International Journal of Early Childhood Special Education (INT-JECSE)
DOI:10.9756/INTJECSE/V14I5.1036 ISSN: 1308-5581 Vol 14, Issue 05 2022

EVALUATION OF PROAPOPTOTIC POTENTIAL OF LIPPIA NODIFLORALEAF EXTRACT ON BREAST CANCER CELL LINE

- **Ashwini. K**

Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences Chennai, Tamilnadu, India -600 077. Email Id: 151801032.sdc@saveetha.com

- **Dr. P. Elumalai***

Department of Pharmacology, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Sciences, Chennai, Tamil Nadu, India -600 077, Email Id:elumalaip.sdc@saveetha.com

- **Dr. S. Raghunandhakumar**

Department of Pharmacology, Saveetha Dental College and Hospital,Saveetha Institute of Medical and Technical Sciences,Chennai, Tamil Nadu, India -600 077, Email Id: raghunandhakumars.sdc@saveetha.com

- **Dr. T. Lakshmi**

Department of Pharmacology, Saveetha Dental College and Hospital,Saveetha Institute of Medical and Technical Sciences,Chennai, Tamil Nadu, India -600 077, Email Id: lakshmi@saveetha.com

- **Dr. Anitha Roy**

Department of Pharmacology, Saveetha Dental College and Hospital,Saveetha Institute of Medical and Technical Sciences,Chennai, Tamil Nadu, India -600 077, Email Id: anitharoy2015@gmail.com

ABSTRACT

Cancer is a disease of gene disorder that occurs in the normal processes of cell division and is one of the most dreaded diseases worldwide. Breast cancer is the most common cancer diagnosed in women causing the highest morbidity. Lippianodiflora leaf extracts have been used as a chemotherapeutic agent due to their antioxidant, anti-inflammatory, anti-bacterial, and anti-tumor effect. The aim of the study is to evaluate the proapoptotic potential of *lippianodiflora* leaf extract on breast cancer cell lines (MCF-7). The proapoptotic potential of *lippianodiflora* leaf extract was assessed using an MTT assay. The cell morphological changes in Lippianodiflora leaf extract-treated cells were observed under a phase-contrast microscope. The proapoptotic effect of*lippianodiflora*leaf extract was examined using DAPI staining. The MTT assay results showed a significant reduction in the viability of breast cancer cells after treatment with different concentrations of *lippianodiflora*leaf extract(5-80 µg/ml) for 24h. We observed the inhibitory concentration at 20 µg/ml. Morphological changes such as reduction in the number of cells, cell shrinkage, and cytoplasmic membrane blebbing indicate the hallmark features of apoptosis were observed in L.nodiflora leaf extract-treated cells. DAPI staining results showed that apoptotic nuclei are stained intensely, fragmented, and have condensation chromatin in treated cells. Therefore it can be concluded that L. nodiflora leaf extracts have a pro-apoptotic potential in the breast cell cancer lines (MCF-7).

Keywords: Lippianodiflora, breast cancer, MCF-7, apoptosis

INTRODUCTION

Cancer is a disease of gene disorder that occurs in the normal processes of cell division and is one of the most dreaded diseases worldwide (1). Breast cancer is the most common cancer diagnosed in women, accounting for more than 1 in 10 new cancer diagnoses each year. It is the second most common cause of mortality among women in the world (2,3). Environmental and lifestyle modifications which are responsible for cancer include ionizing radiation, hormonal therapy, reproductive behavior of women, alcohol consumption, other dietary factors, obesity, and lack of physical activity (4). In recent years, the cancer research field has made splendid improvements in technology.

The current study indicates the important role of apoptosis with cancer cells. Apoptosis or programmed cell death is a process of eliminating normal cells without any inflammatory response which is initiated by intrinsic or extrinsic signals (5). It plays an important role in cancer development and treatment, thus the ability of bioactive compounds to increase the sensitivity of cancerous cells towards cellular damage and activate the proapoptotic response is the most desirable. Induction of apoptosis in tumor cells is the most common anticancer mechanism in the treatment of cancer cells in the body (6). Chemoprevention research has led to the identification of many phytochemicals which are as effective as potential chemopreventive agents (7).

