

Research Article

Antitumor activity of Neem leaf Extract and Nimbolide on Ehrlich Ascites Carcinoma Cells in Mice

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Abstract

Background: *Azadirachta indica* (Neem) has been used traditionally for many centuries. Some impressive therapeutic qualities have been discovered. **Aim:** Our study aims to investigate the *in vivo* antitumor and antioxidant activities of Ethanolic Neem Leaf Extract (ENLE) and its fraction called Nimbolide, a limonoid present in leaves and flowers of the neem tree (*Azadirachta indica*). Also, to study the side effects of the ethanolic Neem leaf extract and Nimbolide fraction on the liver and kidney. **Materials & Methods:** We assessed the effect of nimbolide and ethanolic Neem leaves extract (ENLE) on replicative lifespan prolongation *in vitro* and on the levels of malondialdehyde (MDA), nitric oxide (NO), catalase, glutathione peroxidase (GPx), Caspase-3, and Cytochrome c. Also, our

study estimated their effect on Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Total Protein (TP), Albumin (Alb), bilirubin, urea and creatinine. **Results:** Ethanolic neem leaves extract and nimbolide resulted in increases in replicative lifespan. Also, they showed a significant decrease in malondialdehyde and nitric oxide and an increase in catalase, glutathione peroxidase, caspase-3 activities, and cytochrome c concentration. Hence, it may be possible that Nimbolide and ENLE decrease lipid peroxidation level due to their antioxidant effect and enhance apoptosis. The Neem leaves extract and nimbolide showed no side effects on liver and kidney. Also, they showed a significant protection for both liver and kidney histopathologically.

Introduction

Cancer still represents one of the most serious human health related problems; despite the great progress in understanding its biology and pharmacology. Common therapeutic methods for cancer treatment are individually useful in particular situations and when combined with other remedies, they can offer a more efficient treatment. An analysis of a number of chemotherapeutic drugs and their sources indicates that over 60% of approved drugs are derived from natural compounds (Schottenfeld 2015). Antioxidants are substances that are believed to protect cells from becoming cancerous by stabilizing unstable molecules, known as free radicals. Until now various studies have focused on this relationship between antioxidants and cancer; and these studies have produced various results. The disparity between their conclusions has led some people within the scientific community to question the efficacy of using antioxidant supplementation when attempting to combat or prevent cancer (Schumacker 2015, Basu *et al.* 2018).

In recent years, considerable effort has been

directed towards identifying naturally occurring substances that can protect against oxidative stress (Maha *et al.* 2015). Natural antioxidants have a wide range of biochemical activities, including inhibition of ROS generation, direct or indirect scavenging of free radicals and alteration of intracellular redox potential (Azman *et al.* 2015). Plants are a natural source, producing a wide number of bioactive chemical constituents in a most efficient way and with precise selectivity. Since the middle of the 19th century, different classes of bioactive compounds have been isolated and characterized. Many of these are now used as active ingredients in modern medicine, or as lead compounds for new drug discovery. Several plant derived medicines are rich in phenolics, flavonoids, terpenoids, alkaloids, tannins etc., used in the treatment of various ailments (Dubey 2015).

Azadirachta indica (Neem) which is known as “the mother” of all therapeutical plants has been used extensively many decades ago and still been using for ritual and medicinal purposes. It’s availability and low cost has allowed many people to benefit from this plant. Several studies have been done from the leaves

up to its bark to explore its therapeutical potentials. The phytochemical components such as Azadirachtin, Nimbidin, Nimbin, Nimbinin, Nimbidinin, Nimbolide, Nimbidic acid, Nimbidin and Sodium Nimbidate derived from the Neem plant, possess a variety of pharmacological effects such as antipyretic, antiviral, antioxidant, analgesic, antibacterial, contraceptive as well as hepatoprotective (More *et al.* 2015).

Materials and Methods

Plant materials and extraction

Leaves of *Azadirachta indica* were collected from Al-Qanater Al-Khairyia, Egypt in 2012 and were identified and characterized by Herbarium of Botany department, Cairo University. The leaves were dried and grounded into a uniform powder using a milling machine. Neem extract was prepared according to Chattopadhyay (1998). The extract was suspended in normal saline to obtain its final concentration (Subapriya *et al.* 2005). Extraction of nimbolide was prepared according to Markmee & Saiin (2003).

Animals

120 adult female Swiss albino mice weighing 22-25 g were purchased from the Animal House Colony of the National Research Center, Dokki, Giza, Egypt. The animals were housed at the experimental animal house of the Faculty of Science, Zagazig University. The animals were maintained in a controlled environment of temperature, humidity, light, and fed on a commercial standard diet and tap water *ad libitum*.

