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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 in vivo antitumor, and antioxidant activities of Ethanolic Neem Leaf Extract (ENLE) and its fraction Nimbolide, a limonoid present in leaves and flowers of the neem tree (Azadirachta indica). Also, to study the side effects of Ethanolic Neem leaf Extract and Nimbolide fraction on different organs (Liver/ Kidney). ***Materials & Methods:*** We assessed the effect of nimbolide and Ethanolic Neem Leaves Extract (ENLE) on life span prolongation 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 (T.P), Albumin (Alb), bilirubin, urea and creatinine. ***Results:*** Ethanolic neem leaves extract and nimbolide showed great increase in life span. 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 process. 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 problem; despite the great progress in understanding its biology and pharmacology. The usual therapeutic methods for cancer treatment are individually useful in particular situations and when combined with other remedies, they offer a more efficient treatment for tumors. An analysis of a number of chemotherapeutic drugs and their sources indicates that over 60% of approved drugs are derived from natural compounds [(1)]. Antioxidants are substances that scientists believe that they might protect cells from becoming cancerous by stabilizing unstable molecules, known as free radicals that are thought to contribute to illnesses such as cancer. Till now various studies have been focused on this relationship between antioxidants and cancer; and these studies have produced varied results. The disparity between the conclusions has led some people within the scientific community to question the efficacy of using antioxidant supplementation when attempting to combat or prevent cancer [(2)]. In recent years, considerable effort has been directed towards identifying naturally occurring substances that can protect

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against oxidative stress [3]. 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 [4].

Plants are natural source of producing wide number of bioactive chemical constituents in a most efficient way and with precise selectivity. Since the middle of the 19th century, different class of bioactive compounds have been isolated and characterized. Many of these are used as the active ingredients of the modern medicine, or as the lead compounds for new drugs discovery. Several plant derived medicines, are rich in phenolics, flavonoids, terpenoids, alkaloids, tannins etc., used in the treatment of various ailments [5].

Azadirachta indica (Neem) which is a mother of all therapeutical plants has been used extensively many decades ago and still been using for ritual and medicinal purposes. It's easy availability and low cost has allowed many people to gain benefit from this dynamic plant. Studies have been done widely 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 and hepatoprotective effect and many more[6].

Materials and Methods

Plant materials and extraction

Leaves of *Azadiracthta indica* were collected from Al-Qanater Al-Khaireia, Egypt in 2012 and were identified and proved by Herbarium of Botany department, Cairo University. The leaves were dried and ground into a uniform powder using a milling machine. Neem extract was prepared according to [7]. The extract was suspended in normal saline to obtain a final concentration [8]. Extraction of nimbolide was prepared according to [9].

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 experimental animal house of the Faculty of Science, Zagazig University. The animals were maintained in 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 (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 approximately 2.5 ×106 cells. This process was repeated every 10 days for keeping the strain available throughout the present study [10].

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 [8].

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 [9].

Toxicity study and dose response curve

Approximate LD50 of neem extract was determined according to the method [11]. Dose response curve for ENLE in mice was determined according to the method [12]. Studies 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/kg body wt according to [13]. One tenth of LD50 was used in this work is the most effective dose of nimbolide was first reported by [14].

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 2.5×10^6 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.wt ENLE treatment day by day for 9 days. *Group IV: Preventive group of ENLE:* 15 mice were injected with ENLE treatment before inoculation with EAC cells 2.5×10^6 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 2.5×10^6 cells/0.2 ml before inoculation once with 22.5 mg Nimbolide / kg b.w according to [14] 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 2.5×10^6 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 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 [15], where the total and viable cells (non-stained) were counted at magnification ×40; as the number of cells/ml was determined in the studied groups.

Life span calculation was carried out according to the method described by [16].

Biochemical Investigations

The levels of Malondialdehyde (MDA) were measured according to [17], Nitric Oxide (NO) was determined by [18], catalase enzyme activity (CAT) was estimated according to [19] and Glutathione Peroxidase GPx activity was measured according to [20].

The activity of caspase-3 was determined by the colorimetric caspase-3 kit according to the method of [21], and Cytochrome c was determined according to the method [22].

Total Protein was measured by [23], Albumin according to [24], Alanine Aminotransferase (ALT) and Aminotransferase (AST) were determined according to [25], Serum bilirubin was determined according to [26]**.** Urea was measured by [27] and creatinine was measured according to [28].

Histopathological examination

The flattened sections of liver and kidney were placed on the surface of clean microscope slide according to [29].

Statistical Analysis:

All statistical analyses were done by a statistical for social science package "SPSS" 14.0 for Microsoft Windows, SPSS Inc. and considered statistically significant at a two-sided $P < 0.05$. Numerical data were expressed as mean ± SD. The levels of markers were analyzed by ANOVA but the Mann-Whitney U-test was used for comparisons between independent groups [30].

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 (C24H34O5, 0.76), 385 (C22H25O5, 14), 357 (C21H25O5, 19), 314 (C19H22O4, 9), 283 (C17H15O4, 8), 267 (C15H23O4, 6), 240 (C15H12O3, 4), 186 (C12H10O2, 6), 126 (C7H10O2, 9), 97 (C5H5O2, 8), 59 (C2H3O2, 3), 43 (C3H7, 42) .

H^1NMR (DMSO-d6, ppm) of nimbolide:

δ= 1.12 (s, 3H, CH3-30), 1.28 (s, 3H, CH3-19), 1.39 (s, 3H, CH3-29), 1.60 (s, 3H, CH3-18), 2.00, 1.96 (m, 2H, C16-H), 2.30, 3.10 (dd, 2H, C11-H), 2.86 (d, 1H, C5-αH), 3.36 (s, 3H, COCH3), 5.80 (d, 1H, C2-H), 6.23 (d, 1H, furan- C22-H), 7.27 (d, 1H, C3-H), 7.37 (s, 1H, furan- C21-H), 7.51 (d, 1H, furan- C23-H).

C^{13} 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).

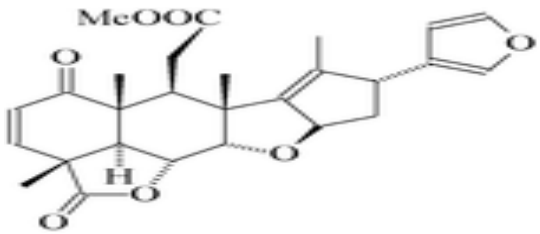

Fig (1): Structure of nimbolide

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 non toxic 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 to positive control mice group as shown in Fig. (2)

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 to positive control mice. Also both showed significant increase in life span in all studied groups compared to positive control group. See table (1).

Biochemical Investigations

Antioxidant assay

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

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

Data from table 4 showed that ENLE administration in mice lowered AST in therapeutic ($p>0.05$) and preventive group ($p<0.001$), ALT ($p<0.001$) activities and bilirubin levels ($p<0.001$) compared to 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 to 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 to positive control group but these values are within normal range in mice so that they showed insignificant change in these enzymes. Data also showed significant increase in T.P levels ($p<0.001$) and albumin activity ($p<0.001$) compared to positive control and these values are within normal range after administration of nimbolide.

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

The two vital organs, (livers and kidneys) were removed from the test groups at the end of the study and were carefully observed macroscopically and revealed no any observable gross lesions when compared with the control group. Microscopically however, the two organs of the test groups revealed some histological changes 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 6), 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 nimbolide studied groups (Table 7), 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) ($p<0.01$).

Discussion

Cancer is a term used to describe a group of diseases that cause the uncontrolled growth, invasion, and spread (metastasis) of abnormal cells. Cancer 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 [(31)].

Extracts of neem leaf have been reported to be non-toxic and non-mutagenic and are found to possess immunomodulatory as well as anti-inflammatory and anticarcinogenic properties [(32)]. There are many studies showed that the ethanolic extract of neem leaves possess anticancer activity. Ethanolic neem leaf extract (ENLE) exhibited anticancer activity against N-methyl-N'-nitro-N- nitrosoguanidine-induced oxidative stress and gastric carcinogenesis [(33)]. ENLE induces 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 [(34, 35)]. Many bioactive compounds are isolated from this plant among which, nimbolide belongs to the limonoid group.

Nimbolide, 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 [(36)]. Nimbolide has numerous types of

biological activity, including antimalarial and anticancer activity [37]. Nimbolide was found to exhibit anticancer activity in a wide variety of tumor cells, including neuroblastoma, osteosarcoma, choriocarcinoma [38], leukemia [39], and melanoma cells, and in macrophages [40].

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 [41]. 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 [42]. 28-Deoxonimbolide exhibited potent cytotoxic activities against HL60 cells [43] and showed high cytotoxicity against a normal lymphocyte cell line [44].

LD50 of nimbolide was reported to be 225 mg⁄Kg body wt in mice according to [12] unlike [45] 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 [46].

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 [47] 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 decreased cell proliferation through the inhibition of the Insulin-like growth factor (IGF) signaling molecules in both MCF-7 and MDA MB-231 cells [48].

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 cell line (HeLa) cancer cells [49]. The therapeutic dose was found to be 22.5 mg/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 [50]. Also, [51], 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 [32]. 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 CAT and GPx. Higher activities of antioxidant enzymes have been observed in malignant tumors compared to control [57].

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 [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 strong [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 many diseases is histopathology. Histopathology refers to the microscopic examination of tissue in order 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 CCl4 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 was 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.

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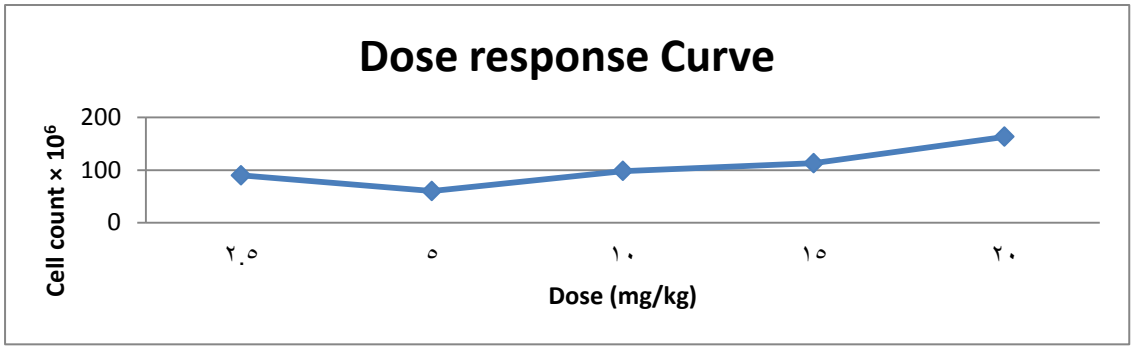


Fig (2):- Dose response curve of total Neem extract

Table(1): EAC count (×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	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). *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

Table(2): Collective table of antioxidant parameters among different study groups

Group	MDA(nmol/ml)	NO(µmol/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

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). *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

Table(4): Collective table of Liver function tests among different study groups

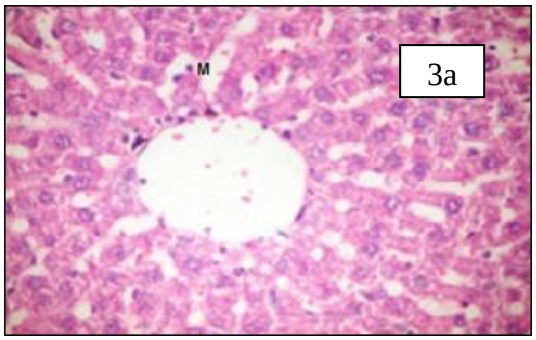
Group	AST(U/L)	ALT(U/L)	Total protein(g/dl)	Albumin(g/dl)	Bilirubin(mg/dl)
Negative control	81.3± 2.36***	41.5± 2.33***	6.86± 0.12***	2.63± 0.11***	0.387±0.04***
Positive control	109.7± 4.62	113± 8.07	5.5± 0.31	1.78± 0.16	0.944± 0.05
Therapeutic Nimbolide	55.2± 2.71***	35.5± 3.22***	6.91± 0.13***	2.77± 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). *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 (no stars)

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). *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



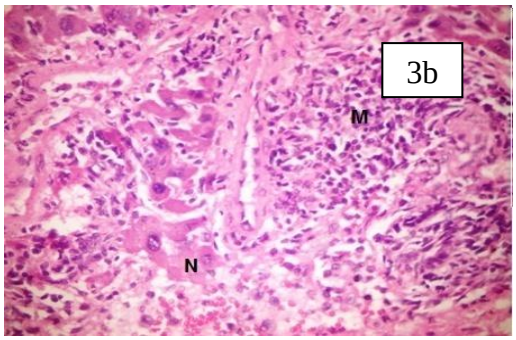
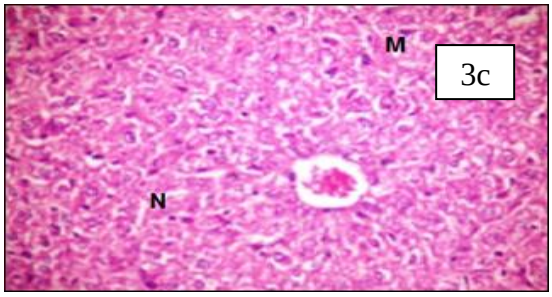