Breast cancer can be usually treated by chemotherapeutic agents if diagnosed at early stages. Nowadays, many plant products are effective as chemotherapeutic agents. The main benefits of using natural formulas are their minor side effects, inexpensive and easy accessibility as compared to chemical drugs (8). The use of medicinal plants for cancer

treatment is improving fast in the last few years. Plant extracts contain materials such as flavonoids and aromatic compounds, which can be effective in decreasing the oxidative stress present in cancerous cells (9). Many natural plant extract and phytochemicals have been reported to induce apoptosis in cancer cell lines (10,11). One of the most conventionally used plant-derived products is *Lippanodiflora.*

In our present study,*lippanodiflora* leaf extracts were selected for the evaluation of proapoptotic potential on breast cancer cell lines (MCF-7).*Lippanodiflora*or frog fruit belongs to the Verbenaceae family. This plant contains various phytochemicals like nodifloretin, nodifloridin, b-sitosterol, stigmasterol, hispidulin, halleridone, hallerone, and eupafolin. Some of these compounds have been known to have anti-diabetic, hypolipidemic, anti-inflammatory, anti-bacterial, and anti-cancerous properties (1). Previous studies also reveal that *Lippanodiflora* extracts cause the formation of DNA laddering in the MCF-7 breast cancer cell lines (12). Our team has extensive knowledge and research experience that has translated into high-quality publications (13). The aim of the study is to evaluate the proapoptotic potential of *lippianodiflora* leaf extract on breast cancer cell lines (MCF-7).

MATERIAL AND METHODS

Reagents

DMEM (Dulbecco's Modified Eagle Medium), Phosphate Buffered Saline (PBS), Trypsin-EDTA, Fetal bovine serum (FBS), were purchased from Gibco, Canada. Dimethyl sulfoxide (DMSO), [3-(4,5-dimethythiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), DAPI, were purchased from Sigma Chemical Pvt Ltd, USA. All other chemicals used were extra pure of molecular grade and were purchased from SRL, India.

Cell line maintenance

Estrogen-dependent (MCF-7) breast cancer cell lines were obtained from the NCCS, Pune. The cells were grown in T25 culture flasks containing DMEM supplemented with 10% FBS and 1% antibiotics. Cells were maintained at 37◦C in a humidified atmosphere containing 5% CO2. Upon reaching confluency, the cells were trypsinized and passaged.

Preparation of the Herbal Extract

Lippianodiflora leaf powder obtained from IMPCOPS (Chennai, India) was used for the present study. About 50g of*lippianodiflora* leaf powder was soaked in 500 mL of 95% ethanol and kept at room temperature for 3 days in a static condition. Then the solution was filtered with crude filter paper followed by whatmann paper. Fine filtrate was subjected to rota evaporation after that 3g of the material was obtained. The total ethanol extract was concentrated in a vacuum evaporate and immediately stored at 4˚C.

Cell viability (MTT) assay

The percentage of breast cancer cell viability after treatment with lippianodiflora leaf extract was assessed by MTT assay. The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. MCF-7 cells were plated in 48 well plates at a concentration of 2x104 cells/well and incubated 24h, then the cells were washed twice with 500µl of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37ºC. After starvation, cells were treated with *lippianodiflora* leaf at different concentrations (0, 5, 10, 20, 40, 60 and 80 µg/ml) for 24 hours. At the end of treatment, the medium from control and *lippianodiflora* leaf treated cells were discarded and 200µl of MTT containing DMEM (0.5 mg/ml) was added to each well. The cells were then incubated for 4h at 37ºC in the CO2 incubator. The MTT containing medium was then discarded and the cells were washed with 1x PBS. The crystals were then dissolved by adding 200µl of solubilization solution and this was mixed properly by pipetting up and down. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (200µl) and incubated in dark for an hour. Then the intensity of the color developed was assayed using a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed as the percentage of control cells cultured in a serum-free medium. Cell viability in the control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells]×100.

Morphology study

Based on MTT assay we selected the optimal doses (IC-50: 20µg/ml) for further studies. Analysis of cell morphology changes by a phase-contrast microscope. 3×104 cells were seeded in 6 well plates and treated with *lippianodiflora* (20µg/ml) for 24h. At the end of the incubation period, the medium was removed and cells were washed once with a phosphate buffer saline (PBS pH 7.4). The plates were observed under a phase-contrast microscope.

Determination of nuclear morphological changes of cells (DAPI staining)

For the nuclear morphological analysis, the monolayer of cells was washed with PBS and fixed with 3% paraformaldehyde for 10 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and incubated with 0.5µg/ml of DAPI for 5 min. The apoptotic nuclei (intensely stained, fragmented nuclei, and condensed chromatin) were viewed under a fluorescent microscope.

Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Student–Newman–Keul's (SNK) tests for comparison between treatment values and control values. Data were expressed as mean ± SEM. The level of statistical significance was set at $p<0.05$.

