

Original Research Article

Regulation of chloride intracellular channel protein 1 and caspase -3 mRNA expression by hydroethanolic extract of *Aegle marmelos* fruit human breast cancer cell line-MCF-7

Running title: *Aegle marmelos* fruit extract in human breast cell lines

ABSTRACT

Introduction: Cancer is the second leading cause of death all over the world where among all types of cancer breast cancer is said to be the leading cancer followed by lung cancer. The aim of this study is to find the regulation of chloride intracellular channel protein 1 and caspase -3 mRNA expression by hydroethanolic extract of *Aegle marmelos* fruit human breast cancer cell line-MCF-7.

Materials and methods: MCF-7 cells were collected from NCCS Pune, India. It is stored in Dubecos Modified Eagle's Medium. The *Aegle marmelos* fruit was collected from the herbal department and its extract was prepared. The extract of *Aegle marmelos* is used in treating MCF-7 cells at different dosages. Isolation of total RNA from MCF-7 cells- The cells will be mixed with total RNA isolation reagent, sonicated and RNA will be isolated as per the standard method. c-DNA conversion and real time polymerase chain reaction. The c-DNA will be synthesized using reverse transcription by commercially available (RT-PCR) kit. Two microlitres of c-DNA will be used for amplification of clic-1 and caspase-3 using gene specific primers by commercially available RT-PCR kit (SyBr kit) and comparative CT method will be used to see the expression of genes. Untreated MCF-7 cells were compared with MCF-7 cells treated with various concentrations of the extract (10, 20 and 40ug).

Result: The given extract inhibits the proliferation of MCF-7 cells therefore said to have antiproliferative activity. Different doses of extract were tested (200ug-500ug) out of which 400ug of extract were preferred. The statistical data were collected from the SPSS software version 21

Conclusion: The given plant extract has anti proliferative properties and hence can be used as a drug to treat breast cancer.

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KEY WORDS: Cancer, MCF-7 cells, *Aegle marmelos*, RNA isolation reagent, c-DNA, RT-PCR kit, Innovative technique.

INTRODUCTION

Cancer is an uncontrollable proliferation of cells which leads to tumor formation and spreads from one organ to another. Cancer is the main cause of death and acts as an important barrier of increasing lifespan (1) (2). Generally cancer cells grow more and then breaks down from original mass of cells and then travels through blood and lymph systems and attaches to other organ and again starts its abnormal growth cycle. This process of leaving an organ and going to other organ and continuing its abnormal growth cycle is called metastasis (3). Such kind of tumor spreads all over the body and is called malignant tumor. For example if the cancerous cells of breast spread to the bone that doesn't mean that the person is suffering from bone cancer it is said to be metastatic breast cancer to bone and if the cancer starts from bones it is said to be bone cancer. Cancer is caused due to exposure to chemicals or toxic substances, ionizing radiation, pathogens and through genes. Chemical exposure to nickel, cadmium, benzene, cigarette smoking (etc) leads to cancer. HBV, EBV, hepatitis B and C (etc) are some of the pathogens which cause cancer. Ionizing radiations like UV rays, alpha, beta, gamma rays and X rays can cause cancer. There are specific kinds of cancer related to human genes like breast cancer cells, ovarian cancer, colorectal cancer, skin cancer, prostate cancer and melanoma cancer. In some people there is a high immune response which controls or eliminates cells that can become cancerous cells (4). Other situations like obesity, lack of exercise, chronic inflammation and hormones lead to cancer. Lump in breast and armpit, nipple pain, bleeding from nipple or any secretions from nipple and redness around nipple are the symptoms of breast cancer. Change in eating habits, sore throat for a long time, continuous cough with bleeding, difficulty in swallowing, persistent fatigue and vomiting are some of the common symptoms of cancer. Based on the stage, tumor location and health of patients, cancer can be treated by surgery, ionizing radiation or chemotherapy (5). They have more side effects which affect their health and these methods of cure are more expensive due to which people prefer alternate methods.

