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Oral wound healing efficacy of 1% *Myristica fragrans* (nutmeg) determined using MTT assay: An *in vitro* study

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Abstract
Wound healing is a complex series of biological processes aimed at tissue regeneration and repair. Traditional therapies can also be combined with modern clinical techniques, biomaterials, and medicines to reduce bacterial resistance and hasten the healing process. *Myristica fragrans* (nutmeg) being a traditional medicinal herb has antimicrobial, anti-inflammatory, anti-oxidant and healing properties. Especially, 1% ethanolic extract of *Mystrica fragrans* has shown to have antibacterial and anti-inflammatory properties whereas its oral wound healing property has not been evaluated. Hence, this *in-vitro* study aims to evaluate the oral wound healing efficacy of 1% ethanolic extract of *Myristica fragrans* (nutmeg) by observing its effect on cell viability, cytotoxicity and proliferation of human gingival fibroblast. The results demonstrated that 1% ethanolic extract of *Myristica fragrans* had statistically significant (p=0.006) increase in the cell proliferation and viability at 24 hours and 72 hours respectively with no cytotoxicity at both time intervals. Present study demonstrated that 1% ethanolic extract of *Myristica fragrans* can be effectively used to enhance oral wound healing without any adverse effects.

Keywords: Cytotoxicity, human gingival fibroblast, *Myristica fragrans*, proliferation, viability, wound healing

1. Introduction
The human body begins a complicated series of biological processes at the time of injury towards the repair and tissue regeneration for lost or injured tissue through wound healing [1]. Fibroblasts are vital cell involved in wound healing. They migrate to the wound area, multiply, and engage in a number of crucial actions while being strictly controlled by injury-mediated factors and the gradually changing environment of the healing wound, which is essential for the wound final stability [2]. Studies have shown that fibroblasts isolated from oral mucosa and skin have a variety of inherent differences, such as a different capacity for tissue remodeling, distinctive secretion patterns for particular extracellular matrix molecules, different migration and cell adhesion properties, and differential expression of extracellular matrix receptors and response to growth factors. These differences could be attributed to the faster and scarless healing of the oral mucosa when compared to skin [3]. Further to minimize bacterial resistance and speed up the healing process, traditional remedies can also be integrated with contemporary clinical procedures, biomaterials, and medications [4]. *Myristica fragrans* (nutmeg) is one of these legendary, age-old foods that has long been used as a spice. Besides this, it was also used as a traditional medicine. *Myristica fragrans* has four parts - The skin, the fruit, the seed and the mace (figure 1). The brown seed has a large circulatory supply which is responsible for its anti-bacterial, anti-inflammatory, anti-collagenolytic and anti-oxidant effects on the gingiva and the periodontium [5]. *Myristica fragrans* contains a volatile oil, a fixed oil, proteins, fats, starch, and mucilage. It yields 5–15% of volatile oil, 25-30% fixed oils [6]. Studies have demonstrated 1% (10mg/ml) ethanolic extract of *Myristica fragrans*, to have antibacterial and anti-inflammatory properties. Malabaricon C and malabaricon B are a major component of *myristica* species which is responsible for its anti-oxidant and healing property due its increased expression of epidermal growth factor (EGF) [7].

Fig 1: Nutmeg fruit

Viability and proliferation of fibroblasts is one of the effective ways of analysing wound healing potential. MTT assay is one of the most commonly used colorimetric assay to assess cytotoxicity, cell viability and proliferation. To the best of our knowledge no studies have been done to evaluate the effect of 1% ethanolic extract of *Myristica fragrans* on the viability, proliferation and cytotoxicity of human gingival fibroblasts.
Hence the aim of the present study is to evaluate the oral wound healing properties of 1% *Myristica fragrans* by observing its effects on Human gingival fibroblasts.

2. Materials and Methods

The study employed an *in vitro* experimental design. The institutional ethics committee of Bapuji Dental College and Hospital, Davangere, granted ethical clearance with reference number BDC Exam/548/2021-22.

2.1 Procurement of material for the study

Florocia Organic 100% Natural Nutmeg (*Myristica fragrans*, Kerala) was procured, dried and powdered finely. HGF (Human gingival fibroblasts) cell lines, media and reagents were supplied by Maratha Mandal Dental College, Belgaum, Karnataka, India.