RESULTS

Effect of *lippianodiflora*leaf extract on cell viability of breast cancer cell line

The cytotoxic potential of *lippianodiflora*leaf extract in breast cancer cells was assessed by an MTT assay. The cells were treated with different concentrations (5-80 µg/ml) of *lippianodiflora*leaf extract for 24h. Lippianodiflora leaf extract treatment significantly decreased the viability of MCF-7 cancer cells compared to control at 24 h time point (Figure 1). The percentage of cell viability reduced gradually with an increase in the concentration. We observed the 50% growth inhibition at (20 µg/ml) concentration. Hence, IC-50 dose (20 µg/ml) was considered for further experiments.

The effect of *lippianodiflora*on cell morphology

The cell morphological analysis of *lippianodiflora* leaf extract treated breast cancer cells was observed in an inverted phase-contrast microscope. The MCF-7 cells were treated with *lippianodiflora*leaf extract (20 µg/ml) for 24h, compared with the untreated cells, treated cells showed significant morphological changes, which are characteristic of apoptotic cells, such as cell shrinkage and reduced cell density were observed in the *lippianodiflora*leaf treated cells (Figure 2). Cells undergoing apoptosis also displayed other types of morphological changes such as rounded up cells that shrink and lose contact with neighboring cells. Some sensitive cells were even detached from the surface of the plates.

Pro-apoptotic effect of *lippianodiflora*leaf extract in breast cancer cells (DAPI staining)

The induction of apoptosis in *lippianodiflora*leaf extract (20 µg/ml) treated cells were analyzed by DAPI staining. After a 24h treatment period, the cells were stained with nuclear staining (DAPI) and observed in fluorescence microscopy. The treated cells clearly showed condensed chromatin and nuclear fragmentation, which are characteristics of apoptosis compared to the control which showed clear round nuclei (Figure 3).