Ehrlich ascites carcinoma cells

EAC cells were initially supplied from the National Cancer Institute, Cairo, Egypt and maintained in female Swiss albino mice through serial intraperitoneal (I.P.) inoculation of 0.2 ml of freshly drawn ascites fluid (diluted to 1:5 saline solution). Each inoculum contained approximately 2.5×10^6 cells. This process was repeated every 10 days for keeping the strain available throughout the present study (Fahim *et al.* 1997).

Neem extract and nimbolide identification

H^1 NMR, C^{13} NMR and Mass spectrum

The H^1 NMR and C^{13} NMR spectra were recorded using Jeol instrument (Japan), at 270 and 125 MHz respectively. The Mass spectrum was recorded on a GCMS-QP 1000 ex spectra mass spectrometer operating at 70 eV. The analyses were carried by the Microanalytical Data Unit at the National Research Center, Giza, Egypt according to Subapriya *et al.* (2005).

High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) analysis of ENLE and nimbolide was performed at the

National Research Center, Giza, Egypt according to Markmee & Saiin (2003).

Toxicity study and dose response curve

The approximate LD50 of Neem extract was determined according to Meier & Theakston (1986). The dose response curve for ENLE in mice was determined according to Crump *et al.* (1976). Studies were carried out for determination of the most effective dose on tumor volume and count. The LD50 value of a single i.p. administration of nimbolide to adult female was 225 mg per kg of body weight according to Rojanapo *et al.* (1985). One tenth of LD50 was used in this work, as it is the most effective dose of nimbolide, reported by Gupta *et al.* (2013).

Experimental design

Ninety adult female albino mice were divided into six groups (each contained 15 mice). *Group I: (Negative control):* 15 mice injected i.p. with sterile saline solution (0.9 NaCl) for 9 days. *Group II: (Positive control):* 15 mice were injected i.p. with EAC cells 2.5×10^6 cells/0.2 ml once for 9 days. *Group III: Therapeutic group of ENLE:* 15 mice were injected with EAC cells; 2.5×10^6 cells/0.2 ml before inoculation with 5 mg/Kg body weight. ENLE treatment day by day for 9 days. *Group IV: Preventive group of ENLE:* mice were injected with ENLE treatment before inoculation with EAC cells; 2.5×10^6 cells/0.2 ml were used. Mice received 5 mg/Kg body weight. ENLE treatments day by day for 9 days. *Group V: Therapeutic group of Nimbolide:* 15 mice were injected i.p. with EAC 2.5×10^6 cells/0.2 ml before inoculation with 22.5 mg Nimbolide/kg b.w according to Gupta *et al.* (2013) for 9 days. *Group VI: Preventive group of Nimbolide:* 15 mice were injected with 22.5 mg Nimbolide / kg body weight; they were then injected once with EAC cells (2.5×10^6 cells/0.2 ml) for 9 days.

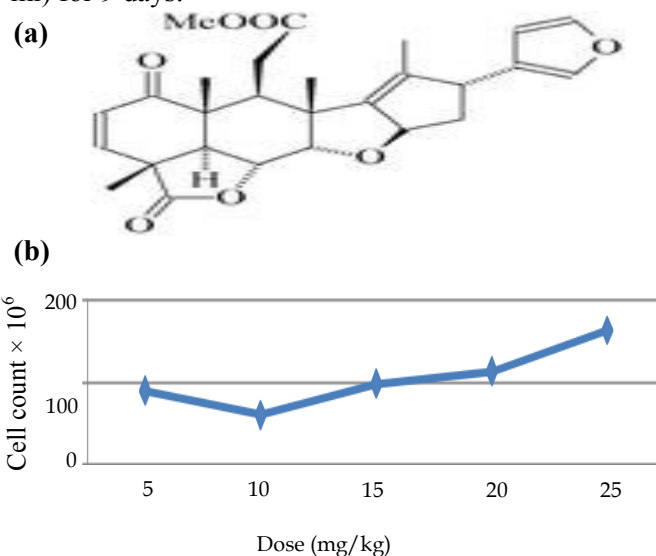


Figure 1. (a) Structure of nimbolide. (b) Dose response curve of total Neem extract.

Table 1. EAC count ($\times 10^6$ cells/ml) and T/C% among different study groups.

