGT) assay. These assays were performed with the help of Qualigens Diagnostic kits (Mumbai, Maharashtra, India) for both cancer and MDR induced and treated groups.

AFP Assay

Pathozyme® Alpha-Fetoprotein (Omega, Scotland, UK) kit was used for the detection of AFP level in the serum of cancer induced and treated animals following the manufacturer's protocol.

DNA Isolation and Analysis of DNA Fragmentation

One-gm liver tissues were chopped and suspended in 5 ml lysis buffer (2% sodium lauryl sulphate, 10 mM EDTA, 10 mM Tris-HCl, pH 8.5) with 0.5 mg/ml proteinase K (HiMedia, Mumbai, Maharashtra, India) and RNase (0.1 mg/ml) and incubated at 55°C for 3 h. After incubation, phenol:chloroform (1:1) washes were given and DNA was precipitated with chilled ethanol. The DNA was separated in 1% agarose gels and visualized by UV illumination after ethidium bromide staining.

Gelatinase Zymography of Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) released into conditioned media was determined by gelatinase zymography according to the method of Patricia et al. (11) with minor modifications. Briefly, gelatinases present in the tissue extracts degrade the gelatin matrix, leaving a clear band after staining the gel for protein. Liver protein of cancer induced and a treated group was electrophoresed under nonreducing conditions on 10% polyacrylamide gels containing 1 mg/ml gelatin (Sigma-Aldrich, Bangalore, Kamataka, India). After electrophoresis, the gels were renatured in 2.5% Triton X-100 (2 × 15 min), then incubated overnight at 37°C in development buffer (50 mMTris–HCl, pH 7.6; 10 mM$CaCl_2$; 50 mM NaCl; 0.05% Brij35 (Invitrogen, Bangalore, Kamataka, India). The gels were stained with 0.5% Coomassie Brilliant Blue R-250.

RT-PCR

Total RNA from liver tissue of both cancer and MDR induced groups were isolated using the TRIzol reagent. One μg of total RNA was used for RT-PCR with NF-*k*B

primers (forward 5′AGCACAGATACCACCAAGAC-3′, reverse 5′TGGTCCCGTGAAATACACCT3′) (12), mdr1 primers (forward 5′GCCTGGCAGCTGGAAGACAAATACACAA3′, reverse 5′CAGCTGACAGTCCAAGAACAGG3′) and actin primers (forward 5′TCACCCACACTGTGCCCATCTACGA3′, reverse 5′CAGCGGAACCGCTCATTGCCAATGG3′) (8), 10× buffer (5.0 μl), cDNA (2.0 μg), 25 mmol/l $MgCl_2$ (3.0 μl), 10 mmol/l dNTPs (1.0 μl), and Taq polymerase (2.5 U). PCR amplification cycles consisted of denaturation at 94°C for 1 min, primer annealing at 57°C for 45 s and extension at 72°C for 45 s, for a total of 30 cycles followed by final extension at 72°C. The PCR product was separated by electrophoresis on 2% agarose gels.

Gas Chromatography-Mass Spectrometry Result of *Eclipta alba* Extract

Gas chromatography-mass spectrometry (GC-MS) analysis of the hydroalcoholic extract of *Eclipta alba* was performed using a Perkin-Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a gas chromatograph interfaced to a mass spectrometer (GC-MS) equipped with an Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused to a capillary column (30 × 0.25 μm ID × 0.25 μmdf). For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 2 μl was used (a split ratio of 10:1). The injector temperature was maintained at 250°C, the ion-source temperature was 200°C, the oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min to 200°C, then 5°C/min to 280°C, ending with a 9-min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min, and the total GC/MS running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The mass-detector used in this analysis was Turbo-Mass Gold-Perkin-Elmer, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver-5.2. The chromatograms of the sample were identified by comparing their mass spectra with National Institute of Standards and Technology library data, and the GC retention time against known standards.

