Effect of Antitumor for *Neem* Leaf Extract and *Nimbolide* on Ehrlich Ascites Carcinoma Cells in Mice

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ABSTRACT

Introduction: Azadirachta Lalandica (*neem*) has traditionally been used for several years. Some impressive therapeutic qualities have been obtained. The objective of the study is to identify antimicrobial agents, antioxidant activities in ENE and *Nimbolide*, lemon extracts in leaves and Indica flowers and Azadirachta. Also, to study the side effects of *neem* ethanol extract and nempolide fraction on different organs (liver / kidney).

Materials and Methods: We evaluated the effect of *neem*bolide leaves and *neem* ethanol (ENLE) on longevity and levels of monolidealdehyde, nitric oxide, catalase, clotathione peroxidase, caspase 3 and cytokrome. The study showed an effect on Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Total Protein (T.P), Albumin, Bilirubin, Urea and Creatinine.

Results: The extracted ethane and nempolid leaves showed a significant increase in shelf life. In addition, decreased malondialdehyde and nitric oxide were significantly increased in catalase enzymes, glucathione peroxidase enzymes, caspase activity 3, and cytochrome c.

Conclusion: thus, *Nimbolide* and ENLE may reduce the lipid peroxide level due to their antioxidant effect and enhance the process of extracting the *neem* leaf extract. The nebulide did not show any side effects on liver and kidneys. Also, as demonstrated, Nympolid and ENLE have significant protection for both liver and kidney dissection.

Keywords: Cancer; Antioxidant; Ehrlich ascites carcinoma cells; *Nimbolide*

Introduction

Despite considerable advances in understanding biology and pharmacy, cancer is one of the most serious problems for human health. The usual treatment methods are considered to be beneficial individually in certain cases, and when combined with other treatments, they are a more efficient treatment for tumors. The results of the analysis of several therapeutic drugs and their sources show that more than 60% of the approved drugs are derived from natural compounds.[1] Scientists believe that antioxidants are substances that may protect cells from becoming cancerous by installing unstable molecules, known as free radicals. It is also believed to contribute to diseases such as cancer. To date, different studies have shown the relationship between antioxidants and cancer. The differences between the findings of some people within the scientific community led to questioning the effectiveness of the use of antioxidant supplements when trying to prevent or combat cancer.[2,3] In recent years, a great effort has been made toward identifying substances that occur naturally and that can protect against oxidative stress.[4] Natural antioxidants contain a wide range of biochemical activities, including inhibition of ROS generation, direct or indirect detection of free radicals, alteration of reduction potentials and intracellular oxidation.[5] Plants are a natural source for the production of a large number of biologically active

chemical components in a multilayered and selectively efficient manner. A different class of biologically active compounds has been isolated and distinguished since the mid-nineteenth century. Much of these are used as the active ingredients of modern medicine, or as the lead compounds for new drugs discovery. Many plant-derived drugs, rich in phenolics, alkaloids, flavonoids, ribenoids, tannins, etc., are used in the treatment of various diseases.[6] Azadirachta indica (N*eem*), a mother of all therapeutic plants, has been used on a large scale for several decades and is still used for ritual and medical purposesDue to its ease of availability and low cost, many people have been able to benefit from this dynamic plant. Studies have been performed on a wide range of leaves until the bark to explore their therapeutic potential. Phytochemical compounds such as azadrachine, nimbidine, nimbin, nimbinin, nimbidine, nimboldate, nimbidic acid, nimbidine and sodium nemptite derived from *neem* plant have a variety of medicinal effects such as antipyretic agents, contraceptives, anti-bacterial antioxidants effect and many more.[7]

Materials and Methods

Plant Materials and Extraction

The Azadirakata indica leaves were collected from Al-Qanater Charitable, Egypt in 2012 and were identified and proven by the Herbalist Department of

Botany, Cairo University. The leaves were sandwiched and grinded to powder using a grinding machine. **Neem** extract prepared by.[8] The extract was extracted in a normal saline solution to obtain a final concentration.[9] Extraction of *nimbolide* was synthesis according to.[10]

Animals

120 Swiss white mice aged 22 to 25 g were purchased from the Animal House Colony at the National Research Center, Dokki, Giza, Egypt. Animals were kept at the Experimental Animal House at the Faculty of Science, Zagazig University. The mice were kept in an appropriate environment of temperature, humidity and light, fed on a standard commercial diet and ad libitum tap.