Group	Positive control	Nimbolide		Extract	
		Therapeutic Group	Preventive Group	Therapeutic Group	Preventive Group
EAC count	2099 \pm 1457	486 \pm 18 ^{***}	No cells	919 \pm 211 ^{***}	684 \pm 195 ^{***}
Survival days	9	14	15	16	17
life span T/C%	--	5555%	6666%	7777%	8888%

Data are expressed as Means \pm SD (n=10) *P value <0.05 was considered significant

*P value <0.01 was considered highly significant *** P value <0.001 was considered very highly significant

NS *P value >0.05 was considered non-significant

Blood Sampling and preparation

At the end of the experimental period, plasma was collected on anticoagulant (EDTA) by centrifuging blood at 3000 rpm for 10 minutes to carry out antioxidant assays (MDA, NO, Catalase and GPx). Serum was collected into a plain tube for determination of liver function tests (ALT, AST, T.P, albumin and bilirubin) and kidney function tests (urea and creatinine).

Tissue sampling

EAC cells were harvested from the peritoneal cavity of each mouse and suspended in sterile isotonic saline for the evaluation of antiapoptotic activity (Caspase-3 and Cytochrome c). Liver and kidney tissues were excised and fixed in 10% formal saline, embedded in paraffin, sectioned and stained with Hematoxylin and Eosin (H&E) for histopathological evaluation.

Viability and life span prolongation

The viability of EAC cells was determined by the *Trypan Blue* Exclusion Method (McLimans *et al.* 1957), where the total and viable cells (non-stained) were counted at magnification $\times 40$; as the number of cells/ml was determined in the studied groups. Replicative life span calculation was carried out according to the method described by Mazumdar *et al.* (1997).

Biochemical analyses

The levels of Malondialdehyde (MDA) were measured according to Satoh (1978), Nitric Oxide (NO) was determined according to Montgomery & Dymock (1961), catalase enzyme activity (CAT) was estimated according to Aebi (1984) and Glutathione Peroxidase GPx activity was measured according to Paglia & Valentine (1967). The activity of caspase-3 was determined by the colorimetric caspase-3 kit according to the method of Casciola-Rosen *et al.* (1996) while Cytochrome c was determined according to Cai *et al.* (1998). Total protein and

albumin were measured according to Doumas *et al.* (1981), Alanine Aminotransferase (ALT) and Aminotransferase (AST) were determined according to Reitman & Frankel (1957). Serum bilirubin was determined according to Jendrassik & Gróf (1938). Urea was measured by Chaney & Marbach (1962) and creatinine was measured according to Murray & Kaplan (1984).

Statistical Analysis

All statistical analyses were done by SPSS 14.0, SPSS Inc. and considered statistically significant at a two-sided $P < 0.05$. Numerical data were expressed as mean \pm SD. The levels of markers were analyzed by ANOVA but the Mann-Whitney U-test was used for comparisons between independent groups (Levesque 2007).

Results

Total extract and nimbolide yield

Following extraction, Neem leaves powder (0.5 Kg yielded 15.8 g of ethanolic Neem leaf extract (a thick green paste) and another 0.5 Kg of leaf powder yielded 1 g of Nimbolide (off-white solid).

Neem extract and nimbolide identification

HPLC was performed for identification of the extract. The retention time (Rt) of standards (Azadirone, 28-Deoxonimbolide, Nimbolide, and Quercetin) were 4.980, 3.548, 2.971, and 7.631 with 100% area. The retention time (Rt) of Azadirone, 28-Deoxonimbolide, Nimbolide, and Quercetin in ethanolic Neem leaves extract were found to be 5.191, 3.790, 2.912 and 7.77 respectively, which are matching with standards Rt values respectively. The amount of Azadirone, 28-Deoxonimbolide, Nimbolide, and Quercetin in ethanolic Neem leaves extract, were found to be 0.4%, 3.4%, 9.4% and 3.2% w/v, respectively. To prove the structure of Nimbolide, mass spectroscopy, H^1 NMR and C^{13} NMR were used, as shown below.

Table 2. Collective table of antioxidant parameters among different study groups.

Group	MDA(nmol/ml)	NO(μ mol/l)	Catalase(U/L)	Glutathione peroxidase(U/L)
Negative control	1199 \pm 079*	1984 \pm 111***	22737 \pm 1210***	20037 \pm 463***
Positive control	4274 \pm 180	3442 \pm 333	15092 \pm 611	10244 \pm 348
Therapeutic Nimbolide	1203 \pm 054***	1327 \pm 191***	71716 \pm 4886***	61448 \pm 1953***
Preventive Nimbolide	13 \pm 075***	1821 \pm 138***	90945 \pm 5107***	77341 \pm 6273***
Therapeutic extract	1961 \pm 145***	2297 \pm 124**	35338 \pm 3560***	35794 \pm 3680***
Preventive extract	1404 \pm 037***	2095 \pm 133***	45408 \pm 6819***	43949 \pm 5510***

M.S (EI): m/z (%) of nimbolide:

Molecular weight= 466 g/mol (M^{+} , 0.25), 402 ($C_{24}H_{34}O_5$, 0.76), 385 ($C_{22}H_{25}O_5$, 14), 357 ($C_{21}H_{25}O_5$, 19), 314 ($C_{19}H_{22}O_4$, 9), 283 ($C_{17}H_{15}O_4$, 8), 267 ($C_{15}H_{23}O_4$, 6), 240 ($C_{15}H_{12}O_3$, 4), 186 ($C_{12}H_{10}O_2$, 6), 126 ($C_7H_{10}O_2$, 9), 97 ($C_5H_5O_2$, 8), 59 ($C_2H_3O_2$, 3), 43 (C_3H_7 , 42).

