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Anti-Tumor Effect of *Azadirachta indica* (Neem) on Murine Solid Ehrlich Carcinoma

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Abstract: Tumor growth can cause antioxidant disturbances in certain tissues of the tumor host. So, we examined the antioxidant system as a possible mechanism through which Neem leaves preparation (NLP) exerts its oncostatic potential. Female Swiss Albino mice were inoculated intramuscularly in the right thigh with Ehrlich ascites carcinoma (EAC) cells. NLP (500 mg/kg body weight) was injected for 20 days intraperitoneally into mice beginning on day 5 of post-EAC cell inoculation. Tumor growth, lipid peroxidation (LPx), glutathione (GSH) contents and the activity of the antioxidant scavenger enzymes were examined. Results indicated that NLP efficiently suppressed the growth of tumors which was associated with normalization of the LPx levels and augmentation of GSH contents. NLP enhanced the activity of the endogenous antioxidant scavenging enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione-S-transferase (GST) in liver and tumor tissue. The effect of NLP was more pronounced when treated as early as day 5 of post-tumor cell inoculation. In conclusion, NLP induced oncostatic activity by modulating lipid peroxidation, augmenting the antioxidant defense system and protecting against oxidative stress.

Key words: Antioxidant enzymes · Ehrlich Carcinoma · GSH · Neem leaf preparation

INTRODUCTION

Tumor growth can cause antioxidant disturbances in certain tissues of the tumor host [1]. One of the characteristics of tumor growth and invasion is the increased flux of oxy-radicals and loss of cellular redox homeostasis. Cancer cells can generate large amounts of hydrogen peroxide, which may contribute to their ability to mutate, damage normal tissues and invade other tissues. This suggests that there is a direct correlation between changes in the rate of cancer cell proliferation and changes in the antioxidant machinery. Furthermore, some anticancer agents can act as antioxidant [2]. Several biological response modifiers (BRMs) have been examined for anticancer activity with limited success due to toxicity. The need for a new cancer therapy with minimal or no side effects is greatly warranted. In most population studies, a correlation was found between high intake/high blood levels of antioxidants and low incidence of different types of cancer [3]. Antioxidants block carcinogenesis by multiple mechanisms that include prevention of procarcinogen activation, inhibition of cell proliferation, invasion and angiogenesis and stimulation of apoptosis [4]. Of late, medicinal plants rich in antioxidant phytochemicals have received growing attention as potential chemo-preventive agents. Several modern anticancer drugs have been developed from traditional medicinal plants [5]. The Neem (*Azadirachta indica*) tree contains different bioactive compounds, which are of interest for their beneficial health effects and considered as an anti-genotoxic and chemo-preventive potential of ethanol extract of Neem leaves against oral and stomach tumors [6-8]. Extracts of Neem leaves have been found to possess

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immunomodulatory and anti-inflammatory properties [8-10]. Therefore, it was in our interests to examine whether NLP might suppress solid Ehrlich carcinoma (SEC) growth through the antioxidant system *in vivo*.

MATERIALS AND METHODS

Animals: The current study was conducted on 32, 8 weeks old female Swiss Albino mice (24 ± 2g). The animals were obtained from the Animal House Colony of the National Research Centre, Dokki, Giza, Egypt and maintained in standard laboratory conditions. The animals were housed in plastic cages at room temperature (25±2°C) and humidity (55%). Mice were controlled constantly with a 12h light dark cycle at National Research Centre, Animal Facility Breeding Colony. They were provided with tap water and standard laboratory diet ad libitum. The standard laboratory diet consists of casein 10%, salts mixture 4%, vitamins mixture 1%, corn oil 10% and cellulose 5% completed to 100 g with corn starch [11]. Animals were allowed to acclimate for two weeks to the housing conditions and received human care in compliance with the guidelines of the Ethical Committee of Medical Research of the National Research Centre, Dokki, Giza, Egypt.

Tumor Cell Line: The murine Ehrlich ascites carcinoma (EAC) cells used in this study were originally obtained from the National Cancer Institute, Cairo University, Cairo, Egypt and maintained *in vivo* by weekly intra-peritoneal (i.p.) passage of 2.5×10^6 cells in female Swiss albino mice. Viability, assessed by the trypan blue dye exclusion method, was found to be 95% or more.