Ehrlich Ascites Carcinoma Cells

EAC cells were initially supplied from the National Cancer Institute, Cairo, Egypt (only for the first transplantation), 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 ~2.5 ×106 cells. This process was repeated every 10 days for keeping the strain available throughout the present study.[11]

Neem Extract and *Nimbolide* Identification

A- H^1 NMR, C^{13} NMR and Mass spectrum: The H^1 NMR and C^{13} NMR spectra were recorded with 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.[9] B- High Performance Liquid Chromatography *HPLC* High Performance Liquid Chromatography (HPLC) analysis of ENLE and *nimbolide* was performed at the National Research Center, Giza, Egypt according to.[10]

Toxicity study and dose response curve Approximate LD50 of *neem* extract was determined according to the method.[12] Dose response curve for ENLE in mice was determined according to the method.[13] Studies performed 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/kg body wt according to.[14] One tenth of LD50 was used in this work is the most effective dose of *nimbolide* was first reported by.[15]

Experimental Design

Ninety adult female albino mice were divided into six groups (each one 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 2.5×106 cells/0.2 ml once for 9 days. Group III: Therapeutic group of ENLE: 15 mice were injected with EAC cells 2.5×106 cells/0.2 ml before inoculation with 5 mg/Kg body.wt ENLE treatment day by day for 9 days. Group IV: Preventive group of ENLE: ice were injected with ENLE treatment before inoculation with EAC cells 2.5×106 cells/0.2 ml. Mice received 5 mg/Kg b.w ENLE treatments day by day for 9 days. Group V: Therapeutic group of *Nimbolide*: 15 mice were injected i.p. with EAC 2.5×106 cells/0.2 ml before inoculation once with 22.5 mg *Nimbolide* cckg b.w according to15 for 9 days. Group VI: Preventive group of *Nimbolide*: 15 mice were injected with 22.5 mg *Nimbolide* / kg b.w once then were injected with EAC cells 2.5×106 cells/0.2 ml for 9 days.

Sampling and Preparation
Blood Sampling

At the end of experimental period, Plasma was collected on anticoagulant (EDTA) by centrifuging blood at 3000 r.p.m. 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 rat and placed in an isotonic saline solution to evaluate the anti-cell activity (Caspase-3 and Cytochrome c). The mouse was dissected and the liver and kidney tissues were removed and placed in a 10% formal saline solution, embedded in paraffin, cut and stained with hematoxylin and iodine (H & E) to evaluate tissues.

Viability and Life Span Prolongation

The validity of the EAC cells was previewed by the Blue Tribal exclusion method,[16] The total and viable cells (untreated) were calculated at 40 × magnification; the number of cells / ml was calculated in the groups studied. Lifetime determination was performed according to the method described by.[17]

Biochemical Investigations

Malondialdehyde (MDA) levels were analyzed and measured according to,[18] Nitric Oxide (NO) was determined by,[19] catalase enzyme activity (CT) was estimated according to[20] and Glutathione Peroxidase GPx activity was measured according to.[21] The activity of caspase-3 was determined the colorimetric caspase-3 kit according to the method of,[22] and Cytochrome c was determined according to the method.[23] Total Protein was measured by,[24] Albumin according to,[25] Alanine Aminotransferase (ALT) and Aminotransferase (AST) were determined according to,[26] Serum bilirubin was determined according to.[27] Urea was measured by[28] and creatinine was measured according to.[29]

Histopathological Examination

The flat parts of the liver and kidneys were examined on the surface of a clean microscope slide according to.[30]

Statistical Analysis

All statistical analyzes were conducted by SPSS 14.0 for Social Sciences, SPSS Inc. And were statistically significant at $p < 0.05$ on two sides. The numerical data was expressed as ± SD. The levels of markers were analyzed by ANOVA but the Mann Whitney test was used for independent comparisons groups.[31]

Results

Total Extract and *Nimbolide* Yield

Neem leaves powder (0.5 Kg) after undergoing extraction, yielded 15.8 g of Ethanolic *Neem* Leaf Extract (Thick green paste) and other (0.5 Kg) of leaf powder yielded 1 g of *Nimbolide* (Off-white solid).

Neem extract and *nimbolide* identification For identification of the extract, HPLC was done. The retention time (Rt) of standards (Azadirone, 28-Deoxonimbolide, *Nimbolide*, and Quercetin) were 4.980, 3.548, 2.971, and 7.631 with100% area. While 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*, it is cleared from Mass spectroscopy, H[1] NMR, C[13] NMR as shown below

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).

H[1]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-αH), 3.36 (s, 3H, $COCH_3$), 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).

