PREVENTIVE EFFECT OF SPIRULINA VERSICOLOR AND ENTEROMORPHA FLEXUOSA ETHANOLIC EXTRACTS AGAINST DIETHYLNITROSAMINE/BENZO(A)PYRENE-INDUCED HEPATOCARCINOCITY IN RATS

OSAMA M. AHMED1,2
MOHAMED B. ASHOUR1
HANAA I. FAHIM1
AYMAN M. MAHMOUD1
NOHA A. AHMED1

1Physiology Division, Dept. of Zoology, Faculty of Science, Beni-Suef University, Egypt
2Faculty of Oral & Dental Medicine, Nahda University, New Beni-Suef City, Beni-Suef, Egypt

ABSTRACT

This study is conducted to assess the preventive effects of Spirulina versicolor and Enteromorpha flexuosa ethanolic extracts on hepatocarcinogenesis induced by diethylnitrosamine (DEN)/benzo(a)pyrene (BP) in albino rats. In vitro anti-proliferative effect of both extracts on hepatocarcinoma cell lines (HepG2) was also evaluated and was found to be moderate. After two weeks of single dose of DEN (200 mg/kg b. wt; intraperitoneal), BP, a promotor of carcinogenesis, was intraperitoneally injected (50 mg/kg b. wt) twice/week for 6 weeks. Spirulina versicolor and Enteromorpha flexuosa ethanolic extracts orally administered, at dose of 25 mg/kg b. wt/day, for 2 and 8 weeks starting from the day of DEN injection successfully counteract the carcinogenic effects of DEN and BP as evidenced by absence of precancerous oval hepatocytes and vesicular nuclei as well as decrease of tumor marker, alpha fetoprotein and proinflammatory cytokine, TNF-α levels in serum. In addition, the treatments improved DEN/BP-induced elevation of AST, ALT, LDH and γ-GGT activities, serum total bilirubin level and liver lipid peroxidation. They increased the lowered serum albumin level and antioxidant enzymes as well. In conclusion, Spirulina versicolor and Enteromorpha flexuosa ethanolic extracts successfully prevented the hepatocarcinogenic and hepatoxic effects of DEN and BP via enhancement of antioxidant defense system and suppression of oxidative stress and inflammation.

KEYWORDS: Dimetylnitrosamine, Benzo(a)pyrene, hepatocarcinogenesis, Spirulina versicolor and Enteromorpha flexuosa.

INTRODUCTION
Hepatocellular carcinoma (HCC) accounts for 70–85% of the primary malignant tumours of the liver and it is the most common cause of cancer-related death in the world (Thorgeirsson
and Grisham, 2002; Jemal et al., 2011). The major risk factors in liver cancer includes hepatitis viral infection, environmental and industrial toxic chemicals, food additives, aflatoxins, alcohol, drugs, water and air pollutants (Farazi and Depinho, 2006; Jemal et al., 2009; Fahim et al., 2008; Ahmed et al., 2008; Ahmed et al., 2013). Diethylnitrosamine (DEN) is a well-known hepatocarcinogenic agent present in cured and fried meals, cheddar cheese, tobacco smoke, water, agriculture chemicals and cosmetics and pharmaceutical products (Sullivan et al., 1991; Reh and Fajen, 1996; Brown, 1999). As established, diethylnitrosamine (N-nitrosodiethylamine; DEN) produces primary metabolic activation resulting in initiation of liver carcinogenesis (Verna et al., 1996; Chen et al., 2012). Other mechanisms may be involved through DNA-adduct formation, mutagenicity, inhibition of many enzymes involved in DNA repair mechanism and tumor initiation (Verna et al., 1996; Bansal et al., 2005).

Benzo(a)pyrene (BP) is a pro-carcinogen, and its mechanism of carcinogenesis depends on its enzymatic metabolism to the ultimate mutagen, benzo(a)pyrene diol epoxide, a molecule that intercalates in DNA, covalently bonding to the nucleophilic guanine nucleic bases at the N2 position. This binding distorts the DNA, inducing mutations by perturbing the double-helical DNA structure (Volk, 2003). There are indications that benzo[a]pyrene diol epoxide specifically targets the protective p53 gene (Shinmura, 2008). This gene is a transcription factor that regulates the cell cycle and hence functions as a tumor suppressor by inducing G (guanine) to T (thymidine) transversions within p53 (Shinmura, 2008).