2.2 Preparation of ethanolic extract of *Myristica fragrans*

The ethanolic extract of *Myristica fragrans* (nutmeg) powder was performed by maceration technique. 200 gm of *Myristica fragrans* (nutmeg) powder was weighed and transferred to a 500 ml beaker. 300 ml of ethanol (95%) was added to the beaker, the contents were mixed thoroughly and were kept undisturbed for 7 days. After 7 days the content was filtered with muslin cloth. The filtrate was collected and allowed to evaporate for another 7 days. Thus 3gm of 100% ethanolic extract of *Myristica fragrans* was obtained (Figure 2) [8].

Fig 2: Ethanolic extract of *Myristica fragrans*

2.3 Preparation of 1% ethanolic extract of *Myristica fragrans*.

Stock solution of *Myristica fragrans* was obtained by treating it with Dimethyl sulphur oxide (DMSO) reagent. Working solution of 1% *Myristica fragrans* was obtained by serial dilution of stock solution in Eagle's minimum essential medium (DMEM) solutions (Figure 3) [9].

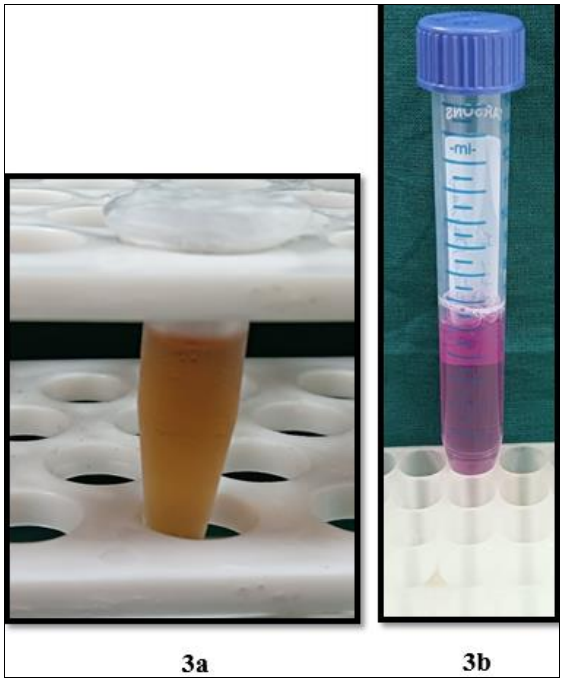

Fig 3: a Stock solution b) Working solution (1% *Myristica fragrans*).

2.4 Human Gingival fibroblast cell culture

The cell line used for the study was Human gingival fibroblasts stored at the repository of central Research Laboratory of Maratha Mandal's NGH Institute of Dental Sciences, Belagavi. The cell line was maintained in 96 wells micro titer plate containing Eagle's minimum essential medium (DMEM) media supplemented with 10% heat inactivated fetal calf serum (FCS), containing 5% of mixture of Gentamicin (10 ug), Penicillin (100 Units/ ml) and Streptomycin (100 µg/ml) in presence of 5% carbon dioxide (CO_2) at 37 ºC for 48-72 hours [9].

2.5 MTT Assay (cellular viability, cytotoxicity and proliferation) [9-10]

Initially the supernatant from the 96 well plate was removed and fresh Eagle's minimum essential medium (DMEM) solution was added. The test group contained working solution of 1% *Myristica fragrans* treated with Eagle's minimum essential medium (DMEM) solution and control group had only DMEM solution without the working solution. The solutions were added to the respective well plates and was incubated for 24 hours and 72 hours at 37 ºC in a humidified atmosphere of 5% Co2, stock solution of MTT was added to each well (20 µl, 5 mg per ml in sterile PBS) for further 4 hours of incubation (Figure 4).

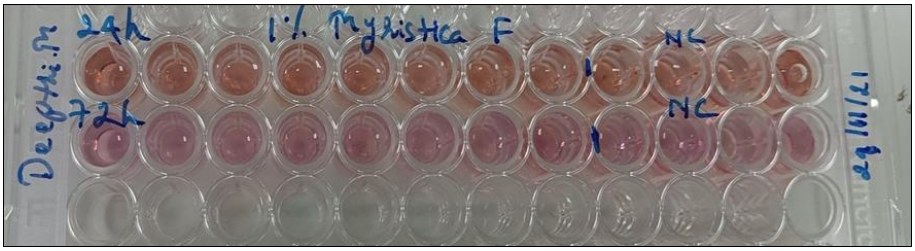

Fig 4: Test (1% *Myristica fragrans*) and negative control (NC) with incubation time (24hrs and 72hrs) being labelled on the 96 well micro titer

