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Cytotoxic Efficacy of Nannochloropsis Extracts on Lung Carcinoma in Mice

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Abstract

Objectives: The natural biomolecules from microalgae were renowned for their biomedical and pharmacological applications. In this study, cytotoxic efficacy of *Nannochloropsis* extracts was investigated on lung cancer induced by benzo(a)pyrene (BaP) in mice. **Methods/Statistical Analysis:** Acute toxicity studies using Ethyl Acetate Extract *Nannochloropsis* Hexane (EAENH) fractionated extract (5 mg/kg, 50 mg/Kg, 300 mg/kg and 2000 mg/kg) were carried out in mice. The*in vivo* study was accomplished in mice generated with lung cancer. **Findings:** The acute toxicity showed EAENH was non-toxic up to 2000 mg/kg devoid of any deaths throughout the study. The *in vivo* study demonstrates upsurge in the final body weight than the tumour-bearing mice group with reduced lung weight in EAENH-treated mice. The haematological and biochemical parameters of EAENH-treated animals gradually reversed to standard values. The antioxidant enzymes generated in EAENH-treated animals initiated apoptosis by oxidative stress. The histopathology of lungs displayed protective efficiency in the EAENH treated groups. It was evident through Western Blot analysis, EAENH turned on caspase 3, up-regulated CYP1A1 and Bax proapoptotic protein, down-regulated Bcl2 antiapoptotic protein in EAENH-treated animals suggesting EAENH promotes apoptosis via Caspase-dependent pathway. RT-PCR involves EAENH-induced apoptotic activity by instigating caspase and impeding phosphorylation of *ERK/Akt* in tumour cells. EAENH repressed the tumour growth in a dose-dependent manner. **Application/Improvements:** The findings suggested that phytochemicals in EAENH could be used successfully as cytotoxic agent for lung cancer.

Keywords: Benzo(a)pyrene, Mice, *Nannochloropsis,* Real Time-Polymerase Chain Reaction **(**RT-PCR), Western Blot

1. Introduction

Lung cancer is the second most widespread one in men and women equally worldwide[1]. Chemotherapy, photodynamic therapy and surgery are the current treatment lines for advanced phases[2,3]. Metastasis occurs in most of the cases during the diagnosis period, thus severely restricting therapeutic possibilities[4]. Phytochemicals identified in microalgae have a high biological demand and considered as alternatives to chemo preventive agents[5].

In tobacco smoke more than 60 carcinogens have been identified. Benzo(a)Pyrene (BaP), a key component

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present in smoke plays a vital part in generating lung carcinogenesis[6]. BaP, a polycyclic aromatic hydrocarbon is metabolically triggered into BaP 7, 8-diol-9, 10-epoxideto develop DNA adduct and disease[7]. The smoke inhalation subject the lung tissue to release raised concentrations of free radical and peroxidation products which build oxidative stress directing carcinogenesis by altering gene expression[8].

The thirst for new biomolecules from natural resources was massive attributed to the scarcity of therapeutic drugs for life-threatening diseases like cancer, AIDS, etc. Only few accounts were registered on the biological activity of microalgal extracts particularly against lung cancer cells. Moreover, presence of carotenoids, fatty acids, flavonoids and saponins in microalgae plus correlation between biochemical and antioxidant capacity has been reported[9]. Researchers have paid great attention to microalgae since their phytochemicals are rich in biomedical properties. The *Chlorella ovalis*, *Nannochloropsis oculata* and Dinoflagellate *Amphidinium carterae* showed anti-proliferative and anti-inflammatory activities[10]. *Nannochloropsis sp*. exhibited strong antioxidant effect in a similar study performed earlier[11]. *Nannochloropsis sp.* is a unicellular spherical green alga belonging to the Eustigmatophyceae class, Chlorophyceae group, which plays a commanding role in the food chain system, and is commonly employed as aqua live feed[12]. It is well known to store carotenoids under stressful conditions. Carotenoids and fatty acids have attained pharmaceutical and nutraceutical importance owing to their enhanced antioxidant abilities[13]. In this research paper, an attempt was rendered to examine the cytotoxic efficacy of EAENH against B(a)P induced lung carcinogenesis.

