Evaluation of Apoptotic Activity of Ethanolic Extract of Andrographis paniculata Linn in Human Hepatocellular Carcinoma Cell Lines

Sneha Kannan a, T. Lakshmi b*, Raghunandha Kumar b# and Anita Roy b#

a Saveetha Dental College, Saveetha Institute of Medical and Technical Science (SIMATS), Saveetha University, Chennai, 77, India.
b Department of Pharmacology, Saveetha Dental College, Saveetha Institute of Medical and Technical Science (SIMATS), Saveetha University, Chennai, 77, India.

Authors’ contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i64B35723

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/77724

Received 20 October 2021
Accepted 27 December 2021
Published 30 December 2021

ABSTRACT

Introduction: Andrographis paniculata is one of the highly used potential medicinal plants in the world. This plant is traditionally used for the treatment of common cold, diarrhoea, fever due to several infective cause, jaundice, as a health tonic for the liver and cardiovascular health, and as an antioxidant. It is also used to improve sexual dysfunctions and serve as a contraceptive. All parts of this plant are used to extract the active phytochemicals, but the compositions of phytoconstituents widely differ from one part to another and with place, season, and time of harvest.

Materials and Methods: All data obtained were analysed by Student t test using MS-Excel. The results were computed statistically using one way ANOVA. The reagents used were MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide]: 0.5 mg MTT/ml of serum-free DMEM, Solubilization solution: Dimethyl sulfoxide (DMSO) and Phosphate Buffered Saline (PBS; pH 7.4). The cytotoxic effects of lycopene Ag NP on 3T3 cells. Cells were treated with Lycopene Ag NP(10, 20, 30, 50, 100 and 200 μM) for 24 h, and cell viability was evaluated by MTT assay. Data are shown as means ± SD (n = 3). * compared with the control-blank group, p < 0.001.

* Associate Professor;
*Corresponding author: E-mail: lakshmi@saveetha.com;
Kannan et al., JPRI, 33(64B): 332-341, 2021; Article no.JPRI.77724

Results: The results were evaluated using three tests namely, MTT assay, Dual staining (acridine orange/ethidium bromide) and morphological changes.

Conclusion: Different stages of apoptosis revealed the importance of A.paniculata in hepatocellular carcinoma cell lines. Andrographolide and analogues have been subjected to extensive chemico-biological investigations for anticancer drug development. Several andrographolide analogues have shown superior anticancer activities in both in vitro and in vivo models. Further clinical and biomedical studies are required to confirm the pharmacological, pharmaceutical and toxicological properties of andrographolide.

Keywords: Andrographis paniculata; carcinoma; apoptosis; viability.

1. INTRODUCTION

Medical plant is an integral part of human life to combat the sufferings from the dawn of civilization [1]. It is estimated that more than 80,000 of total plant species have been identified and used as medicinal plants around the world [2]. Liver cancer is that the third leading explanation for cancer death worldwide and regarding half the patients with liver disease need adjuvant medical care when surgical operation [3,4]. Therefore, development of novel agents to eradicate cancer cells might represent a viable approach to treat patients with liver disease [5]. Andrographolide, a diterpenoid lactone isolated from Andrographis paniculata, is understood to possess potent inhibitor, medication, antineoplastic and antiviral properties [6]. During this study, we tend to investigate the cytotoxic impact of andrographolide on human liver disease cells and explore the necrobiosis mechanism [7]. Andrographolide iatrogenic a necrobiosis distinct from cell death in multiple human liver disease cells [8]. The death was characterised by autophagy as proven by the buildup of LC3 II and autophagosomes, and therefore the formation of puncta GFP-LC3 [9-15]. (‘Molecular structure and vibrational spectra of 2,6-bis(benzylidene)cyclohexanone: A density functional theoretical study’, 2011) [16-28]. This autophagy similarly as toxicity caused by andrographolide [29] may well be effectively prevented by 3-methyladenine (a chemical substance of autophagy) [30,31].

