CYTOTOXIC EFFECT OF *Lippia nodiflora* LEAF EXTRACT AGAINST THE PROSTATE CANCER CELL LINE

Running title - Anticancer activity of L. *nodiflora* leaf flower extract against prostate cancer cell

ABSTRACT

Prostate cancer is the second most common cause of cancer deaths for men. L. *nodiflora* has been used as a natural remedy for various diseases, because of its antioxidant, anti-inflammatory, anti-bacterial, and anti-tumor effect. This study was to investigate the cytotoxic effect of *Lippia. Nodiflora (L. nodiflora)* ethanolic leaf extract in prostate cancer cell lines (PC-3). The growth inhibitory effect of L. nodiflora ethanolic leaf extract was assessed by MTT assay. The cell morphological changes in *L. nodiflora* leaf extract-treated cells were observed using an inverted phase-contrast microscope. Apoptosis induction by nodiflora was determined by AO/EtBr (acridine orange and ethidium bromide) dual staining. The MTT assay results showed that dose-dependent (10-120µg/ml) cell growth inhibition was observed in L. nodiflora leaf extract treated PC-3 cells. The IC-50 dose was observed at 40µg/ml. Morphological changes such as reduction in the number of cells, cell shrinkage, and cytoplasmic membrane blebbing were observed in the treated cells. Induction of apoptosis by *Lippia nodiflora* (40µg/ml) treated cells showed an increased number of early apoptotic and late apoptotic cells. The above data indicate that L. nodiflora inhibits cell proliferation and induces apoptosis in prostate cancer cells. Therefore, it can be concluded that *L. nodiflora* exhibits anti-cancer activity, and thus it raises new hope for its use in anti-cancer therapy.

Keywords: *L. nodiflora*, Prostate cancer, Cytotoxicity, Apoptosis.

INTRODUCTION

Cancer has become one of the major causes of the death of people worldwide (1). There are various types of cancer occurring all over the body such as Oral cancer, breast cancer, colon cancer, prostate cancer, etc. Oral cancer has also become the leading cause of morbidity and mortality (2),(3). Prostate cancer is the second most common malignancy in men that leads to death. The increase in the incidence of cancer in various countries is due to the widespread adoption of the Western diet and lifestyle (4). In recent years cancer research has made splendid improvements in our basic understanding and technique of cancer studies and cancer biology.

Comment [PC1]: (2,3)

Conventional anti-cancer therapies with standard chemotherapeutic agents have various adverse effects (5). It includes nausea, vomiting, metallic taste, hair loss, etc (6). Nanotechnology has played an important role in cancer treatment (7),(8). Current studies indicate the important role of apoptosis in the improvement of therapeutic agents and treatment of cancer. The primary aim of cancer remedy is to kill the cancer cells without causing too much harm to normal cells (1,9). The knowledge about the mechanisms of apoptosis has made us understand the origination and progression of cancers. Various studies have been done to understand the cytotoxic and apoptotic effect of different leaf and seed extract and their use in anti-cancer therapy (10)-(11). It has also made us understand that cancer remedies can work in two ways, via induction of apoptosis and direct toxicity to cancer cells (12). Therefore, in the improvement of therapeutic agents and treatment of cancer, regulators of cell death and cell cycle should be targeted (13). The present study was designed to broaden a novel therapeutic agent from natural resources to exploit our knowledge of apoptotic mechanisms to promote apoptosis of most cancer cells and limit the concurrent dying of normal cells. The medicinal flowers which are used in conventional medication in growing countries include an extensive range of phytochemicals that may be used to deal with cancer (14). One such plant to have a curative effect is *L. nodiflora*.

Jalpapli, which is also known as Phyla nodiflora, is a small herb that is a native of California and it also grows widely in India (15). The plant contains triterpenoids, flavonoids, phenols, steroids, etc. It has various pharmacological values (16). It is used for lack of bowel movement, in swollen cervical glands, and also used for pain in knee joints, in ulcers, and boils (17). It has been used in treating various skin diseases and as a cosmetic agent (18). It is also used in gonorrhea and hepatitis. It also has antibacterial effects against various bacteria such as *E.coli*, *Pseudomonas*, *Staphylococcus*. It has been the source of medicine for liver disorders, dandruff control, indigestion in children, an anticonvulsant effect, etc. Furthermore, studies reveal that L. nodiflora has antimicrobial, antifungal, antitumor, antidiuretic, anti-inflammatory, antiurolithiatic, antidiabetic, and neuropharmacological activity (19). Our team has extensive knowledge and research experience that has translated into high quality publications (20). This study was done to investigate the cytotoxic effect of L. nodiflora leaf extract in the prostate cancer line.