Neem Leaf Preparation (NLP): Fresh matured leaves of a Neem tree were collected from the garden of Al-Obour City, Cairo, Egypt. The samples were identified in the Botany Department, Faculty of Science, Helwan University, Cairo, Egypt. The leaves were cleaned, dried and powdered; the powder was used for the preparation of crude methanolic extract according to the procedure described by Manikandan *et al.* [12] with some modification. Air-dried powder (100g) of Neem leaves were mixed with 100 ml methanol (70%) and kept in a refrigerator for 24 hours. The extract of Neem leaves was concentrated and dried under vacuum evaporator. The residue was dissolved in distilled water, filtered and used in the experiment.

Experimental Design: After 2 weeks of adaptation, mice were randomly assigned into four groups (8 mice/group) as follows:

Group (1): mice were fed on standard control diet and injected intra peritoneal with phosphate buffer saline (PBS) daily for 20 days. This group set as healthy control group.

Group (2): fed standard control diet and injected with 500 mg/kg body weight NLP daily for 20 days, according to Balasenthil *et al.* [13].

Groups (3-4): were intramuscularly injected with 0.2ml of EAC, which contained 2.5×10^6 cell in the right thigh of the lower limb for production of solid tumors. After 5 days of EAC cells injection.

Group (4): (SEC+NLP) was treated with 500 mg/kg body weight NLP daily for 20 days, while group 3 (SEC) served as positive control.

Sample Collection: At the end of the experiment (day 25), animals were fasted for 16 h then were anesthetized. Liver and tumor tissues were excised and washed in ice-cold normal saline, blotted dry and weighed. Each tumor was weighted individually (TW/g). A 10% w/v homogenate was prepared in ice-cold phosphate buffer (0.1M, pH 7.4) using homogenizer for both liver and tumor tissues.

Body Weight Changes: Body weight (BW/g) and was monitored throughout the experimental time course. BW was examined for initial, final and net BWs at day 25. The net final BW = final BW-tumor weight. BW gain was determined as the difference between initial and net final BW.

Evaluation of the Antioxidant Activity

Determination of Glutathione (GSH): Reduced glutathione (GSH) was determined using method of Ellman *et al.* [14]. The method was based on the reduction of 5, 5` dithiobis 2-nitrobenzoic acid (DTNB) with GSH producing a yellow compound which can be measured at 405 nm.

Determination of Thiobarbituric Acid Reactive Substances: Thiobarbituric acid reactive substances (TBARS) were assayed calorimetrically in liver and tumor

homogenate according to the method of Ohkawa *et al.* [15]. In briefly, 1ml of trichloroacetic acid 10% and 1ml of thiobarbituric acid 0.67% were heated in a boiling water bath for 30 min. TBARS was formed and the absorbance measured at 535 nm.

Assay for Glutathione-S-transferase Activity: Glutathione-S-transferase (GST) activity was assayed according to method of Habig *et al.* [16]. That method was based on conjugation of 1-chloro-2, 4-dinitrobenzene (CDNB) with reduced glutathione. The conjugation is accompanied by an increase in absorbance at 340 nm. The rate of increase is directly proportional to the GST activity in the sample.

Assay for Glutathione Peroxidase Activity: Glutathione peroxidase (GPx) activity was measured by the method of Paglia and Valentine [17]. The assay is an indirect measure of the activity of GPx. Oxidized glutathione (GSSG), produced upon reduction of organic peroxide by GPx, is recycled to its reduced state by the enzyme glutathione reductase. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm.

Assay for Glutathione Reductase Activity: Glutathione reductase (GR) activity was assayed by the method of Factor *et al.* [18]. GR catalyses the reduction of glutathione in the presence of NADPH, which was oxidized to $NADPH^+$. The decrease in absorbance was measured at 340 nm.