Ayurveda is an old traditional medicinal system followed in India. *Aegle marmelos* is one of the most used ayurvedic medicines used in India (6)(7)(8). It is also called bael fruit, japanese bitter fruit, wood apple (etc) and is available in the Indian subcontinent, South Asia (9)(3)(9). It contains chemicals like tannins, flavonoids, coumarins which reduce swelling and proliferation of cells. *Aegle marmelos* contains antimicrobial, antidiarrhoeal,

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antiviral, radio protective, chemopreventive, antipyretic, ulcer healing, antigenotoxic, diuretic, antifertility which plays an important role in curing diseases(4)(10). *Aegle marmelos* is also used in the treatment of asthma, eye infections, used as snake bite antidote, antiemetic drug, treating constipation, peptic ulcer, antigenotoxic drug (etc)(9)(11)(3).. The root of the plant possesses astringent activity as a home remedy to cure ear problems. The root is dipped in neem oil and lighted and the oil dripped from the burning end is an effective medicine for ear problems. The astringent extract of root combines with the antiseptic properties of neem which is helpful in curing ear infection, pus discharge and chronic inflammation(12). The juice of the leaves are also used in treatment of ear infection(9). The powdered leaf extract is effective to cure leprosy. The oil extract from the seeds was said to be a very effective fungal infection. The ethanolic extract of the plant is very effective against *Curvularia lunata*, *Aspergillus niger* and *Rhizopus nodulans*. The bark of the tree is very effective in preventing dandruff, hairfall and scaly skin of the scalp(12).

Our team has extensive knowledge and research experience that has translated into high quality publications (13–15)(16–21),(22)(23),(24)(25),(26)(27)(28–32).

The aim of the study is to regulate chloride intracellular channel protein 1 and caspase-3 mRNA expression by hydroethanolic extract of *Aegle marmelos* fruit in human breast cancer cell.

MATERIALS AND METHODS

Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Trypsin-EDTA, fetal bovine serum (FBS), antibiotics-antimycotics, RPMI 1640 medium and phosphate buffered saline (PBS). (5,5,6,6-tetrachloro-1,1,3,3-tetraethyl benzimidazole carbocyanine iodide) and Real Time PCR kit.

Cell line and cell culture:

MCF-7 cells will be obtained from NCCS, Pune, India and grown in DMEM (Dulbecco's Modified Eagles Medium) and after 80% of confluency of the cells it will be passed to T-25 culture flask. Different doses of plant extract will be extracted, will be treated and kept for 24 hours and then the cells will be trypsinised and used in various parameters.

Cell viability by MTT assay:

The cell viability was seen using purple formazan crystals. The MCF-7 Human breast cancer cell line were treated with different concentration of Bael fruit extract for about 48 hours. Then at the end 100ul of 0.5mg/ml MTT solution were added to concentrated solution and were incubated at 37 degree celsius for an hour. The formazan crystals were dissolved in dimethyl

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sulfoxide and was incubated in dark for an hour. Then the intensity of the color developed was seen using a micro ELISA plate reader at 570nm.

Gene expression analysis by real time PCR:

Each sample was submerged in 2ml Trizol for RNA extraction and stored at -80 degree celsius until further process. cDNA was synthesized by performing on 2ug RNA in a 10ul sample volume using reverse transcriptase. Then 2ug RNA is used in amplification of CLIC-1 and caspase-3 using genes specific primers by commercially available RT-PCR kit (SyBr kit) and comparative CT method will be used to see the expression of genes.

Statistical analysis:

The statistical analysis followed is one way analysis variance (ANOVA) and Duncan's multiple range test with computer based software to find the significance of individual variations. The significance was considered at $p < 0.05$.

RESULT

Cell viability was studied by MTT assay. And the result showed that there was maximum inhibition of cancer cells at 300 and 400 $\mu\text{g/ml}$ suggesting the cytotoxic effect ($p < 0.05$) (figure 1). Effect of *Aegle marmelos* fruit extract on CLIC-1 mRNA expression in MCF-7 cells. The result showed that extract has significantly decreased the CLIC-1 mRNA expression in MCF-7 cancer cells and caspase 3 mRNA expression ($p < 0.05$) (Figure 2 and 3). 400 $\mu\text{g/ml}$ concentration of the extract is preferred from the different doses of extract since less concentration have very less effects on cancer cells and more concentration of the extract leads to diarrhoea, bowel movements (etc).