Statistical Analysis

Values were expressed as mean ± SEM and the data were analyzed with one-way analysis of variance (ANOVA) or two-way ANOVA. Post hoc analyses involving Dunnett's tests with significance set at $P < 0.05$.

RESULTS

Histopathological Studies

Thin sections of liver obtained after autopsy schedule were stained with Harris hematoxylin and eosin and were subsequently observed under microscope. Histology of liver section of normal control animal showed well-defined cytoplasm, prominent nucleus and central vein, whereas that of the cancer-induced group animal showed total loss of hepatic architecture with centrilobular hepatic necrosis, toxicity vacuolization, and congestion of sinusoids compared to control group. The histological sections following treatment with EAE (500 mg/kg body weight) showed normal lobular pattern as compared to cancer induced animals with minimal pooling of blood in the sinusoidal spaces. EAE treated animals histological sections showed almost complete recovery comparable to that of the control group (Figure 1).

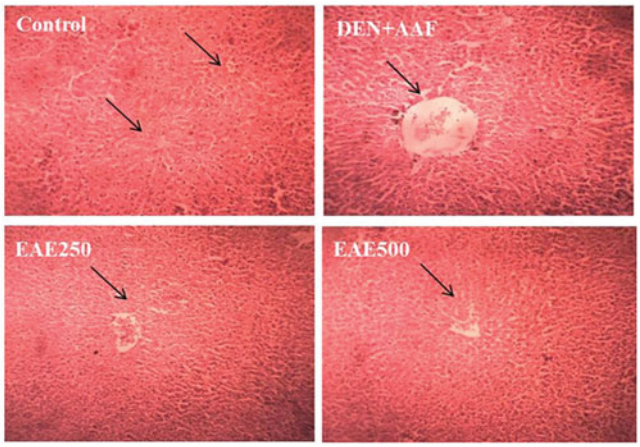

FIG. 1. Histopathology of liver sections of control, diethylnitrosamine (DEN) + 2-acetylaminofluorene (AAF) and different doses of *Eclipta alba* extract (EAE; 250 and 500 mg/kg body weight) treated animal groups. (Color figure available online.)

Serum Profiling

The level of liver marker enzymes, serum ALT, AST, bilirubin, HDL cholesterol, and GGT in the control group showed a normal range. Nevertheless, the DEN-treated group showed a high level of liver enzymes; the elevation of these enzymes was an indicative of the disrupted cells. The efficacy of EAE was dose-dependent, with the 500 mg/kg body weight being more effective than 250 mg/kg body weight. Alternatively, the EAE-treated group showed a very interesting result: EAE treatment significantly reduced the raised levels of liver enzymes than the DEN-treated group (Table 1). EAE administration helped in reverting back to control levels. For MDR study we selected EAE 500 mg/kg body weight concentration only, based on the anticancer results. In the MDR study the levels of ALT, AST, bilirubin and GGT of the MDR-induced groups also showed a significant difference when compared to the control groups after 3 days, 7 days, and 14 days of induction (Table 2). Administration of EAE 500 mg/kg body weight caused a significant reduction in the value of ALT, AST, bilirubin, and GGT.

TABLE 1
Serum profiling for different parameters of control, DEN + AAF and EAE treated groups

