

**ANTI-PROLIFERATIVE EFFECT OF HYDROETHANOLIC LEAF  
EXTRACT OF *CITRULLUS COLOCYNTHIS* (L) ON  
RETINOBLASTOMA CELL LINE**

**ABSTRACT**

**Introduction**

*Citrullus colocynthis* has many pharmacological, biological, and therapeutic properties making it a very important medicinal plant. The cucurbitacin produced/derived from the plant play an important role in anticancer activities. The retinoblastoma Y-79 cell lines are studied in attachment culture. A proper study of these cells can pave the way for control of its proliferative property. Cell viability was assayed using a modified colorimetric technique that is based on the ability of the live cells to convert MTT, a tetrazolium compound into purple formazan crystals. There was an effect on the proliferation and gene expressions of the retinoblastoma Y-79 cell line. Bioactive extracts of these plants are being majorly used as a potential chemopreventive element as an alternative approach for cancer progression management.

**Materials and Method**

Human retinoblastoma cell line (Y-79) cell line was purchased from the National Centre for Cell Sciences (NCCS), Pune, India. Cell viability was assayed using a modified colorimetric technique that is based on the ability of live cells to convert MTT.

**Result**

The experimental study showed antiproliferative effects against the retinoblastoma Y-79 cells. The experimental study showed the effect on cell viability, Bcl2 mRNA expression and BclxL mRNA expression in Y-79 cells.

**Conclusion**

The hydroethanolic leaf extract obtained from the *Citrullus colocynthis* exhibited an antiproliferative effect on the retinoblastoma Y-79 cell line by affecting the expression of the Bcl2 mRNA and BclxL mRNA and hindering the cell viability.

**Keywords:**

Anti-proliferative effect, *Citrullus colocynthis*, retinoblastoma cell line

**INTRODUCTION**

*Citrullus colocynthis*, a perennial plant, has the ability to propagate both by vegetative and generative means. *Citrullus colocynthis* in general is used in the treatment of various diseases like diabetes, asthma, leprosy, jaundice, constipation, bronchitis, joint pain, cancer, and mantitis (1). Very little is known regarding the other parts of the plant. Many studies have reported antioxidant and antibacterial activities of the leaf extract of *Citrullus colocynthis*. Leaf extracts give cucurbitacin derivatives and their glycosides which have been studied the most. The cucurbitacins induce arrest in growth and apoptosis in colorectal cancer cells targeting the JAK/STAT3 signaling pathway (2). The cucurbitacin glycosides extracted from *Citrullus colocynthis* leaves had an anti-proliferative effect on human breast cancer cell growth which reveals that cucurbitacin glycosides cause both cell cycle arrest and apoptosis due to its pleiotropic effect on cells (3–4) The leaf extract of *Citrullus colocynthis* has the highest antioxidant potency, The extract showed free-radical-scavenging and antioxidant abilities to its maximum (5–6).

Flavonoids, glycosides, alkaloids, and saponosides were reported to be present in the ethanolic and aqueous extract obtained from *Citrullus colocynthis* leaves and fruits (7). Leaf extract of *Citrullus colocynthis* has reported moderate antimicrobial activity (8–13). The silver nanoparticle synthesized from the extract of calli of *Citrullus colocynthis* has biomedical potential in causing cell death through apoptosis hence paving a way to potentially eliminate the use of expensive drugs for cancer treatment (14). Retinoblastoma are tumors occurring inside the eye, i.e., intraocular tumors which normally appear in childhood for which proneness can be inherited. (15–16)

The Y-79 cells derived from retinoblastoma tumors have been used for various detailed experimental studies (17). Human Y-79 retinoblastoma cells are studied in attachment culture. A proper study concerning the normal retinal cells will pave a way for the control of

retinoblastoma growth and development (18). This study research will help in bringing forward the anticancer activity of leaf extract of *Citrullus colocynthis* on these retinoblastoma Y-79 cell lines. *Citrullus colocynthis* contains many useful properties along with antioxidation, it's also a useful anti-inflammatory source that helps in preventing subsequent harm or damage to the adjacent cells (19). The apoptosis effect induced by the cucurbitacin glycoside combination causes loss of membrane polarity followed by translocation of membrane (20)(21)(20). Hence this research will shed light on the antiproliferative effect of *Citrullus colocynthis* leaf extract on retinoblastoma cell lines.