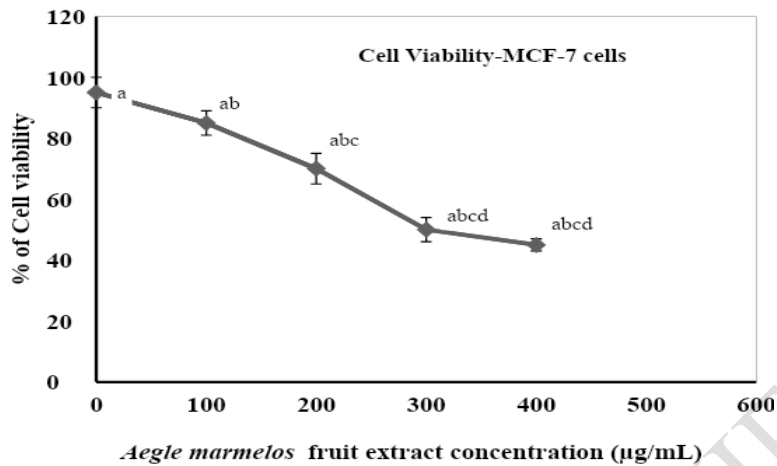


Figure 1: Assessment of cell viability. Effect of *Aegle marmelos* fruit extraction cell viability in MCF-7 cells. Each bar represents mean \pm SEM of 6 observations. Significance at $p < 0.05$, a- compared with untreated control cells, b- compared with 100ug MCF-7 cells, c-compared with 200ug treated MCF-7 cells.

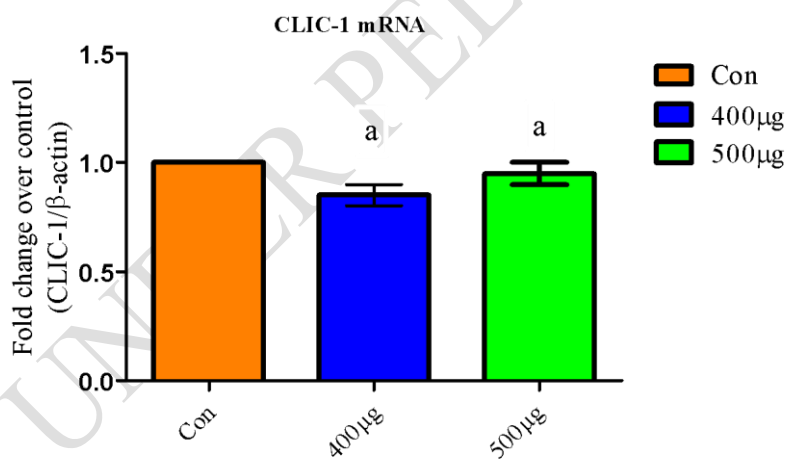


Figure 2: CLIC-1mRNA expression (Fold change over control). Orange colour denotes control, Blue color denotes 400µg and green denotes 500µg. Effect of *Aegle marmelos* fruit extract CLIC-1 mRNA expression in MCF-7cells. Each bar represents the mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells, b-compared with 200µg treated MCF-7 cells.

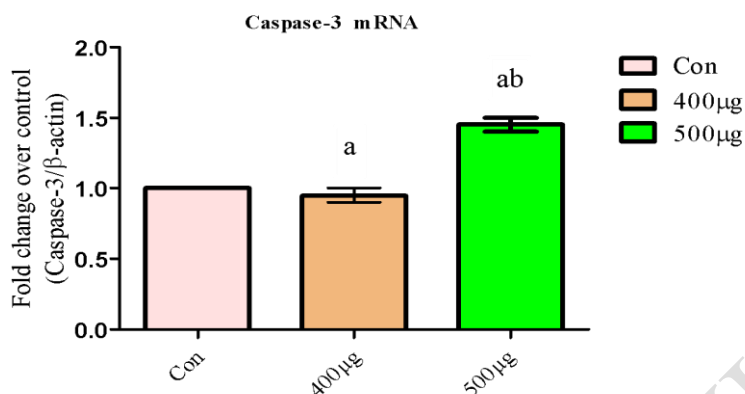


Figure 3: Caspase-3 mRNA expression (Fold change over control). Orange colour denotes control, Blue color denotes 400 μ g and green denotes 500 μ g. Effect of *Aegle marmelos* fruit extract on Caspase-3 mRNA expression in MCF-7 cells. Each bar represents a mean \pm SEM of 6 observations. Significance at $p < 0.05$, a-compared with untreated control cells, b-compared with 200 μ g treated MCF-7 cells.