	40 days plant extract				60 days plant extract			
Parameters	Control	DEN + AAF	EAE (250)	EAE (500)	Control	DEN + AAF	EAE (250)	EAE (500)
SGOT (U/L)	24.9 ± 1.34	$38.9 \pm 1.44^{**}$	$34 \pm 1.32^{***}$	$30 \pm 1.56^{**}$	25.1 ± 1.3	$50.9 \pm 1.54^{**}$	$32 \pm 1.53^{**}$	$29.83 \pm 1.25^{***}$
SGPT (U/L)	35.8 ± 1.79	$51.6 \pm 2.58^{*}$	$45.6 \pm 3^{*}$	$40.7 \pm 1.32^{*}$	36.08 ± 1.80	$64.01 \pm 3.2^{*}$	$58.00 \pm 1.38^{*}$	$41.8 \pm 1.8^{**}$
Bilirubin (g%)	0.33 ± 0.016	$1.1 \pm 0.045^{**}$	$0.61 \pm 0.019^{*}$	$0.38 \pm 0.04^{**}$	0.32 ± 0.01	$1.4 \pm 0.06^{*}$	$0.42 \pm 0.06^{**}$	$0.38 \pm 0.05^{***}$
HDL (g%)	447.8 ± 22.3	$569.4 \pm 28.4^{**}$	$495.7 \pm 30^{**}$	$470.4 \pm 27.8^{**}$	460.3 ± 23.0	$660.5 \pm 33.0^{***}$	$551.7 \pm 16^{**}$	$520.3 \pm 24^{**}$
GGT (U/L)	5.2 ± 0.26	$10.6 \pm 0.5^{**}$	$8.0 \pm 0.46^{**}$	$5.8 \pm 0.45^{**}$	5.74 ± 0.28	$20.5 \pm 1.02^{*}$	$14.8 \pm 0.67^{*}$	$09.4 \pm 0.47^{*}$

DEN = diethylnitrosamine; AAF = 2-acetylaminofluorene; EAE = *Eclipta alba* extract; SGOT = Serum glutamic oxaloacetic transaminase; SGPT = Serum glutmaic pyruvic transaminase; HDL = high-density lipoprotein; GGT = gamma-glutamyl-transferase. Values are mean ± SEM for 4 animals in each observation.

$^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$, as compared with control group.

$^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$, as compared with DEN induced group.

TABLE 2
Serum analysis for biochemical parameters for MDR reversal in control, AAF and EAE treated groups

	3 days plant extract			7 days plant extract			14 days plant extract		
Parameters	Control	AAF	EAE (500)	Control	2AAF	EAE (500)	Control	AAF	EAE (500)
SGOT(U/L)	23.09 ± 0.34	$38.8 \pm 2.9^{*}$	$28.98 \pm 1.2^{**}$	25.05 ± 0.55	$44.5 \pm 3.7^{**}$	$31.965 \pm 1.36^{*}$	24.9 ± 0.7	$51.05 \pm 0.15^{**}$	$31.35 \pm 0.45^{**}$
SGPT(U/L)	36.98 ± 0.9	$58 \pm 1.9^{**}$	$50.5 \pm 1.45^{**}$	37.15 ± 1.35	$60.35 \pm 1.75^{*}$	$41 \pm 1.52^{**}$	37.25 ± 0.85	$64.65 \pm 0.35^{**}$	$37.35 \pm 0.75^{**}$
Bilirubin (g%)	0.43 ± 0.019	$0.72 \pm 0.048^{*}$	$0.55 \pm 0.05^{**}$	0.425 ± 0.025	$0.84 \pm 0.06^{**}$	$0.5 \pm 0.02^{**}$	0.41 ± 0.02	$1.1 \pm 0.1^{***}$	$0.495 \pm 0.005^{*}$
GGT(U/L)	5.4 ± 0.2	$6.98 \pm 0.07^{**}$	$6.09 \pm 0.41^{**}$	5.1 ± 0.1	$7.95 \pm 0.05^{**}$	$5.65 \pm 0.15^{**}$	5.35 ± 0.25	$8.7 \pm 0.1^{**}$	$5.65 \pm 0.25^{**}$

AAF = 2-acetylaminofluorene; EAE = *Eclipta alba* extract; SGOT = Serum glutamic oxaloacetic transaminase; SGPT = Serum glutmaic pyruvic transaminase. Values are mean ± SEM for 4 animals in each observation.

$^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$, as compared with control group.

$^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$, as compared with DEN induced group.

TABLE 3
Alpha-fetoprotein content of different treatment groups

Groups	40 days(ng/ml)	60 days(ng/ml)
Control	06.0 ± 0.16	06.4 ± 0.15
DEN	50.1 ± 0.135**	62.8 ± 0.145**
EAE 250	23 ± 0.125*	20.9 ± 0.132*
EAE 500	21.2 ± 0.143**	16.8 ± 0.136**

DEN = diethylnitrosamine; EAE = *Eclipta alba* extract. Values are mean ± SEM for 4 animals in each observation.