## **MATERIALS AND METHODS**

Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Pvt Ltd, USA. Trypsin-EDTA, fetal bovine serum (FBS), antibiotics-antimycotics, RPMI 1640 medium and phosphate buffered saline (PBS) were purchased from Gibco, Canada. (5,5,6,6-tetrachloro-1,1,3,3 - tetraethylbenzimidazolocarboyanine iodide) and Real Time PCR kit was purchased TAKARA (Meadowvale Blvd, Mississauga, ON L5N 5S2, Canada).

### **Cell lines and cell culture**

Human retinoblastoma cell line (Y-79) cell line was purchased from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in DMEM medium (Thermo Fisher Scientific, CA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, CA, USA) at 37°C with 5% CO<sub>2</sub>.

### **Cell viability by MTT assay**

Cell viability was assayed using a modified colorimetric technique that is based on the ability of live cells to convert MTT, a tetrazolium compound into purple formazan crystals by mitochondrial reductases (Mosmann, 1983). Briefly, the cells ( $1 \times 10^4$ /well) were exposed to different concentrations of *Citrullus colocynthis* leaf extract (100-500µg/ml) with Y-79 cells for 48 h. At the end of the treatment, 100 µl of 0.5 mg/ml MTT solution was added to each well and incubated at 37 °C for an hour. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (100 µl ) and incubated in the 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 the control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability =  $[A_{570 \text{ nm of treated cells}}/A_{570 \text{ nm of control cells}}] \times 100$ .

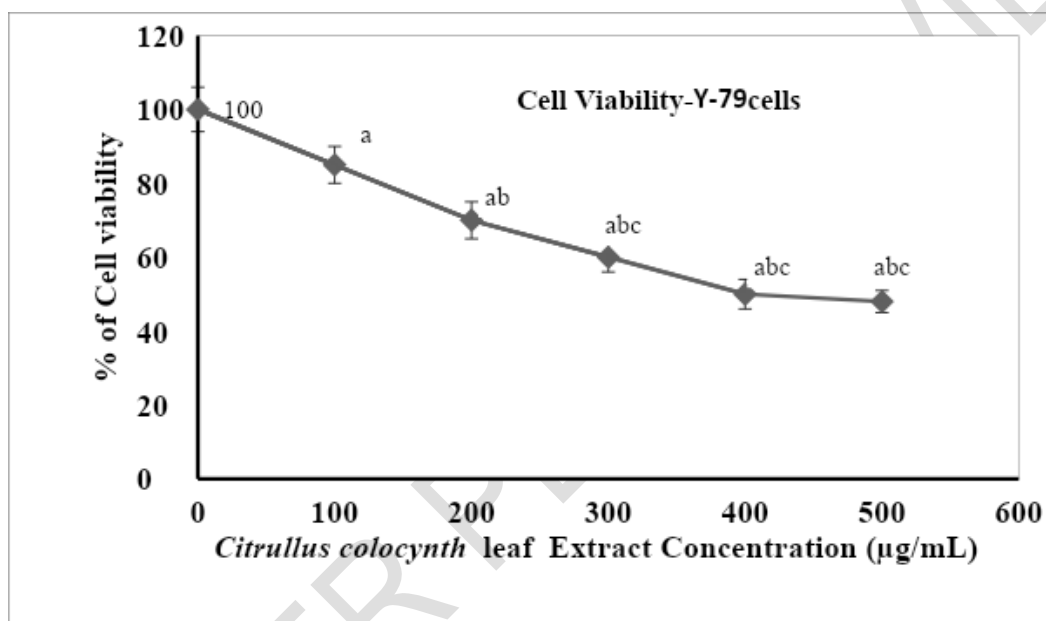
### **Gene expression analysis by Real Time-PCR**