DISCUSSION

Aegle marmelos contain marmenol, marmelin, marmesin, marmelosin(etc) which is used as an anticancer drug where the effect is mediated through inhibition of cell proliferation of cancer cells(6)(33). The present study proved that the given extract contains anti-inflammatory and antiproliferative properties and therefore can be used in the treatment of breast cancer.

Aegle marmelos belong to the family of Rutaceae. Bael is a slow growing, tough, medium sized subtropical tree and the tree produces fruits once in a year. According to ayurveda it is a healing tree as all its parts cure all kinds of diseases and are edible in nature. The bael fruit pulp also contains vitamin A, c, thiamine, riboflavin, niacin and minerals such as calcium, phosphorus (etc). Unripe fruits aid digestion and stomach irritation and are used in the treatment of diarrhoea and dysentery (etc)(34). Sweet drink is prepared from bael which cures bacillary dysentery. It is also used in treating rheumatism and gout. The *Aegle marmelos* leaf extract is said to be very effective against various tumor lines including breast cancer cell lines MCF-7. The *Aegle marmelos* extract is found to have antiinflammatory and

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antiproliferative activity(35) (4). The bael leaf is also said to inhibit the growth of breast cancer cell lines (MCF-7), leukemia K562,T Lymphoid Jurkat, Melanoma colo38, erythroleukemia HEL. The roots of the plant prevent dysentery, fever and the leaves reduce bowel movements, bleeding piles and diarrhoea(36). The leaves extract of the plant can be used in treating kidney problems and the paste of ground roots in bael in butter is used in treating insomnia. The pulp of the fruit has detergent properties, therefore can be used as a herbal soap for allergic patients. The study needs to be further continued for 2-3 months and hence the study will be more effective.

In the present study, *Aegle marmelos* fruit extract has effects on MCF-7 breast cancer cell lines and showed anti-inflammatory and antiproliferative activity. The activity has been proven only fruit extract, further studies required to confirm which part of the plant has potent anti-inflammatory and antiproliferative activity.

CONCLUSION

From this study it has been proved that *Aegle marmelos* have effects on MCF-7 breast cancer cell lines. It showed anti-inflammatory and antiproliferative activity, has less side effects and therefore can be used as a drug to treat breast cancer.

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.

REFERENCE:

1. SAGE Journals: Your gateway to world-class research journals [Internet]. [cited 2021 Mar 8]. Available from: <https://journals.sagepub.com/action/cookieAbsent>
2. Wang X, Ricciuti B, Nguyen T, Li X, Rabin MS, Awad MM, et al. Association between smoking history and tumor mutation burden in advanced non-small cell lung cancer. *Cancer Res* [Internet]. 2021 Jan 1 [cited 2021 Mar 8]; Available from: <https://cancerres.aacrjournals.org/content/early/2021/03/02/0008-5472.CAN-20-3991.abstract>
3. Zhang C, Gao Y, Du C, Markowitz GJ, Fu J, Zhang Z, et al. Hepatitis B-induced IL-8 Promotes Hepatocellular Carcinoma Venous Metastasis and Intrahepatic Treg Accumulation. *Cancer Res* [Internet]. 2021 Jan 1 [cited 2021 Mar 8]; Available from: <https://cancerres.aacrjournals.org/content/early/2021/03/02/0008-5472.CAN-20->

Comment [CT6]: Limitations of the Study?
Recommendations for future studies...