*$P < 0.05$. **$P < 0.01$. ***$P < 0.001$, as compared with control group.

*$P < 0.05$. **$P < 0.01$. ***$P < 0.001$, as compared with DEN induced group.

AFP Determination

There was a significant increase in value of AFP from control to DEN-induced animals from 06.4 ± 0.15 to 62.8 ± 0.145 ng/ml. The values were noted to decline appreciably following the plant extract treatment towards to control group animals. After 60 days with EAE 500 mg/kg body weight treatment, 62.8 ± 0.145 ng/ml of AFP in DEN-induced animals goes down to the 16.8 ± 0.136 ng/ml. AFP <20 ng/ml is considered to be normal and we got 16 ng/ml of AFP in case of EAE 500 mg/kg treated group, almost equal to the normal (Table 3).

DNA Fragmentation

DNA fragmentation reflecting the endonuclease activity characteristic of apoptosis was analyzed. As shown in Fig. 2A, treatment with EAE at the different doses resulted in the formation of DNA fragments that could be visualized via electrophoretic examination as a characteristic ladder pattern. There was no evidence of any fragmentation in the livers of the cancer-induced group. EAE at concentrations of 250 mg/kg and 500 mg/kg body weight were able to induce apoptosis.

Gelatin Zymography

Zymographic detection on substrate gels is often a good choice for the initial characterization of a proteinase activity. We performed gelatin zymography for the initial characterization of MMPs. After zymography, gelatinolytic band was observed of MMP-2. The MMP-2 activity was significantly higher in the cancer-induced group as compared to the control group. EAE treatment led to significant reduction in MMP-2 activity as compared to the cancer-induced group (Fig. 2B and 2C).

NF-*k*B Expression

NF-*k*B expression was analyzed by RT-PCR in cancer-induced and EAE-treated groups. NF-*k*B was highly expressed in cancer-induced groups. The EAE was able to inhibit the expression of NF-*k*B at transcriptional level in a dose-dependent manner. EAE at concentration of 500 mg/kg body weight almost revert back its expression comparable to the control. NF-*k*B suppression follows antiproliferative effects of EAE. The quantity of mRNA in each lane was normalized by β-actin expression (Fig. 3A and 3B).