A promising new approach to cancer prevention, which is termed chemoprevention, aims to halt, prevent or reverse the development and progression of pre-cancerous cells by administering non-cytotoxic nutrients and/or pharmacological agents during the time period between tumor initiation and malignancy (Sporn et al., 1976; Bishayee et al., 1995; Sayed-Ahmed et al., 2010, Zhang et al., 2013). Numbers of investigations are being conducted worldwide, to discover natural products that can suppress or prevent the process of carcinogenesis during the initiation, promotion or progression stages (Lee et al., 2002; Aggarwal et al., 2003; Cheng et al., 2004; Zhang et al., 2013).

Edible seaweeds have historically been consumed by coastal populations across the globe. Epidemiological evidence suggests regular seaweed consumption may protect against a range of diseases of modernity (Brownlee et al., 2012). Therefore, the current study was designed to evaluate the protective effect of both Spirulina versicolor and Enteromorpha flexuosa hydroethanolic extracts against the cytotoxic effects of DEN and BP.
MATERIALS AND METHODS:

**Chemicals:**
Diethyl Nitrosamine (DEN) and Benzo(a)Pyrene (BP) were purchased from Sigma Chemical Company, USA. All other chemicals used for the investigation were of analytical grade.

**Collection of seaweeds and preparation of extracts:**
*Enteromopha flexuosa* (*E. flexuosa*) was obtained from El Koseir area on Red Sea (Egypt). It was washed several times with tap water and finally with distilled water, then air-dried in shade. *Spirulina versicolor* (*S. versicolor*) was obtained from Harraz medicinal plant company, Cairo, Egypt (www.harrazegypt.com). Both algae were authenticated by staff members of the Phycology Division, Botany Department, Faculty of Science, Beni-Suef University, Egypt. Both algae were powdered with an electric grinder and extracted by maceration in 80% aqueous ethanol until exhaustion at room temperature. After filtration, the filtrate was concentrated under vacuum in a rotary evaporator. The residue obtained was stored at -20°C till use in biological evaluation.

**In vitro antitumor assay:**
Hepatocarcinoma cell lines (HepG2) were obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.) and were maintained in Dulbeco's Modified Eagle's Medium (DMEM) with 10% foetal calf serum, sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C and 5% CO₂ at Pharmacology Unit, Cancer Biology Department, National Cancer Institute, Cairo University, Egypt by serial sub-culturing. Hepatocarcinoma cell lines were seeded in flat-bottomed microtiter 96-well plate at a cell concentration of 1x 10⁴ cells per well in 200 µl of growth medium. Cytotoxicity of *S. versicolor* and *E. flexuosa* ethanolic extracts was tested using the method of Skeha et al. (1990). The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO₂ for a period of 48 h. Three wells were used for each concentration of the tested sample (0, 5, 12.5, 25 and 50 µg/ml). Control cells were incubated without test sample and with the equivalent volume of DMSO (0.1%) for 48 hr. At the end of the incubation period, the cells were fixed and stained with sulforhodamine B dissolved in acetic acid. Unbound stain was removed by washing four times with 1% acetic acid and the protein bound dye was extracted with tris–EDTA buffer.

www.jiarm.com
Absorbance was measured in an ELISA reader. The relation between surviving fraction and compound concentration was plotted to get the survival curve and the concentration of an agent that causes a 50% growth inhibition for each tested extract using each cell line was obtained from the survival curve.

**In vivo investigation:**

**Experimental animals:**

Male albino rats weighting about 120-150g were used as experimental animals in the present investigation. They were obtained from National Research Center (NRC), Dokki, Giza. They were kept under observation before the onset of the experiment to exclude any intercurrent infection. The chosen animals were housed in plastic good aerated cages at normal atmospheric temperature (25±5°C) as well as normal 12 hours light/dark cycle. Rats were given access of water and supplied daily with standard diet of known composition. All animal procedures are in accordance with the recommendations of Canadian Committee for care and use of animals (Canadian Council on Animal Care (CCAC), 1993).