Samples from each group were submerged in 2 ml Trizol (Invitrogen, Carlsbad, CA, USA) for RNA extraction and stored at  $-80^{\circ}\text{C}$  until further processed. cDNA synthesis was performed on 2  $\mu\text{g}$  RNA in a 10  $\mu\text{l}$  sample volume using Superscript II reverse transcriptase (Invitrogen) as recommended by the manufacturer. Real-time PCR array analysis was performed in a total volume of 20  $\mu\text{l}$  including 1  $\mu\text{l}$  cDNA, 10  $\mu\text{l}$  qPCR Master Mix 2x (Takara, USA) and 9  $\mu\text{l}$  ddH<sub>2</sub>O. Reactions were run on an CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) using universal thermal cycling parameters (95 $^{\circ}\text{C}$  for 5 min, 40 cycles of 15 sec at 95 $^{\circ}\text{C}$ , 15 sec at 60 $^{\circ}\text{C}$  and 20 sec at 72 $^{\circ}\text{C}$ ; followed by a melting curve: 5 sec at 95 $^{\circ}\text{C}$ , 60 sec at 60 $^{\circ}\text{C}$  and continued melting). For quality control purposes, melting curves were acquired for all samples. The specificity of the amplification product was determined by melting curve analysis for each primer pair. The data were analyzed by comparative CT method and the fold change is calculated by  $2^{-\Delta\Delta\text{CT}}$  method described by Schmittgen and Livak (2008) using CFX Manager Version 2.1 (Bio Rad, USA).

### **Statistical analysis**

The obtained data were analyzed statistically by one-way analysis of variance (ANOVA) and Duncan's multiple range test with a computer-based software (Graph Pad Prism version 5) to analyze the significance of individual variations among the control and experimental groups. The significance was considered at  $p < 0.05$  level in Duncan's test

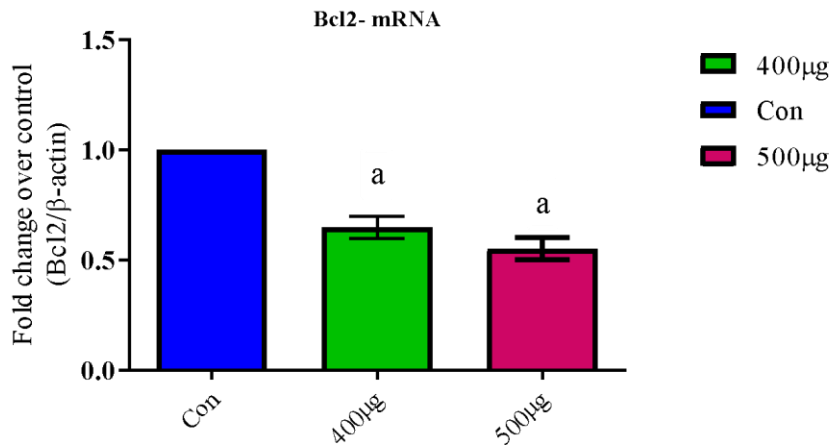
## RESULTS AND DISCUSSION



**Figure 1:** Effect *Citrullus colocynthis* leaf extract on cell viability in Y-79 cells. Each bar represents a mean  $\pm$  SEM of 6 observations. Significance at  $p < 0.05$ , a-compared with untreated control cells, b-compared with 100 micron M treated Y-79 cells.