Comment [PC2]: (7,8)

Comment [PC3]: Clastogenic effect?

Comment [PC4]: (10,11)

Comment [PC5]: Suggestion to remove all etc.

Comment [PC6]: , added

MATERIALS AND METHODS

Reagents

DMEM (Dulbecco's Modified Eagle Medium), Phosphate Buffered Saline (PBS), Trypsin-EDTA, Fetal bovine serum (FBS), were purchased from Gibco, Canada. Acridine orange (AO), ethidium bromide (EtBr), Dimethyl sulfoxide (DMSO), [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), 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

Prostate cancer cell lines (PC-3) were obtained from the National Centre for Cell Science (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

L. nodiflora Leaf powder obtained from The Indian Medical Practitioners Co-operative Pharmacy and Stores Ltd (IMPCOPS) (Chennai, India) was used for the present study. About 50g of L. nodiflora 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 Whatman paper. The 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 cell viability of L. nodiflora extract-treated PC-3 cells 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. PC-3 cells were plated in 48 well plates at a concentration of 2x104 cells/well 24 hours after plating, 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 L. nodiflora at different concentrations (10-120µg/ml) for 24 hours. At the end of treatment, the medium from control and L. nodiflora 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 a 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 the MTT assay we selected the optimal doses (IC-50: 40µg/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 *Phyla nodiflora* (40µg/ml for PC-3 cells) 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.

Comment [PC7]: mL

Determination of mode of cell death by acridine orange (AO)/ethidium bromide (EtBr) dual staining

The effects of *L. nodiflora* in PC-3 cell death were also determined by AO/EtBr dual staining as described previously (Cury-Boaventura et al., 2004). The cells were treated with L. nodiflora for 24 h and then the cells were harvested, washed with ice-cold PBS. The pellets were resuspended in 5 µl of acridine orange (1 mg/mL) and 5 µl of EtBr (1 mg/mL). The apoptotic changes of the stained cells were then observed by using a fluorescence microscope.

Comment [PC8]: L

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 *L.nodiflora* extract on cell viability of prostate cancer cell line:

MTT assay was used to assess the cytotoxic potential of *L.nodiflora* extract in the prostate cancer cell line. The cells were treated with different concentrations (10, 20, 40, 80, 100, and 120µg/ml) *L* for 24 hours. With a gradual increase in concentration, the percentage of cell

viability was reduced. 50% growth inhibition was observed at 40 µg/ml concentration and hence IC-50 dose was considered for further experiments.

The effect of *L.nodiflora* on cell morphology:

The cell morphological analysis of *L.nodiflora* extract-treated under prostate cancer was observed in a phase-contrast microscope. The PC-3 cells were treated with *L.nodiflora* extract (40 µg/ml) for 24h and on comparison with untreated cells, treated cells showed significant morphological changes. There was a decrease in the number of cells and cells exhibited cell shrinkage and cytoplasmic membrane blebbing.

Comment [PC9]: mL

Pro-apoptotic effect of *L.nodiflora* extract in prostate cancer cells (AO/EtBr dual staining):

To confirm the induction of apoptosis in *L.nodiflora* extract AO\EtBr dual staining was used. For evaluating the nuclear morphology of apoptotic cells AO\EtBr dual staining was used. The cells were treated with *L.nodiflora* extract (40 µg/ml) for 24h. The cells were stained with both AO/EtBr staining after treatment and it was observed under fluorescence microscopy. The results say that AO stained both live and dead cells and EtBr stains only the cells that have lost their membrane integrity. Cells stained uniform bright green nucleus represent viable cells, early apoptotic cells having bright orange areas of condensed or fragmented chromatin in the nucleus and late apoptotic cells having a uniform bright red nucleus. In the present study, control cells showed a uniform green color, and in *L.nodiflora* extract-treated cells showed orange and red color. From the above result, it can be confirmed that *L.nodiflora* extract induces apoptosis in prostate cancer cells.