Mechanistic study indicated that andrographolide induced autophagic necrobiosis by disruption of mitochondrial [32] transmembrane potential and elevation of reactive element species [33,34] that were related with mitochondrial porosity transition pore inhibition of cyclophilin D (a element of MPTP) by cyclosporin A or repeal of its expression by tiny meddlesome ribonucleic acid considerably suppressed the toxicity of andrographolide [35], suggesting that cyclophilin D could play a very important role in mediating andrographolide-induced toxicity [36,37]. Taken along, our findings unveil a unique mechanism of drug action by andrographolide in cancer of the liver cells [38] and counsel that andrographolide could represent a promising novel agent within the treatment of cancer of the liver [39,40]. The aim of this study is to evaluate apoptotic activity of A. paniculata in controlling hepatocellular carcinoma.

2. MATERIALS AND METHODS

2.1 Chemicals and Antibodies

DMEM medium, 0.25% Trypsin-EDTA solution, sodium bicarbonate solution, bovine serum albumin (BSA), low melting agarose, MTT from Sigma Chemicals Co., St. Louis, USA. fetal bovine serum (FBS) and antibiotic/antimycotic solution, DMSO were from Himedia, Sodium phosphate monobasic and dibasic, sodium chloride, sodium hydroxide, sodium carbonate, hydrochloric acid and methanol were purchased from Sisco Research Laboratories (SRL) India.

2.2 Cell Culture Reagents

2.2.1 DMEM

Commercially available DMEM contains 7.5% sodium bicarbonate solution. To 500ml of DMEM, 5ml of penicilin/streptomycin solution and 0.5ml of amphotericin B solution was added. Then the medium was sterile filtered (0.22μ) inside the hood. The medium was then dispensed into sterile containers and stored at 4°C.

2.2.2 Growth medium (DMEM with 10% FBS)

10ml of FBS was made up to 100ml using sterile DMEM. It was stored in a sterile container in cool and aseptic condition.
2.2.3 Phosphate buffered saline (PBS; pH 7.4)

0.63 g of sodium phosphate monobasic (NaH₂PO₄), 0.17 g of sodium phosphate dibasic (Na₂HPO₄) and 4.5 g of sodium chloride (NaCl) were dissolved in 500 ml of double autoclaved millQ water. The pH was then adjusted to 7.4 using 1 N HCl and 1 N NaOH, sterile filtered (0.22 μ) and then stored in a sterile container.

2.2.4 Trypsin-EDTA

Trypsin was purchased as 1 x with EDTA (0.5% trypsin, 5.3 mM EDTA sodium salt). (Note: Freeze-thaw process does not affect the enzyme activity. Thawing is done at room temperature).

2.2.5 Cell line maintenance

Human breast cancer cell line was procured from the National Centre for Cell Science (NCCS, Pune), India. The cells were grown in T25 culture flasks containing DMEM medium supplemented with 10% FBS. Upon reaching confluence, the cells were detached using Trypsin-EDTA solution.

2.2.6 Passaging of cells

Upon reaching the confluence cell line and were passed as follows.

The medium from the culture flask was aspirated. The flask was rinsed with 2ml of PBS and aspirated again quickly. 1.5-ml of trypsin-EDTA solution was added and incubated at 37ºC for about 2 minutes until cells started detaching. As soon as the cells were detached, transfer the trypsinized medium containing cells using a transfer pipette into a 15 ml falcon tube and it was centrifuged at 1000 rpm for 5 min. The medium was carefully aspirated off and care was taken not to put the pipette tip in the bottom of the tube, where the cells were pelleted. The cells were gently resuspended in the DMEM medium with 10% FBS by pipetting up and down 5-8 times gently. From the cell suspension, a drop was placed on the edge of the coverslip of neubauer hemocytometer. The drop was led to run under the cover slip by capillary action. Care was taken not to force the liquid and the entry of air bubble was avoided. Then the cells from the E1, E2, E3, E4 and E5 squares were counted under microscope. The cells were then gently resuspended and transferred to sterile culture flasks and the volume of medium was made up to required volume with growth medium per flask.

2.2.7 Cell proliferation (MTT) assay

The proliferation of MCF-7 cells was assessed by MTT assay Safadi et al. [41].