Assay for Catalase Activity: Catalase (CAT) activity was assayed by the method of Aebi [19]. CAT reacts with a known quantity of H_2O_2. The reaction was stopped after exactly one minute with catalase inhibitor. In the presence of peroxidase (HRP), remaining H_2O_2 reacts with 3,5 dichloro-2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with color intensity inversely proportional to the amount of CAT in the original sample.

Assay for Superoxide Dismutase Activity: Superoxide dismutase (SOD) activity was assayed by the method of Nishikimi *et al.* [20]. This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye.

Statistical Analysis: Data were expressed as the mean ± standard error of the mean (SEM). For the comparison of significance between groups, Mann Whitney U-test was used. Significance level was recorded at $p<0.05$. All statistics were done according to the statistical program software "Medical version 11.0".

RESULTS

Effect of NLP Treatment Tumor Weight (TW): Data in Table 1 showed that, on day 25 of the experiment, the mean tumor weights of untreated group (SEC) was markedly increase (4.54 ± 0.24 g) compared to tumor bearing animal and treated with NLP (SEC + NLP) which record 3.43 ± 0.43g. Treatment with NLP recorded a 24.44% reduction in TW.

Effect of NLP Treatment on Body Weight (BW): The effect of NLP treatment on body weight (BW) was investigated weekly, for 25 days after tumor challenge. As shown in Table 1, control mice had a BW change of 13.4%. In addition, mice not inoculated with EAC and treated with NLP had a BW gain of 5.25%. Meanwhile, untreated mice bearing SEC and treated mice bearing SEC showed a BW loss of 13.9% and 5.27%, respectively.

Table 1: Effect of administration of NLP on body weight (gm) in mice-bearing Ehrlich solid carcinoma

Parameter	Control	NLP	SEC	SEC + NLP
Initial body weight (g)	25.36 ± 0.65	26.06 ± 0.26	24.54 ± 1.67	24.98 ± 0.85
Last body weight (g)	28.76 ± 0.56	27.43 ± 0.65	25.65 ± 0.43	26.98 ± 0.43
% of change	-	-	-	-24.44%
Net final body weight (g)	28.76 ± 0.56	27.43 ± 0.65[a]	21.11 ± 1.01[a]	23.55 ± 0.54[b]
Body weight change (g)	+ 3.4	+ 1.37	-3.43	-1.43
% of change from the initial BW	+ 13.40 %	+ 5.25 %	-13.9 %	-5.72 %

Each value represents the mean ± SE

Net final body weight = (Last body weight-Tumor weight)

Body weight change = (Net final body weight-Initial body weight)

a: $p < 0.01$ as compared with control group

b: $p < 0.01$ as compared with SEC group

Table 2: Effect of NLP administration on MDA and GSH content in liver and tumor tissue of different experimental groups

	MDA		GSH	
Animal groups	Liver nmol/g tissue	Tumor nmol/g tissue	Liver µmol/g tissue	Tumor µmol/g tissue
NLP	49.23±0.72[a]	--	2.26±0.04b	--
*% of change	-19%	--	18.9%	--
SEC	104.11±3.8[a]	90.98 ±3.9	1.38±0.02[a]	1.00±0.01
*% of change	71.2%	--	-27.36%	--
SEC+ NLP	71.01±2.79[c]	67.74±1.5[c]	1.56±0.05[b]	1.81±0.04[c]
**% of change	-31.79%	-25.54%	13%	81%

Each value represents the mean ± SE
a: $p < 0.01$ as compared with control group
b: $p < 0.05$ as compared with control group
c: $p < 0.01$ as compared with SEC group
*: percentage of change from control
**: percentage of change from SEC group

Table 3: Effect of NLP administration on antioxidant enzymes, SOD and catalase activity in liver and tumor tissue of different experimental groups.