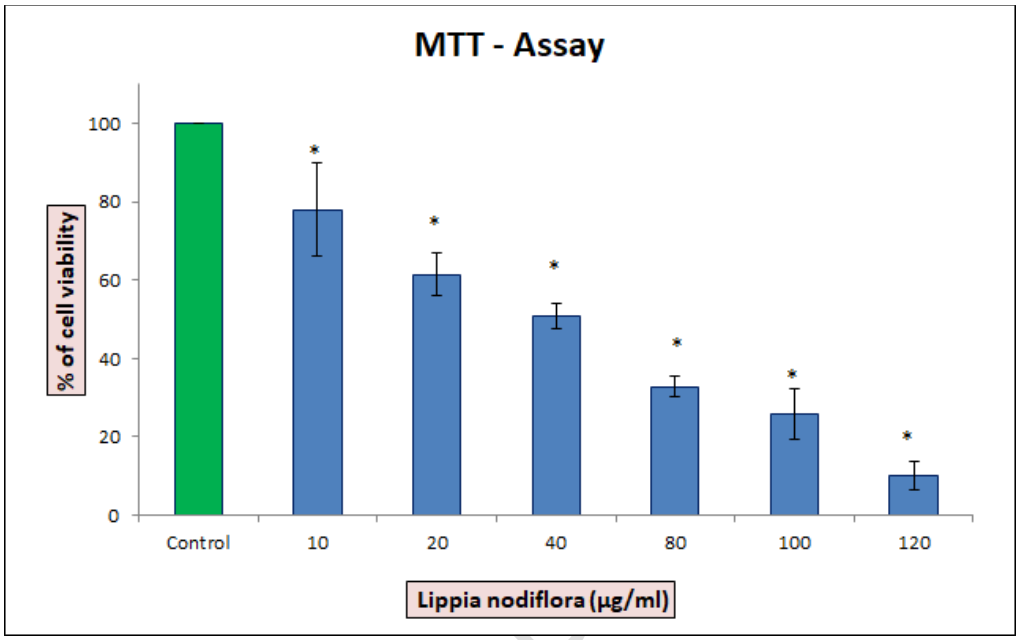

*Figure 1: Cytotoxic effect of L. nodiflora leaf extract against the prostate cancer cell line. The cells were treated with different concentrations (10, 20, 40, 80, 100, and 120µg/ml) L. nodiflora leaf extract for 24hrs. The 50% of inhibition observed at 40 µg/ml (p-value: 0.0037) concentration, which has been taken as inhibitory concentration (IC-50) dose value and fixed for further experiments. * represents statistical significance between control versus treatment groups at p < 0.05 level using Student's–Newman–Keuls test.*

(A) *(B)*

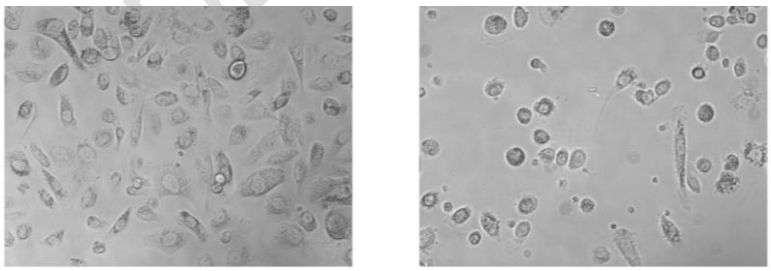

Figure 2: Effect of L. nodiflora leaf extract on cell morphological changes in the prostate cancer cell line. After treatment with L. nodiflora leaf extract 40 µg/mL for 24hrs and observed under

phase-contrast microscope at 20x magnification. (A) Control cells, (B) lippia nodiflora leaf extract (40µg/ml). Here, the number of cells was decreased after treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing.

(A)

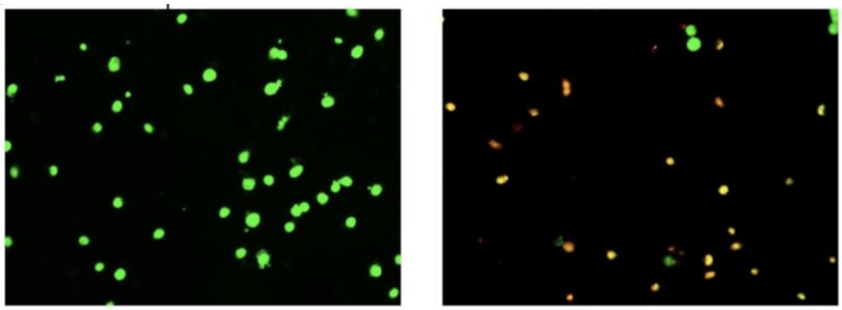

Figure 3: Induction of apoptosis in L. nodiflora leaf extract treated prostate cancer cell line. The PC-3 cells treated with L. nodiflora leaf extract at 40 µg/mL concentration for 24hrs, treatment cells were stained with and viewed under fluorescence microscope at 20x magnification. (A) Control cells, (B) lippia nodiflora leaf extract (40µg/ml). The viable cells are possessing a uniform bright green nucleus, early apoptotic cells having bright orange areas of condensed or fragmented chromatin in the nucleus and late apoptotic cells having a uniform bright red nucleus.