2.2.8 Principle

The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. Only live cells are able to take up the tetrazolium salt. The enzyme (mitochondrial dehydrogenase) present in the mitochondria of the live cells is able to convert internalized tetrazolium salt to formazan crystals, which are purple in colour. Then the cells are lysed using a 20% SDS solution, which releases the formazan crystal [42]. These crystals are solubilized by DMF present in the solubilizer. The colour developed is then determined in an ELISA reader at 620 nm.

2.2.9 Reagents

- MTT [3-(4,5-dimethylthiazol-2-yl) 2.5-diphenyl tetrazolium bromide]: 0.5 mg MTT/ml of serum-free DMEM.
- Solubilization solution: Dimethyl sulfoxide (DMSO)
- Phosphate Buffered Saline (PBS; pH 7.4).

2.2.10 Procedure

A549 and MCF-7 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 [43]. After starvation, cells were treated with Compound Name different concentrations for 24 hours. At the end of treatment, the medium from control and Compound Name 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 [44].

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 formed crystals 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 serum-free medium. Cell viability in 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] x 100.

2.3 Morphology Study

Based on MTT assay we selected the optimal doses (---) 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 Compound Name (concentration for MCF-7 cells) for 24 h [45,46]. 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.

2.4 Ethidium Bromide/Acridine Orange Staining (Ao/Eb Staining Or Dual Staining)

A549/HepG2 cells were plated at a density of 5 x 10⁴ in 6 well plates containing sterile coverslips. They were allowed to grow at 37°C in a humidified CO2 incubator until they are 70 - 80% confluent [43].

Then cells were treated with synthesized (50µM) for 24hrs. The culture medium was aspirated from each well and the cells were gently rinsed twice with PBS at room temperature [43]. Then the coverslips were taken and kept on glass slides and stained with 100 µl of dye mixture (1:1 of EB and AO), immediately viewed under the fluorescence microscope.

Viable cells had green fluorescent nuclei with an organized structure. The early apoptotic cells had yellow chromatin in nuclei that were highly condensed or fragmented. Apoptotic cells also exhibited membrane blebbing [44]. The late apoptotic cells had orange chromatin with nuclei that were highly condensed and fragmented. The necrotic cells had bright orange chromatin in round nuclei. Only cells with yellow, condensed, or fragmented nuclei were counted as apoptotic cells in a blinded, non biased manner [16]. For each sample, random 300 cells were observed in a fluorescent microscope (EVOS FL Cell Imaging System, Thermo Fisher Scientific, USA) and observed at x400 magnification.at least 500 cells/well and 4 wells/condition were counted, and the percentage of apoptotic cells was determined [% of apoptotic cells = (total number of apoptotic cells/total number of cells counted) x 100]. Dual acridine orange/ethidium bromide (AO/EB) staining method was repeated 3 times at least.

3. RESULTS

3.1 MTT assay : Fig. 1

The cytotoxic effects of lycopene Ag NP on 3T3 cells. Cells were treated with Lycopene Ag NP(10, 20, 30, 50, 100 and 200 µM) for 24 h, and cell viability was evaluated by MTT assay [46]. Data are shown as means ± SD (n = 3). * compared with the control-blank group, p < 0.001.

3.2 AO/EB: Fig. 2

Acridine orange/Ethidium bromide (AO/EtBr) staining of MDA-MB-231 cells after treatment of IC50 concentration of andrographolide for 24h and compared with untreated control cells using fluorescence microscopy [47]. Cells showing bright orange fluorescence indicate apoptosis in comparison to control cells showing green fluorescence.

3.3 Morphology: Fig. 3

Assessment of cell morphology of MDA-MB-231 treated without or with andrographolide. Cells were treated with Lycopene Ag NP (100 µM) for 24 h along with the control group [47,48]. Images were obtained using an inverted Phase contrast microscope.

3.4 Statistical Analysis

All data obtained were analyzed by Student's-t-test using MS-Excel, represented as mean ± SD for triplicates. The results were computed statistically (SPSS/10 Software Package; SPSS Inc., Chicago, IL, USA) using one-way ANOVA. The level of statistical significance was set at p<0.05.
Fig. 1. Bar graph represents the percentage of cell viability of HepG2 cells against *A. paniculata*, where x axis represents *Andrographis paniculata* and y axis represents percentage of cell viability. The control cell against *A. paniculata* showed 100% cell viability. MTT assay shows that when there is an increase in *A. paniculata* Lin, there is subsequent decrease in cell viability.