C13 NMR (DMSO-d6, ppm) of *nimbolide*:

1. = 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) (Fig. 1)
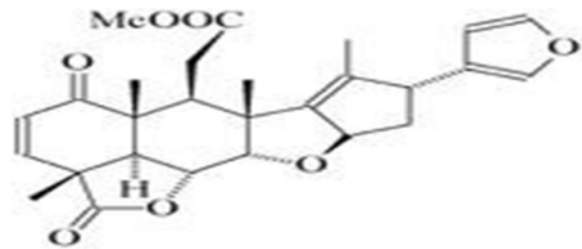
Figure 1. Structure of nimbolide Toxicity study and dose response curve.

For determination of 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 it is cleared that 5 mg ENLE/Kg mice was found to be the most effective dose as it reduced the number of EAC cells in treated mice group to 76% of EAC cells compared with positive control mice group as shown in Fig. 2.
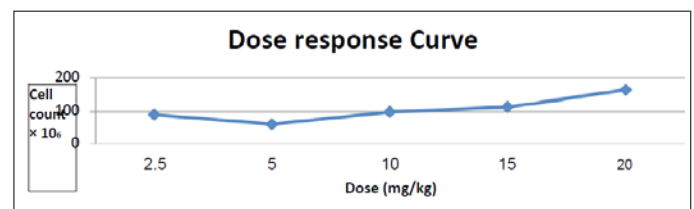
Figure 2. Dose response curve of total Neem extract.

Viability and Life Span Prolongation

From our results, it has been demonstrated that ENLE and *nimbolide* from *neem* leaves have display anticancer activity as they decreased EAC count in mice bearing EAC in all groups ($p < 0.001$) compared with positive control mice. Also both showed significant increase in life span in all studied groups compared with positive control group. See Table 1.

Biochemical Investigations
Antioxidant Assay

The effects of extracting and *nimbolide* have been tested on antioxidants. Data in Table 2 The results showed that the use of ethanol extract significantly reduced the levels of malondialdehyde (MDA) and nitric oxide (NO) ($p < 0.001$)in preventive groups and therapeutic. It also significantly increased the activity of catalase and lutathione and peroxidase enzymes in both treatment and preventive groups ($p < 0.001$). The results also showed that taking nempolid resulted in a significant reduction in levels of malondialdehyde (MDA) and nitric oxide (NO) ($p < 0.001$)in preventive groups and therapeutic. It also significantly increased the activity of catalase enzymes and asotathione peroxidase in both treatment and preventive groups ($p < 0.001$).

Table 1. EAC count (×106 cells/ml) and T/C% Among Different Study Groups

Group	Positive control	*Nimbolide*		Extract	
		Therapeuti c Group	Preventiv e Group	Therapeuti c Group	Preventiv Groupe
EAC count	209.9± 14.57	48.6± 1.8***	No cells	91.9 ± 2.11***	68.4 ± 1.95***
Survival days	9	14	15	16	17
life span T/C%	–	55.55%	66.66%	77.77%	88.88%

Data are expressed as Means ± SD. (*n* = 10).
N.S. P value >0.05 was considered non-significant.
*P value <0.05 was considered significant.
**P value <0.01 was considered highly significant.
*** P value <0.001 was considered very highly significant.

Table 2. Collective Table of Antioxidant Parameters Among Different Study Groups

Group	MDA(nmol/ml)	NO(mmol/l)	Catalase(U/L)	Glutathione peroxidase(U/L)
Negative control	11.99± 0.79*	19.84± 1.11***	227.37 ± 12.10***	200.37± 4.63***
Positive control	42.74± 1.80	34.42± 3.33	150.92± 6.11	102.44± 3.48
Therapeutic *Nimbolide*	12.03± 0.54***	13.27± 1.91***	717.16± 48.86***	614.48± 19.53***
Preventive *Nimbolide*	13± 0.75***	18.21± 1.38***	909.45± 51.07***	773.41± 62.73***
Therapeutic extract	19.61± 1.45***	22.97± 1.24**	353.38± 35.60***	357.94± 36.80***
Preventive extract	14.04± 0.37***	20.95± 1.33***	454.08± 68.19***	439.49± 55.10***

Data are expressed as Means ± 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. N.S. P value >0.05 was considered non-significant.

Apoptotic Effect

Data in Table 3 revealed that administration of the extract showed significant increase in caspase-3 activity and cytochrome c concentration in both therapeutic and preventive groups ($p < 0.001$). Also, *nimbolide* administration showed significant increase in caspase-3 and cytochrome c activities in therapeutic group ($p < 0.001$). While, in *nimbolide* preventive group the cells were absent.