After 4 hours, the supernatant was carefully aspirated, the precipitated crystals of "Formazan blue' were solubilized by adding Dimethyl sulphur oxide (DMSO) (100 µl). The Optical density was then measured at a wavelength of 570 nm by using BIO-RAD. The mean of five readings was considered for calculation of result. The following formula was used, where OD stands for optical density:

$$\text{Surviving cells (\%)} = \frac{\text{Mean OD of test compound}}{\text{Mean OD at control}} \times 100$$

The same procedure is repeated after 72hours of incubation and the surviving cells (%) are obtained by the above mentioned formula. Results of cell viability, cytotoxicity and proliferation was tabulated for both 24hrs and 72hrs of incubation period.

3. Results

After 24hours incubation of human gingival fibroblast with 1% *Myristica fragrans* at 37 ºC it had 109% cell viability, 9% cell proliferation and 0% cell cytotoxicity whereas control group(only DMEM solution) showed 100% cell viability and 0% cytotoxicity this difference was statistically significant (p<0.01).

After 72 hours incubation of human gingival fibroblast with 1% *Myristica fragrans* at 37 ºC it had 104% cell viability, 4% cell proliferation and 0% cytotoxicity whereas control group (only DMEM solution) showed 100% cell viability and 0% cytotoxicity this difference was also statistically significant (p<0.01) (Table 1).

Table 1: Cell viability, cell death and cell proliferation between the two groups at different time intervals.

	1% *Myristica fragrans*	Control
After 24 hours		
Cell viability	109%	100%
Cell death	0	0
Cell proliferation	9%	0%
After 72 hours		
Cell viability	104%	100%
Cell death	0	0
Cell proliferation	4%	0%

3.1 Intragroup comparison

A statistically significant increase in the mean OD in 1% *Myristica fragrans* group was observed; from 0.446±0.003 at 24 hours to 0.681±0.003 at 72 hours (p=0.006). However, a decrease was observed in control group, from 0.853±0.001 to 0.593±0.003, which was not statistically significance (p=0.125) (Table 2).

Table 2: Intragroup Comparison of Effect 1% *Myristica fragrans* on Human Gingival Fibroblast at 24 And 72 Hours.

Time	N	Mean	Std. Deviation	Test statistic[a]	p-value
1% *Myristica fragrans*					
After 24 hours	4	0.44688	0.003182	-1.452	0.006*
After 72 hours	4	0.68138	0.003335		
Control					
After 24 hours	4	0.85375	0.001708	-1.826	0.125
After 72 hours	4	0.59300	0.003162		

3.2 Intergroup comparison

Intergroup comparison shows a higher mean OD at 24 hours in control group (0.853±0.001) as compared to 1% *Myristica fragrans* (0.446±0.003) this difference was statistically significant (p=0.006). However, at 72 hours, the mean OD of 1% *Myristica fragrans* was 0.681±0.003, and mean OD of control group was 0.593±0.003. This difference was also statistically significant at p=0.006 (Table 3).

Table 3: Intergroup comparison of effect 1% *Myristica fragrans* on human gingival fibroblast at 24 and 72 hours.

Group	N	Mean	Std. Deviation	Test statistic[a]	p-value
After 24 hours					
1% *Myristica fragrans*	4	0.44688	0.003182	-2.132	0.006*
Control	4	0.85375	0.001708		
After 72 hours					
1% *Myristica fragrans*	4	0.68138	0.003335	-2.123	0.006*
Control	4	0.59300	0.003162		

4. Discussion

The basic goal of integrative wound treatment is to speed up healing while causing the least amount of pain and discomfort [3]. Many substances have been discovered to promote wound healing. The primary and essential source of contemporary medicine around the world is thought to be herbal remedies and their by-products. Many herbal medicines are utilised to promote healing in order to overcome the shortcomings of conventional medications, such as drug allergy and resistance [11-12]. Consequently, a variety of herbal products are currently being researched in this approach. Recent research has identified *Myristica fragrans* (nutmeg) as one such compound among these herbal products.