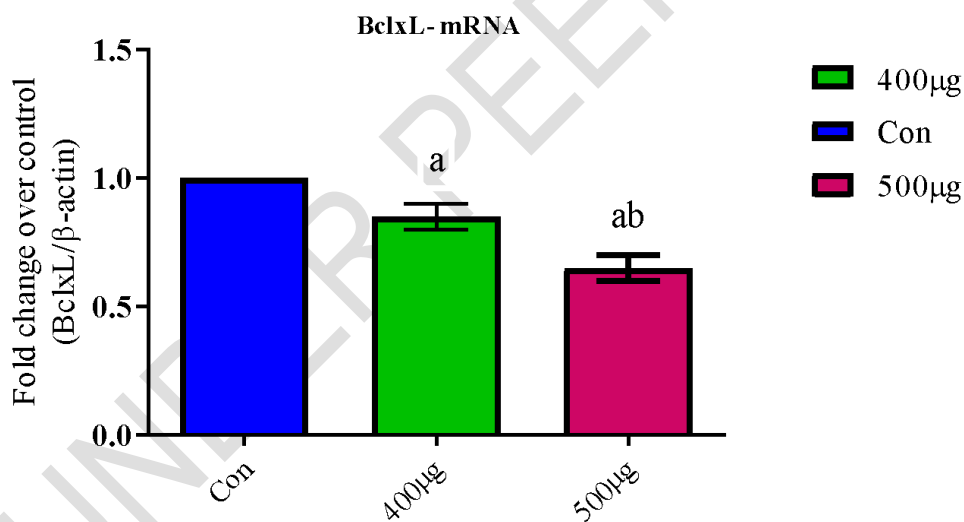
### Gene expression analysis

Bcl2 mRNA expression (Fold change over control)



**Figure 2:** Effect of *Citrullus colocynthis* leaf extract on Bcl2 mRNA expression in Y-79 cells. Each bar represents a mean  $\pm$  SEM of 6 observations. Significance at  $p < 0.05$ , a-compared with untreated control cells.

BclxL mRNA expression (Fold change over control)



**Figure 3:** Effect of *Citrullus colocynthis* leaf extract on BclxL mRNA expression in Y-79 cells. Each bar represents a mean  $\pm$  SEM of 6 observations. Significance at  $p < 0.05$ , a-compared with untreated control cells, b-compared with 400µg treated cells.

The experimental study showed antiproliferative effects against the retinoblastoma Y-79 cells. The experimental study showed the effect on cell viability, Bcl2 mRNA expression and BclxL mRNA expression in Y-79 cells.

The anticancer effect of *Citrullus colocynthis* has been via a variety of pathways which include apoptotic pathway, anti-inflammatory traits, and antioxidant pathway, etc (19). Bioactive extracts of plants are being majorly considered as potential chemopreventive elements as an alternative approach for cancer progression management (22). Treatment with AMF and its constituent for 24 hours improved the cell motility and also the keratinocyte expression drastically in the cell lines in the experiment conducted by Azmi et al (23). Similarly, the expression of the genes Bcl2 mRNA and BclxL mRNA in the Y-79 cells were affected along with the cell viability (24–26). In this study, the extract obtained from the leaves were used to treat the cells. In the study conducted by Tehila Tannin-spitz et al., The cucurbitacins obtained exhibited inhibition of growth of the cell line (20) which was exhibited by the inhibition of growth in the present study in the retinoblastoma cells. Many derivatives from different plants such as taxol from the Pacific yew tree (27), combrestastian from the South African bush willow, and Camptothecin obtained from the Chinese ‘Happy Tree’ have shown worldwide use in drugs employed in chemotherapy (28). Herbal medicine is becoming more and more attentive as it has shown many potential cancer therapeutic and preventive agents. In a cross-sectional study, a great number of people were taking herbal medicine for cancer treatment (29). *Citrullus colocynthis* has shown many therapeutic effects in treating many diseases. It has many pharmacological activities like antidiabetic, antineoplastic, antiallergic, etc. (5). The study by M A Campbell et al stated the support in an origin of Y-79 cells from the pluripotent retinal stem cell. (17). According to the study by Gerald J Chaer in his experimental research, stated that when retinoblastoma cells are treated with specific agent inducing partial neuronal or glial-like morphology, they begin to express other specific markers by ‘maturation’ and start losing inappropriate markers (18),(30)(18) this ability can be hindered by the use of the cucurbitacins derivative which hinders the expression of mRNA in retinoblastoma cells, extracted from the leaves of *Citrullus colocynthis* (L) ((31–35) The present study had studied a small proportion of the sample. A much more and bigger scale study can help understand the effects even better and also help for other related issues.

## CONCLUSION

The hydroethanolic leaf extract obtained from the *Citrullus colocynthis* exhibited an antiproliferative effect on the retinoblastoma Y-79 cell line by affecting the expression of the Bcl2 mRNA and BclxL mRNA and hindering the cell viability. Hence, it can be a great scope for using the extract into medicinal properties with further studies to improve herbal medicine for cancer over expensive drugs.