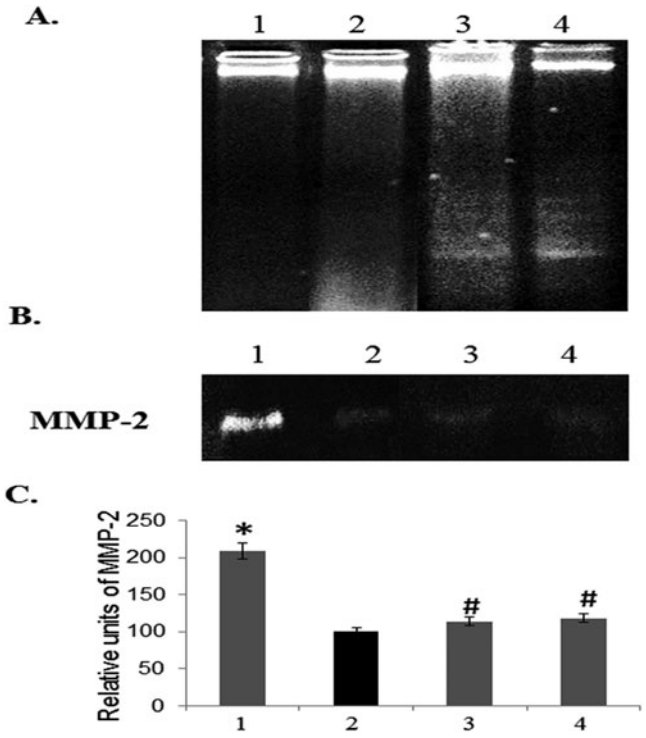

FIG. 2. DNA fragmentation assay (A) and gelatin zymogram **(B)** for matrix metalloproteinases (MMPs) was done in the liver tissue homogenates of control, diethylnitrosamine (DEN) + 2-acetylaminofluorene (AAF) and different doses of *Eclipta alba* extract (EAE; 250 and 500 mg/kg body weight) treated animal groups after 60 days. C:Relative units of MMP-2 were quantified in comparison to the control group using AlphaEase FC software (Alpha Innotech) and plotted as histogram. Lane 1-DEN + AAF, Lane 2-control, Lane 3-EAE 250 mg/kg body weight, Lane 4-EAE 500 mg/kg body weight. * represents the statistical significant difference between control group and all other treated groups. # represents the statistical difference between DEN + AAF treated groups with the EAE treated groups. * and # = $P < 0.05$.

mdr1 Expression

RT-PCR was performed to detect the change in mRNA levels of mdr1 gene. On the basis of cancer study, we selected only EAE 500 mg/kg body weight for MDR reversal study. The mRNA of mdr1 was significantly upregulated in MDR-induced animals, which was reduced near to the normal levels by the administration of EAE 500 mg/kg body weight. The quantity of mRNA in each lane was normalized by β-actin expression (Fig. 3C and D).

GC-MS

The results relating to the GC-MS analysis are given in Fig. 4 and Table 4. Three compounds were detected in hydroalcoholic extract of *Eclipta alba* leaf. The results revealed that glycine [N-methyl-N-(1-oxododecyl); 62.19%] was found as major compound followed erythritol (1,2,3,4-tetrahydroxybutane; 32.71%) and oleic acid ester (9-octadecenoic acid; 5.10%). The

TABLE 4
Phytocomponents identified in hydroalcoholic leaf extract of *Eclipta alba* using gas chromatography-mass spectrometry

No.	RT	% area	Name of the compound	Molecular weight	Nature of the compound	Activity
1	10.957	32.71	1,2,3,4-tetrahydroxybutane	120.1039	Erythritol	Food additive, antioxidant
2	16.206	62.19	Glycine, N-methyl-N-(1-oxododecyl)	420.5838	Sodium lauroylsarcosinate	Inhibit the initiation of DNA transcription
3	17.495	5.10	9-octadecenoic acid (Z)	282.4614	Oleic acid ester	Antiinflammatory, Cancer preventive, cosmetic, flavor

identified compounds were belonging to different fatty acids and their derivatives (aldyhydes, esters, and alcohols).

DISCUSSION

In the present study, 2-stage carcinogenesis was accompanied by single i.p. administration of DEN followed by 2-AAF orally, which are bioactivated to form DNA adducts in the liver (13). Apoptosis has been used to describe a form of cell death in an active and inherently controlled manner that eliminates no longer wanted cells. Cleavage of chromosomal DNA into oligonucleosomal size fragments is a biochemical assurance of apoptosis (14). The apoptosis resistance of hepatic cells was reported to be one of the significant factors for hepatocarcinogenesis or tumor progression in HCC (15). DNA isolated from liver cancer-induced groups did not show any fragmentation because of apoptosis resistance potency of HCC cells. Treatment with EAE showed increased apoptosis cancer-induced liver carcinogenesis as evident by ladder formation.