Liver function Tests

The results from Table 4 showed that taking ENLE in mice has decreased AST in therapeutic (P> 0.05) and preventive group ($p < 0.001$), ALT ($p < 0.001$) bilirubin levels and activities ($p < 0.001$) compared with positive control group but these values are within normal range in mice so that they showed insignificant change in these enzymes Data showed also insignificant increase in T.P ($p < 0.05$) and albumin activity ($p < 0.05$) compared with positive control and these values are within normal range after administration of extract. Also administration of *nimbolide* showed significant decrease in AST ($p < 0.001$), ALT ($p < 0.001$) and bilirubin ($p < 0.001$) compared with positive control group but these values are within normal range in mice so that they showed insignificant change in these enzymes. The results also showed a significant increase in levels of T.P ($p < 0.001$) and albumin activity ($p < 0.001$) compared with positive control and these values are within normal range after administration of *nimbolide*.

Table 3. Collective Table of Apoptosis Parameters Among Different Study Groups

Group	Caspase-3(ng/ml)	(ng/ml)Cytochrome c
Positive control	0.834± 0.06	1.061± 0.09
Therapeutic *Nimbolide*	3.462± 0.22***	4.74± 0.35***
Therapeutic extract	2.188± 0.14***	2.48± 0.28***
Preventive extract	2.956± 0.24***	3.32± 0.36***

Data are expressed as Means ± SD. (*n* = 10).
N.S. P value >0.05 was considered non significant
*P value <0.05 was considered significant.
**P value <0.01 was considered highly significant.
***P value <0.001 was considered very highly significant.

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	81.3±2.36***	41.5±2.33***	6.86±0.12***	2.63±0.11***	0.387 ± 0.04***
Positive control	109.7±4.62	113± 8.07	5.5± 0.31	1.78± 0.16	0.944± 0.05
Therapeutic *Nimbolide*	55.2±2.71***	35.5±3.22***	6.91±0.13***	2.77±0.17***	0.201±0.06***
Preventive *Nimbolide*	41.7±5.17***	23.8±3.18***	6.76±0.20	2.82±0.13***	0.138±0.05***
Therapeutic extract	102.8±7.99	75.7±5.33***	5.85±0.32**	2.21±0.14*	0.289±0.06***
Preventive extract	84.5±3.38***	73.5±4.43***	6.32±0.34*	2.23±0.12*	0.415±0.07***

Data are expressed as Means ± SD. (*n* = 10).
N.S. P value >0.05 was considered non significant (no stars)
*P value <0.05 was considered significant.
**P value <0.01 was considered highly significant.
***P value <0.001 was considered very highly significant.

Kidney Function Tests

Our study showed that administration of ENLE had significant decrease effect on urea ($p < 0.001$) and creatinine activities ($p < 0.001$) in all studied groups and these values are within normal range. Also, administration of *nimbolide* showed significant decrease in urea ($p < 0.001$) and creatinine activities ($p < 0.001$) in all studied groups and these values are within normal range.

Histopathological Examination

After the study and the test group, the vital organs (liver and kidney). The microscope was carefully observed and did not reveal any significant lesions that could be observed when compared with the control group. Microscopic, the device detected some histological changes from the test groups when compared with the negative control group.

Correlations Between Different Parameters in the Studied Groups

To confirm our results, correlations between parameters in the studied groups were done, as there were positive and negative correlations between different parameters in the all studied groups. Among ENLE studied groups (Table 4), there were strong positive correlations between MDA and NO (r= 0.95) ($p < 0.01$), GPx and (Catalase and Cytochrome c) (r= 0.9) and (r= 0.8) ($p < 0.01$), Caspase-3 with (Cytochrome c and catalase) (r= 0.97) and (r= 0.84) ($p < 0.01$) and Catalase with cytochrome c (r= 0.82) ($p < 0.01$). Also there were strong negative correlations between MDA and (GPx and Catalase) (r= -0.6), (r= -0.6) ($p < 0.01$), NO and (GPx and Catalase) (r= -0.6) and (r= -0.6) ($p < 0.01$). Among studied groups (Table 5), 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), (r= 0.95) and (r= 0.97) ($p < 0.01$) and Caspase with Cytochrome c (r= 0.99) ($p < 0.01$). Also there were strong negative correlations between MDA and (GPx and Catalase)

(r= -0.6), (r= -0.6) ($p < 0.01$), NO and (GPx, Catalase, caspase-3 and Cytochrome c) (r= -0.7), (r= -0.7), (r= -0.5) and (r= - 0.5) ($p < 0.01$).