## 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. Rani A, Goyal A, Arora S. A Brief Review on *Citrullus colocynthis*- Bitter Apple [Internet]. Vol. 8, Archives of Current Research International. 2017. p. 1–9. Available from: <http://dx.doi.org/10.9734/acri/2017/35158>
2. Chawech R, Jarraya R, Girardi C, Vansteelandt M, Marti G, Nasri I, et al. Cucurbitacins from the Leaves of *Citrullus colocynthis* (L.) Schrad. *Molecules*. 2015 Sep 30;20(10):18001–15.
3. Al-Snafi PDAE, Prof Dr Ali. Medicinal plants with antioxidant and free radical scavenging effects (part 2): plant based review [Internet]. Vol. 06, IOSR Journal of Pharmacy (IOSRPHR). 2016. p. 62–82. Available from: <http://dx.doi.org/10.9790/3013-06726282>
4. Barabadi H, Mojab F, Vahidi H, Marashi B, Talank N, Hosseini O, et al. Green synthesis, characterization, antibacterial and biofilm inhibitory activity of silver nanoparticles compared to commercial silver nanoparticles [Internet]. Vol. 129, Inorganic Chemistry Communications. 2021. p. 108647. Available from: <http://dx.doi.org/10.1016/j.inoche.2021.108647>
5. Hussain AI, Rathore HA, Sattar MZA, Chatha SAS, Sarker SD, Gilani AH. *Citrullus colocynthis* (L.) Schrad (bitter apple fruit): a review of its phytochemistry, pharmacology, traditional uses and nutritional potential. *J Ethnopharmacol*. 2014 Aug 8;155(1):54–66.
6. Egbuna C, Mishra AP, Goyal MR. Preparation of Phytopharmaceuticals for the Management of Disorders: The Development of Nutraceuticals and Traditional Medicine. Academic Press; 2020. 574 p.
7. Benariba N, Djaziri R, Bellakhdar W, Belkacem N, Kadiata M, Malaisse WJ, et al. Phytochemical screening and free radical scavenging activity of *Citrullus colocynthis* seeds extracts [Internet]. Vol. 3, Asian Pacific Journal of Tropical Biomedicine. 2013. p. 35–40. Available from: [http://dx.doi.org/10.1016/s2221-1691\(13\)60020-9](http://dx.doi.org/10.1016/s2221-1691(13)60020-9)
8. Rajakumari R, Volova T, Oluwafemi OS, Rajesh Kumar S, Thomas S, Kalarikkal N. Grape seed extract-soluplus dispersion and its antioxidant activity. *Drug Dev Ind Pharm*. 2020 Aug;46(8):1219–29.
9. Clarizia G, Bernardo P. Diverse Applications of Organic-Inorganic Nanocomposites: Emerging Research and Opportunities: Emerging Research and Opportunities. IGI Global; 2019. 237 p.
10. Prakash AKS, Devaraj E. Cytotoxic potentials of *S. cumini* methanolic seed kernel extract in human hepatoma HepG2 cells [Internet]. Vol. 34, Environmental Toxicology. 2019. p. 1313–9. Available from: <http://dx.doi.org/10.1002/tox.22832>
11. Tahmasebi S, Qasim MT, Krivenkova MV, Zekiy AO, Thangavelu L, Aravindhan S, et al. The effects of oxygen-ozone therapy on regulatory T-cell responses in multiple

sclerosis patients. *Cell Biol Int*. 2021 Jul;45(7):1498–509.