**Experimental design:**

The animals were divided into 4 groups, each is 12 rats. Six rats of each group were sacrificed at the end of 2 weeks and others at the end of 8th week. The assigned groups were designated as follow:

- **Group 1 (Normal):** It intraperitoneally (ip) received the equivalent volume of normal saline (vehicle 1) at 1st day and olive oil (vehicle 2) at the end of 2nd week of the experiment. It was also orally administered the equivalent volume of 1% carboxymethylcellulose (CMC) (vehicle 3) daily for 8 weeks.
- **Group 2 (DEN/BP Control):** The rats of this group received a single ip dose of 200mg/kg b.wt. DEN (dissolved in 0.9% saline) (Saleem et al., 2013) and benzo(a)pyrene (dissolved in corn oil) at dose level of 50 mg/kg b.wt (Lutz and Schlatter, 1979) twice/week for 6 weeks beginning from the 3rd week of the experiment. The rats of this group were orally administered the equivalent volume of 1% CMC (vehicle 3) daily for 8 weeks beginning from the starting period of the experiment.
- **Group 3 (DEN/BP + S. versicolor ethanolic extract):** It received 200mg/kg b.wt DEN and 50 mg/kg b.wt benzo(a)pyrene twice/week as group 2 and orally supplemented S. versicolor ethanolic extract (25mg/kg b.wt/day) (AbouZid et al., 2013) dissolved in 5ml 1% CMC through the entire experimental period.
Group 4 (DEN/BP + *E. flexuosa* ethanolic extract): The rats of this group received 200mg/kg b.wt DEN and 50 mg/kg b.wt benzo (a) pyrene twice/week and orally supplemented *E. flexuosa* ethanolic extract (25mg/kg b.wt/day) (AbouZid *et al.*, 2013) dissolved in 5ml 1% CMC through the entire experimental period.

At the end of the specified periods, rats from each group were sacrificed under mild diethylether anaesthesia. Blood samples were collected and left to coagulate then centrifuged at 3000 r.p.m for 15 minutes. Sera were quickly removed and kept at -20°C till used. Liver were quickly excised and perfused with ice-cold saline for each rat, 0.5 gm of liver was homogenized in 5 ml 0.9% NaCl (10% w/v) using Teflon homogenizer (Glas-Col, Terre Haute, USA). The homogenate was centrifuged at 3000 r.p.m and the supernatant was kept at -20°C pending estimation of oxidative stress parameters and anti-oxidant defense markers.

**Biochemical analysis:**

Serum tumour necrosis factor alpha (TNF-α) and alpha fetoprotein (AFP) were determined using reagent kit purchased from R&D Systems (USA) according to the manufacturer instructions.

Serum ALT (Tietz, 1986), AST (Murray, 1984), γ-GT (Persijn and van derSlik, 1976) and LDH (Tietz, 1986) were determined using reagent kits from Spinreact (Spain). Total bilirubin (Kaplan and Pesce, 1984) and albumin (Webster, 1974) were determined using reagent kits purchased from Diamond Diagnostic Chemical Company (Egypt).

Liver lipid peroxidation, reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (G-S-T) were determined according to the methods of Preuss *et al.* (1998), Beutler *et al.* (1963), Matkovics *et al.* (1998), Marklund and Marklund (1974), Cohen *et al.* (1970) and Mannervik and Gutenberg (1981), respectively.

**Histological study:**

After dissection, autopsy samples were taken from the liver of rats of different groups and fixed in 10% formal saline for twenty four hours. Fixed samples were transferred to the Pathology Department, National Cancer Institute, Cairo University, Egypt for embedding in wax after dehydration, sectioning and staining with haematoxylin and eosin (Banchroft *et al.*, 1996).
Statistical analysis:
One way ANOVA (PC – STAT, 1985) was used for data analysis. Results were expressed as mean ± standard error (SE) followed by LSD at 5% and LSD at 1% to compare between the different groups. Values of P>0.05 were considered statistically non-significantly different, while values of P<0.05, P<0.01 and P<0.001 were significantly, highly significantly and very highly significantly different respectively.

RESULTS:
Data describing the effect of *S. versicolor* and *E. flexuosa* ethanolic extracts on serum TNF-α and AFP concentrations were represented in table 1. DEN/BP- administered rats showed a highly significant (P<0.01; LSD) elevation in serum TNF-α and AFP concentrations recording percentage increase of 123.58 and 260.78 % respectively as compared to normal control after 8 weeks. Treatment of DEN/BP- administered rats with either *S. versicolor* or *E. flexuosa* potentially (P<0.01; LSD) improved the altered serum levels of TNF-α and AFP, with more potent effect offered by *E. flexuosa*.