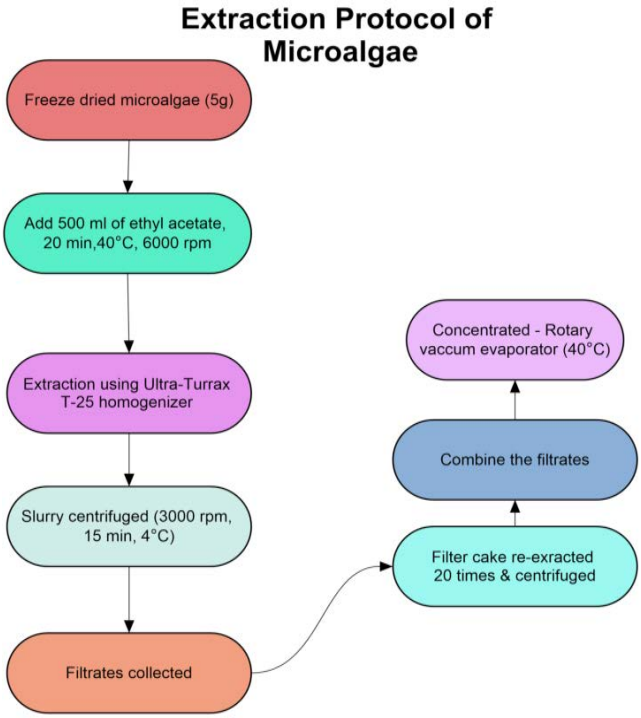
2. Materials and Methods

2.1 Materials

BaP, enzymes and enzymes were purchased from Sigma Aldrich, Mumbai. All other reagents and chemicals used were of analytical grade.

2.2 Microalgal Extraction

The biomass dry weight was noticed to be 5 g and the percentage yield of ethyl acetate extract was 38.88% (1.9 g). The dried resulting ethyl acetate extract was partially purified further using an open silica column chromatography; eluted with mixture of hexane:ethyl acetate, ethyl acetate:methanol and toluene:ethyl acetate; active fractions collected using Thin Layer Chromatography; evaporated resulting in a concentrated thick residue i.e., EAENH powder[14].



2.3 Animals

Healthy Swiss albino mice (6–8 weeks old) weighing 25–30 g were obtained from GIET School of Pharmacy, Rajahmundry, Andhra Pradesh and housed in cages of polypropylene. The animals were maintained under standard temperature and light conditions (25±2°C, 12 h light and dark cycle) and accustomed to laboratory conditions. They were provided standard pellet diet and had free access to water. Ethical clearance (for handling of animals and the procedures used in study) was obtained from the Institutional Animal Ethical Committee (GSP/IEAC/2018/02/02) before conducting the *in vivo* study.

2.4 Acute Toxicity Study

In order to acclimatize to the laboratory conditions, the animals were kept in the microlon cages for 5 days prior to dosing. The animals were weighed one day before dosing after the fasting period. During the first 30 minutes after dosage, animals were observed individually at least once, regularly during the first 24 h, whereas special attention was given during the first 4 h, and daily there-

after for a total of 14 days. A set of three animals were then administered orally using gavage tube for each dosage with EAENH (5 mg/kg, 50 mg/Kg, 300 mg/kg and 2000 mg/kg dissolved in olive oil) (OECD 2001). The animals were weighed and humanely slaughtered after the experimental phase. The histopathological examination of their vital organs including heart, lungs, liver, kidneys and brain were done according to OECD 423 guidelines[15].

2.5 *In-vivo* study on BaP Induced Lung Carcinoma in Swiss Albino Mice

Animal grouping was carried out to avoid statistical differences in the body weight of mice. The experimental animals were divided into six groups with six animals in each group.

Group I: Control, received olive oil throughout the course of the experiment (0.1%).

Group II: Induced lung cancer (2 X10^6) with BaP [50 mg/kg dissolved in olive oil] orally, twice a week (1st and 4th day) for four successive weeks

Group III: BaP [50 mg/kg dissolved in olive oil] along with Cisplatin [20 mg/kg body weight] orally twice a week for 16 weeks after the first dose of BaP administration.

Group IV: BaP [50 mg/kg dissolved in olive oil] along with EAENH [50mg/kg body weight dissolved in olive oil]; EAENH treatment began one week after the first dose of BaP administration and continued daily for 16 weeks.

Group IV: BaP [50 mg/kg dissolved in olive oil] along with EAENH [100mg/kg body weight dissolved in olive oil]; EAENH treatment began one week after the first dose of BaP administration and continued daily for 16 weeks**.**

Group VI: EAENH (20mg/kg body weight dissolved in olive oil), given daily for 16 weeks using gavage tube to assess EAENH-induced cytotoxicity (if any). Each mouse was fed as required by the experimental groups with the aid of a fine pipette. Based on acute toxicity studies, the dosing regimen for the experimental animals was fixed[16].

All methodologies were carried out at low temperatures. Blood was collected for haematological and biochemical study parameters. The animals were sacrificed by cervical decapitation at the end of the experimental period. The lung tissues were immediately expunged, rinsed in ice cold saline, blotted dry, weighed and homogenized in 0.1 mol/L Tris-Hydrochloride buffer (pH 7.4). The homogenate was used for biochemical and immunoblotting studies. Any change of deduction was identified by gross histomorphological examination of lung tissues.

2.5.1 Hematological Analysis

The blood was collected through retro-orbital puncture from mice under slight anaesthesia using diethyl ether. The haematological parameter such as Red Blood Cells (RBC), White Blood Cells (WBC), Differential Count (DC), and haemoglobin (Hgb) were measured by cell analyzer. The differential count of WBC was determined with Leishman stained blood smear[17].