DISCUSSION

Apoptosis is a programmed cell death that serves as an important mechanism for tissue homeostasis and cell eradication. The use of medicine-derived bioactive compounds as a replacement to anti-cancer and chemotherapeutic medicines is becoming increasingly popular. Plant-derived products are being tested as apoptotic inducers in anticancer research (23, 36). Several cancer treatments based on plant-derived bioactive compounds, such as flavonoids and limnoids have recently been proposed. By inhibiting metabolic activation, boosting detoxification, or offering alternate targets for electrophonic metabolites, these substances aid in the prevention of carcinogenesis (13, 20).

The cytotoxic effect of *L.nodiflora* extract on cell lines was calculated by micro-culture tetrazolium assay (MTT). The multiple concentration of L.nodiflora extract was used and IC50

doses were calculated. The MTT assay results showed that dose-dependent (10-120µg/ml) cell growth inhibition was observed in *L. nodiflora* leaf extract treated against PC-3 cells. The IC-50 dose was observed at 40µg/ml. Since *L.nodiflora* has better cytotoxic activity against PC-3 cells it was used for further investigation.

Morphological investigation of apoptosis revealed that *L.nodiflora* extract at a concentration of 20µg/ml induced cell death in the PC-3 cell line by apoptosis (figure 2). Phenotypically apoptosis is characterized by cell shrinkage, DNA fragmentation, chromatin condensation, plasma membrane blebbing, and collapse of the cell into small membranes. The phase-contrast image shows that *L.nodiflora* extract has shown morphological changes such as reduction in the number of cells, cell shrinkage, and cytoplasmic membrane blebbing in the treated cells when compared with untreated cells. Isolated compound-like flavonoids exhibited exceptional antioxidant activity in all antioxidant tests and significantly reduced lipid peroxidation at a concentration of 50 g/ml, according to prior research (41). The findings showed that extracts or phytocompounds derived from L. nodiflora might be utilised as a bioactive source of natural antioxidants with health benefits (42).

AO\EtBr (acridine orange and ethidium bromide) dual staining was performed to determine if exposure to L. nodiflora leaf extract causes cell death by apoptosis in PC-3 cell lines. In the AO/EtBr staining, the viable cells will possess a uniform bright green nucleus. The early apoptotic cell will have bright orange areas of condensed or fragmented chromatin in the nucleus. Late apoptotic cells will have a uniform bright red nucleus. It was found that untreated cells were mostly green with an intact nucleus (Figure 3). AO\EtBr analysis showed that L. nodiflora leaf extract was cytotoxic towards PC-3 cells through apoptosis when treated with IC50 concentration (40 µg/mL). In the AO\EtBr analysis, cells treated with IC50 value of L. nodiflora leaf extract showed a red color nucleus, which further confirmed the induction of late apoptosis in PC-3 cells by *L.nodiflora* extract. This finding was characterized by membrane blebbing and nuclear shrinkage. The above data indicate that *L. nodiflora* inhibits cell proliferation and induces apoptosis in prostate cancer cells.

CONCLUSION

In summary, the present results indicate that *L. nodiflora* leaf extract induced apoptosis which was further confirmed by characteristic morphological changes and cytotoxic effect.

Therefore, *L. nodiflora* leaf extract leaf extracts exhibit anti-cancer activity and can be used for developing anti-cancer agents(11) (21) (22) (23) (24) (25) (26) (27) (28) (29) (30) (31) (32) (33) (34). However, further investigations and studies are required to employ them as anticancer drugs for prostate cancer.