Fig. 2. Acridine orange/Ethidium bromide (AO/EtBr) staining of HepG2 cells after treatment of IC50 concentration of andrographolide for 24h and compared with untreated control cells using fluorescent microscopy. Cells showing bright orange fluorescence indicate apoptosis in comparison to control cells showing green fluorescence.
Fig. 3. Morphological changes are visible between control and *Andrographis paniculata* Lin. It shows shrunken nucleus, chromatin and membrane blebbing seen in *Andrographis paniculata* Lin compared to that with viable cells

4. DISCUSSION

Kumar et al. fractionated the methanol extract of *A. paniculata* into dichloromethane, petroleum ether and aqueous extracts and found that only the dichloromethane fraction significantly inhibited the proliferation of HT-29 colon cancer cells. They further fractionated the dichloromethane extract and yielded three diterpene compounds, namely andrographolide, 14-deoxyandrographolide and 14-deoxy-11,12-didehydro andrographolide [49]. Andrographolide showed the greatest anti-cancer activity on a range of cancer cells. The *A. paniculata* ethanol extract showed cytotoxic activities against human cancer cell lines, such as Jurkat (lymphocytic), PC-3 (prostate), HepG2 (hepatoma) and Colon 205 (colonic) cells [50]. An in vivo study demonstrated that *A. paniculata* 70% ethanol extract and andrographolide increased the life spans of mice injected with thymoma cells. Isolated from 85% ethanol extract of *A. paniculata*, andrographolide and isoandrographolide exhibited higher antiproliferative activities in human leukaemia HL-60 cells than other 16 ent-labdane diterpenoids with IC50’s of 9.33 and 6.30 μM [51].

In study conducted by Wei, we investigated the effects of andrographolide on human liver cancer cells and further examined the cell death mechanism [52]. Our observations demonstrated that andrographolide-induced cytotoxicity was attributed to autophagy but not apoptosis in human liver cancer cells and that this autophagy-inducing activity was closely associated with the cyclophilin D-mediated mitochondrial permeability transition pore (MPTP) [53]. The preclinical studies conducted by Karl suggest that andrographolide could be useful for the treatment of liver cancer [54]. Andrographolide suppresses apoptosis of human umbilical vein endothelial cells (HUVECs) induced by growth factor deprivation via the activation of PI3/Akt pathway.