2.5.2 Biochemical Analysis

The blood was collected, centrifuged and serum was utilized to estimate the parameters like Serum Glutamate Oxaloacetate Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), Alka-Line Phosphatase (ALP) levels[18]. The lung homogenate was used for detecting the levels of Lipid Per-Oxidation (LPO), Super-Oxide Dismutase (SOD), CATalase (CAT), Glutathione Peroxidase (GP) and Glutathione Reductase (GR) levels[17].

2.5.3 Histopathological Examination of Lungs, Tumour Incidence and Measurement of Body Weight and Lung Weight

Histopathological evaluation was conducted to confirm the tumour-induction in mice treated with BaP and to establish the EAENH effect on the tumour. After slaying the mice, lungs were collected, cleaned recurrently in Phosphate Buffered Saline (PBS) and immersed in blotting paper for blood removal. The fixation of tissues was ensured using 10% neutral buffered formalin for 24 h. The tissue samples were desiccated in escalating concentrations of ethanol, cleaned in xylene and implanted in paraffin to prepare the block. For microscopical analysis, serial section of lungs was excised, stained with hematoxylene and eosin, then photomicrographs were captured[16,19]. After dissection, the weight of entire body and lungs including tumour incidence were measured in all mice[19].

2.5.4 Western Blot Analysis

The first step was to isolate protein prior to western blot analysis. From all groups lungs were collected and homogenized in lysis buffer. The homogenized material was centrifuged for 15 minutes at 13,000 *g* at 4ºC; then collect the supernatant identified as cytoplasmic extract. The stored supernatant was used for western blot analysis and Lowry's method was employed to quantify the amount of protein. The density was established by Gel Doc System for quantitative assay of each band. Sodium Dodecyl Sulphate (SDS) polyacrylamide gel electrophoresis separated aliquots comprising 20–50 µg proteins; then electrotransferred to a nitrocellulose membrane. The membranes were exposed to immunoblot testing and using Enhanced Chemi-Luminescence (ECL) method the protein bands were observed[20].

2.5.5 Real-Time Polymerase Chain Reaction (RT-PCR) for Detecting mRNA Expression of Different Genes

The total RNA from the lung tissue was extracted using Trizol reagent following the standard protocol. After PCR, 5-µL sample aliquots were subjected to 1% (w/v) agarose gel electrophoresis for 20–30 min, stained with ethidium bromide and photographed. Densitometry was carried out using Total Lab software. The internal loading control amplified was β-actin mRNA[21]. The internal loading control amplified was beta-actin mRNA since it has been used in a similar research related to evaluation of anticancer agents of Ficus glomerata extract against lung, breast and colon human tumour cell lines.

2.6 Statistical Analysis

All values were represented as the mean ± standard deviation of triplicates (n = 3) of each experiment. The data were analyzed using ANalysis Of VAriance (ANOVA). The results with $P \leq 0.05$ were determined to be statistically significant. The data were statistically calculated by Microsoft Excel 2007 and linear regression analysis using Graph Pad Prism (Windows version 6.01, Graph Pad Software, La Jolla, California, USA).

3. Results

3.1 Phytochemical, Biochemical Contents Antioxidant and in Vitro Cytotoxicity Assay of EAENH

The phytochemical screening of *Nannochloropsis sp.*primarily comprises saponins, terpenoids, flavonoids, and phenols which were confirmed by HPTLC, FT-IR and GC-MS analysis. The EAENH fraction showed 40.61 mg GAE/g, 68.77 mg QE/g, 5.73 mg/g, and 57.38 mg CHL/g for total phenolic, flavonoid, carotenoid, and sterol content, respectively. Moreover, antioxidant activities were evaluated for the extract showing high flavonoid and phenolic contents after partial purification with hexane. The half inhibitory concentration (IC_{50}) values for EAENH was found to be 13.9, 21.22, and 14.58 µg/mL for 1,1diphenyl2picrylhydrazyl radical, hydrogen peroxide and reducing power assays respectively. The cytotoxic activity of EAENH on human nonsmall lung cancer cell line (A549) IC_{50}value was 175 µg/mL using 3(4,5 dimethylthiazol2yl) 2,5diphenyltetrazolium bromide in vitro assay[14].

3.2 Acute Toxicity Studies

According to OECD 423 Guidelines, no mortality was documented among the mice treated orally with 2000 mg/kg of EAENH in the 14 days observation period kept under standard housing conditions. During this study phase, the mice showed no mortality and variations in the

Table 1. Clinical observations of acute toxicity for EAENH

Sl. No.	Observation	5 mg/Kg	50 mg/Kg	300 mg/Kg	2000 mg/Kg
1.	Tremor	- ve	- ve	- ve	- ve
2.	Convulsion	- ve	- ve	- ve	- ve
3.	Salivation	NAD	NAD	NAD	NAD
4.	Pilo-erection	- ve	- ve	- ve	- ve
5.	Lethargy	- ve	- ve	- ve	- ve
6.	Sleep	NAD	NAD	NAD	NAD
7.	Coma	- ve	- ve	- ve	- ve

general appearance. The evaluation of clinical signs (Table 1) presented no aberrant symptoms that could be caused by EAENH treatment. Only some agonal changes were detected at necropsy and absence of extract associated gross lesions in the histopathology studies of all dosage studies (Figure 1).