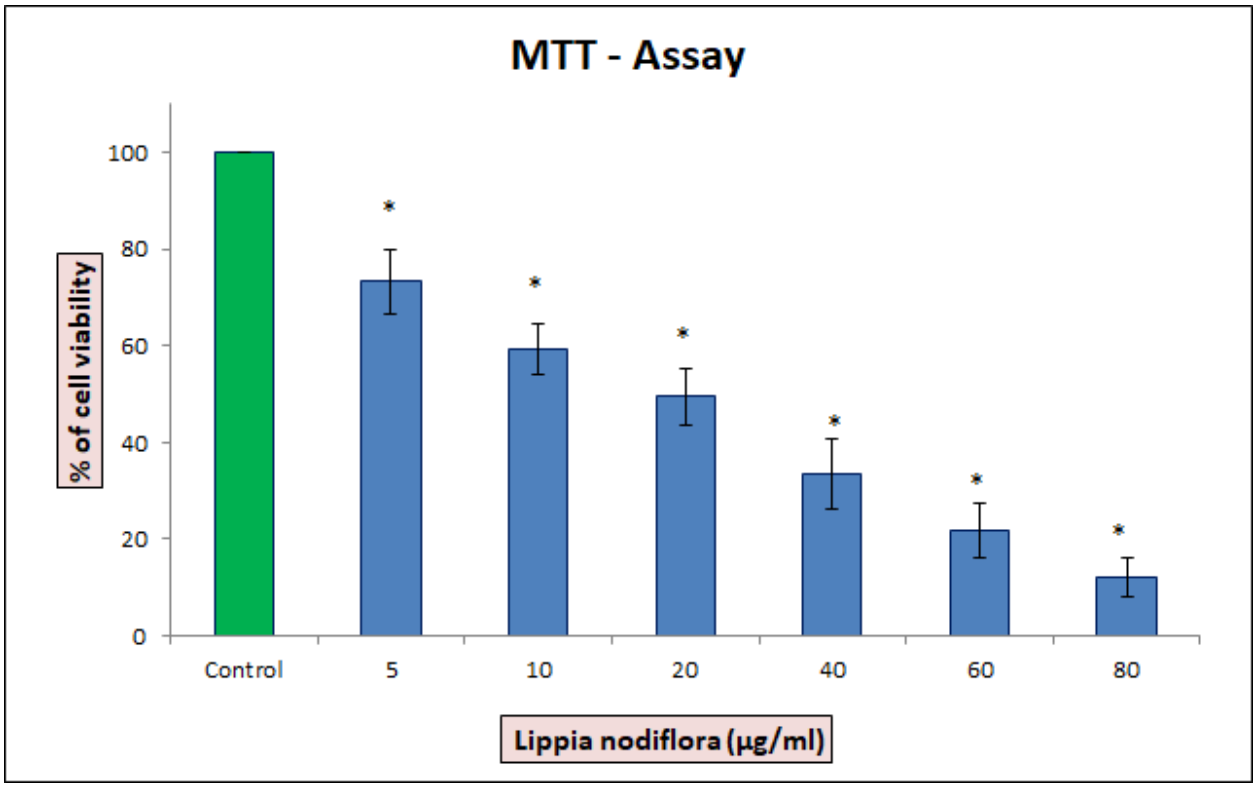
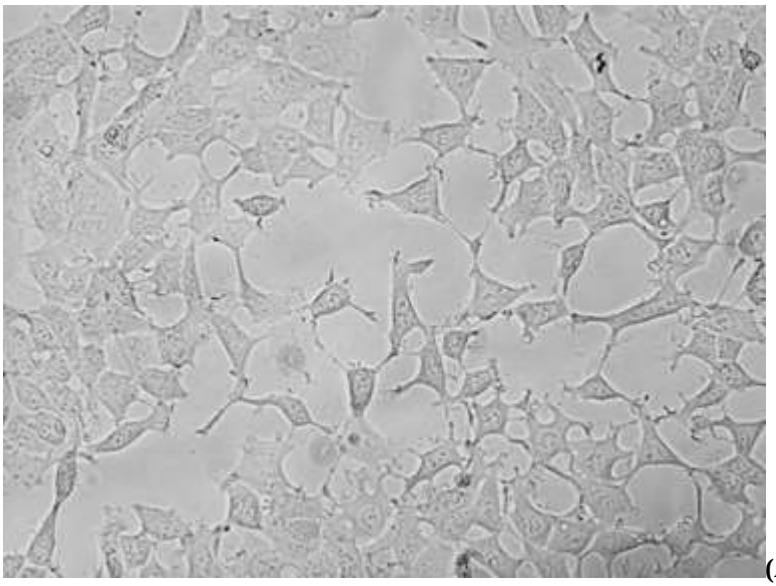
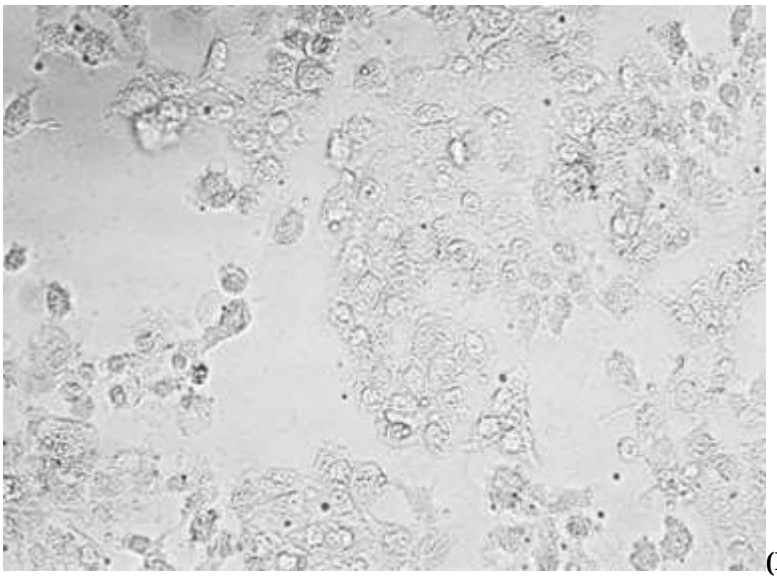


*Figure 1: Effect of lippianodiflora leaf extract on the viability of breast cancer cells was determined by MTT assay. The cells were treated with different concentrations (0,5,10,20,40,60 and 80 µg/ml) for 24 hrs. Inhibitory concentration (IC-50) dose: 20µg/ml (p value: 0.0027). *represents statistical significance between control versus treatment groups at p< 0.05 level using Student's-Newman-Keul's test.*



(A)



(B)

Figure 2: Effect of lippianodiflora leaf extracts on cell morphological changes in breast cancer cell line (MCF-7) visualised in a phase-contrast microscope at 20x magnification. (A) Control cells; (B) lippianodiflora leaf extract (20µg/ml). The number of cells decreased after the treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing.