Table 5. Collective Table of Kidney Function Tests Among Different Study Groups

Group	Urea(mg/dl)	Creatinine(mg/dl)
Negative control	21± 3.97***	0.385± 0.17***
Positive control	62.1± 3.38	1.663± 0.16
Therapeutic *Nimbolide*	***24.9± 2.21	0..903± 0.06***
Preventive *Nimbolide*	20.5± 2.72***	0.758± 0.09***
Therapeutic extract	29.2± 1.24***	0.658± 0.10***
Preventive extract	31.6± 1.62***	0.509± 0.09***

Data are expressed as Means ± SD. (n = 10).
N.S. P value >0.05 was considered non-significant
*P value <0.05 was considered significant.
**P value <0.01 was considered highly significant.
***P value <0.001 was considered very highly significant.

Discussion

Cancer is a word that expresses the term to describe a range of diseases that cause irregular growth, invasion, and the spread of (malignant tumor) of abnormal cells. Cancer occurs due to external factors such as malnutrition, environmental conditions, tobacco use, radiation, infectious organisms, lack of exercise, as well as internal factors such as mutations, genetics, and hormones.[32] *Neem* leaf extracts have been reported to be non-mutagenic and non-toxic, and have been found to have immune, anti-cancer and anti-inflammatory properties.[33] There are many studies that have shown that the extract of ethanol from *neem* leaves has an anti-cancer activity. Ethylene *Neem* Extract (ENLE) showed an anti-cancer activity against infectious carcinogenesis caused by nitrosoganidine and methane-n.[34] ENLE induces apoptosis in a-prostate cancer cell line (PC-3) by up-regulating the pro-apoptotic protein Bax and lessening the level of Bcl-2

protein resulting In dividing the DNA into prostate cancer cells.[35,36] Many biologically active compounds are isolated from this plant. Among them, *nimbolide* belongs to the lemongrass group. The main component of the *neem* leaves is *Nimbolide*, a tetrahydrate consisting of a lemon skeleton with an α, β-unsaturated ketone system and a δ-lactone ring.[37] *Nimbolide* has many types of biological activity, including anti-cancer and anti-malarial activity.[38] *Nimbolide* was obtained to have an anticancer activity in a wide range of cancer cells, including neuromuscular neural cell carcinoma, bone sarcoma, placental carcinoma,[39,40] leukemia,[41] and melanoma cells, and in macrophages[41] ENLE contains several antioxidants and anticarcinogens including terpenoids, limonoids, quercetin and sitosterols. *Neem* leaf components such as *nimbolide*, 28-deoxonimbolide, and azadirone were obtained because they had a cytotoxic activity against different cancer cell lines.[42] Quercetin, a *neem* bioflavonoid highly soluble in ethanol and potent antioxidants, was obtained to prevent the growth and regulation of cancer cells in malignant cell lines the expression of Bcl-2 and mutant p53 protein.[43] 28-Deoxonimbolide exhibited potent cytotoxic activities against HL60 cells[44] and showed high cytotoxicity against a normal lymphocyte cell line.[45] LD50 of *nimbolide* was reported to be 225 mg/Kg body wt in mice according to[13] unlike[46] who suggested that LD50 of *nimbolide* in amice was reported to be 280 mg/Kg body wt. this difference was supposed to be due individual variation.[47] The acute toxicity was estimated by intraperitoneal administration of the compounds (ENLE) to determine the median lethal dose (LD50). Our results revealed that, dose up to 2000 mg /kg was considered safe for ENLE. Our results showed that, 5 mg /kg was considered to be the most effective dose of ENLE. Our results are in agreement with[48] 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 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 *neem* tree have display anticancer activity as the decreased EAC count in mice in all treated groups. It showed 100% absence of tumor cells in *nimbolide* preventive group. ENLE has helped reduce cell proliferation by inhibiting an insulin-like growth factor (IGF) signaling molecules MCF-7 and MDA MB-231.[49] In another study, the antidepressant activity of the ENLE extract alone or in combination with cisplatin was demonstrated by a cell survival test on human breast (MCF-7) and cervical human epithelial adenocarcinoma cell line (HeLa) cancer cells.[50] The therapeutic dose was found to be 22.5mg/Kg b.w. which is one tenth of the LD50 and the effective dose according to[14] who reported that administration of *nimbolide* at 20 mg/kg of body weight reduced tumor growth by almost 90% of human