MMPs activation is associated with basement membrane remodeling that occurs in injured tissues and during tumor invasion (16). High levels of MMPs were reported in HCCs, cholangiocarcinomas, and liver metastases, compared with their low levels of expression in normal liver (17). Gelatin zymography is mainly used for the detection of the gelatinases (MMP-2 and MMP-9). Five classes of MMP have been identified so far, based on their structure and/or substrate specificity, including collagenases, gelatinases, stromelysins, elastases, and membrane-type metalloproteinases. MMP-2 (gelatinase A, 72 kDa type IV collagenase) is a widely studied matrix metalloproteinase. It was first described and purified from highly metastatic murine tumors (18) and cultured human melanoma cells (19). In our study, zymograph reveals 1 band of 72 kDa represents MMP-2. The expression of MMP-2 was observed to be elevated in cancer-induced animals. The treatment with EAE was able to reduce the levels of MMP-2 significantly implicating its antiinvasive role in cancer. In our previous study on in vitro efficacy of the EAE on HepG2 cell lines, the extract was able to decrease the MMP-2 and MMP-9 activity to a significant level in the HepG2. Thus EAE can act as an potent antimetastasis agent (7).

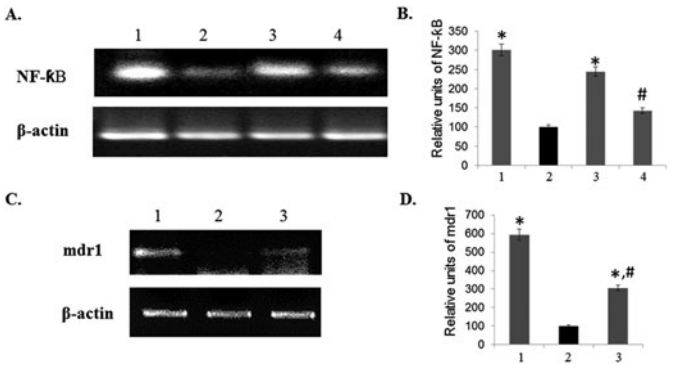

FIG. 3. **A: Nuclear factor** (NF)-kB and β-actin mRNA expression analysis using RT-PCR in the liver tissue homogenates of control, diethylnitrosamine (DEN) + 2-acetylaminofluorene (AAF) and different doses of *Eclipta alba* extract (EAE; 250 and 500 mg/kg body weight) treated animal groups after 60 days. B: Relative units of NF-kB mRNA expression were quantified relative to the β-actin expression in comparison to the control group using AlphaEase FC software (Alpha Innotech) and plotted as histogram. Lane 1-DEN + AAF, Lane 2-control, Lane 3-EAE 250 mg/kg body weight, Lane 4-EAE 500 mg/kg body weight. * represents the statistical significant difference between control group and all other treated groups. # represents the statistical difference between DEN + AAF treated groups with the EAE-treated groups. * and # = $p < 0.05$. C: mdr1 and β-actin mRNA expression analysis using RT-PCR in the liver tissue homogenates of control, AAF, and EAE 500 mg/kg body weight) treated animal groups after 14 days. D: Histogram represents units of mdr1 mRNA expression relative to the β-actin expression in comparison to the control group using AlphaEase FC software (Alpha Innotech). Lane 1-AAF, Lane 2-control, Lane 3-EAE 500 mg/kg body weight. * represents the statistical significant difference between control group and all other treated groups. # represents the statistical difference between AAF-treated group with the EAE 500 mg/kg body weight treated groups. * and # = $P < 0.05$.

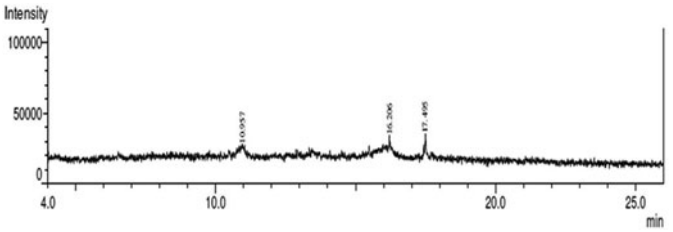

FIG. 4. Gas chromatography-mass spectrometry chromatogram of hydroalcoholic leaf extract of *Eclipta alba*.