12. Wadhwa R, Paudel KR, Chin LH, Hon CM, Madheswaran T, Gupta G, et al. Anti-inflammatory and anticancer activities of Naringenin-loaded liquid crystalline nanoparticles in vitro. *J Food Biochem*. 2021 Jan;45(1):e13572.
13. Vivekanandhan K, Shanmugam P, Barabadi H, Arumugam V, Raj DDRD, Sivasubramanian M, et al. Emerging Therapeutic Approaches to Combat COVID-19: Present Status and Future Perspectives [Internet]. Vol. 8, *Frontiers in Molecular Biosciences*. 2021. Available from: <http://dx.doi.org/10.3389/fmolb.2021.604447>
14. Satyavani K, Gurudeeban S, Ramanathan T, Balasubramanian T. Biomedical potential of silver nanoparticles synthesized from calli cells of *Citrullus colocynthis* (L.) Schrad. *J Nanobiotechnology*. 2011 Sep 26;9:43.
15. Kamath SM, Manjunath Kamath S, Jaison D, Rao SK, Sridhar K, Kasthuri N, et al. In vitro augmentation of chondrogenesis by Epigallocatechin gallate in primary Human chondrocytes - Sustained release model for cartilage regeneration [Internet]. Vol. 60, *Journal of Drug Delivery Science and Technology*. 2020. p. 101992. Available from: <http://dx.doi.org/10.1016/j.jddst.2020.101992>
16. Gowhari Shabgah A, Ezzatifar F, Aravindhan S, Olegovna Zekiy A, Ahmadi M, Gheibihayat SM, et al. Shedding more light on the role of Midkine in hepatocellular carcinoma: New perspectives on diagnosis and therapy. *IUBMB Life*. 2021 Apr;73(4):659–69.
17. Campbell MA, Karras P, Chader GJ. Y-79 retinoblastoma cells: Isolation and characterization of clonal lineages [Internet]. Vol. 48, *Experimental Eye Research*. 1989. p. 77–85. Available from: [http://dx.doi.org/10.1016/0014-4835\(89\)90021-3](http://dx.doi.org/10.1016/0014-4835(89)90021-3)
18. Chader GJ. Multipotential differentiation of human Y-79 retinoblastoma cells in attachment culture [Internet]. Vol. 20, *Cell Differentiation*. 1987. p. 209–16. Available from: [http://dx.doi.org/10.1016/0045-6039\(87\)90436-2](http://dx.doi.org/10.1016/0045-6039(87)90436-2)
19. Abdulridha MK, Al-Marzoqi A-H, Ghasemian A. The Anticancer Efficiency of *Citrullus colocynthis* Toward the Colorectal Cancer Therapy [Internet]. Vol. 51, *Journal of Gastrointestinal Cancer*. 2020. p. 439–44. Available from: <http://dx.doi.org/10.1007/s12029-019-00299-6>
20. Tannin-Spitz T, Grossman S, Dovrat S, Gottlieb HE, Bergman M. Growth inhibitory activity of cucurbitacin glucosides isolated from *Citrullus colocynthis* on human breast cancer cells [Internet]. Vol. 73, *Biochemical Pharmacology*. 2007. p. 56–67. Available from: <http://dx.doi.org/10.1016/j.bcp.2006.09.012>
21. Bharath B, Perinbam K, Devanesan S, AlSalhi MS, Saravanan M. Evaluation of the anticancer potential of Hexadecanoic acid from brown algae *Turbinaria ornata* on HT–29 colon cancer cells [Internet]. Vol. 1235, *Journal of Molecular Structure*. 2021. p. 130229. Available from: <http://dx.doi.org/10.1016/j.molstruc.2021.130229>
22. Verma S, Bahorun T, Singh RK, Aruoma OI, Kumar A. Effect of *Aegle marmelos* leaf extract on N-methyl N-nitrosourea-induced hepatocarcinogenesis in Balb/c mice. *Pharm*

Biol. 2013 Oct;51(10):1272–81.