colorectal cancer xenografts which supports 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.[51] Also,[38] suggested that *Nimbolide*, a triterpenoid extracted from the flowers of the *neem* tree (Azadirachta indica), was found to have antiproliferative activity against some cancer cell lines resulted in cell cycle disruption Living organisms possess intrinsic antioxidant defense mechanisms against free radicals, which are sufficient to prevent oxygen radical cytotoxic effects.[52] In our study MDA, NO and other major antioxidants were chosen as lipid peroxidation indicators. Our results showed significant decrease in MDA and NO levels while it showed increase in the activities of catalase and glutathione peroxidase. Hence, it may be possible that ENLE and *nimbolide* decrease lipid peroxidation level due to their antioxidant effect. Our results agreed with[53] who suggested that *Neem* leaf aqueous extract, flower and stem bark ethanol extracts exhibited higher free radical scavenging. The leaf aqueous extract is significantly decreased malondialdehyde (MDA) levels by the thiobarbituric acid reactive substances (TBARS) method for cure of diabetes. Another study showed an increase in MDA and NO levels after treatment with 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 normalcy[54] A. indica (*neem*) leaf extract has been shown to enhance the activity of GPx in various tissues of mice and rats.[55] Another study showed that A. indica leaf extract elevated GPx activity in benzo (a)pyrene-induced forestomach tumorigenesis in mice.[56] 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.[33] This may be may be attributed to enhanced antioxidant capacities. Increased generation of ROS such as $O_2^{\bullet-}$ and H_2O_2 is recognized to induce CT and GPx. Higher activities of antioxidant enzymes have been observed in malignant tumors compared with control.[57] Apoptosis is an ordered and orchestrated cellular operation 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.[58] Caspase-3 is also required for some typical hallmarks of apoptosis.[59] Cytochrome c is also involved in initiation of apoptosis. Upon release of cytochrome c to the cytoplasm, the protein binds apoptotic protease activating factor.[60] Cytochrome c was reported to be higher in cancer tissues than in non-diseased organs.[61] Our results showed significant increase in Caspas-3 activity and Cytochrome c level compared with positive control group.[62] investigated that *nimbolide*; a limonoid

present in leaves and flowers of the *neem* tree (Azadirachta indica) 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. Results In vivo showed that NLE treatment induced morphological apoptotic changes were associated with increased caspase 3 in oocyte.[63] Also, our study is in agreement with[64] who reported that *nimbolide* increase Cytochrome c levels while treating human colon cancer cells with *neem* extract and suggested that Cytochrome c is a controlled form of cell death used to kill cells in the process of development or in response to infection or DNA damage. Liver is the most important key organ in the metabolism, detoxification and secretary functions in the body and it is highly affected primarily by toxic agents thus the following parameters were studied for assessment of the liver damage: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Total protein (T.P), Albumin (Alb) and Bilirubin.[65] Our study showed that ethanolic *neem* leaves extract and *nimbolide* showed insignificant changes in all parameters in the studied groups which prove the non-hepatotoxic nature of *neem* according to normal values reported in.[66] The non-hepatotoxic nature of *neem* was proved in the study performed by,[67] who was 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,[68] 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, Azadirachta Indica (Neem) aqueous leaf extract significantly prevented changes in the serum levels of bilirubin, total protein, alanine aminotransferase, aspartate aminotransferase after induction with antitubercular drugs in albino rats.[69] The kidneys are important because they keep the composition, or make up, of the blood stable, which lets the body functions like: prevent the buildup of wastes and extra fluid in the body, keep levels of electrolytes stable, such as sodium, potassium, and phosphate, make hormones that help, regulate blood pressure, make red blood cells and bones stay intensive.[70] Our results showed insignificant decrease in Urea and Creatinine levels with respect to positive control group and these values are within normal values according to[66,71] 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 Azadirachta indica (neem) leaves against cisplatin induced nephrotoxicity. Neem leaves 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; co and post-treatment can prevent the nephrotoxicity induced by cisplatin.[72] This was in accordance with another study which reported insignificant change in urea and creatinine of aqueous Azadirachta indica leaf extract (ALE) as a study for the performance serum biochemistry parameters of broiler chicks.[73] The gold standard for diagnosis and staging of much diseases is histopathology. Histopathology refers to the microscopic examination of tissue to study the manifestations of disease specifically, in clinical medicine.[74] The livers of the positive group showed very clear pathological changes, such as focal large area of necrotic cells and 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 Figs. 3a, 3b, 3c and 3d. The kidneys of the positive group showed very clear pathological changes, such as mononuclear cells 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 as shown in Figs. 4a, 4b, 4c, and 4d. 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[65] who reported that *nimbolide* protected the liver tissue against CCl_4 toxicity with mild hepatocellular degeneration, less inflammatory cell infiltration and well preserved hepatocytes were observed in most areas and the recovery from degeneration of hepatic cells of *nimbolide* pretreated were comparable to that of standard Silymarin. Paracetamol induced liver necrosis was found to be reduced as observed macroscopically and histologically after treating with aqueous leaf extract of Azadirachta indica (A. indica)[68] To confirm our results, correlations between parameters in the studied groups were calculated, as there were positive and negative correlations between different parameters in the all studied groups. In conclusion, the present study demonstrated that ethanolic neem leaves extract and *nimbolide* provided a significant antiproliferative activity against Ehrlich ascites carcinoma cells and had potent antioxidant activity and good inducer for apoptosis by stimulation of caspase-3 and releasing of cytochrome c with no side effects on liver or kidney.