5. CONCLUSION

The three findings confirm that andrographolide induces apoptosis effectively in HepG2 human hepatoma cellular carcinoma cells *In vitro* [54-60]. Taken together, our findings unveil a novel mechanism of drug action by andrographolide in liver cancer cells and suggest that andrographolide may represent a promising novel agent in the treatment of liver cancer. Further investigations revealed needed at pre-
clinical and clinical levels for establishing it as a potential agent for cancer therapy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Alamgir ANM. Therapeutic Use of Medicinal Plants and Their Extracts: Pharmacognosy. Springer. 2017:1.
2. Rajeshkumar S, et al. Biosynthesis of zinc oxide nanoparticles using Mangifera indica leaves and evaluation of its antioxidant and cytotoxic properties in lung cancer (A549) cells' Enzyme and Microbial Technology. 2018;117:91–95.
3. Duke JA. Duke’s Handbook of Medicinal Plants of the Bible. CRC Press; 2007.
4. Gheena S, Ezharasani D. Syringic acid triggers reactive oxygen species-mediated cytotoxicity in HepG2 cells. Human & Experimental Toxicology. 2019;38(6):694–702.
5. Nandhini JT, Ezharasani D, Rajeshkumar S. An ecofriendly synthesized gold nanoparticles induces cytotoxicity via apoptosis in HepG2 cells. Environmental Toxicology. 2020;36(1):24–32.
6. Sen S, Chakraborty R. Herbal Medicine in India: Indigenous Knowledge, Practice, Innovation and Its Value. Springer Nature; 2019.
7. Vairavel M, Devaraj E, Shanmugam R. An eco-friendly synthesis of Enterococcus sp.-mediated gold nanoparticle induces cytotoxicity in human colorectal cancer cells. Environmental science and Pollution Research International. 2020;27(8):8166–8175.
8. Tang W, Eisenbrand G. Chinese Drugs of Plant Origin: Chemistry, Pharmacology, and Use in Traditional and Modern Medicine. Springer Science & Business Media; 2013.
9. Malik Z, et al. Anticancer potential of andrographolide from Andrographis paniculata (Burm.f.) Nees and its mechanisms of action. Journal of Ethnopharmacology. 2021;272:113936.
10. Ezharasani D. Oxidative stress is bane in chronic liver diseases: Clinical and experimental perspective. Arab Journal of Gastroenterology: The official publication of the Pan-Arab Association of Gastroenterology. 2018;19(2):56–64.
11. Ezharasani D, Apoorva VS, Ashok VN. Syzygium cumini extract induced reactive oxygen species-mediated apoptosis in human oral squamous carcinoma cells. Journal of oral pathology & medicine: official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology. 2019;48(2). DOI: 10.1111/jop.12806.
12. Ezharasani D, Apoorva VS, Vardhan NA. Syzygium cumini extract induced reactive oxygen species-mediated apoptosis in human oral squamous carcinoma cells. Journal of Oral Pathology & Medicine; 2018. DOI: 10.1111/jop.12806.
13. Ezharasani D, Sokal E, Najimi M. Hepatic fibrosis: It is time to go with hepatic stellate cell-specific therapeutic targets', Hepatobiliary & pancreatic diseases international: HBPD INT. 2018;17(3):192–197.
14. Danda AK, Krishna TM, et al. 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’, Journal of oral and maxillofacial surgery: Official Journal of the American Association of Oral and Maxillofacial Surgeons. 2010;68(2). DOI: 10.1016/j.joms.2009.04.060.
15. Danda AK, Muthusekhar MR, et al. Open versus closed treatment of unilateral subcondylar and condylar neck fractures: a prospective, randomized clinical study’, Journal of oral and maxillofacial surgery: official journal of the American Association of Oral and Maxillofacial Surgeons. 2010;68(6). DOI: 10.1016/j.joms.2009.09.042.
16. Ramadurai N, et al. Effectiveness of 2% Articaine as an anesthetic agent in children: randomized controlled trial’, Clinical Oral Investigations. 2019;23(9). DOI: 10.1007/s00784-018-2775-5.
17. Sathivel A, et al. Anti-peroxidative and anti-hyperlipidemic nature of Ulva lactuca crude polysaccharide on D-galactosamine induced hepatitis in rats', Food and chemical toxicology: An international journal published for the British Industrial Biological Research Association. 2008; 46(10). DOI: 10.1016/j.fct.2008.07.016.

18. Panda S, et al. Additive effect of autologous platelet concentrates in treatment of intrabony defects: a systematic review and meta-analysis', Journal of Investigative and Clinical Dentistry. 2016;7(1). DOI: 10.1111/jicd.12117.

19. Neelakantan P, et al. The impact of root dentine conditioning on sealing ability and push-out bond strength of an epoxy resin root canal sealer', International endodontic Journal. 2011;44(6). DOI: 10.1111/j.1365-2951.2010.01848.x.

20. Neelakantan P, et al. Continuous chelation irrigation improves the adhesion of epoxy resin-based root canal sealer to root dentine. International Endodontic Journal. 2012;45(12). DOI: 10.1111/j.1365-2951.2012.02073.x.

21. Neelakantan P, Grotra D, Sharma S. Retractability of 2 mineral trioxide aggregate-based root canal sealers: a cone-beam computed tomography analysis. Journal of Endodontia. 2013; 39(7):893–896.

22. Govindaraju L, Neelakantan P, Gutmann JL. Effect of root canal irrigating solutions on the compressive strength of tricalcium silicate cements', Clinical Oral Investigations. 2017;21(2). DOI: 10.1007/s00784-016-1922-0.

23. Sekhar CH, Narayanan V, Baig MF. Role of antimicrobials in third molar surgery: prospective, double blind, randomized, placebo-controlled clinical study. The British journal of Oral & Maxillofacial Surgery. 2001;39(2). DOI: 10.1054/bjom.2000.0557.