23. Azmi L, Shukla I, Goutam A, Allaiddin, Rao CV, Jawaid T, et al. In vitro wound healing activity of 1-hydroxy-5,7-dimethoxy-2-naphthalene-carboxaldehyde (HDNC) and other isolates of *Aegle marmelos* L.: Enhances keratinocytes motility via Wnt/ $\beta$ -catenin and RAS-ERK pathways [Internet]. Vol. 27, Saudi Pharmaceutical Journal. 2019. p. 532–9. Available from: <http://dx.doi.org/10.1016/j.jsps.2019.01.017>
24. Saraswathi I, Saikarthik J, Senthil Kumar K, Madhan Srinivasan K, Ardhanaari M, Gunapriya R. Impact of COVID-19 outbreak on the mental health status of undergraduate medical students in a COVID-19 treating medical college: a prospective longitudinal study. PeerJ. 2020 Oct 16;8:e10164.
25. Santhakumar P, Roy A, Mohanraj KG, Jayaraman S, Durairaj R. Ethanolic Extract of *Capparis decidua* Fruit Ameliorates Methotrexate-Induced Hepatotoxicity by Activating Nrf2/HO-1 and PPAR $\gamma$  Mediated Pathways. Ind J Pharm Educ. 2021 Mar 19;55(1s):s265–74.
26. Nambi G, Kamal W, Es S, Joshi S, Trivedi P. Spinal manipulation plus laser therapy versus laser therapy alone in the treatment of chronic non-specific low back pain: a randomized controlled study. Eur J Phys Rehabil Med. 2018 Dec;54(6):880–9.
27. Chakravarthi BV, Sujay R, Kuriakose GC, Karande AA, Jayabaskaran C. Inhibition of cancer cell proliferation and apoptosis-inducing activity of fungal taxol and its precursor baccatin III purified from endophytic *Fusarium solani* [Internet]. Vol. 13, Cancer Cell International. 2013. p. 105. Available from: <http://dx.doi.org/10.1186/1475-2867-13-105>
28. Marzouk B, Mussi F, Jamali C, Galati S, Bekkouche K, Aouni M, et al. Inhibitory Activity of Leaves Extracts of *Citrullus colocynthis* Schrad. On HT29 Human Colon Cancer Cells [Internet]. Vol. 12, European Journal of Medicinal Plants. 2016. p. 1–10. Available from: <http://dx.doi.org/10.9734/ejmp/2016/22888>
29. Pei S, Yang X, Wang H, Zhang H, Zhou B, Zhang D, et al. Plantamajoside, a potential anti-tumor herbal medicine inhibits breast cancer growth and pulmonary metastasis by decreasing the activity of matrix metalloproteinase-9 and -2. BMC Cancer. 2015 Dec 16;15:965.
30. Ezhilarasan D. Critical role of estrogen in the progression of chronic liver diseases. Hepatobiliary Pancreat Dis Int. 2020 Oct;19(5):429–34.
31. Sridharan G, Ramani P, Patankar S, Vijayaraghavan R. Evaluation of salivary metabolomics in oral leukoplakia and oral squamous cell carcinoma. J Oral Pathol Med. 2019 Apr;48(4):299–306.
32. R H, Hannah R, Ramani P, Ramanathan A, Jancy MR, Gheena S, et al. CYP2 C9 polymorphism among patients with oral squamous cell carcinoma and its role in altering the metabolism of benzo[a]pyrene [Internet]. Vol. 130, Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology. 2020. p. 306–12. Available from: <http://dx.doi.org/10.1016/j.oooo.2020.06.021>
33. J PC, Pradeep CJ, Marimuthu T, Krithika C, Devadoss P, Kumar SM. Prevalence and

measurement of anterior loop of the mandibular canal using CBCT: A cross sectional study [Internet]. Vol. 20, *Clinical Implant Dentistry and Related Research*. 2018. p. 531–4. Available from: <http://dx.doi.org/10.1111/cid.12609>

34. Wahab PUA, Abdul Wahab PU, Madhulaxmi M, Senthilnathan P, Muthusekhar MR, Vohra Y, et al. Scalpel Versus Diathermy in Wound Healing After Mucosal Incisions: A Split-Mouth Study [Internet]. Vol. 76, *Journal of Oral and Maxillofacial Surgery*. 2018. p. 1160–4. Available from: <http://dx.doi.org/10.1016/j.joms.2017.12.020>
35. Mudigonda SK, Murugan S, Velavan K, Thulasiraman S, Krishna Kumar Raja VB. Non-suturing microvascular anastomosis in maxillofacial reconstruction- a comparative study. *Journal of Cranio-Maxillofacial Surgery*. 2020 Jun 1;48(6):599–606.

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