Also, the present study demonstrated that *nimbolide* provided a significant protective effect against Ehrlich ascites when administered as it showed 100% absence of tumor cells. The anticancer activity of *nimbolide* could be due to presence of α,β-unsaturated ketone structural element and δ-lactone ring so, suggests that *nimbolide* represent a high potential for antitumor activity than neem extract.
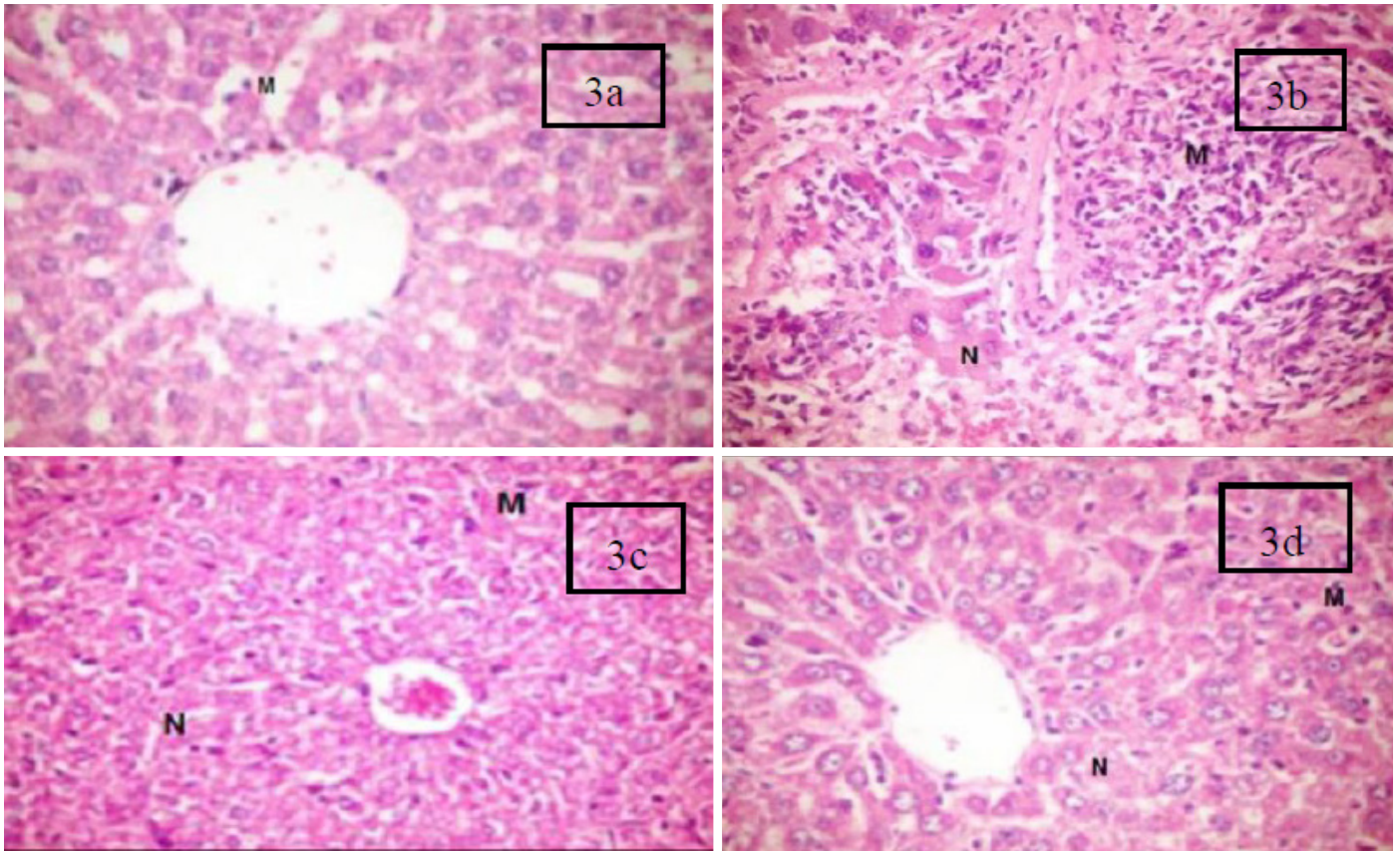
Figure 3. (a): A photomicrograph of Liver "Negative Control Group" showing normal healthy hepatic parenchyma and **(b):** A photomicrograph of Liver "Positive Control Group" showing focal large area of necrotic cells [N] infiltrated with mononuclear cells [M] (Hx& E X400). **(c):** A photomicrograph of liver "Extract preventive and therapeutic