	SOD		Catalase	
Animal groups	Liver U/g tissue	Tumor U/g tissue	Liver U/g tissue	Tumor U/g tissue
Control	2.17±0.04	--	0.72±0.01	--
NLP	2.62±0.06	--	0.9±0.01	--
*% of change	20.7%	--	25%	--
SEC	1.87±0.03[a]	2.49±0.005	0.40±0.01[a]	0.33±0.03
*% of change	-13.8%	--	-44.4%	--
SEC+ NLP	3.43±0.06[b]	3.35±0.004[b]	0.75±0.0[b]	0.5±0.06[b]
**% of change	83.4%	34.5%	87.5%	51.5%

Each value represents the mean ± SE
a: $p < 0.01$ as compared with control group
b: $p < 0.01$ as compared with SEC group
*: percentage of change from control
**: percentage of change from SEC group

Table 4: Effect of NLP administration on antioxidant enzymes, GPx, GST activity in liver and tumor tissue of different experimental groups

	Gpx		GST	
Animal groups	Liver U/g tissue	Tumor U/g tissue	Liver µmol/h/g tissue	Tumor µmol/h/g tissue
Control	0.87±0.02	--	0.73±0.01	--
NLP	1.17±0.02[a]	--	0.90±0.02	--
*% of change	34.5%	--	32.3%	--
SEC	0.76±0.06[a]	1.40±0.01	0.55±0.01[a]	0.36±0.01
*% of change	-12.6%	--	-24.6%	--
SEC+ NLP	1.18±0.03[b]	1.79±0.01[b]	0.90±0.01[b]	0.62±0.06[b]
**% of change	55.3%	27.8%	23.3%	72.2%

Each value represents the mean ± SE
a: $p < 0.01$ as compared with control group
b: $p < 0.01$ as compared with SEC group
*: percentage of change from control
**: percentage of change from SEC group

Effect of NLP on Antioxidant Status: Lipid Peroxidation (Malondialdehyde) Content: The effect of NLP treatment on the level of lipid peroxidation was measured in term of malondialdehyde (MDA) in liver and tissues of tumor was depicted in Table 2. Untreated mice bearing a solid tumor (SEC group) showed a marked elevation in MDA level in liver (71.2%, $p < 0.01$) as compared to the normal animals. Treatment with NLP on day 5 post-tumor cell inoculation in SEC-bearing mice returned MDA levels to be close to control values in liver (-31.79%) and tumor (-25.54%), as compared with the untreated control SEC-bearing mice.

Glutathione (GSH) Level: As summarized in Table 2, treatment with NLP solely revealed significant change in liver GSH level (18.9%, p>0.01) when compared with normal control values. A significant depletion in GSH level was recorded in the liver of animal bearing tumor group (-27.36%, p<0.01) as compared to their corresponding normal controls. Treatment with NLP significantly restored GSH content in the liver to the normal values and elevated its level in tumor tissue above the values of the untreated mice bearing SEC (81%, p<0.01).

The Activities of Antioxidant Scavenger Enzymes: The effect of NLP on the activity of antioxidant scavenger enzymes in normal and SEC tumor-bearing mice was examined. These enzymes include glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD), Glutathione reductase (GR) and catalase (CAT). Data in Table 3 showed that, administration of NLP solely revealed insignificant change in SOD and CAT activities of liver when compared to the normal control group. The activities of these enzymes in the liver and tumor tissue of the SEC group was significantly lower (p<0.01) than that of their corresponding control. Treatment with NLP to SEC-bearing mice elevated SOD and CAT activity in liver to be comparable with the normal levels. In addition, treatment with NLP markedly augmented SOD and CAT activity in the tumor tissue (p<0.01) when compared with the untreated SEC values. Table 4 indicated that, administration of NLP solely revealed significant increase in GPx level in liver (34.5%, p<0.01) when compared with the normal control. SEC group showed a great depletion in liver activities of Gpx and GST [(-12.6%, p<0.01) and (-4.6%, p<0.01), respectively] compared with normal control group. Treatment with NLP markedly augmented GPx and GST activity in the tumor tissue (p<0.01) when compared with the untreated SEC values [(27.8%, p<0.01) and (72.2%, p<0.01), respectively)].