 1H NMR (DMSO-d₆, ppm) of nimbolide:

δ = 1.12 (s, 3H, CH_3 -30), 1.28 (s, 3H, CH_3 -19), 1.39 (s, 3H, CH_3 -29), 1.60 (s, 3H, CH_3 -18), 2.00, 1.96 (m, 2H, C_{16} -H), 2.30, 3.10 (dd, 2H, C_{11} -H), 2.86 (d, 1H, C_5 - α H), 3.36 (s, 3H, COCH₃), 5.80 (d, 1H, C_2 -H), 6.23 (d, 1H, furan- C_{22} -H), 7.27 (d, 1H, C_3 -H), 7.37 (s, 1H, furan- C_{21} -H), 7.51 (d, 1H, furan- C_{23} -H).

 ^{13}C NMR (DMSO-d₆, ppm) of nimbolide:

δ = 198.46 (C-1), 126.10 (C-2), 153.51 (C-3), 49.11 (C-4), 45.29 (C-5), 77.88 (C-6), 84.86 (C-7), 51.71(C-8), 39.84 (C-9), 53.96 (C-10), 32.20 (C-11), 136.33 (C-13), 130.89 (C-14), 83.04 (C-15), 40.01 (C-16), 45.29 (C-17), 14.79 (C-18), 17.56 (C-19), 110.84, 125.32, 139.36, 143.84 (C-furan), 176.36 (C-28), 23.15 (C-29), 18.50 (C-30), 171.76 (C=O, acetate), 53.90 (CH₃-acetate).

For determination of the median lethal dose (LD₅₀) of ENLE, all doses up to 2000 mg/Kg mice were found to be nontoxic as no deaths were recorded, which suggests that ENLE may be a safe mixture. For dose-response curve, 5 mg ENLE/Kg mice was found to be

Table 3. Collective table of apoptosis parameters among different study groups

Group	Caspase-3(ng/ml)	(ng/ml) Cytochrome c
Positive control	0834 \pm 006	1061 \pm 009
Therapeutic Nimbolide	3462 \pm 022***	474 \pm 035***
Therapeutic Extract	2188 \pm 014***	248 \pm 028***
Preventive Extract	2956 \pm 024***	332 \pm 036***

the most effective dose as it reduced the number of EAC cells in the treated mice group to 76% compared to positive control mice group as shown in Figure 1.

Viability and life span prolongation

Our results demonstrated that ENLE and nimbolide from Neem leaves display anticancer activity as they decreased EAC count in mice bearing EAC in all groups ($p < 0.001$) compared to positive control mice. Also both showed significant increase in life span in all studied groups compared to positive control group (Table 1).

Biochemical Analyses*Antioxidant assay*

The effects of extract and nimbolide on antioxidants were examined. Data in Table 2 showed that administration of ethanolic extract revealed a significant decrease in malondialdehyde (MDA) and nitric oxide (NO) levels ($p < 0.001$) in therapeutic and preventive groups. It also showed a significant increase in activities of catalase and glutathione peroxidase enzymes in the same groups ($p < 0.001$). In addition, it showed that administration of nimbolide resulted in significant decrease in malondialdehyde (MDA) and nitric oxide (NO) levels ($p < 0.001$) in the therapeutic and preventive groups. It also showed significant increase in activities of catalase and glutathione peroxidase enzymes in the same groups ($p < 0.001$).

Apoptotic effect

Data in Table 3 show that administration of the extract causes a significant increase in Caspase-3 activity and Cytochrome C concentration in both the therapeutic and preventive groups ($p < 0.001$). Also, nimbolide administration showed significant increase in Caspase-3 and Cytochrome C activities in the therapeutic group ($p < 0.001$).