The effects of *S. versicolor* and *E. flexuosa* on liver function enzymes in serum, after 2 and 8 weeks of DEN administration, were represented in figures 2-5. The recorded data showed a significant (P<0.05; LSD) elevation of ALT activity in DEN-intoxicated rats after 2 weeks in comparison to normal control rats. On the other hand, the elevation of serum AST, LDH and γGT was highly significant (P<0.01; LSD) in DEN-intoxicated rats when compared with normal ones. The deleterious effects on these enzymes activities were more pronounced as the period extended from 2 weeks to 8 weeks. Treatment with *S. versicolor* produced a non-significant (P>0.05; LSD) effect on serum ALT, LDH and GGT activities after 2 weeks of DEN administration, while the effect on serum AST was highly significant (P<0.01; LSD). On the other hand, *E. flexuosa* effect on ALT and LDH was highly significant (P<0.01; LSD) and was significant (P<0.05; LSD) regarding GGT. Conversely, treatment with *E. flexuosa* showed a non-significant (P>0.05; LSD) effect on serum AST after 2 weeks period. After 8 weeks experimental period, all assayed serum enzymes were highly significantly elevated (P<0.01; LSD) in DEN-BP intoxicated rats when compared to the normal control group. Serum ALT, AST, LDH and GGT showed a highly significant (P<0.01; LSD) alleviation following treatment with either *S. versicolor* or *E. flexuosa*.

Regarding serum total bilirubin, there was a non-significant (P>0.05) difference between the studied groups after 2 weeks. After 8 weeks, DEN-BP administered rats showed a highly significant (P<0.01; LSD) elevation in serum total bilirubin concentration when compared to
normal control rats. Treatment with both tested extracts produced a highly significant (P<0.01; LSD) amelioration of serum total bilirubin concentration in comparison to DEN-BP control rats. After 2 weeks, serum albumin of DEN-induced rats was highly significantly (P<0.01; LSD) decreased in comparison to normal control group. *S. versicolor* ethanolic extract protected rats against the decline of serum albumin while the effect of *E. flexuosa* extract was non-significant (P<0.05; LSD) as depicted in table 1. At the end of the 8th week, DEN-BP administered rats showed a highly significant (P<0.01; LSD) reduced serum albumin in comparison with the normal control group. On the other hand, both treatment agents produced a highly significant (P<0.01; LSD) amelioration of serum albumin concentration (Table 1).

The effect of *S. versicolor* or *E. flexuosa* extracts on liver oxidative stress and antioxidant defense system were represented in table 3. At both experimental periods, liver lipid peroxidation (MDA content) showed a highly significant (P<0.01; LSD) increase in DEN-BP administered rats when compared with normal control rats. Treatment of DEN-intoxicated rats with either *S. versicolor* or *E. flexuosa* extracts produced a marked (P<0.01; LSD) amelioration of the elevated MDA. Liver GSH content of DEN control rats showed a non-significant change (P>0.05; LSD) after 2 weeks when compared to normal control rats. The treatment of DEN-administered rats for 2 weeks with *S. versicolor* produced a significant (P<0.01; LSD) increase in GSH content, while *E. flexuosa* extract produced a non-significant effect (P<0.05; LSD) as compared to DEN control. After the 8 weeks of treatment, there was a non-significant (P>0.05) difference in GSH content between all experimental groups. Liver SOD, GPx, CAT and GST activities showed a highly significant decline in DEN-administered rats (P<0.01; LSD), at the end of 2 weeks, in comparison to normal control rats. *S. versicolor* supplementation for 2 weeks produced a highly significant (P<0.01; LSD) increase in the activities of SOD, GPx, CAT and G-S-transferase activities. On the other hand, *E. flexuosa* administration for 2 weeks significantly (P<0.01; LSD) enhanced the activities of GST and SOD while it did not significantly affect GPx and CAT. After intraperitoneal administration of BP and as the period extended to 8 weeks, liver activities of GPx and SOD were more deteriorated than those at the 2nd week since the recorded percentage decreases were 58.81 and 65.42 % at the 8th weeks and -46.94% and -47.97% at the 2nd week respectively. Supplementation of DEN/BP-administered rats with *S. versicolor* extract produced a significant amelioration of the altered enzyme activities. The treatment with *E. flexuosa* extract, on the other hand, notably alleviated (P<0.01; LSD) the activities of SOD, GPx and CAT and non-significantly (P>0.05; LSD) affect GST activity.
Histopathological examination of the liver of normal control rats showed a normal histologic structure of the hepatic lobule (Fig. 6a-c). On the other hand, liver of the DEN/BP intoxicated group revealed dissociation of hepatocytes, oval cells hyperplasia, vacuolated hepatocytes, karyomegaly with vesicular nuclei as precancerous changes (Fig. 7a), cytomegalic hepatocytes with foamy cytoplasm (Fig. 7b-c). The group supplemented with *S. versicolor* as revealed in figure 8(a-d) fatty changes of hepatocytes, kupffer cells activation and vacuolization of some hepatocytes. In *E. flexuosa*-treated group, liver tissue showed inflammatory cells infiltration, kupffer cell activation, binucleation of hepatocytes and vacuolization of some hepatocytes (Fig 9a-c)