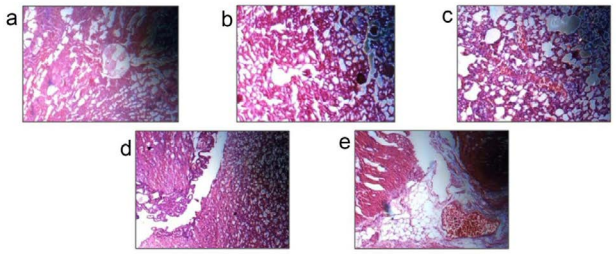


Figure 1. Histopathological studies of Lungs, Liver, kidney, heart, and brain showing normal architecture of cells. Biopsy of (a) Lungs, (b) Liver, (c) Kidney, (d) Heart and (e) Brain in acute toxicity test (2000 mg/kg).

3.3 Tumour Incidence and Measurement of Body Weight and Lung Weight

The tumour incidence, body weight and lung weight of all groups of mice that were killed after 16 weeks study were showed in Table 2. Overall, not only Group V mice (Figure 2e) showing less tumour nodules than that of Group IV (Figure 2d) mice, but also low number of tumour nodules was noted when compared with the vigorously grown tumours found in Group II mice (Figure 2b). No considerable changes was noted in Group VI mice (Figure 2f) ($p<0.05$) (treated only with EAENH) when matched with control Group I mice (Figure 2a). In Bap-induced mice treated with EAENH, the final body weight was substantially increased and lung weight was effectively reduced in group V mice ($p<0.05$) than Group IV mice. Group III Cisplatin-treated mice (Figure 2c) exhibited very fewer tumour nodules than Group V.

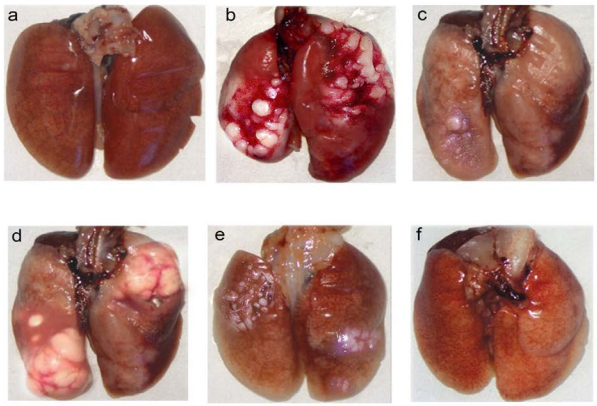


Figure 2. Tumor incidence in the experimental animals; a. Group 1: Control; b. Group 2: BaP+ olive oil; c. Group 3: BaP+ olive oil + Cisplatin; d. Group 4: BaP+ olive oil + 50 mg/kg bw of EAENH; e. Group 5: BaP+ olive oil + 100 mg/kg bw of EAENH; f. Group VI: 20mg/kg bw of EAENH + olive oil.

A sudden drop in body weight might be attributed to cancer cachexia in Group II tumour-bearing mice. Cancer cachexia leads to gradual weight loss, noticed in cancer patients that implies weak prognosis and reduces their life expectancy[22]. The steady rise in body weight in EAENH treatment (group IV and V) suggested direct anticancer effect of EAENH. There were no considerable differences in mice treated only with EAENH (Group VI). The enormous increase in lung weight in tumour-bearing animals could be attributed to the immense proliferation of the cancer cells (Group II).

The slow reversal of RBC count and haemoglobin values in mice treated with BaP plus EAENH strongly

Table 2. Effect of EAENH on the body weight and lung weight of the experimental animals

Parameters	A-549 lung cancer cells					
	Control (Olive oil)	Tumour Control (B(a)P 50 mg/kg)	Standard (Cisplatin 20 mg/kg)	EAENH (50 mg/kg + B(a)P 50 mg/kg)	EAENH (100 mg/kg + B(a)P 50 mg/kg)	EAENH (50 mg/kg)
Body weight (g)	30.21±1.12	18.7±0.51	30.13±1.11	25.73±1.03	28.09±0.33	27.73±1.01
Lung weight (g)	0.268.11±0.003	0.395.08±0.004	0.267.05±0.007	0.258.21±0.002	0.262.03±0.005	0.261.01±0.001

Values represent the mean ± SD for six mice Statistical significance at $P \leq 0.05$, as compared with groups