Groups" showing healthy hepatic parenchyma while **(d):** A photomicrograph of liver "*Nimbolide* preventive and therapeutic Groups" showing healthy hepatic parenchyma. (Hx& E X400).
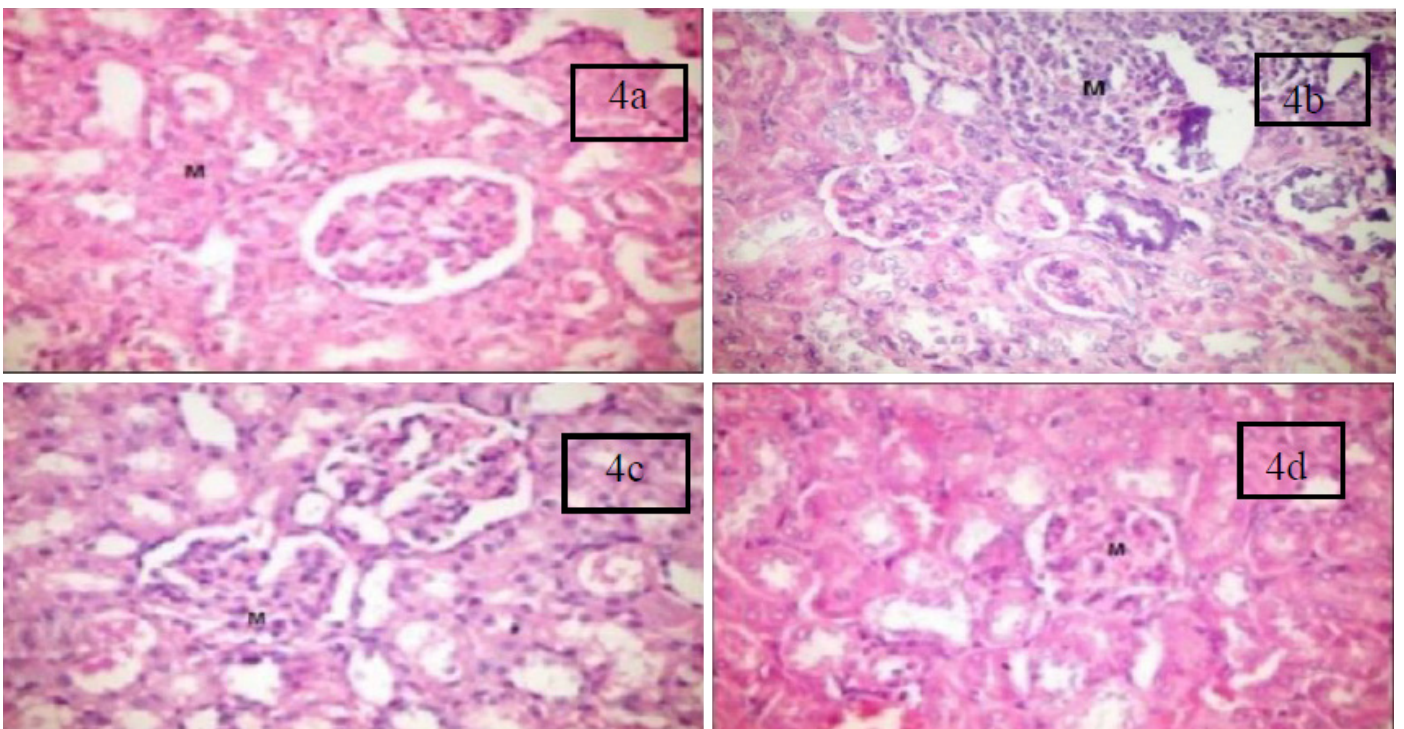
Figure 4. (a): A photomicrograph of kidney "Negative control Group" showing normal glomeruli and renal tubules and **(b):** A photomicrograph of kidney "Positive control Group" showing mononuclear cells infiltration [M]. (Hx& E X400). **(c):** A photomicrograph of kidney "Extract preventive and therapeutic Groups" showing normal glomeruli and renal tubules while **(d):** A photomicrograph of kidney "*Nimbolide* preventive and therapeutic Groups" showing normal glomeruli and renal tubules. (Hx& E X400).

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