Table 4. Collective table of Liver function tests among different study groups

Group	AST(U/L)	ALT(U/L)	Total protein(g/dl)	Albumin(g/dl)	Bilirubin(mg/dl)
Negative control	813±236***	415±233***	686±012***	263±011***	0387±004***
Positive control	1097±462	113± 807	55± 031	178± 016	0944± 005
Therapeutic Nimbolide	552±271***	355±322***	691±013***	277±017***	0201±006***
Preventive Nimbolide	417±517***	238±318***	676±020	282±013***	0138±005***
Therapeutic extract	1028±799	757±533***	585±032**	221±014*	0289±006***
Preventive extract	845±338***	735±443***	632±034*	223±012*	0415±007***

Liver function tests

Data from Table 4 showed that ENLE administration in mice lowered AST in the therapeutic and preventive group ($p<0.001$), ALT activities ($p<0.001$) and bilirubin levels ($p<0.001$) compared to the positive control group. Data also showed an insignificant increase in T.P and albumin activity, compared to positive controls, while these values are within normal range after administration of the extract. In addition, administration of nimbolide showed a significant decrease in AST ($p<0.001$), ALT ($p<0.001$) and bilirubin ($p<0.001$) compared to the positive control group but these values are within normal range in mice. Data also showed a significant increase in T.P levels ($p<0.001$) and albumin activity ($p<0.001$) compared to positive control, while, again, these values are within normal range after administration of nimbolide.

Kidney function tests

Our study showed that administration of ENLE significantly decreased urea ($p<0.001$) and creatinine levels ($p<0.001$) in all studied groups, while these values remain within the normal range. Administration of nimbolide had the same effect.

Histopathological examination

The two vital organs (livers and kidneys) were removed from the test groups at the end of the study and carefully observed macroscopically. No observable gross lesions were identified, when compared with the control group. Microscopically however, the two organs of the test groups revealed some histological changes compared to the negative control group.

Correlations between different parameters among study groups

To confirm our results, correlations between parameters in the studied groups were done. Among ENLE studied groups there were strong positive correlations between MDA and NO ($r=0.95$) ($P<0.01$), GPx and Catalase as well as Cytochrome c ($r=0.9$ and

0.8, respectively) ($p<0.01$), Caspase-3 with Cytochrome C and catalase ($r=0.97$ and 0.84 , respectively) ($p<0.01$) and catalase with Cytochrome C ($r=0.82$) ($p<0.01$). Also there were strong negative correlations between MDA and GPx as well as Catalase ($r=-0.6$ for both cases) ($p<0.01$), NO and GPx as well as Catalase ($r=-0.6$ for both) ($p<0.01$). Among nimbolide studied groups, there were strong positive correlations between MDA and NO ($r=0.92$) ($P<0.01$), GPx and Catalase, Caspase-3 and Cytochrome c ($r=0.99$, 0.95 and 0.97 respectively) ($p<0.01$) and Caspase with Cytochrome c ($r=0.99$) ($p<0.01$). Also there were strong negative correlations between MDA and GPx as well as Catalase ($r=-0.6$ in both cases) ($p<0.01$), NO and GPx, Catalase, Caspase-3 and Cytochrome c ($r=-0.7$, -0.7 , -0.5 , -0.5 , respectively) ($p<0.01$).

Discussion

Cancer is a term used to describe a group of diseases that cause the uncontrolled growth, invasion, and spread (metastasis) of abnormal cells. It is caused by external factors such as environmental conditions, radiation, infectious organisms, poor diet, lack of exercise, and tobacco use, as well as internal factors such as genetics, mutations, and hormones (Peter 2008). Extracts of neem leaf have been reported to be non-toxic and non-mutagenic and have been found to

Table 5. Collective table of kidney function tests among different study groups.

Group	Urea(mg/dl)	Creatinine(mg/dl)
Negative control	21± 397***	0385± 017***
Positive control	621± 338	1663± 016
Therapeutic Nimbolide	***249± 221	0903± 006***
Preventive Nimbolide	205± 272***	0758± 009***
Therapeutic extract	292± 124***	0658± 010***
Preventive extract	316± 162***	0509± 009***