Table 3. Effect of EAENH on the Hematological parameters of the experimental animals

Haemato-logical Para-meters	A-549 lung cancer cells					
	Control (Olive oil)	Tumour Control (B(a) P 50 mg/kg)	Standard (Cisplatin 20 mg/kg)	EAENH 50 mg/kg + B(a) P 50 mg/kg)	EAENH (100 mg/kg + B(a) P 50 mg/kg)	EAENH (50 mg/kg)
Hb (g%)	12.41±1.07	6.7±0.82	12.13±1.01	10.73±1.03	11.96±0.33	11.73±1.03
RBC (million/ mm^3)	5.21±0.65	2.5±0.61	5.14±0.12	4.13±0.32	4.91±0.02	5.11±0.32
Hematocrit (%)	12.02±0.41	22.51±0.32	13.05±0.21	15.09±0.09	14.11±0.05	14.03±0.09
MCV (fL)	35.32±0.12	63.11±0.41	37.28±0.03	40.13±0.08	41.21±0.13	42.13±0.08
MCH (pg)	55.05±0.23	50.68±0.32	54.17±0.05	53.07±0.09	57.71±0.11	54.15±0.09
MCHC (g/dL)	18.55±0.61	20.13±0.33	18.11±0.42	17.12±0.07	17.36±0.12	17.21±0.07
PLT (10^3/mm^3)	34.12±0.13	38.05±0.15	34.02±0.07	32.13±0.11	33.01±0.03	34.03±0.05
RDW (%)	25.03±0.23	17.62±0.51	23.51±0.33	18.45±0.19	24.11±0.15	24.31±0.13
WBC (10^3 cells/mm^3)	6.81±0.52	14.05±0.22	6.92±0.41	5.77±0.12	5.03±0.09	6.87±0.15
DC-Lym (%)	70.32±0.61	35.11±0.16	67.21±0.19	69.13±0.08	72.91±0.11	68.71±0.11
DC-Neutro (%)	18.42±0.81	51.34±0.16	19.13±0.41	16.09±0.17	15.11±0.15	19.11±0.17
DC-Mono (%)	0.93±0.92	0.45±0.35	0.91±0.33	0.91±0.18	0.98±0.07	0.88±0.33
DC Granu-locytes (%)	15.31±0.19	5.51±0.27	14.15±0.45	14.13±0.22	15.05±0.13	15.43±0.15

Values represent the mean ± SD for six mice Statistical significance at $P \leq 0.05$, as compared with groups

Table 4. Effect of EAENH on the biochemical parameters of the experimental animals

Para-meters	A-549 lung cancer cells					
	Control (Olive oil)	Tumour Control (B(a) P 50 mg/kg)	Standard (Cisplatin 20 mg/kg)	EAENH (50 mg/kg + B(a) P 50 mg/kg)	EAENH (100 mg/kg + B(a) P 50 mg/kg)	EAENH (50 mg/kg)
Proteins (%)	7.95±0.34	6.12±0.71	7.16±0.35	10.21±0.43	7.53±0.18	9.32±0.14
SGPT (U/L)	26.11±1.12	56.41±2.31	31.25±1.17	30.17±2.11	33.22±0.87	31.11±1.05
SGOT (U/L)	33.55±2.15	61.36±1.22	39.12±2.11	31.25±1.32	36.09±2.09	32.15±1.21
ALP (U/L)	80.15±1.65	117.03±3.15	80.02±2.16	85.13±1.52	83.05±2.19	82.11±1.15
LPO (nmol MDA/ mg protein)	0.57±0.013	0.98±0.032	0.73±0.071	0.67±0.056	0.51±0.015	0.53±0.018
SOD (µmol/ mg. min protein)	4.19±3.12	2.71±1.33	4.13±2.31	4.35±1.53	4.17±1.05	4.27±1.13
CAT (µmol/ mg. min protein)	249.31±0.043	129.16±0.011	245.32±0.052	235.97±0.031	241.65±0.012	232.16±0.013
GP (µmol/ mg. min protein)	43.19±3.12	21.71±1.33	41.13±2.31	37.35±1.53	40.17±1.05	35.15±1.22
GR (µmol/ mg. min protein)	2.97±0.013	1.67±0.032	2.95±0.071	2.81±0.056	2.93±0.015	2.77±0.055

Values represent the mean ± SD for six mice Statistical significance at $P \leq 0.05$, as compared with groups

suggests that the *Nannochloropsis* extract could have lowered the hypoxic state in lung cancer, thus lessening the degree of carcinogenesis. The EAENH- treated mice groups slowly restored back the haematological parameters than the tumour-bearing groups. Nevertheless, the Cisplatin-treated animals provided recuperated results than the EAENH-treated animals (Table 3).