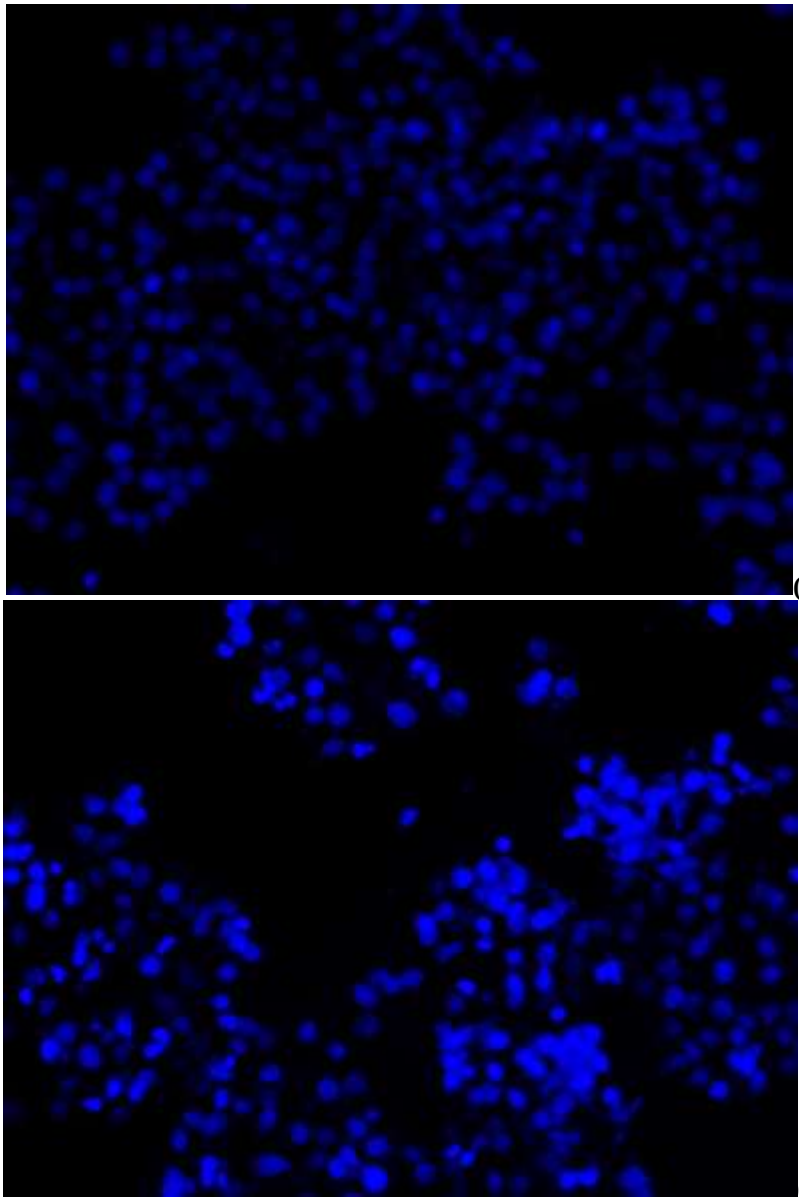


Figure 3: Induction of apoptosis in lippianodiflora leaf extract treated breast cancer cell line (MCF-7) visualised under a fluorescent microscope at 20x magnification. (A) Control cells ; (B) lippianodiflora leaf extract (20µg/ml).The nuclei were stained with DAPI and observed under a fluorescent microscope. The treated cells clearly showed condensed chromatin and nuclear fragmentation.

DISCUSSION

Apoptosis is a physiological process that functions as an essential mechanism of tissue homeostasis and the elimination of unwanted cells. Current trends include the use of medicine-derived plant products as the alternative for anti-cancer and chemotherapeutic agents. Screening plant-derived products as apoptotic inducers play a major role in anticancer research. Plants also have the opportunity to supply newer therapies. Recently, numerous treatments have been proposed for cancer, which uses plant-derived materials. Medical plants contain bioactive compounds which include tannins, sugars, hormones, and flavonoids. These compounds help in preventing carcinogenesis by blocking metabolic activation, increasing detoxification, or providing alternative targets for electrophonic metabolites (14).

The present study focuses on the ability of *lippianodiflora* leaf extracts against breast cancer cell lines and the pro-apoptotic activity. The leaf extracts significantly inhibited the proliferation of breast cancer cell lines after an incubation period of 24 h and the pro-apoptotic potential was evaluated by DAPI staining. The MTT assay showed that the pro-apoptotic potential of *lippianodiflora* leaf extracts was selective towards MCF-7 cells. Morphological investigations reveal that apoptosis in the breast cell cancer line is characterized when *lippianodiflora* leaf extracts were at an inhibitory concentration of 20µg/ml (**Figure 1)**. Morphologic changes observed showed cell shrinkage, cytoplasmic membrane

blebbing, and collapse of cells into small membranes when viewed in a phase-contrast microscope (**Figure 2)**. There is an interplay of caspase substrate cleavage during apoptosis (15). The membrane blebbing is due to the Rho effector protein ROCK I, which contributes to phosphorylation of myosin light chains, myosin ATPase activity, and coupling of actin-myosin filaments to the plasma membrane. It is cleaved during apoptosis to generate an active form. ROCK proteins are necessary and sufficient for the formation of membrane blebs and for the re-localization of fragmented DNA into blebs and apoptotic bodies (16). These morphological changes could be seen after 24 h treatment with extracts and positive control from the surface.