Comment [PC10]: (11, 21-34)

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES:

1. Anderson DJ, Anderson RG, Moug SJ, Baker MJ. Liquid biopsy for cancer diagnosis using vibrational spectroscopy: systematic review. BJS Open [Internet]. 2020 May 19; Available from: http://dx.doi.org/10.1002/bjs5.50289

2. Ezhilarasan D, Lakshmi T, Subha M, Deepak Nallasamy V, Raghunandhakumar S. The ambiguous role of sirtuins in head and neck squamous cell carcinoma. Oral Dis [Internet]. 2021 Feb 11; Available from: http://dx.doi.org/10.1111/odi.13798

3. Abijeth B, Ezhilarasan D. Syringic acid induces apoptosis in human oral squamous carcinoma cells through mitochondrial pathway. J Oral Maxillofac Pathol. 2020 Jan;24(1):40–5.

4. Sohaib M, Ezhilarasan D. Carbamazepine, a Histone Deacetylase Inhibitor Induces Apoptosis in Human Colon Adenocarcinoma Cell Line HT-29. J Gastrointest Cancer. 2020 Jun;51(2):564–70.

5. Anirudh BVM, Ezhilarasan D. Reactive Oxygen Species-Mediated Mitochondrial Dysfunction Triggers Sodium Valproate-Induced Cytotoxicity in Human Colorectal Adenocarcinoma Cells. J Gastrointest Cancer [Internet]. 2020 Sep 3; Available from: http://dx.doi.org/10.1007/s12029-020-00505-w

6. Rithanya P, Ezhilarasan D. Sodium Valproate, a Histone Deacetylase Inhibitor, Provokes Reactive Oxygen Species–Mediated Cytotoxicity in Human Hepatocellular Carcinoma Cells [Internet]. Vol. 52, Journal of Gastrointestinal Cancer. 2021. p. 138–44. Available from: http://dx.doi.org/10.1007/s12029-020-00370-7

7. Shathviha PC, Ezhilarasan D, Rajeshkumar S, Selvaraj J. β-sitosterol Mediated Silver Nanoparticles Induce Cytotoxicity in Human Colon Cancer HT-29 Cells. Avicenna J Med Biotechnol. 2021 Jan;13(1):42–6.

8. R KR, Kathiswar RR, Ezhilarasan D, Rajeshkumar S. β-Sitosterol-assisted silver nanoparticles activates Nrf2 and triggers mitochondrial apoptosis via oxidative stress in human hepatocellular cancer cell line [Internet]. Vol. 108, Journal of Biomedical Materials Research Part A. 2020. p. 1899–908. Available from: http://dx.doi.org/10.1002/jbm.a.36953

9. Bell R. Cancer and Its Remedy. 1909. 137 p.

10. Solai Prakash AK, Devaraj E. Cytotoxic potentials of S. cumini methanolic seed kernel extract in human hepatoma HepG2 cells. Environ Toxicol. 2019 Dec;34(12):1313–9.

11. 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/

12. Teoh PL, Liau M, Cheong BE. Phyla nodiflora L. Extracts Induce Apoptosis and Cell Cycle Arrest in Human Breast Cancer Cell Line, MCF-7 [Internet]. Vol. 71, Nutrition and Cancer. 2019. p. 668–75. Available from: http://dx.doi.org/10.1080/01635581.2018.1559942

13. 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

14. El-Shemy H. Aromatic and Medicinal Plants: Back to Nature. BoD – Books on Demand; 2017. 298 p.

15. Chaudhary BA, Jabeen M, Jillani U, Uzair M. PHYLA NODIFLORA (VERBENACEAE): A REVIEW [Internet]. Vol. 2, Pakistan Journal of Pharmaceutical Research. 2016. p. 49. Available from: http://dx.doi.org/10.22200/pjpr.2016149-54

16. Sharma V. A mini review on medicinally important plant Lippia nodiflora [Internet]. Vol. 11, Asian Journal of Research in Chemistry. 2018. p. 176. Available from: http://dx.doi.org/10.5958/0974-4150.2018.00036.6

17. Cheng L-C, Murugaiyah V, Chan K-L. Flavonoids and phenylethanoid glycosides from Lippia nodiflora as promising antihyperuricemic agents and elucidation of their mechanism of action. J Ethnopharmacol. 2015 Dec 24;176:485–93.

18. Shue YJ, Chen PC, Wang MC, Ko HH. Tyrosinase inhibitory effect and antioxidant activity of Formosan Phyla nodiflora for cosmetic use [Internet]. Vol. 74, Planta Medica. 2008. Available from: http://dx.doi.org/10.1055/s-0028-1084682

19. 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

20. Rajeshkumar S, Kumar SV, 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. Enzyme Microb Technol. 2018 Oct;117:91–5.

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. 2011 Jan 1;78(1):113–21.

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. 2013 Jul;39(7):893–6.

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