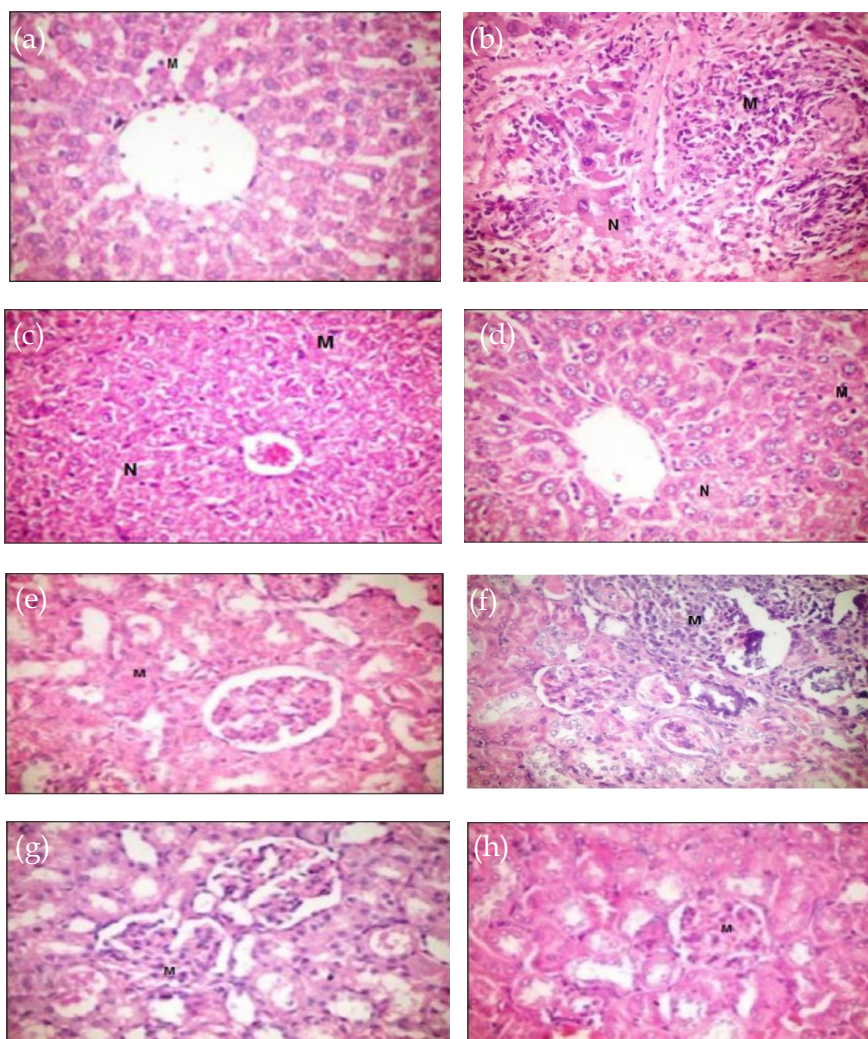


Figure 2. Liver and kidney H&E photomicrographs. (a) Photomicrograph from the liver “negative control” group showing normal healthy hepatic parenchyma and (b) from the liver “positive control” group showing a focal large area of necrotic cells [N] infiltrated with mononuclear cells [M]. (c): Photomicrograph of liver “extract preventive and therapeutic” groups showing healthy hepatic parenchyma and (d) photomicrograph from the liver “Nimbolide preventive and therapeutic” groups showing healthy hepatic parenchyma. (e) Photomicrograph from kidney “negative control” group showing normal glomeruli and renal tubules. (f) Photomicrograph from kidney “positive control” group showing infiltration of mononuclear cells [M]. (g) Photomicrograph from kidney “extract preventive and therapeutic” groups showing normal glomeruli and renal tubules while (h) shows normal glomeruli and renal tubules from kidney “Nimbolide preventive and therapeutic” groups.

possess immunomodulatory as well as anti-inflammatory and anticarcinogenic properties (Subapriya & Nagini 2005). There are many studies showing that the ethanolic extract of Neem leaves possess anticancer activity. Ethanolic Neem leaf extract (ENLE) has exhibited anticancer activity against N-methyl-N'-nitro-N-nitrosoguanidine-induced oxidative stress and gastric carcinogenesis (Subapriya & Nagini 2003). ENLE has been reported to induce apoptosis in a prostate cancer cell line (PC-3) by up-regulating the pro-apoptotic protein Bax and decreasing the level of Bcl-2 protein resulting in DNA fragmentation in prostate cancer cells (Kumar *et al.* 2006, Gunadharini *et al.* 2011). Many bioactive compounds are isolated from this plant including nimbolide, which belongs to the limonoid group. Nimbolide is a major component of Neem leaves and a tetranortriterpenoid that consists of a classic limonoid skeleton with an α , β -unsaturated ketone system and a δ -lactone ring (Anitha *et al.* 2007). Nimbolide has numerous types of biological activity, including antimalarial and anticancer activity (Roy *et al.* 2007). It has been found to exhibit anticancer activity in a wide variety of tumor cells, including neuroblastoma,

osteosarcoma and choriocarcinoma (Phillips *et al.* 2006), leukemia (Gerhauser 2008), melanoma cells as well as macrophages (Neto 2007). ENLE contains a number of antioxidants and anticarcinogens including terpenoids, limonoids, quercetin and sitosterols. Constituents of Neem leaf such as nimbolide, 28-deoxonimbolide and azadirone have been found to possess cytotoxic activity against various cancer cell lines (Naundri *et al.* 2003). Quercetin, a highly ethanol soluble Neem bioflavonoid and potent antioxidant has been reported to inhibit the growth of tumor cells in malignant cell lines and down regulate the expression of Bcl-2 and mutant p53 protein (Nguyen *et al.* 2004). Similarly, 28-Deoxonimbolide exhibited potent cytotoxic activities against HL60 cells (Kikuchi *et al.* 2011) and showed high cytotoxicity against a normal lymphocyte cell line (Pati *et al.* 2008). The LD50 of nimbolide was reported to be 225 mg/Kg body weight in mice according to Crump *et al.* (1976) whereas Glinsukon *et al.* (1986) reported it to be 280 mg/Kg body wt. This difference was proposed to be due to individual variation (Casarez 2001).