**DISCUSSION**

Most of medical remedies for liver diseases generally have limited efficacy and hence the use of complementary and alternative medicines (CAMs) that utilize herbal medicines have increased, and these CAMs have attracted considerable interest from patients for treating liver diseases (Seeff *et al.*, 2001; Strader *et al.*, 2002). DEN is a potent hepatocarcinogen influencing the initiation stage of carcinogenesis during a period of enhanced cell proliferation accompanied by hepatocellular necrosis and induces DNA carcinogen adducts, DNA-strand breaks and in turn hepatocellular carcinomas without cirrhosis through the development of putative preneoplastic focal lesions (Tatematsu *et al.*, 1988; Chen *et al.*, 2012).

The current study revealed a significant increase in serum TNF-α and AFP of DEN/BP-administered rats. It has been reported that TNF-α is produced by macrophages and they play an important role in tumor conditions (Moon *et al.*, 1999). In addition, TNF-α is an essential factor in tumor promotion (Suganuma *et al.*, 2000). Indeed, Barton *et al.* (2001) stated that TNF-α plays a causal role in the development of liver injury. Alleviation of serum TNF-α levels produced by supplementation of either *E. flexuosa* or *S. versicolor* in DEN/BP-intoxicated rats, may be attributed to their ant-inflammatory and antitumor activities. Alpha fetoprotein is a fetal specific glycoprotein which falls rapidly after birth, high level of alpha fetoprotein is suspicious of hepatocellular carcinoma but may be elevated in chronic viral hepatitis (Patil *et al.*, 2013). Its decrease in DEN/BP-administered rats treated with *E. flexuosa* or *S. versicolor* ethanolic extract may provide evidence for decreased probability for hepatocellular carcinoma as compared with DEN/BP-administered control rats which showed profound elevation of serum AFP.
Reuben (2004) reported that elevation in transaminase levels in conjunction with a rise in bilirubin level to more than double of its normal level, is considered as an ominous marker for hepatotoxicity. In addition, it is well known that the elevation of ALT and ALP is credited to hepatocellular damage and reflects the pathological alteration in biliary flow (Bulle et al., 1990; Sivaramakrishnan et al., 2008). In the current study, serum AST, ALT, GGT and bilirubin levels were significantly increased following DEN and BP administration. An elevated level of serum indices of hepatocellular damage has been previously reported in many models of DEN-induced hepatocellular degeneration (Ha et al., 2001; Lee et al., 2002; Barbisan et al., 2003; Bansal et al., 2005; Sayed-Ahmed et al., 2010).

Supplementation of either *E. flexuosa* or *S. versicolor* significantly prevented the increase in serum liver function markers, suggesting that both agents may have protective effect against DEN/BP-induced liver damage by stabilizing the hepatocyte membrane. The current data run parallel to the study of Ismail et al. (2010) who demonstrated the chemoprevention of rat liver toxicity and carcinogenesis by Spirulina platensis. In addition, Amin et al. (2006) reported the hepatoprotective effect of Spirulina against cadmium-induced hepatotoxicity. Moreover, Kumar et al (2005) stated that consumption of Enteromorpha rich diets reversed DEN-hepatocellular carcinoma in rats.