In tumour-bearing animals, the SGOT, SGPT and ALP values were higher in the serum while EAENH-treated groups and Cisplatin-treated group produces slow

restoration of these values to normal range. The substantial rise in LPO levels was parallel to the reduction of antioxidant enzymes (SOD, CAT, GP and GR) in tumour-bearing groups and vice versa i.e., lowering of LPO and enhanced antioxidant enzymes levels were noticed in EAENH-treated groups (Table 4). The ROS production in EAENH could result in the initiation of apoptosis. There was no considerable variation noted for the animals treated only with EAENH and control animals.

3.4 Histological Studies

The histological examination of the control and experimental group lung sections were displayed in Figure 3. The control animals (Group I), presented small regular nuclei with standard cellular architecture (Figure 3a). The tumour-bearing animals (Group II) showed alveolar damage, irregular architecture as well as hyperchromatic nuclei in the alveolar cells (Figure 3b). In BaP induced EAENH-treated animals (Group V) and BaP plus Cisplatin treated mice (Group III) (Figure 3c), less alveolar damage with nearby normal architecture was observed (Figure 3e). In BaP induced EAENH-treated animals (Group IV), somewhat decreased alveolar damage was noted (Figure 3d). The group VI animals treated only with EAENH revealed no considerable variation from the control animals in this study (Figure 3f).

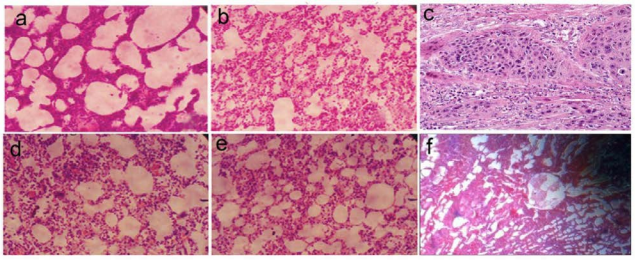
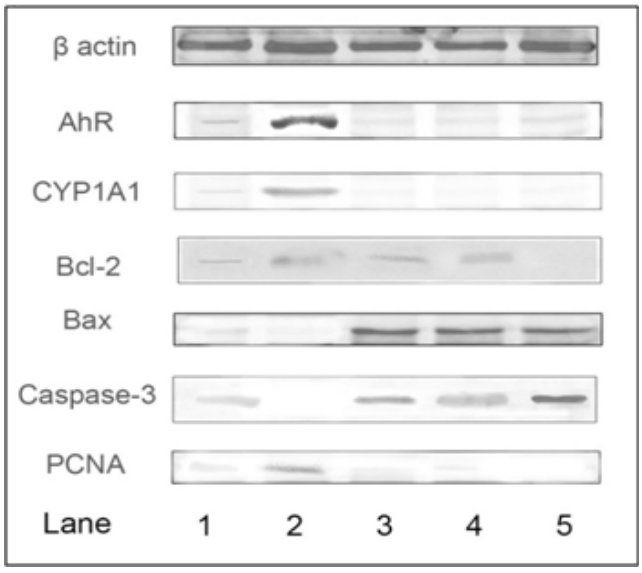


Figure 3. Histopathological studies of lungs in the experimental groups; a. Group 1: Control; b. Group 2: BaP+ olive oil; c. Group 3: BaP+ olive oil + Cisplatin; d. Group 4: BaP+ olive oil + 50 mg/kg bw of EAENH; e. Group 5: BaP+ olive oil + 100 mg/kg bw of EAENH; f. Group VI: 20mg/kg bw of EAENH + olive oil.

3.5 Western Blot Analysis

The down-regulation of Cytochrome P450 1A1 (CYP1A1) and Aryl hydrocarbon receptor (Ahr) was noted in EAENH-treated animals, signifying that the DNA adduct formation was obstructed (Figure 4a). The reports reveal that EAENH intervenes through alteration of caspase-3 expression in programmed cell death processes. The densitometry results were shown in Figure 4b.



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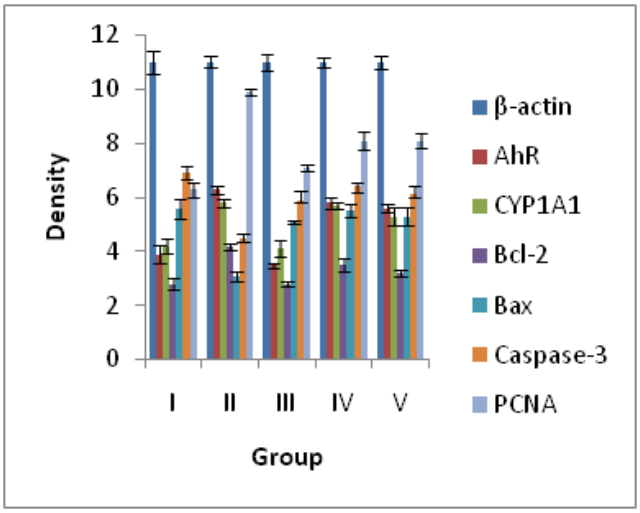

Figure 4. Effect of EAENH on the expression of β-actin (internal control), AhR, CYP1A1, Bcl-2, Bax, Caspase-3, PCNA during mouse lung carcinogenesis induced by BaP; Lane 1: Control; lane 2: BaP+ olive oil; lane 3: BaP+ olive oil + Cisplatin; lane 4: BaP+ olive oil + 50 mg/kg bw of EAENH; lane 5: BaP+ olive oil + 100 mg/kg bw of EAENH.