DISCUSSION

Administration of NLP at day 5 post-tumor cell inoculation to tumor-bearing mice showed a marked and progressive suppression of the tumor growth. The antitumor activity is exemplified by a significant reduction in TW. The biological activity of Neem leaves was due to its rich content of flavonoids [21]. Flavanoids have been reported to possess both antioxidant activity [22] and anti-inflammatory activities [23, 24] via scavenging free radicals [25] and inhibition of lipid peroxidation [21]. Antitumor activity of flavonoids, isolated from several sources other than Neem, has been reported by Horvathova *et al.* [26]. That compounds have been shown to have anti-proliferative effects on human squamous cell carcinoma [27]. Six phenolic compounds including gallic acid, benzoic acid, p-coumaric acid, p-hydroxybenzoic acid, vanillic acid and trans-cinamic acid were isolated and identified in both Neem bark and leaves. Polyphenolics are known for their potent antioxidant and free radical scavenging properties [28]. The present results revealed marked depletion in GSH content as well as the activities of the antioxidant scavenger enzymes, GPx, GST, SOD and CAT in the liver and tumor tissues of tumor-bearing mice. The relationship among cancer growth, GSH content and the antioxidant system has been also studied. GSH, a potent inhibitor of the neoplastic process, plays an important role as an endogenous antioxidant system that is found in particularly high concentration in the liver and is known to have a key function in the protective process [29].

Reduced activities of the GSH and GSH related enzymes such as GPx in cancer patients were also reported by Balasubramaniyan *et al.* [30] and Wong *et al.* [31]. It was reported that during cancer growth, glutathione redox (GSH/GSSG) decreases in the blood of Ehrlich ascites tumor-bearing mice, mainly due to an increase in blood GSSG levels as a result of oxidative stress. This increase may be caused by an increase in peroxide production by tumor cells that can lead to GSH oxidation within the red blood cells and increased GSSG release from different tissues into the blood [32]. GSH-Px plays an important role in metabolizing lipid peroxides in the liver and this enzyme decrease is potentially ascribable to inactivation by the increase in ROS or lipid peroxide formations [33]. In the current study, we also observed that the detoxifying enzyme glutathione-S-transferase (GST) activity was significantly dropped in the liver of the SEC-bearing mice. Our results were in accordance with others who detected low liver GST activity in SEC-bearing mice [34] and in lung cancer-bearing animals [35]. SOD, CAT and GPx are involved in the clearance of superoxide and hydrogen peroxide. SOD catalyses the diminution of superoxide into H_2O_2, which has to be eliminated by GPx and/or CAT [36]. The decline in SOD activity was observed in different tissues of SEC-bearing mice [2, 37, 38]. It is worth mentioning that SOD activity plays an important role in the antitumor effects of active oxygen-forming anticancer agents. However, when the oxidative damage is extreme as

a result of tumor growth, ROS scavenging enzymes such as SOD and catalase are degraded [39]. The inhibition of CAT activity in different tissues of mice bearing an Ehrlich tumor as a result of tumor growth was also reported by several investigators [2, 40].

Results of the present study demonstrated the antioxidant and free radical scavenging property of NLP, as exemplified by the ability of NLP to increase activity of the antioxidant enzymes in the cells of normal animals and in animals bearing tumors. In addition, NLP normalized the level of LPx in liver and tumor tissue in animals bearing tumors. In the present finding, the free radical scavenging GSH content in liver and tumor tissue of mice treated with NLP was found to be significantly higher than that in the untreated SEC-bearing mice. Elevation in the activity of GST, help in subsequent initiation of the apoptotic process in tumor cells have enormous clinical significance for immunotherapy of various forms of cancer with a completely nontoxic therapy [41, 42]. An ethanolic extract of Neem has been shown to induce cell death in prostate cancer cells (PC-3) by inducing apoptosis as evidenced by a dose-dependent increase in DNA fragmentation and a decrease in cell viability [43]. Further, their studies indicated that treatment with Neem extract could decrease level of Bcl-2, which is an anti-apoptotic protein and at the same time, increased expression of pro-apoptotic Bax protein. Moreover NLP was able to cause tumor regression in SEC-bearing mice through the induction of cancer cell apoptosis via its immunomodulatory effect [44].

In conclusion, our results strongly suggest that, NLP represent a high potential for antitumor activity *in vivo* via its radical scavenging effect by encountering free radicals after tumor cells inoculation and in the future it may be used as adjuvant chemotherapy.

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