Recently, many studies confirmed the contribution of oxidative stress during development of hepatocarcinogenesis and promotion of liver cancer (Gayathri et al., 2009; Al-Rejaie et al., 2009; Janani et al., 2010; Mizukami et al., 2010; Taha et al., 2010; Yang et al., 2010). The current data revealed a significant increase of liver MDA content with a concomitant reduction of GSH content and activities of the antioxidant enzymes, GPx, SOD and CAT, in DEN-BP intoxicated rats when compared with normal control rats at both experimental periods. Treatment of DEN and BP-administered rats with either *S. versicolor* or *E. flexuosa* extracts produced marked reduction of the elevated MDA accompanied with improved antioxidant system. We assumed that, the hepatoprotective effects of the tested algal extracts were due to their antioxidant properties and their ability to reduce lipid peroxidation. In this regard, Evanprince et al. (2009) reported the hepatoprotective potential of *S. fusiformis* in acetaminophen-induced hepatotoxicity in mice and attributed this finding to the antioxidant effects of Spirulina and to its ability to potentiate the antioxidant system. In addition, Spirulina supplementation has reduced lipid peroxidation and increased activity of the antioxidant system in the liver of cadmium-intoxicated rats as reported by Amin et al. (2006). Similarly, Enteromorpha has been found to exhibit potent antioxidant activity (Shanab et al., 2011;
Narasimhan et al., 2013) and to protect against the hepatotoxic effects of DEN (Kumar et al., 2005). In conclusion, both *S. versicolor* or *E. flexuosa* prevented DEN/BP-induced hepatotoxicity and hepatocarcinogenesis through their anti-inflammatory, anti-carcinogenic and antioxidant efficacies.

Table 1: Serum TNF-α and AFP Effects of *S. versicolor* and *E. flexuosa* ethanolic extracts on DEN /BP administered rats at the 8th week.

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>TNF-α (pg/ml)</th>
<th>% change</th>
<th>AFP (ng/ml)</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td>32.1 ± 1.23d</td>
<td>-</td>
<td>0.51 ± 0.05c</td>
<td>-</td>
</tr>
<tr>
<td>DEN/BP Control</td>
<td></td>
<td>71.77 ± 1.40a</td>
<td>+123.58</td>
<td>1.84 ± 0.10a</td>
<td>+260.78</td>
</tr>
<tr>
<td>DEN/BP + <em>S. versicolor</em></td>
<td></td>
<td>50.5 ± 0.44b</td>
<td>-29.64</td>
<td>0.97 ± 0.09b</td>
<td>-47.28</td>
</tr>
<tr>
<td>DEN/BP + <em>E. flexuosa</em></td>
<td></td>
<td>44.33 ± 1.97c</td>
<td>-38.23</td>
<td>0.79 ± 0.06b</td>
<td>-57.07</td>
</tr>
</tbody>
</table>

F-Probability: P<0.001  LSD at 5% level: 3.31  LSD at 1% level: 4.51

- Data are expressed as mean ± SE. Number of animals in each group is six.
- Means, which share the same superscript symbol (s), are not significantly different.

Table 2: Effect of *S. versicolor* and *E. flexuosa* ethanolic extracts on serum albumin and total bilirubin concentrations of DEN /BP administered rats at the 2nd and 8th weeks.

<table>
<thead>
<tr>
<th>periods</th>
<th>Group</th>
<th>Parameter</th>
<th>Total bilirubin (mg/dl)</th>
<th>% change</th>
<th>Albumin (g/dl)</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Weeks</td>
<td>Normal control</td>
<td></td>
<td>0.52 ± 0.02d</td>
<td>-</td>
<td>3.95 ± 0.02a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DEN control</td>
<td></td>
<td>0.69 ± 0.03d</td>
<td>+32.69%</td>
<td>2.80 ± 0.15c</td>
<td>-29.11%</td>
</tr>
<tr>
<td></td>
<td>DEN + <em>E. flexuosa</em></td>
<td></td>
<td>0.58 ± 0.03d</td>
<td>-15.94%</td>
<td>3.60 ± 0.40ab</td>
<td>+28.57%</td>
</tr>
<tr>
<td></td>
<td>DEN + <em>S. versicolor</em></td>
<td></td>
<td>0.62 ± 0.03d</td>
<td>-10.14%</td>
<td>2.93 ± 0.10bc</td>
<td>+4.64%</td>
</tr>
<tr>
<td>8 Weeks</td>
<td>Normal control</td>
<td></td>
<td>0.59 ± 0.07d</td>
<td>-</td>
<td>3.92 ± 0.25a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DEN/BP control</td>
<td></td>
<td>1.67 ± 0.15a</td>
<td>+183.05%</td>
<td>2.43 ± 0.14c</td>
<td>-38.01%</td>
</tr>
<tr>
<td></td>
<td>DEN/BP + <em>S. versicolor</em></td>
<td></td>
<td>1.17 ± 0.19b</td>
<td>-29.94%</td>
<td>3.68 ± 0.13a</td>
<td>+51.44%</td>
</tr>
<tr>
<td></td>
<td>DEN/BP + <em>E. flexuosa</em></td>
<td></td>
<td>1.01 ± 0.13bc</td>
<td>-39.52%</td>
<td>3.67 ± 0.24a</td>
<td>+51.03%</td>
</tr>
</tbody>
</table>