3.6 Effect of EAENH on Proapoptotic and Antiapoptotic Protein Expression

After validating that EAENH extract stimulates apoptosis in tumour-bearing animals, the next step was to detect whether the extract has influence on apoptosis-related

gene expression. Since various proapoptotic and anti-apoptotic proteins play a vital role in apoptosis, it was necessary to examine whether EAENH can affect the expression of Bax, a proapoptotic protein along with Bcl-2, an antiapoptotic protein in mice. It was also observed that EAENH could increase the Bax expression and decreases the Bcl-2 expression in BaP plus EAENH-treated animals after 16 weeks study as shown. In EAENH-treated animals, down-regulation of PCNA expression established anticancer affects which restricts the spread of cancer cells.

3.7 Effect of EAENH on Gene Expression by RT-PCR

The mRNA expression of different genes such as *AKT*, *ERK* and *p53* has been depicted in Figure 5 with β-actin as internal control. The *AKT*, *ERK* and *p53* expression levels have been increased in the EAENH-treated animal groups.

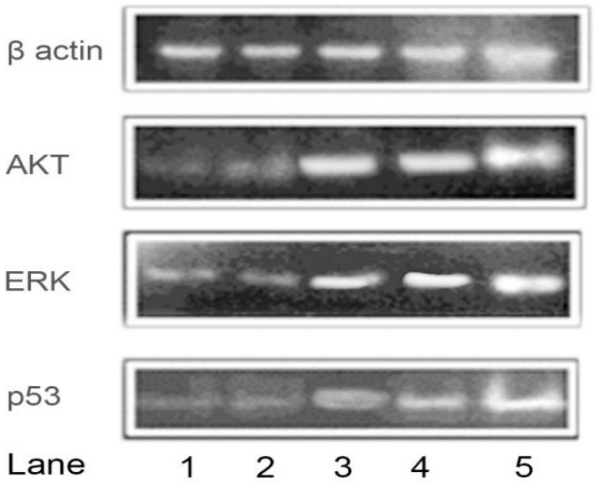

Figure 5. mRNA gene expression of AKT, ERK and p53 with internal control β-actin; Lane 1: Control; lane 2: BaP+ olive oil; lane 3: BaP+ olive oil + Cisplatin; lane 4: BaP+ olive oil + 50 mg/kg bw of EAENH; lane 5: BaP+ olive oil + 100 mg/kg bw of EAENH.

4. Discussion

It was suggested that organic free radical intermediates and Reactive Oxygen Species (ROS) produced from different carcinogens might be engaged in the start and progression of carcinogenesis[23] cancer, inflammatory joint disease, asthma, diabetes, senile dementia and degenerative eye disease. The process of biological ageing might also have a free radical basis. Most free radical damage to cells involves oxygen free radicals or, more generally, activated oxygen species (AOS. The lung was significantly at risk for the ROS lethal effects since it interfaces with numerous oxidants, such as cigarette smoke and environmental pollutants that were the main sources of BaP[24]. BaP, a procarcinogen agent involve in the activity of incomplete pyrolysis of organic materials and induce enormous amounts of ROS and free radicals[20]. The detrimental outcomes of BaP includes immunotoxicity, neurotoxicity, teratogenicity and carcinogenicity of various experimental animals[25]. One of the promising approaches for chemoprevention was to lessen oxidative stress associated with all stages of carcinogenesis.

Due to the promising influence of antioxidants in cancer therapy and since *Nannochloropsis* sp. evidently possessed remarkable antioxidant effect in our previous work, the in vivo study was conducted to determine the chemopreventive effect of EAENH extract[26,27]. The predominant active constituents of EAENH were found to be saponins, terpenoids and flavonoids. EAENH comprises mainly Octadecanoic acid (10.9%) followed by Hexadecanoic acid (8.32%), Octadecanoic acid, ethenyl ester (6.87%) 1, 4 - epoxynaphthalene - 1 (2H) - methanol, 4,5,7-tris (1,1 - dimethylethyl) - 3,4 – dihydro - (7.1%), 5-Methyl-Z-5-docosene (3.24%), 1,2-Benzenedicarboxylic acid (5.61%) and 1,2-Cyclopentanediol (4.81%)[14]. The phytochemicals present in the EAENH fractionated extract account for the noteworthy antioxidant activity of the microalgae.