In this regard, the acute toxicity was estimated by intraperitoneal administration of ENLE

to determine the median lethal dose (LD50). Our results revealed that a dose up to 2000 mg /kg was considered safe. In addition, we show that 5 mg /kg is considered to be the most effective dose of ENLE. Our results are in agreement with Ghimeray *et al.* (2009) who reported that toxicological investigation of *A. indica* leaf extracts at 0.6 - 2.0 g/kg body weight did not possess any lethal effects on hematology, enzyme levels and affect any histopathological parameters of experimental animals. For the evaluation of antiproliferative activity of the ethanolic extract and nimbolide, it has been demonstrated that nimbolide and ENLE from the Neem tree have anticancer activity, as they decreased EAC count in mice in all treated groups. There was complete absence of tumor cells in the nimbolide preventive group. ENLE decreased cell proliferation through the inhibition of the Insulin-like growth factor (IGF) signaling molecules in both MCF-7 and MDA MB-231 cells (Elumalai *et al.* 2012). Another study proved the antiproliferative activity of ethanolic Neem leaves extract (ENLE) alone or in combination with cisplatin by cell viability assay on human breast (MCF-7) and cervical human epithelial adenocarcinoma cells (HeLa) (Chhavi *et al.* 2014). The therapeutic dose was found to be 22.5mg/Kg body weight, which is one tenth of the LD50 and the effective dose according to Rojanapo *et al.* (1985) who reported that administration of nimbolide at 20 mg/kg of body weight reduced tumor growth by almost 90% of human colorectal cancer xenografts, further supporting our results. Nimbolide has effectively inhibited proliferation of WiDr colon cancer cells and has been shown to exert its antiproliferative effects in various cell lines (Babykutty *et al.* 2011). Also, Roy *et al.* (2007) suggested that Nimbolide, a triterpenoid extracted from the flowers of the Neem tree was found to have antiproliferative activity against some cancer cell lines.

Living organisms possess intrinsic antioxidant defense mechanisms against free radicals, which are sufficient to prevent oxygen radical cytotoxic effects (Yoshikawa & Naito 2002). In our study MDA, NO and other major antioxidants were chosen as lipid peroxidation indicators. Our results showed a significant decrease in MDA and NO levels while an increase in the activities of catalase and glutathione peroxidase was shown. Hence, it may be possible that ENLE and nimbolide decrease lipid peroxidation level due to their antioxidant effect. Our results agree with Dutta *et al.* (2011) who suggested that Neem leaf aqueous extract exhibited significant free radical scavenging. Another study showed an increase in MDA and NO levels after treatment with the methanolic leaves extract of *Azadirachta indica* (MLEN, 500 mg/Kg b.w) on cisplatin- (CP) induced nephrotoxicity and oxidative stress in rats. However, the oral administration of MLEN to CP-intoxicated rats for 5 days brought back MDA, NO production, and enzymatic and non-enzymatic antioxidants to near

normal levels (Ahmed *et al.* 2014).

Neem leaf extract has been shown to enhance the activity of GPx in various tissues of mice and rats (Dasgupta *et al.* 2004). Another study has shown that the extract elevated GPx activity in benzo(a)pyrene-induced forestomach tumorigenesis in mice (Gangar *et al.* 2006). GPx activity was significantly increased in the stomach after treatment with ethanolic Neem leaf extract against N-methyl -N'- nitro-N nitrosoguanidine-induced gastric carcinogenesis in Wistar rats (Subapriya & Nagini 2005). This may be attributed to enhanced antioxidant capacities. Increased generation of ROS such as $O_2^{\cdot-}$ and H_2O_2 is recognized to induce CAT and GPx. Higher activities of antioxidant enzymes have been observed in malignant tumors compared to controls (Kumaraguruparan *et al.* 2002).