F-probability: P<0.001  LSD at 5% level: 0.34  LSD at 1% level: 0.46

- Data are expressed as mean ± SE. Number of animals in each group is six.
- Means which share the same superscript symbol(s) are not significantly different.
Table 3: Effect of *E. flexuosa* and *S. versicolor* ethanolic extracts on oxidative stress and antioxidant defense markers in liver of DEN/BP-administered animals at the 2nd and 8th weeks.

<table>
<thead>
<tr>
<th>Periods</th>
<th>Parameter</th>
<th>LPO (nmol MDA/100mg tissue)</th>
<th>% change</th>
<th>GSH (nmol/100mg tissue)</th>
<th>% change</th>
<th>GST (mU/100mg tissue)</th>
<th>% change</th>
<th>GPx (mU/100mg tissue)</th>
<th>% change</th>
<th>CAT (k × 10^2)</th>
<th>% change</th>
<th>SOD (U/g tissue)</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Weeks</td>
<td>Normal control</td>
<td>67.25 ± 0.46^b</td>
<td>-</td>
<td>19.46 ± 0.43^b</td>
<td>-</td>
<td>91.6 ± 8.10^b</td>
<td>-</td>
<td>18.94 ± 1.69^b</td>
<td>-</td>
<td>40.68 ± 2.14^b</td>
<td>-</td>
<td>18.26 ± 0.29^b</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DEN control</td>
<td>87.25 ± 0.39^a</td>
<td>+29.74%</td>
<td>18.79 ± 0.13^b</td>
<td>-3.44%</td>
<td>64.67 ± 1.54^d</td>
<td>-29.42%</td>
<td>10.05 ± 0.32^c</td>
<td>-46.94%</td>
<td>15.28 ± 0.41^d</td>
<td>-62.44%</td>
<td>9.50 ± 0.35^c</td>
<td>-47.97%</td>
</tr>
<tr>
<td></td>
<td>DEN + <em>S. versicolor</em></td>
<td>66.63 ± 0.32^e</td>
<td>-23.63%</td>
<td>19.47 ± 0.54^b</td>
<td>+3.62%</td>
<td>91.46 ± 3.04^b</td>
<td>+41.43%</td>
<td>21.07 ± 1.81^d</td>
<td>+109.65%</td>
<td>37.61 ± 2.04^e</td>
<td>+146.14%</td>
<td>18.75 ± 0.22^a</td>
<td>+97.37%</td>
</tr>
<tr>
<td></td>
<td>DEN + <em>E. flexuosa</em></td>
<td>64.88 ± 1.25^c</td>
<td>-25.64%</td>
<td>36.07 ± 4.88^a</td>
<td>+91.96%</td>
<td>85.47 ± 4.66^b</td>
<td>+32.16%</td>
<td>15.67 ± 0.68^b</td>
<td>+55.92%</td>
<td>20.69 ± 0.62^d</td>
<td>+35.41%</td>
<td>15.20 ± 0.31^d</td>
<td>+60%</td>
</tr>
<tr>
<td>8 Weeks</td>
<td>Normal control</td>
<td>71.03 ± 1.38^b</td>
<td>-</td>
<td>20.34 ± 0.22^b</td>
<td>-</td>
<td>105.4 ± 2.53^a</td>
<td>-</td>
<td>16.07 ± 1.26^bc</td>
<td>-</td>
<td>40.46 ± 3.89^b</td>
<td>-</td>
<td>17.93 ± 0.63^abc</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DEN/BP control</td>
<td>91.98 ± 3.68^a</td>
<td>+29.49%</td>
<td>16.49 ± 0.09^b</td>
<td>-18.93%</td>
<td>76.11 ± 1.88^ad</td>
<td>-27.79%</td>
<td>6.62 ± 0.57^c</td>
<td>-58.11%</td>
<td>31.54 ± 1.47^e</td>
<td>-22.05%</td>
<td>6.20 ± 0.70^f</td>
<td>-65.42%</td>
</tr>
<tr>
<td></td>
<td>DEN/BP + <em>S. versicolor</em></td>
<td>66.95 ± 1.90^c</td>
<td>-27.21%</td>
<td>21.37 ± 0.15^b</td>
<td>+29.59%</td>
<td>89.43 ± 2.72^b</td>
<td>+17.50%</td>
<td>19.63 ± 2.60^b</td>
<td>+196.53%</td>
<td>43.31 ± 0.56^ab</td>
<td>+37.32%</td>
<td>16.47 ± 0.60^ed</td>
<td>+165.65%</td>
</tr>
<tr>
<td></td>
<td>DEN/BP + <em>E. flexuosa</em></td>
<td>67.02 ± 1.38^c</td>
<td>-27.14%</td>
<td>21.96 ± 0.22^b</td>
<td>+33.17%</td>
<td>85.19 ± 1.83^b</td>
<td>+11.93%</td>
<td>13.60 ± 1.66^c</td>
<td>+105.44%</td>
<td>48.60 ± 2.10^c</td>
<td>+54.09%</td>
<td>16.6 ± 0.66^med</td>
<td>+167.74%</td>
</tr>
<tr>
<td>F-probability</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSD at 5% level</td>
<td>5.70</td>
<td>5.83</td>
<td>12.70</td>
<td>5.02</td>
<td>6.59</td>
<td>1.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSD at 1% level</td>
<td>7.62</td>
<td>7.85</td>
<td>17.37</td>
<td>6.76</td>
<td>8.87</td>
<td>2.27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Data are expressed as mean ± SE. Number of animals in each group is six.
- Means, which share the same superscript symbol(s), are not significantly different.