24. DeSouza SI, et al. Mobile phones: the next step towards healthcare delivery in rural India?', Plos One. 2014(9). DOI: 10.1371/journal.pone.0104895.

25. Nasim I, et al. Color stability of microfilled, microhybrid and nanocomposite resins—an in vitro study', Journal of Dentistry. 2010;38(Suppl 2). DOI: 10.1016/j.jdent.2010.05.020.

26. 'Molecular structure and vibrational spectra of 2,6-bis(benzylidene)cyclohexanone: A density functional theoretical study'. Spectrochimica acta. Part A, Molecular and Biomolecular Spectroscopy. 2011; 78(1):113–121.

27. Putchala MC, et al. Ascorbic acid and its pro-oxidant activity as a therapy for tumours of oral cavity – A systematic review’, Archives of Oral Biology. 2013; 563–574. DOI: 10.1016/j.archoralbio.2013.01.016.

28. Suresh P, et al. Optimization of machining parameters in turning of Al-SiC-Gr hybrid metal matrix composites using grey-fuzzy algorithm’, Transactions of Nonferrous Metals Society of China. 2014;2805–2814. DOI: 10.1016/s1003-6326(14)63412-9.

29. Dua K, et al. The potential of siRNA based drug delivery in respiratory disorders: Recent advances and progress’, Drug Development Research. 2019;714–730. DOI: 10.1002/ddr.21571.

30. Gomathi AC, et al. Anticancer activity of silver nanoparticles synthesized using aqueous fruit shell extract of Tamarindus indica on MCF-7 human breast cancer cell line', Journal of Drug Delivery Science and Technology. 2020;101376. DOI: 10.1016/j.jddst.2019.101376.

31. Gomathi M, et al. Green synthesis of silver nanoparticles using Gymnema sylvestre leaf extract and evaluation of its antibacterial activity', South African Journal of Chemical Engineering. 2020;1–4. DOI: 10.1016/j.sajce.2019.11.005.

32. Rajasekaran S, et al. Collective influence of 1-decanol addition, injection pressure and EGR on diesel engine characteristics fueled with diesel/LDPE oil blends', Fuel. 2020;118166. DOI: 10.1016/j.fuel.2020.118166.

33. Raj RK, DE, SR. β-Sitosterol-assisted silver nanoparticles activates Nrf2 and triggers mitochondrial apoptosis via oxidative stress in human hepatocellular cancer cell line’, Journal of biomedical materials research. Part A. 2020;108(9): 1899–1908.

34. Worakunpanich W, et al. Safety of Andrographis paniculata: A systematic review and meta-analysis. Pharmacoepidemiology and Drug Safety; 2020. DOI: 10.1002/pds.5190.

35. Santhoshkumar J, et al. Toxicology evaluation and antidermatophytic activity of
silver nanoparticles synthesized using leaf extract of Passiflora caerulea, South African Journal of Chemical Engineering. 2019;17:23.

36. Singh AK. Cultivation of Kalmegh Andrographis Paniculata in India; 1999.

37. Saravanan M, et al. Synthesis of silver nanoparticles from Phenerochaete chrysosporium (MTCC-787) and their antibacterial activity against human pathogenic bacteria, Microbial Pathogenesis. 2018;68-72.

38. Raghunandhakumar S, et al. Thymoquinone inhibits cell proliferation through regulation of G1/S phase cell cycle transition in N-nitrosodimethylamine-induced experimental rat hepatocellular carcinoma, Toxicology Letters. 2013;223(1):60-72.

39. Asokkumar S, et al. Antiproliferative and antioxidant potential of beta-ionone against benzo(a)pyrene-induced lung carcinogenesis in Swiss albino mice, Molecular and Cellular Biochemistry. 2012;363(1-2):335-345.

40. Anandakumar P, et al. Capsaicin inhibits benzo(a)pyrene-induced lung carcinogenesis in an in vivo mouse model, Inflammation research: official journal of the European Histamine Research Society. 2012;61(11):1169-1175.

41. Ramesh A, et al. Comparative estimation of sulfiredoxin levels between chronic periodontitis and healthy patients: A case-control study, Journal of Periodontology. 2018;89(10):1241-1248.