These hallmark features of morphological changes suggested that L. nodiflora extracts caused early apoptotic changes in MCF-7 breast cancer cell lines (17). The nuclei were stained with DAPI and observed under a fluorescent microscope. The apoptotic nuclei were intensely stained, fragmented, and had condensed chromatin (**Figure 3)**. AO/EtBr staining shows apoptotic rate was positively correlated with the cytotoxic activity of the extracts. Previous studies state that L. nodiflora extracts when assessed for antitumor activity using Ehrlich's ascites carcinoma (EAC) bearing swiss albino mice were found to bear good antitumor activity, which was supposed to be due to the increased antioxidant activity (18). These effects could be due to the free radical-scavenging efficiency and reducing power, as a result of their phenolic and/or non-phenolic constituents in triggering different cellular mechanisms involved in cancer and apoptosis. The results of our study are supported by Teoh et al. confirming Lippanodiflora leaf extracts as one of the most potent chemotherapeutic agents against breast cancer cell lines with pro-apoptotic potential (19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)(30)(31)(32)(33)(34)

CONCLUSION

Overall, the present study results demonstrated that the lippianodiflora leaf extracts were cytotoxic and induced apoptosis to the breast cancer cells at 20µg/mlconcentration and 24h incubation period. However, more research is needed to understand the mechanisms of cytotoxicity of Lippianodiflora leaf extracts.

ACKNOWLEDGEMENT

The authors are thankful to Saveetha Dental College for providing a platform to express our knowledge.

CONFLICT OF INTEREST

The author declares no conflict of interest.

REFERENCES

1. 'adawiyah MAR 'atul, Rabi 'atul 'adawiyah M, Cheong BE, Teoh PL. Antiproliferative and apoptotic effects of Phyla nodiflora extracts on human breast cancer cell line [Internet]. Vol. 4, Asian Pacific Journal of Tropical Disease. 2014. p. 238. Available from: http://dx.doi.org/10.1016/s2222-1808(14)60540-5
2. Alkabban FM, Ferguson T. Breast Cancer. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020. Available from: https://www.ncbi.nlm.nih.gov/pubmed/29493913
3. Parkin DM, Fernández LMG. Use of statistics to assess the global burden of breast cancer. Breast J [Internet]. 2006 Jan;12 Suppl 1:S70–80. Available from: http://dx.doi.org/10.1111/j.1075-122X.2006.00205.x
4. Lee JW, Jaffar Z, Pinkerton KE, Porter V, Postma B, Ferrini M, et al. Alterations in DNA methylation and airway hyperreactivity in response to in utero exposure to environmental tobacco smoke. Inhal Toxicol [Internet]. 2015 Nov 2;27(13):724–30. Available from: http://dx.doi.org/10.3109/08958378.2015.1104402
5. Hannun YA. Apoptosis and the Dilemma of Cancer Chemotherapy [Internet]. Vol. 89, Blood. 1997. p. 1845–53. Available from: http://dx.doi.org/10.1182/blood.v89.6.1845
6. Koff J, Ramachandiran S, Bernal-Mizrachi L. A Time to Kill: Targeting Apoptosis in Cancer [Internet]. Vol. 16, International Journal of Molecular Sciences. 2015. p. 2942–55. Available from: http://dx.doi.org/10.3390/ijms16022942
7. Azad MRH, Tabassum J, Bharali R. Chemopreventive Potential ofGlycosmis pentaphyllaCorrea on Hepatic Carcinogen Metabolizing Enzymes and Antioxidant Defense Mechanisms in Mice [Internet]. Vol. 13, Journal of Herbs, Spices & Medicinal Plants. 2008. p. 1–14. Available from: http://dx.doi.org/10.1300/j044v13n03_01
8. Moattar FS, Sariri R, Giahi M, Yaghmaee P, Ghafoori H, Jamalzadeh L. Antioxidant and Anti-Proliferative Activity of Calamintha officinalis Extract on Breast Cancer Cell Line MCF-7 [Internet]. Vol. 15, Journal of Biological Sciences. 2015. p. 194–8. Available from: http://dx.doi.org/10.3923/jbs.2015.194.198
9. Wang SY, Lewers KS, Bowman L, Ding M. Antioxidant Activities and Anticancer Cell Proliferation Properties of Wild Strawberries [Internet]. Vol. 132, Journal of the American Society for Horticultural Science. 2007. p. 647–58. Available from: http://dx.doi.org/10.21273/jashs.132.5.647
10. Seo W-G, Pae H-O, Oh G-S, Chai K-Y, Yun Y-G, Chung H-T, et al. Ethyl Acetate Extract of the Stem Bark of Cudrania Tricuspidata Induces Apoptosis in Human Leukemia HL-60 Cells [Internet]. Vol. 29, The American Journal of Chinese Medicine. 2001. p. 313–20. Available from: http://dx.doi.org/10.1142/s0192415x01000332
11. Bold RJ, Termuhlen PM, McConkey DJ. Apoptosis, cancer and cancer therapy [Internet]. Vol. 6, Surgical Oncology. 1997. p. 133–42. Available from: http://dx.doi.org/10.1016/s0960-7404(97)00015-7