www.jiarm.com
Fig. 1: Cytotoxicity effects of *S. versicolor* and *E. flexuosa* ethanolic extracts against HepG2 cell line. IC\(_{50}\) for *S. versicolor* = 45.62 µg/ml, IC\(_{50}\) for *E. flexuosa* = 44.95 µg/ml

Fig. 2: Effect of *S. versicolor* and *E. flexuosa* ethanolic extracts on serum AST activity of DEN/BP-intoxicated rats. LSD at 5% level: 11.77; LSD at 1% level: 15.85.
Fig. 3: Effect of *S. versicolor* and *E. flexuosa* ethanolic extracts on serum ALT activity of DEN/ BP-intoxicated rats. LSD at 5% level: 10.11; LSD at 1% level: 13.62.

Fig. 4: Effect of *S. versicolor* and *E. flexuosa* ethanolic extracts on serum LDH activity of DEN/ BP-intoxicated rats. LSD at 5% level: 59.33; LSD at 1% level: 79.90.

Fig. 5: Effect of *S. versicolor* and *E. flexuosa* ethanolic extracts on serum γGT activity of DEN/BP-intoxicated rats. LSD at 5% level: 7.13; LSD at 1% level: 9.60.
Legend for figures from 6 to 9

Fig. 6a-c: Photomicrographs of liver sections of normal control groups showing the normal histological structure of hepatic lobule, CV: (central vein); S: (sinusoids); T: (trabecula); KC: (Kupffer cells). (H and E x400).

Fig. 7: Photomicrographs of liver sections of DEN- BP administered rats showing dissociation of hepatocytes, oval cells hyperplasia (OV), vacuolated hepatocytes (Vh), karyomegaly with vesicular nuclei as precancerous changes. (Fig.7a), cytomegalic hepatocytes (CM) with foamy cytoplasm. (Fig.7b) and vesicular nuclei as precancerous changes (Fig.7c) (H and E x400).

Fig. 8a-d: Photomicrographs of liver sections of DEN/BP administered rats treated with S. versicolor showing fatty change (FC) of hepatocytes, kupffer cells (KC) activation and vacuolization (V) of some hepatocytes (H and E X400).

Fig. 9: Photomicrographs of liver sections of DEN/BP administered rats treated with E. flexuosa showing inflammatory cells infiltration (IF) in hepatic sinusoids (Fig. 9a), kupffer cell (KC) activation and binucleation of hepatocytes (Fig. 9b) and kupffer cell activation and vacuolization (V) of some hepatocytes (Fig. 9c). (H and E X400).
REFERENCES:


