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Cytotoxic Potential of Neem (*Azadirachta indica* A. Juss) Oil

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ABSTRACT

An increasing interest is emerging in identifying natural products to overcome drug resistance in cancer patients. In this context, the present study was conducted to investigate the cytotoxic effects of neem plant (*Azadirachta indica*) oil in three different biological models (breast cancer cell lines, *Allium cepa* root tip, and mice vital organs). The cytotoxic potential of the neem oil was evaluated with two human cell lines (MCF7 and MDA-MB231) and an *Allum cepa* root tip bioassay. Histopathological analysis was conducted on the neem oil-treated and untreated control mice. The results revealed an anti-proliferative effect for neem oil on both estrogen receptor-positive (MCF7) and estrogen receptor-negative (MDA-MB231) breast cancer cell lines with an IC_{50} value of 45.7 and 60 µg/ml, respectively. Also, a significant ($p \leq 0.001$) reduction in the average mitotic index and elevated levels of cytogenetic abnormalities were observed in *A. cepa* root tips treated with different concentrations of neem oil extract compared to untreated roots. Moreover, very low to undetectable histopathological changes in the vital organs (liver and spleen) of mice administered with 15 mg/ml neem oil were also observed. The findings of this study suggest that neem oil extract has the potential to inhibit the growth of different rapidly dividing biological models (breast cancer cell lines and *A. cepa* root tips) with minimal toxicity to the vital organs of the exposed mice. This could be explored further for the development of novel therapeutics to address the problem of drug resistance associated with different malignancies.

Keywords: *Allium cepa* root tips, Breast cancer cell lines, Cytotoxic potential, Neem oil

Introduction

Drug resistance has become a significant problem in a number of agricultural and health sectors. Natural compounds with the potential to address this issue have recently attracted the attention of researchers. In light of the diversity of bioactive chemicals that are required for this function, medicinal plants offer a rich source for a variety of possible therapeutic agents.[1,2] The neem plant, *Azadirachta indica*, a member of the Meliaceae family, has a high antioxidant content that may have health-promoting potential, particularly in preventing the proliferation of quickly dividing cells.[3] The cytotoxic effects of plant extracts have been evaluated using a variety of experimental models. One of these biological assays, known as the *Allium cepa* root tip test, is frequently used to assess how the plant's cell genome reacts to various exogenous toxicants by measuring DNA and chromosomal damages, such as disruptions in the mitotic cycle. The *Allium cepa* root tip test (AT) is an appropriate cellular model for evaluating cytotoxic and antimitotic effects for a number of practical reasons. These include low cost, simplicity in handling and growth, prompt reaction to stimuli, sizeable chromosomes, and few chromosomes.[4] As a result, information about the cytotoxic effects of test substances or extracts are observed through chromosomal aberrations, cell cycle arrest, and proliferation inhibition in AT provides very useful information about how effective such treatments are in terms of the development of potential anticancer drugs.

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Neem has been shown to have anti-cancer properties, and studies have shown that its extract can modulate the activity of important tumour suppressor genes.

Several lines of evidence have reported apoptotic and autophagy effects on cancer cell lines via the upregulation of the gene expression of related genes, such as *TP53*,[5] *BCL-2*,[6] and *pTEN*.[7] Additionally, PC-3 and LNCaP prostate cell lines treated with neem leaf extract had significant inhibition of the PI3K/Akt pathway, which induced apoptosis.[8, 9] Studies have shown how safe neem is for essential organs and healthy tissues, in addition to its cytotoxicity to pathogenic microbes and other quickly proliferating bio-experimental models (such as the *Allium* root tip assay and cancer cell lines). However, thorough safety analyses of the application of neem leaf formulations have not yet been carried out. When used in high concentrations over extended periods of time, neem leaf extract has been shown in previous studies to affect liver and renal functions.[10,11] According to some findings, neem leaf extract has the power to prevent kidney and liver damage.[12] Additionally, studies have highlighted the antioxidant potential of neem oil by restoring disrupted antioxidants closer to their normal levels in the liver.[13,14]

The present study was conducted to examine the potential cytotoxicity of neem oil against drug-resistant breast cancer cell lines as well as its influence on the mitotic activity of *A. cepa* root tips in light of the problem of drug resistance that is prevalent in the majority of human malignancies. An assessment of the potential cytotoxic effects of neem oil on the liver and spleen of mice was also investigated.

Materials and Methods

Source of experimental animals

Swiss albino male mice, 5–6 weeks old, weighing 20–25g, were purchased from the Biotechnology Research Center at Al-Nahrain University. They were housed in plastic cages in the animal house of the Veterinary Medicine College, University of Baghdad. Before beginning the studies, the mice were given ten days to acclimate.

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Ethical clearance
Ethical clearance for this study was obtained from the Research Ethics Committee, College of Science, University of Baghdad, with a seal of approval (Ref. No. CSEC/0922/0081).

Cytotoxicity assay
Two human cell lines (MCF7 and MDA-MB231) derived from breast cancer were used to test the cytotoxic effects of neem oil using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide). MTT solution (5 mg/ml) was prepared by combining MTT powder with sterile PBS (Macklin, Shanghai, China). Before incubating with neem oil, $1x10^4$ cells were seeded in each of the assigned 96-well plates and left for 24 hours to ensure cell adherence. After that, cells were exposed to increasing concentrations of neem oil extract (1, 10, 25, 50, and 100 µg/ml) and incubated for 48 hours. The media was then aspirated from the entire well after incubation, and 20 µl of the prepared MTT solution was mixed with an equal volume of fresh serum-free RMPI-1640 media in each well. The plate was then incubated at 37°C for 3 hours in the dark before adding 50 µl of DMSO to dissolve the formazan that had formed (i.e., from MTT). In the end, the plate was read by a microplate reader at a wavelength of 620 nm. The estimated percentage viability was calculated according to the equation below:

$$\text{Viability \%} = \frac{A\ test - A\ blank}{A\ control - A\ blank} * 100$$

Where A is the absorbance. The growth inhibitory concentration that reduces the cells' viability to 50% (IC_{50}) was measured from the viability curve plotted by GraphPad Prism software.

In vivo cytotoxic effect of neem oil on the cell division of onion root tips
Onion bulbs were collected from *A. cepa* purchased from the local market at Al-Karkh, Baghdad, Iraq. Old roots and dry scales were removed and allowed to germinate by placing them in suitable glass containers containing tap water. Onion bulbs were left for 48 hours to germinate until the roots reached 2-3 cm in length. Root meristems were subjected to increased concentrations (0.0, 5, 10, 15, 20, and 25 mg/ml) of neem oil extract for four hours to evaluate their cytotoxic potential by the *A. cepa* root tip bioassay.[15,16] Mitotic squash was prepared for all the examined root tips (neem oil-treated and untreated control groups) for mitotic index estimation.[17] Each experiment was repeated twice with three replicates of each test concentration and control treatment, with at least five micro-glass slides for each parameter. Roots initiated by placing onion bulbs in freshwater were used as a control. The root tips were stabilized in Carnoy fluid to study cell division. The frequency of the mitotic phase was also determined via cytogenetic analysis of the mitotic squash.

Toxicity assay and histopathological analysis
The mice were thereafter divided into two groups of four each. For 14 days, group 1 (the control) received 0.1 mg of the physiological solution by oral syringe, while group 2 was given 15 mg/ml of neem oil orally. Diethyl ether was used to kill the test mice, and they were then vivisected to remove their liver and spleen for the histological investigation. Before inspection, the retrieved liver and spleen organs were preserved in formalin.

Statistical analysis
GraphPad Prism version 6 (GraphPad Software Inc. in San Diego, CA, USA) was utilized for cytotoxicity data analysis. In addition, Statistical Package for Social Sciences (SPSS; version 21) was used to analyze the remaining data. Differences were considered significant only if $p < 0.05$ (95% confidence level).

Results and Discussion

Cytotoxic effects of neem oil on breast cancer cell lines
Using the MTT test, the cytotoxic effects of neem oil on MCF7 and MDA-MB231 cells were investigated. Following incubation with higher concentrations of neem oil, the results demonstrated a concentration-dependent reduction in viability of both estrogen receptor-positive (MCF7) and estrogen receptor-negative (MDA-MB231) breast cancer cell lines. The highest reduction in the viability (reaching just 18.85%) of this cell line was achieved following incubation with 100 µg/ml of neem oil. The vitality of MCF7 cells dropped by more than half (viability reached 42%) upon exposure to 50 µg/ml of neem oil extract (Figure 1a). MDA-MB231 cells were more sensitive to neem oil; viability was reduced to 36.6 and 9.66% with concentrations of 50 and 100 µg/ml, respectively, of neem oil as shown in Figure 1b. In addition, the IC_{50} values of neem oil indicated that MCF7 cells (45.7 µg/ml) were more sensitive than MAD-MB231 cells (60 µg/ml). However, MDA-MB231 cells showed greater sensitivity than MCF7 to the highest concentration of neem oil (100 µg/ml).
Breast tissue from patients with metastatic adenocarcinoma served as the initial source of breast cancer cell lines. It is recognized that metastatic breast cancer is very difficult to eradicate. It necessitates the use of systemic medications that combat cancer throughout the body, such as chemotherapy, hormone therapy, targeted therapies, and immunotherapy.[18] Interestingly, this study found that exposure of triple-negative (MDA-MB231) and estrogen receptor-positive (MCF7) breast cancer cell lines to neem oil had an anti-proliferative effect. The triple-negative breast cancer (TNBC) cell line and mouse model treated with neem leaf extract showed decreased expression of the c-MYC oncogene.[19] In line with the findings of the present investigation, another study found that neem extracts suppressed the proliferation and growth of tumour cells by disrupting cell cycle progression.[20 21] Such potential anti-cancer effects are believed to be attributed to the rich phytochemicals content of neem oil extract. Moreover, another study speculated that the anti-proliferative and pro-apoptotic effects of methanolic neem leaf extract were shown to be mediated via the modulation of the expression of the nuclear factor-κB.[22]

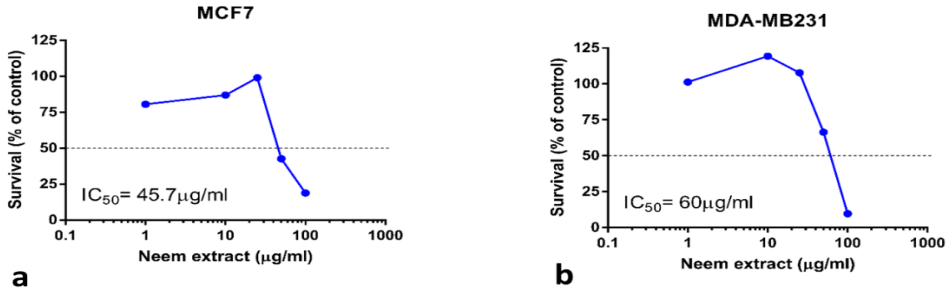

Figure 1: Cytotoxic effect of neem oil extract on breast cancer cell lines assessed by MTT assay. The viability of both MCF7 (a) and MDA-MB231 (b) cell lines dropped significantly 48 hrs post incubation with an increasing concentration (1, 10, 25, 50, and 100 µg/ml) of neem oil. The survival curve was plotted by GraphPad Prism, and IC_{50} was calculated by the same software. Each point represents the mean viability percentage of three replicate wells.

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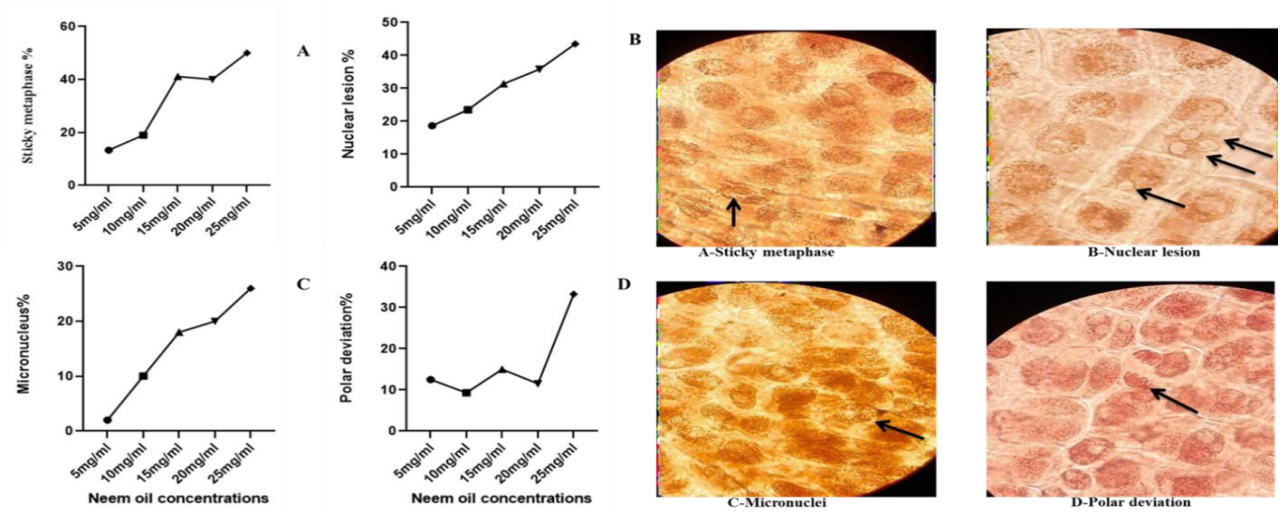

Figure 2: The percentages and representative of different cytogenetic abnormalities in *Allium cepa* root tips upon the treatment with increased concentrations of neem oil.

A: Sticky metaphase; B: Nuclear lesion; C: Micronuclei; D: Polar deviation.

Table 1: Mitotic index, frequencies of mitotic phase, and cytogenetic abnormalities of *Allium cepa* root tips subjected to different concentrations of neem oil.

Treatment (mg/ml)	MI%	Phase index %				Cytogenetic abnormalities %			
		Prophase	Meta-phase	Ana-phase	Telo-phase	Sticky metaphase	Nuclear-lesion	Micro nucleus	Polar deviation
Control	8.76	9.3	3.2	1.7	2	00.0	00.0	00.0	0>00
5	8.49	9.44	1.8	3	5	13.3	18.7	2	12.5
10	7.78	9.07	2.7	4.1	2.4	19	23.5	10	9.3
15	6.3	9.13	2.6	3.9	2.6	41.1	31.4	18	15
20	6.59	9.36	2.4	3.5	2.2	40	35.8	20	11.5
25	5.66	9.18	2.2	2.3	1.9	50	43.5	26	33.3

Cytotoxic effects of neem oil extracts on onion root tips

The results of the *A. cepa* mitotic index assay showed a significant ($p \leq 0.001$) reduction in the average mitotic index in all root tips treated with different concentrations of neem oil extract (Table 1 and Figure 2). The reduction was observed to be concentration-dependent, where the mitotic index was reduced by 33.33% in *A. cepa* root tips subjected to the highest concentration (25 mg/ml) of neem oil extract compared to those exposed to the lowest concentration (5 mg/ml) with values of 5.66% and 8.49%, respectively. All the test concentrations showed relatively comparable values during prophases to that of untreated root in terms of the phase index percentage values after exposure to neem oil extract (Table 1). In contrast, lower metaphase index values were observed in root tips treated with increased concentrations of neem oil compared to the control treatment (1.8, 2.7, 2.6, 2.4, 2.2, and 3.2, respectively) as presented in Table 1. Furthermore, the anaphase index was also higher in root tips treated with the increased neem oil concentration than that of the untreated root tip (Table 1). However, the telophase index showed higher values in root tips treated with neem oil, especially at the concentration of 5 mg/ml (2.5-folds increase) than that of the untreated root tips (Table 1).

Neem oil treatment was found to cause an intense concentration-dependent increase in the occurrence of various cytogenetic abnormalities when chromosomal aberrations in the examined root tips were assessed. The highest levels of cytogenetic aberrations were recorded for sticky metaphase and nuclear lesion (Table 1 and Figure 2) that reached 50 and 43.5%, respectively, when *A. cepa* root tips were treated with the highest concentration of neem oil (25 mg/ml). In contrast, micronuclei and polar deviation abnormalities peaked at 26 and 33.3%, respectively, when *A. cepa* root tips were grown with 25 mg/ml of neem oil (Table 1 and Figure 2). Collectively, the antimitotic activity of neem oil caused a significant reduction in the mitotic index in *A. cepa* root tips, along with the profound effect in inducing chromosomal abnormalities. These results suggest a broad potential destructive impact of the investigated neem oil extract on the mitotic machinery of the dividing cells. Such observed cytogenetic and chromosomal aberrations, including sticky metaphase, nuclear lesion, micronuclei, and polar deviation, are indications for damaged or disrupted genetic material caused by the treatment with neem oil extract. One proposed explanation of such effects is that neem influences mitosis similarly to colchicine.[23]

Histotoxic potential of neem oil on some vital organs

The toxicity of neem oil (15 mg/ml) was also investigated *in vivo* by evaluating the histological changes in some vital organs (liver and spleen) in experimental mice. Hepatic cells of mice dosed with neem oil extract showed no noticeable changes compared to the untreated controls. The liver showed a slight dilation of central veins, vascular degeneration of the hepatocytes, and neutrophil infiltration adjunct to the central veins wall at 7 days post administration (Figure 3A). The spleen of the neem oil-treated mice showed proliferation of megakaryocytes in the red pulp (Figure 3B). The histopathological changes of the liver and spleen are shown in Figure 3. Considering the relatively very low to undetectable histopathological changes in the vital organs (liver and spleen) of mice treated with neem oil observed in the present study, neem extracts can be further investigated *in vivo* for their preventive and protective potential against infections and tumorigenesis. Medicinal plants have revealed a wide range of beneficial effects,[24] and a high potential for safeguarding human health since ancient times.[23,25] Considering the urgent need to develop novel therapeutic agents to combat the increasing incidence of malignant diseases,[26] identifying natural products that have the potential to cure many of today's untreatable diseases is a promising research area.

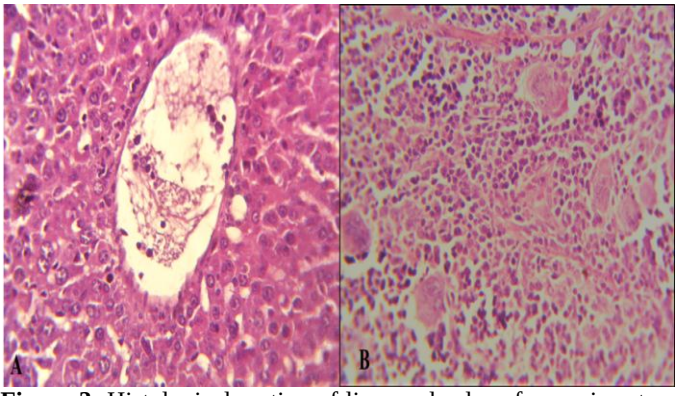

Figure 3: Histological section of liver and spleen from mice at day seven post-treatment with neem oil (15 mg/ml): A: Vascular degenerating of the hepatocytes and neutrophils infiltration adjunct to the central veins wall (H & E; 400x); B: Spleen cells on day 7, the proliferation of megakaryocytes in the red pulp (H & E Stain; 100x).

Conclusion

The findings of the present study suggest that neem oil extract has the potential to inhibit the growth of these different rapidly-dividing biological models (namely breast cancer cell lines and *A. cepa* root tip), with minimal toxicity on vital organs of mice. This could be explored further for the development of novel therapeutics to address the problem of drug resistance associated with cancer.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

References

1. Okolie NP, Falodun A, Davids O. Evaluation of the antioxidant activity of root extract of pepper fruit (Dennetia tripetala), and it's potential for the inhibition of lipid peroxidation. . Afr J Trad Compl and Altern Med. 2014; 11(3):221-227.
2. Nazir S, Abdel-Ghani N, Atia MA, El-Sherif AA. Exploring the Effectiveness of Lepidium sativum for Inhibiting Human Hepatocellular Carcinoma Cell Lines: In vitro Analysis, Molecular Docking, and Pathway Enrichment Analysis. Egypt J Bot 2023; 63(1):251-264.
3. Yunarto N, Helentina ED, Sulistyowati I, Reswandaru UN, Alegantina S, Isnawati A. Antioxidant Activity and Inhibition of HMG CoA Reductase Enzyme by Bay Leaf (*Syzygium polyanthum* Wight) Extract as a Treatment for Hyperlipidemia. Trop J Nat Prod Res. 2022; 6(11): 1798–1801.
4. Bonciu E, Firbas P, Fontanetti CS, Wusheng J, Karaismailoğlu MC, Liu D, Menicucci F, Pesnya DS, Popescu A, Romanovsky AV. An evaluation for the standardization of the *Allium cepa* test as cytotoxicity and genotoxicity assay. Caryologia. 2018; 71(3):191-209.
5. Jaiswara PK, Gupta VK, Sonker P, Rawat SG, Tiwari RK, Pathak C, Kumar S, Kumar A. Nimbolide induces cell death in T lymphoma cells: Implication of altered apoptosis and glucose metabolism. Enviro Toxicol. 2021; 36(4):628-641.
6. Sophia J, Kowshik J, Dwivedi A, Bhutia SK, Manavathi B, Mishra R, Nagini S. Nimbolide, a neem limonoid inhibits cytoprotective autophagy to activate apoptosis via modulation of the PI3K/Akt/GSK-3β signalling pathway in oral cancer. Cell Death Dis. 2018; 9(11):1-18.
7. Li H, Xia B, Chen W, Zhang Y, Gao X, Chinnathambi A, Alharbi SA, Zhao Y. Nimbolide prevents myocardial damage by regulating cardiac biomarkers, antioxidant level, and apoptosis signaling against doxorubicin-induced cardiotoxicity in rats. J Biochem Molec Toxicol. 2020; 34(9):e22543.
8. Gunadharini DN, Elumalai P, Arunkumar R, Senthilkumar K, Arunakaran J. Induction of apoptosis and inhibition of PI3K/Akt pathway in PC-3 and LNCaP prostate cancer cells by ethanolic neem leaf extract. J Ethnopharmacol. 2011; 134(3):644-650.
9. Kumar D, Haldar S, Gorain M, Kumar S, Mulani FA, Yadav AS, Miele L, Thulasiram HV, Kundu GC. Epoxyazadiradione suppresses breast tumor growth through mitochondrial depolarization and caspase-dependent apoptosis by targeting PI3K/Akt pathway. BMC Cancer. 2018; 18(1):1-17.
10. Adamu KM, Aliyu-Paiko M, Abdullahi F, Mustapha A. Effects of Azadirachta indica Leaf Powder on some Biochemical Parameters of the African catfish (Clarias gariepinus). NJBAS. 2017; 25(2):41-50.
11. Nwobodo EI, Nwosu DC, Ogbodo SO, Ugwuene FO, Ihim AC, Ani NO, Nnodim JK, Ani O. Effects of Azadirachta indica leaf aqeous extract on the antioxidant enzymes in paracetamol-induced hepatotoxicity in Wistar rats. Int J Bioll Chem Sci. 2018; 12(1):1-10.
12. Seriana I, Akmal M, Darusman D, Wahyuni S, Khairan K, Sugito S. Neem leaf (*Azadirachta indica* A. Juss) ethanolic extract on the liver and kidney function of rats. SWJ. 2021; 7970424
13. Iman M, Taheri M, Bahari Z. The anti-cancer properties of neem (Azadirachta indica) through its antioxidant activity in the liver: its pharmaceutics and toxic dosage forms. A literature review. J Complem Integ Med. 2022; 19(2):203-211.
14. Gaafar AA, Salama ZA, El-Baz FK. A Comparative study on the active constituents, antioxidant capacity and anti-cancer activity of cruciferous vegetable residues. BSJ. 2020; 17(3):0743-0743.
15. Mohammed RK. Cytogenetic in Vivo Effects of the aqueous extract of *Raphanus sativus* l. leaves in mitosis of the meristematic cells of\onion roots. IJS. 2020; 2189-2195.
16. Jaddoa NTM, Mohammed RK. Evaluating the anti-*Pseudomonas aeroginosa* efficacy and potential cytotoxicity of *Nerium oleander* alcoholic extract. Ind J Foren Med Toxicol. 2021; 15(2):3026-3033.
17. Barman A, Ray S. Mitotic index reduction and cytotoxic effects of leaf aqueous extract of *Maesa macrophylla* (Wall.) A. DC. in *Allium cepa* root tip cells. Cytologia. 2022; 87(2):81-85.
18. Bailleux C, Eberst L, Bachelot T. Treatment strategies for breast cancer brain metastases. Br J Cancer. 2021; 124(1):142-155.
19. Hu M-H, Wu T-Y, Huang Q, Jin G. New substituted quinoxalines inhibit triple-negative breast cancer by specifically downregulating the c-MYC transcription. Nucleic Acids Res. 2019; 47(20):10529-10542.
20. Nagini S, Nivetha R, Palrasu M, Mishra R. Nimbolide, a neem limonoid, is a promising candidate for the anticancer drug arsenal. J Med Chem. 2021; 64(7):3560-3577.
21. Sudhakaran G, Velayutham M, Aljarba NH, Al-Hazani TM, Arokiyaraj S, Guru A, Arockiaraj J. Nimbin (N1) and analog N3 from the neem seeds suppress the migration of osteosarcoma MG-63 cells and arrest the cells in a quiescent state mediated via activation of the caspase-modulated apoptotic pathway. Mol Biol Rep. 2023; 7970424:1-13.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

22. Rahmani A, Almatroudi A, Alrumaihi F, Khan A. Pharmacological and therapeutic potential of neem (*Azadirachta indica*). Pharmacog Rev. 2018; 12(24):250-255.
23. Latif MJ, Hassan SM, Mughal SS, Aslam A, Munir M, Shabbir N, Mushtaq M, Perveiz S. Therapeutic potential of *Azadirachta indica* (neem) and their active phytoconstituents against diseases prevention. J Chem Cheml Sci. 2020 ;10(3):98-110.
24. Falodun A, Siraj R, Choudhary MI. GC-MS analysis of insecticidal leaf essential oil of *Pyrenacantha staudtii* Hutch and Dalz (Icacinaceae). Trop J. Pharm Res 2009; 8(2): 139-143.
25. Alzohairy MA. Therapeutics role of Azadirachta indica (Neem) and their active constituents in diseases prevention and treatment. Evid-Based Comp Alternat Med. 2016; 7382506.
26. Metwally MA, Ali S, Khatab I, El-Sayed MK. Antibacterial potential of some seaweeds species to combat biofilm-producing multi-drug resistant *Staphylococcus aureus* of Nile Tilapia. Egypt J Bot. 2020; 60(1):9-24.