Based on acute toxicity reports, the dosage structure for in vivo studies was fixed as 50 mg/kg and 100 mg/kg orally. EAENH treatment at different doses did not produce any toxic symptoms or alter the weight gain, food consumption or water intake in the mice.

In Bap induced mice treated with EAENH the final body weight was substantially increased; tumour nodules and lung weight was effectively reduced in group V mice ($p<0.05$) than Group IV mice which correlates with the previous studies[28].

The reduction of lung weight with EAENH treatment groups may be characteristic of the EAENH's inhibitory action on tumour proliferation that helps to prevent the spread of cancer cells validating anticancer effect of *Nannochloropsis* fractionated extract. The EAENH-treated mice groups slowly restore back the haematological and biochemical parameters to normal levels, suggesting that

increased levels of antioxidant enzymes induce apoptosis attributable to ROS production.

In this study, there was a decline in RBC count and haemoglobin in tumour-bearing animals, which infers anaemia due to tumour hypoxia[29]. The hypoxia can lead to cellular alterations considered by increased ability for local penetration, enhanced malignant development, progressive tumor multiplication, malignant progression, resistance to therapy and it has thus become a central issue in tumor physiology and cancer treatment by biochemists, clinicians as well as physiologists. Restoration of Haemoglobin and RBC contents in mice receiving B(a)P-plus-EAENH indicate that EAENH might have reduced the hypoxia during lung carcinogenesis, thereby reducing tumour dissemination. Also rise in WBC count and variations in differential count (neutrophils, lymphocytes and monocytes) have been considered as an important feature of carcinogenesis[30]. In BaP-induced tumour-bearing animals, the reduction in lymphocyte and monocyte counts along with increased WBC counts and neutrophils comply with the earlier reports.

The B(a)P was a very effective carcinogen which induce massive amounts of free radicals, that reacts with lipids causing LPO[31]. The present study unveiled that EAENH treatment lowered ALT and AST in the serum, probably due to escalation in antioxidant enzymes which may reflect its significant antioxidant activity like the water and ethanol extracts of fennel (Foeniculum vulgare).

Antioxidant enzymes were supported by the ROS defense team and found to be decreased in B[a]p induced tumour-bearing animals[33]. Antioxidant enzymes use ROS to protect biopolymers and reduce oxidative damage to DNA[34]. Flavonoids generally have strong antioxidant effect on several oxidation pathways, such as lipid peroxidation scavenging free radicals[35].

The histological lung examination of BaP induced EAENH-treated animals showed less alveolar damage with near−normal architecture when compared to tumour-bearing animals[36].

The Western Blot illustrated that Bcl-2 protein has antiapoptotic properties and it's over expression was extensive in adenomatous hyperplasia cells that correlate with the intracellular ratio of Bax protein and spread around 70%[37]. It has been registered that p53 either upregulates the transcription factors of Bax proapoptotic genes transcription or downregulates the transcription of Bcl-2 antiapoptotic gene leading to apoptosis[38]. Proliferating Cell Nuclear Antigen (PCNA) was highly expressed through vigorously propagating cells, quickly degrading as the cell enters the non-proliferative stage and considered as a proliferation marker[39]. The decreased expression of PCNA in EAENH-treated animals revealed the antiproliferative/ cytotoxic efficacy of EAENH.

In NSCLC, the dysregulation of *Akt* or *ERK* occurs, which was a pronounced feature of several human cancers[40]. Therefore, the *Akt* and *ERK* phosphorylation regulator can result in p53 activation, which in turn switches on the pro-apoptotic signalling pathways viz. Caspase activation[41]. The RT-PCR findings support that EAENH induces apoptosis via inhibition of *Akt* and *ERK* and initiation of caspases in A549 cells. This study suggests the direct anticancer properties of the EAENH and the potential protective effects of the EAENH evidenced by blocking the expression of cancer related genes, DNA damage and suppressing biochemical alterations.

The previous papers of *Nannochloropsis sp.* did not show much considerable evidence for anticancer properties[42-44] Caspase-3 activated the apoptosis induction directed by intrinsic or extrinsic pathway in a cell[45]. The findings specify that EAENH has apoptotic effect on A549 cells *in vitro* and BaP-induced lung cancer *in vivo* with moderate cytotoxic efficacies. It was evident that EAENH activated the caspase 3, elevated CYP1A1 and Bax protein in A549 cells, firmly demonstrating that EAENH stimulates apoptosis mainly through the Caspase-dependent pathway.

5. Conclusions

Our study illustrates that EAENH induced apoptotic activity via caspase activation, *ERK/AKT* inhibition and markedly increased the ratio of Bax/Bcl-2 protein as one of the potent anti-tumour constituents. Thus, it can be thought that EAENH definitely exhibited anticancer activity through the synergistic action of these anti-tumour phytochemicals in the present study. This experimental data confirms that EAENH can be efficaciously utilized as a chemopreventive/ cytotoxic agent for lung cancer treatment.

6. References

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