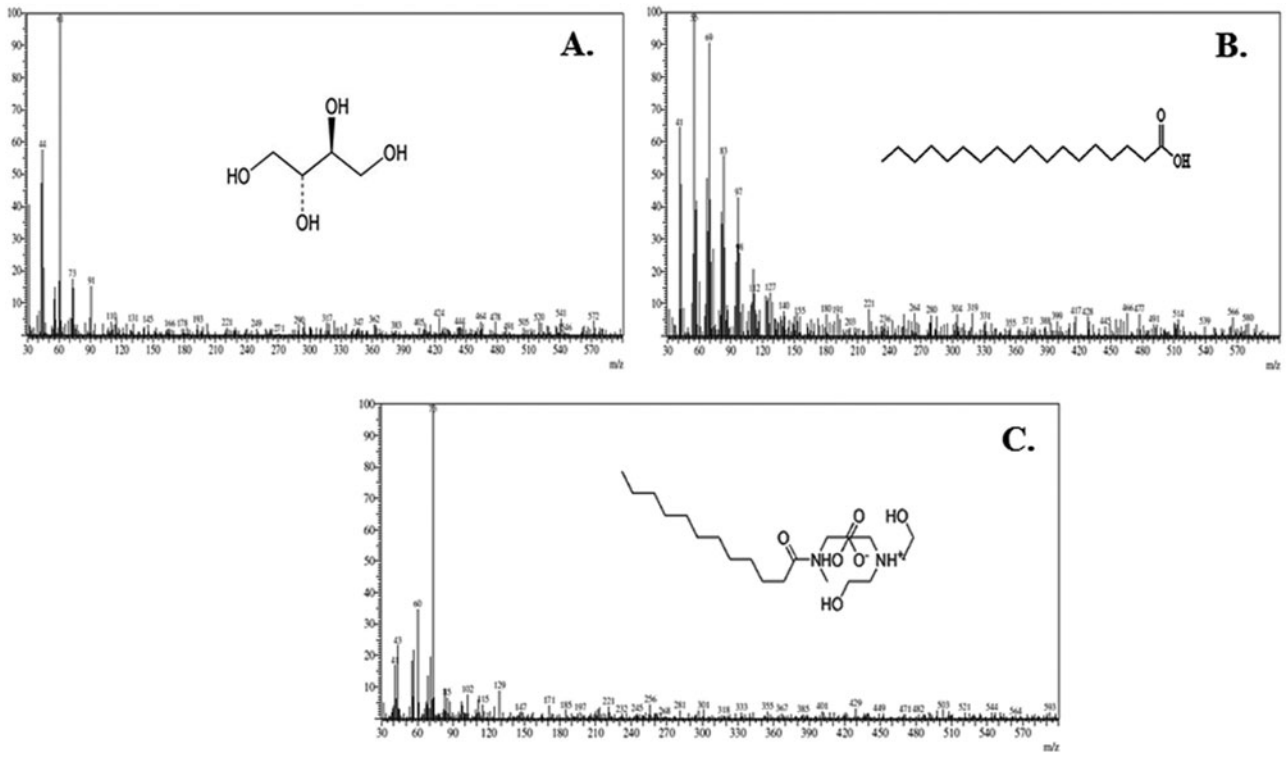

FIG. 5. A: Mass spectra of erythritol (1,2,3,4-Tetrahydroxybutane). B: Mass spectra of oleic acid ester (9-octadecenoic acid). C) Mass spectra of glycine (N-methyl-N-(1-oxododecyl).

NF-*k*B proteins are a family of transcription factors that play important roles in cell development, cell growth, survival, angiogenesis, and proliferation and is involved in many pathological conditions (20). In the present study, mRNA has been isolated and checked for the expression of NF-*k*B by using specific primers. Results indicated that the level of expression increased in liver cancer-induced animals because of activation of NF-*k*B signaling pathway. There is a decrease in the expression level of these mRNA in EAE-treated animals in a dose-dependent manner. In our earlier studies, the NF-*k*B expression was also decreased in the in vitro analysis following EAE treatment on HepG2 cell lines (7).

MDR of cancer cells is often associated with overexpression of mdr1 gene encode P-gp, a plasma membrane transporter that extrudes chemotherapeutic drugs by using ATP hydrolysis as the energy source. The xenobiotics like AAF mainly use MDR inducers in animal model (21). Sensitization of mouse liver with AAF for 3 consecutive days resulted in enhanced expression of mdr1 gene and production of ROS. Intracellular ROS is involved in regulation of P-gp expression in cancer cells (22). ALT, AST, bilirubin, albumin, and GGT level were elevated in MDR-induced as compared to the control. EAE was able to revert back MDR induced to control levels. In addition, EAE 500 mg/kg body weight administration lowered down the mRNA expression of mdr1 gene. In our previous findings, when drug-resistant HepG2 cells were treated with EAE 20 μg/ml, the level of MDR1 mRNA and P-gp was decreased to almost the same level as nonresistant HepG2 cells (8). Reduction of the expression of mdr1 levels may certainly be proposed as one of the mechanisms for certain modulators or agents to reverse MDR phenotype (23).

Histopathological examination of the liver illustrated the high damage in the connective tissue, portal blood supply, and clumps of hepatocytes. The distance between the hepatocytes was also observed to be increased, indicating hampered liver functioning. Recovery was observed in all tissue treated with EAE. The intrahepatocyte spacing was observed to be reduced, and re-appearance of the connective tissue was seen. Liver damage caused by DEN and AAF generally reflects instability of liver cell metabolism, which leads to distinctive changes in the serum enzyme activities. It is also reported that the generation of reactive oxygen species (ROS) by DEN and AAF causes carcinogenic effects. ROS are potentially dangerous by-products of cellular metabolism that have direct effect on cell development, growth, and survival (24). Serum ALT, AST, bilirubin, HDL cholesterol, and GGT are representative of liver function; their increased levels are indicators of liver damage (25). The cotreatment with EAE attenuated the increased activities of these enzymes. It is suggested that EAE aids in parenchymal cell regeneration in the liver, thus protecting membrane integrity and thereby decreasing enzyme leakage.

AFP is normally produced during fetal and neonatal development by the liver, yolk sac, and in small concentrations by the gastrointestinal tract. After birth, serum AFP concentrations decrease rapidly and thereafter only trace amounts are normally detected in serum. AFP screening, at present, is the most sensitive, convenient, and economical method for detecting early liver cancers (26). Analysis of AFP following the DEN and AAF treatment, the value was observed to be extreme high. On treatment with EAE, the concentration of the AFP was observed to reduce, indicating the control over the cancerous condition and facilitate in reverting back to the control levels.

In the present study, 3 compounds have been identified from hydroalcoholic extract of the leaf of *Eclipta alba* by GC-MS analysis. Among the identified phytochemicals, erythritol (1,2,3,4-tetrahydroxybutane; 32.71%) have the property of antioxidant activity (27). Glycine, N-methyl-N-(1-oxododecyl), and sodium lauroylsarcosinate inhibit the initiation of DNA transcription (28). Oleic acid ester (9-octadecenoic acid) has the property of antiinflammatory, antiarthritic, hypocholesterolemic, cancer preventive, cosmetic flavor (29,30). Thus, this type of GC-MS analysis is the first stage to understand the nature of active values in this medicinal plant. These results suggest that EAE was very effective at reversing cancer and MDR in vivo. Further studies into the pharmacological significance of *Eclipta alba* and their diversity and detailed phytochemistry may add new information in the traditional medical systems.

CONCLUSION

In conclusion, our study provides important inferences about new potential therapeutic options for hepatocellular cancers and MDR. EAE is expected to be promising alternative or complementary measures to conventional medical treatments for cancer treatment as well as MDR reversal. EAE may give good drug leads for cancer treatment, its systematic study and understanding is worth considering by pharmaceutical industries to develop their active ingredients as allopathic drugs for cancer therapy.

FUNDING

The authors are thankful to Nirma Education and Research Foundation, Ahmedabad, India for financial support.

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