12. Liau M, Cheong BE, Teoh PL. ANTIOXIDANT AND ANTICANCER PROPERTIES OF SOLVENT PARTITIONED EXTRACTS OF PHYLA NODIFLORA L [Internet]. Vol. 7, Journal of Microbiology, Biotechnology and Food Sciences. 2017. p. 42–6. Available from: http://dx.doi.org/10.15414/jmbfs.2017.7.1.42-46
13. Rajeshkumar S, Venkat Kumar S, Ramaiah A, Agarwal H, Lakshmi T, Roopan SM. Biosynthesis of zinc oxide nanoparticles usingMangifera indica leaves and evaluation of their antioxidant and cytotoxic properties in lung cancer (A549) cells [Internet]. Vol. 117, Enzyme and Microbial Technology. 2018. p. 91–5. Available from: http://dx.doi.org/10.1016/j.enzmictec.2018.06.009
14. Anandakumar P, Kamaraj S, Jagan S, Ramakrishnan G, Asokkumar S, Naveenkumar C, et al. Capsaicin inhibits benzo(a)pyrene-induced lung carcinogenesis in an in vivo mouse model. Inflamm Res [Internet]. 2012 Nov;61(11):1169–75. Available from: http://dx.doi.org/10.1007/s00011-012-0511-1
15. Hu W, Kavanagh JJ. Anticancer therapy targeting the apoptotic pathway [Internet]. Vol. 4, The Lancet Oncology. 2003. p. 721–9. Available from: http://dx.doi.org/10.1016/s1470-2045(03)01277-4
16. Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I [Internet]. Vol. 3, Nature Cell Biology. 2001. p. 339–45. Available from: http://dx.doi.org/10.1038/35070009
17. Vanajothi R, Sudha A, Manikandan R, Rameshthangam P, Srinivasan P. Luffa acutangula and Lippia nodiflora leaf extract induces growth inhibitory effect through induction of apoptosis on human lung cancer cell line [Internet]. Vol. 2, Biomedicine & Preventive Nutrition. 2012. p. 287–93. Available from: http://dx.doi.org/10.1016/j.bionut.2012.03.002
18. Ashokkumar D, Mazumder UK, Gupta M, Thamil Selvan V. Effect on Inhibition of Proliferation and Antioxidant Enzyme Level of Lippia nodiflora in EAC Cell Line Treated Mice [Internet]. Vol. 6, Journal of Complementary and Integrative Medicine. 2009. Available from: http://dx.doi.org/10.2202/1553-3840.1233
19. Teoh PL, Liau M, Cheong BE. Phyla nodiflora L. Extracts Induce Apoptosis and Cell Cycle Arrest in Human Breast Cancer Cell Line, MCF-7. Nutr Cancer [Internet]. 2019 Jan 19;71(4):668–75. Available from: http://dx.doi.org/10.1080/01635581.2018.1559942
20. Ezhilarasan D, Apoorva VS, Ashok VN. Syzygium cumini extract induced reactive oxygen species-mediated apoptosis in human oral squamous carcinoma cells. J Oral Pathol Med [Internet]. 2019 Feb [cited 2021 Sep 15];48(2). Available from: https://pubmed.ncbi.nlm.nih.gov/30451321/
21. Danda AK, Krishna TM, Narayanan V, Siddareddi A. Influence of primary and secondary closure of surgical wound after impacted mandibular third molar removal on postoperative pain and swelling--a comparative and split mouth study. J Oral Maxillofac Surg [Internet]. 2010 Feb [cited 2021 Sep 15];68(2). Available from: https://pubmed.ncbi.nlm.nih.gov/20116700/
22. Ramadurai N, Gurunathan D, Samuel AV, Subramanian E, Rodrigues SJL. Effectiveness of 2% Articaine as an anesthetic agent in children: randomized controlled trial. Clin Oral Investig [Internet]. 2019 Sep [cited 2021 Sep 15];23(9). Available from: https://pubmed.ncbi.nlm.nih.gov/30552590/
23. Sathivel A, Raghavendran HR, Srinivasan P, Devaki T. Anti-peroxidative and anti-hyperlipidemic nature of Ulva lactuca crude polysaccharide on D-galactosamine induced hepatitis in rats. Food Chem Toxicol [Internet]. 2008 Oct [cited 2021 Sep 15];46(10). Available from: https://pubmed.ncbi.nlm.nih.gov/18706469/