Fig (3a): A photomicrograph of Liver "Negative Control Group" showing normal healthy hepatic parenchyma and Fig. (3b): A photomicrograph of Liver "Positive Control Group" showing focal large area of necrotic cells [N] infiltrated with mononuclear cells [M] (Hx& E X400).



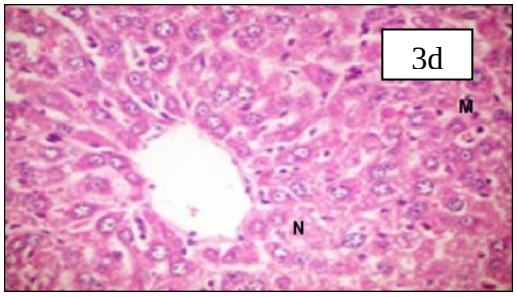
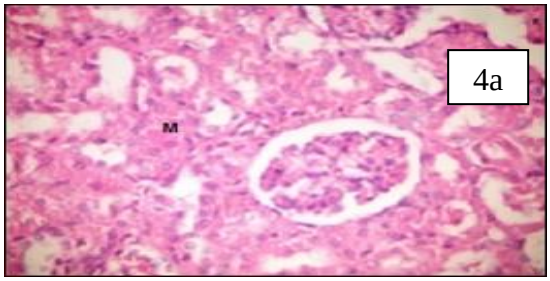


Fig (3c): A photomicrograph of liver "Extract preventive and therapeutic Groups" showing healthy hepatic parenchyma while Fig. (3d): A photomicrograph of liver"Nimbolide preventive and therapeutic Groups" showing healthy hepatic parenchyma. (Hx& E X400).



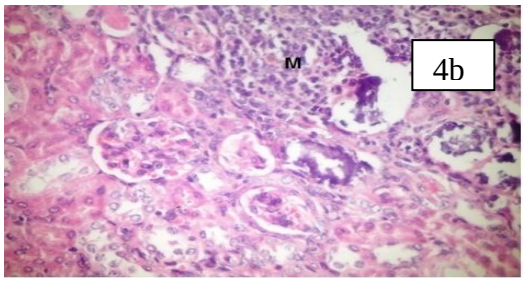
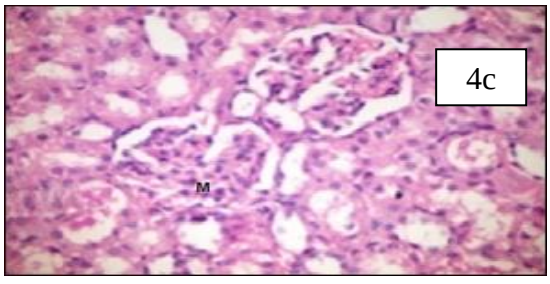


Fig (4a): A photomicrograph of kidney "Negative control Group" showing normal glomeruli and renal tubules and Fig. (4b): A photomicrograph of kidney "Positive control Group" showing mononuclear cells infiltration [M]. (Hx& E X400).



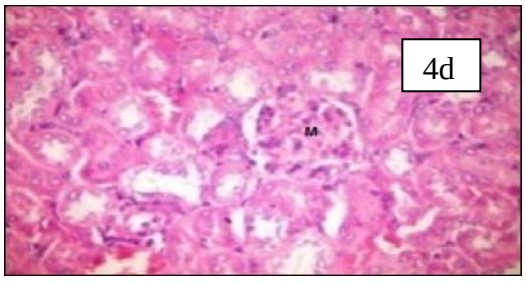


Fig (4c): A photomicrograph of kidney "Extract preventive and therapeutic Groups" showing normal glomeruli and renal tubules while Fig (4d): A photomicrograph of kidney "Nimbolide preventive and therapeutic Groups" showing normal glomeruli and renal tubules. (Hx& E X400).