Similarly, apoptosis is an ordered and orchestrated cellular process that occurs in physiological and pathological conditions. Apoptosis plays an important role in the treatment of cancer as it is a popular target of many treatment strategies (Wong 2011). Caspase-3 is also required for some hallmarks of apoptosis (Porter & Jänicke 1999). Cytochrome c is also involved in the initiation of apoptosis. Cytochrome c was reported to be higher in cancer tissues than in non-diseased organs (Sánchez-Alcázar *et al.* 2000). Our results showed significant increase in Caspase-3 activity and Cytochrome c level compared with the positive control group. Elumalai *et al.* (2012) has previously shown that nimbolide activates caspase 3. Also, the mitochondrial pathway is engaged by the release of apoptogenic factors like cytochrome c from the mitochondrial inter membrane space into the cytosol. Additionally, NLE treatment-induced morphological apoptotic changes have been associated with increased caspase-3 in oocytes (Anima *et al.* 2011). Our study is in agreement with Gupta *et al.* (2011), who reported that nimbolide increases Cytochrome c levels while treating human colon cancer cells with Neem extract and suggested that it is a controlled form of cell death.

Liver is the most important key organ in the metabolism, detoxification and secretory functions in the body and it is highly affected primarily by toxic agents. In this regard, the following parameters were studied for assessment of liver damage: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Total protein (T.P), Albumin (Alb) and Bilirubin (Baligar *et al.* 2014). Our study showed that ethanolic Neem leaf extract and nimbolide have no significant changes in all parameters in the studied groups, highlighting the non-hepatotoxic nature of Neem extracts. These results are in agreement with Haque *et al.* (2006), who found unaltered and normal activities of serum ALT and AST following prophylactic treatment on Swiss albino mice with neem leaf preparation against Ehrlich's carcinoma. Also, Bhanwra *et al.* (2000) reported the hepatoprotective role of Neem leaves extract against

paracetamol-induced hepatic damage in albino rats as indicated by stable serum activity of ALT and AST and histopathological observations of liver tissues. Similarly, the extract has been reported to prevent changes in the serum levels of bilirubin, total protein, alanine aminotransferase and aspartate aminotransferase after induction with antitubercular drugs in albino rats (Kale *et al.* 2003).

Concerning the kidneys, our results showed insignificant decreases in urea and creatinine levels with respect to the positive control group, while these values are within normal range. Mallick *et al.* (2013) reported that Neem leaf glycoprotein (NLGP) is non-toxic even in higher doses and showed apparently normal urea and creatinine levels. Thus, it can be recommended for human use in anti-cancer therapy. Another study investigated the protective effect of the leaves against cisplatin induced nephrotoxicity. They showed significant protection as evidenced by the decrease of elevated urea. This improvement of physiological function was associated with high protection against histopathological injury induced by cisplatin on kidney. This result suggests that neem leaves pre- and post-treatment can prevent the nephrotoxicity induced by cisplatin (Doaa *et al.* 2011). This is in accordance with another study which reported insignificant changes in urea and creatinine of aqueous *Azadirachta indica* leaf extract (ALE) (Nnenna & Okey 2013).

With regards to histopathology, the livers of the positive group showed very clear pathological changes, such as focal large areas of necrotic cells infiltrated with mononuclear cells, while the negative control group showed normal healthy hepatic parenchyma. The therapeutic and the preventive groups of Nimbolide and ENLE also showed normal healthy hepatic parenchyma as shown in Figure 2a-d. The kidneys of the positive group showed very clear pathological changes, such as mononuclear cell infiltration while the negative group showed normal glomeruli and renal tubules. The therapeutic and the preventive groups of Nimbolide and ENLE also showed normal glomeruli and renal tubules (Figure 2e-h). It was found that histopathological changes were remarkably reversed in graded doses of nimbolide pretreated rats with lesser vacuolar degeneration and hepatic necrosis. Our results are in accordance with Baligar *et al.* (2014) who reported that nimbolide protected the liver tissue against CCl₄ toxicity with mild hepatocellular degeneration and less inflammatory cell infiltration. Similarly, paracetamol-induced liver necrosis was found to be reduced as observed macroscopically and histologically after treatment with aqueous leaf extract of *A. indica* (Bhanwra *et al.* 2000). To confirm our results, correlations between parameters in the studied groups were calculated.

In conclusion, the present study demonstrates that ethanolic Neem leaves extract and nimbolide

provide a significant antiproliferative activity against Ehrlich ascites carcinoma cells, have potent antioxidant activity and induce apoptosis through caspase-3 and the release of cytochrome c, with no side effects on the liver or kidney. In addition, the study shows that nimbolide provides a significant protective effect against Ehrlich ascites. The anticancer activity of nimbolide could be due to the presence of α,β -unsaturated ketone structural elements and a δ -lactone ring. Therefore, nimbolide could represent a high potential antitumoral compound.

Conflict of Interest

The authors declare that there is no conflict of interest to disclose.

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