24. Panda S, Doraiswamy J, Malaiappan S, Varghese SS, Del Fabbro M. Additive effect of autologous platelet concentrates in treatment of intrabony defects: a systematic review and meta-analysis. J Investig Clin Dent [Internet]. 2016 Feb [cited 2021 Sep 15];7(1). Available from: https://pubmed.ncbi.nlm.nih.gov/25048153/
25. Neelakantan P, Varughese AA, Sharma S, Subbarao CV, Zehnder M, De-Deus G. Continuous chelation irrigation improves the adhesion of epoxy resin-based root canal sealer to root dentine. Int Endod J [Internet]. 2012 Dec [cited 2021 Sep 15];45(12). Available from: https://pubmed.ncbi.nlm.nih.gov/22612994/
26. Govindaraju L, Neelakantan P, Gutmann JL. Effect of root canal irrigating solutions on the compressive strength of tricalcium silicate cements. Clin Oral Investig [Internet]. 2017 Mar [cited 2021 Sep 15];21(2). Available from: https://pubmed.ncbi.nlm.nih.gov/27469101/
27. Sekhar CH, Narayanan V, Baig MF. Role of antimicrobials in third molar surgery: prospective, double blind,randomized, placebo-controlled clinical study. Br J Oral Maxillofac Surg [Internet]. 2001 Apr [cited 2021 Sep 15];39(2). Available from: https://pubmed.ncbi.nlm.nih.gov/11286448/
28. DeSouza SI, Rashmi MR, Vasanthi AP, Joseph SM, Rodrigues R. Mobile phones: the next step towards healthcare delivery in rural India? PLoS One [Internet]. 2014 Aug 18 [cited 2021 Sep 15];9(8). Available from: https://pubmed.ncbi.nlm.nih.gov/25133610/
29. Nasim I, Neelakantan P, Sujeer R, Subbarao CV. Color stability of microfilled, microhybrid and nanocomposite resins--an in vitro study. J Dent [Internet]. 2010 [cited 2021 Sep 15];38 Suppl 2. Available from: https://pubmed.ncbi.nlm.nih.gov/20553993/
30. Danda AK, Muthusekhar MR, Narayanan V, Baig MF, Siddareddi A. Open versus closed treatment of unilateral subcondylar and condylar neck fractures: a prospective, randomized clinical study. J Oral Maxillofac Surg [Internet]. 2010 Jun [cited 2021 Sep 15];68(6). Available from: https://pubmed.ncbi.nlm.nih.gov/20303209/
31. Molecular structure and vibrational spectra of 2,6-bis(benzylidene)cyclohexanone: A density functional theoretical study. Spectrochim Acta A Mol Biomol Spectrosc [Internet]. 2011 Jan 1 [cited 2021 Sep 15];78(1):113–21.

Available from: http://dx.doi.org/10.1016/j.saa.2010.09.007

32. Putchala MC, Ramani P, Herald J. Sherlin, Premkumar P, Natesan A. Ascorbic acid and its pro-oxidant activity as a therapy for tumours of oral cavity – A systematic review [Internet]. Vol. 58, Archives of Oral Biology. 2013. p. 563–74. Available from: http://dx.doi.org/10.1016/j.archoralbio.2013.01.016
33. Neelakantan P, Grotra D, Sharma S. Retreatability of 2 mineral trioxide aggregate-based root canal sealers: a cone-beam computed tomography analysis. J Endod [Internet]. 2013 Jul;39(7):893–6. Available from: http://dx.doi.org/10.1016/j.joen.2013.04.022
34. Suresh P, Marimuthu K, Ranganathan S, Rajmohan T. Optimization of machining parameters in turning of Al-SiC-Gr hybrid metal matrix composites using grey-fuzzy algorithm [Internet]. Vol. 24, Transactions of Nonferrous Metals Society of China. 2014. p. 2805–14. Available from: http://dx.doi.org/10.1016/s1003-6326(14)63412-9