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4. Akhouri V, Kumari M, Kumar A. Therapeutic effect of Aegle marmelos fruit extract against DMBA induced breast cancer in rats. *Sci Rep* [Internet]. 2020 Oct 22 [cited 2021 Mar 8];10(1). Available from: <https://pubmed.ncbi.nlm.nih.gov/33093498/>
5. Bhatti R, Singh J, Saxena AK, Suri N, Ishar MPS. Pharmacognostic standardisation and antiproliferative activity of Aegle marmelos (L.) Correa leaves in various human cancer cell lines. *Indian J Pharm Sci.* 2013;75(6):628.
6. Phytochemical profile and pharmacological activity of Aegle marmelos Linn. *J Integr Med.* 2018 May 1;16(3):153–63.
7. Rajaram A, Vanaja GR, Vyakaranam P, Rachamalla A, Reddy GV, Anilkumar K, et al. Anti-inflammatory profile of Aegle marmelos (L) Correa (Bilva) with special reference to young roots grown in different parts of India. *J Ayurveda Integr Med.* 2018;9(2):90.
8. Aegle Marmelos [Internet]. [cited 2021 Mar 8]. Available from: <https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/aegle-marmelos#:~:text=Aegle%20marmelos%20commonly%20known%20as,to%20golden%20orange%20when%20ripe.&text=Its%20edible%20fruit%2C%20leaf%2C%20root,the%20Ayurvedic%20medicine%20in%20India.>
9. SAGE Journals: Your gateway to world-class research journals [Internet]. [cited 2021 Mar 8]. Available from: <https://journals.sagepub.com/action/cookieAbsent>
10. Mujeeb F, Bajpai P, Pathak N. Phytochemical Evaluation, Antimicrobial Activity, and Determination of Bioactive Components from Leaves of Aegle marmelos. *Biomed Res Int* [Internet]. 2014 May 11 [cited 2021 Mar 9];2014. Available from: <https://www.hindawi.com/journals/bmri/2014/497606/>
11. Nigam VG, Nambiar VS. Knowledge, Practice And Use Of Aegle Marmelos (L.) Correa Leaves Among Naturopathy And Ayurvedic Practitioners Of Vadodara City and Desk Review On Various Commercial Formulations available In Health And Disease Specially Diabetes. *Phytomedicine.* 2017 Sep 2;9(3):451.
12. Rishabha M, Ajay K, Anupama S, Kulkarni GT. Pharmacological Screening, Ayurvedic values and Commercial Utility of Aegle Marmelos. *International Journal of Drug Development and Research* [Internet]. 2012 [cited 2021 Mar 8];4(1). Available from: <https://www.ijddr.in/abstract/pharmacological-screening-ayurvedic-values-and-commercialutility-of-aegle-marmelos-4949.html>
13. 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.
14. 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 γ Mediated Pathways. *Ind J Pharm Educ.* 2021 Mar 19;55(1s):s265–74.

15. 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.
16. 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.
17. Clarizia G, Bernardo P. Diverse Applications of Organic-Inorganic Nanocomposites: Emerging Research and Opportunities: Emerging Research and Opportunities. IGI Global; 2019. 237 p.
18. 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>
19. 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.
20. 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.
21. 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>
22. Ezhilarasan D. Critical role of estrogen in the progression of chronic liver diseases. *Hepatobiliary Pancreat Dis Int*. 2020 Oct;19(5):429–34.
23. 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.
24. 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>
25. 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>
26. 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>

27. 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.
28. 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.
29. 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>
30. 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>
31. 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>
32. 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.
33. Atul NP, Nilesh VD, Akkatai AR, Kamlakar SK. A review on Aegle marmelos: a potential medicinal tree. *International Research Journal of Pharmacy*. 2012 Aug 1;3(8):86–91.
34. Pathirana CK, Madhujith T, Eeswara J. Bael (Aegle marmelos L. Corrêa), a Medicinal Tree with Immense Economic Potentials. *Advances in Agriculture* [Internet]. 2020 Dec 9 [cited 2021 Mar 9];2020. Available from: <https://www.hindawi.com/journals/aag/2020/8814018/>
35. Aegle Marmelos [Internet]. [cited 2021 Mar 8]. Available from: <https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/aegle-marmelos>
36. Brijesh S, Daswani P, Tetali P, Antia N, Birdi T. Studies on the antidiarrhoeal activity of Aegle marmelos unripe fruit: Validating its traditional usage. *BMC Complement Altern Med*. 2009 Nov 23;9(1):1–12.

UNDER PEER REVIEW