42. Paramasivam A, et al. In Vitro Anti-Neuroblastoma Activity of Thymoquinone Against Neuro-2A Cells via Cell-cycle Arrest, Asian Pacific journal of cancer prevention: APJCP. 2015; 16(18):8313-8319.

43. Arumugam P, George R, Jayaseelan VP. Aberrations of m6A regulators are associated with tumorigenesis and metastasis in head and neck squamous cell carcinoma. Archives of Oral Biology. 2021;122:105030.

44. Duraisamy R, et al. Compatibility of Nonoriginal Abutments With Implants, Implant Dentistry. 2019;289-295.

45. Koka P, et al. Uncoupling Warburg effect and stemness in CD133+ve cancer stem cells from Saos-2 (osteosarcoma) cell line under hypoxia, Molecular Biology Reports. 2018;45(6):1653-1662.

46. Gnanavel V, Roopan SM, Rajeshkumar S. Aquaculture: An overview of chemical ecology of seaweeds (food species) in natural products, Aquaculture. 2018;1-6.

47. Markov A, et al. Mesenchymal stem/stromal cells as a valuable source for the treatment of immune-mediated disorders, Stem Cell Research & Therapy; 2021.

48. Menon S, Agarwal H, Rajeshkumar S. Investigating the Antimicrobial Activities of the Biosynthesized Selenium Nanoparticles and Its Statistical Analysis, Bio Nano Science; 2020.

49. Mohapatra S, et al. Assessment of Cytotoxicity, Anti-Inflammatory and Antioxidant Activity of Zinc Oxide Nanoparticles Synthesized Using Clove and Cinnamon Formulation - An In-Vitro Study, Journal of Evolution of Medical and Dental Sciences. 2020;1859-1864.

50. Barma MD. Synthesis of triphala incorporated zinc oxide nanoparticles and assessment of its antimicrobial activity against oral pathogens: An in-vitro study, Bioscience Biotechnology Research Communications. 2021;13(7):74-78.

51. Vikneshan M, et al. Algal biomass as a source for novel oral nano-antimicrobial agent, Saudi Journal of Biological Sciences. 2021;27(12):3753-3758.

52. Nandhini NT, Rajeshkumar S, Myhili S. The possible mechanism of eco-friendly synthesized nanoparticles on hazardous dyes degradation, Biocatalysis and Agricultural Biotechnology. 2019;101138.

53. Rajendran R, et al. Comparative Evaluation of Remineralizing Potential of a Paste Containing Bioactive Glass and a Topical Cream Containing Casein Phosphopeptide-Amorphous Calcium Phosphate: An In vitro Study, Pesquisa Brasileira em Odontopediatria e Clinica Integrada. 2019;19(0):4668.

54. Ashok BS, Ajith TA, Sivanesan S. Hypoxia-inducible factors as neuroprotective agent in Alzheimer’s disease, Clinical and Experimental Pharmacology & Physiology. 2017;44(3).

55. 340
55. Malli SN, et al. Concentrated Growth Factors as an Ingenious Biomaterial in Regeneration of Bony Defects after Periapical Surgery: A Report of Two Cases', Case Reports in Dentistry; 2019. DOI: 10.1155/2019/7046203.

56. Mohan M, Jagannathan N. Oral field cancerization: an update on current concepts', Oncology Reviews. 2014;8(1). DOI: 10.4081/oncol.2014.244.

57. Samuel SR, Acharya S, Rao JC. ‘School Interventions-based Prevention of Early-Childhood Caries among 3-5-year-old children from very low socioeconomic status: Two-year randomized trial', Journal of Public Health Dentistry. 2019;80(1).

58. Praveen K, et al. Hypotensive anaesthesia and blood loss in orthognathic surgery: a clinical study', The British Journal of Oral & Maxillofacial Surgery. 2001;39(2). DOI: 10.1054/bjom.2000.0593.

59. ‘Oligonucleotide therapy: An emerging focus area for drug delivery in chronic inflammatory respiratory diseases’. Chemico-biological Interactions. 2019;308: 206–215.

60. Kumar MS, et al. Expression of matrix metalloproteinases (MMP-8 and -9) in chronic periodontitis patients with and without diabetes mellitus’, Journal of periodontology. 2006;77(11):1803–1808.