Myristica fragrans (nutmeg) have shown to have anti-bacterial, anti-inflammatory, and anti-oxidant effects on the gingiva and the periodontium. Among all extracts tested, ethanolic extract of flesh was found to have the highest significant inhibitory effect against Gram-positive and Gram-negative bacteria and also highest bactericidal effects. According to a study done by Varghese *et al*, 2019 among the different concentrations of ethanolic extract of *Myristica fragrans*, 1% (10 mg/ml) has shown to have a wide antimicrobial spectrum against *Porphyromomas gingivalis*, *S. mutans*, *A. viscosus* and *S. sanguis* [13].

Phenolic compounds are important plant constituents with

redox properties responsible for antioxidant activity. This antioxidant activity is determined through ferric reducing antioxidant power (FRAP) assay which measures the antioxidant potential in samples through the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by antioxidants present in the samples. Studies have demonstrated highest total phenolic content (TPC) and ferric reducing antioxidant power (FRAP) in ethanolic extracts of *Myristica fragrans* owing to its antioxidant property [14]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in virtually all eukaryotic cells. These molecules control a number of physiological processes, such as differentiation, hypertrophy, proliferation, and metabolism, but when they are present in excess, they interact with lipids, proteins, and nucleic acids to change the structural and functional characteristics of their targets, resulting in severe tissue dysfunction and injury. Thus its benefit/risk ratio must be optimal for favoring the vitality of the tissues. Studies have identified highly favourable benefit/risk ratio of reactive oxygen species and reactive nitrogen species (ROS/RNS) was attainable with *Myristica fragrans* species which attributed to its anti-inflammatory property [15]. Malabaricone B and malabaricone C, the major constituents of *Myristica* species are responsible for its antioxidant property, increased expression of EGF (epidermal growth factor) receptors accounts to their healing capacities [16]. As *Myristica* serves all the properties essential for wound healing and as 1% ethanolic extract in particular had antimicrobial property it was essential to analyse its impact on wound healing in oral cavity through its effect on human gingival fibroblast [13].

The human gingival fibroblasts (hGFs) are crucial cells of periodontal connective tissue. They aid in the development of new tissues through proliferation, differentiation, and remodelling which promotes wound healing in the oral cavity [17].

The commonly used assay to determine the cell viability, proliferation and cytotoxicity is MTT assay/Cell viability assay, it is a non-radioactive colorimetric assay technique, to detect cellular metabolic activity as a sign of cell viability, proliferation, and cytotoxicity using a MTT reagent [18].

Our study results reveal that at both 24 hours and 72 hours incubation of human gingival fibroblast with 1% *Myristica fragrans* had statistically significant increase in the cell proliferation and with no cytotoxicity. A recent study by Karpinska *et al*, 2022, evaluated the effect of *Myristica fragrans* on human skin fibroblast (Hs68 cells) incubated for 48hrs using MTT assay. This study concluded that the cells were viable and *myristica* had no cytotoxic effect at a concentration range between 1 µM -10µM. Our study results showed similarity to this work but on human gingival fibroblast by maintaining cell viability and no cytotoxicity [19].

Previous study by Varghese *et al* 2019, assessed the effect of different concentration of neem extract and chlorhexidine (CHX) on human gingival fibroblast (hGF) using MTT assay. It was revealed that cytotoxicity assay showed 10% concentration of CHX is responsible for maximum cell death. Neem extract showed maximum cytotoxicity on hGF at concentration of 75%. Whereas our study had revealed that *Myristica fragrans* had no cytotoxic effect on hGF at 1% concentration of ethanolic extract, this could be attributed to its antimicrobial property and anti-inflammatory property thus yielding to its acceptance by the cells for wound healing [13].

Similar studies were performed in which cytotoxic activity of the *Myristica fragrans* with a concentration ranging between 25 to 125 µg/mL on human oral epidermal carcinoma KB cell lines and 50 to 100 µg/mL on human leukemia T cell line incubated for 24hours and 48hours respectively was assessed. These studies concluded cytotoxic effect of *Myristica fragrans* on human oral epidermal carcinoma KB cell lines and human leukemia T cell line thus suggesting its anticancer potential. In our study also cytotoxic activity of *Myristica fragrans* is determined but on human gingival fibroblast, which showed no cytotoxic effect but rather increased proliferation of fibroblast cell lines which owes to its healing potential [20]. To conclude, 1% ethanolic extract of *Myristica fragrans* have already proven to be an efficient antimicrobial agent owing to the activity of malabaricon C and malabaricon B. The current study adds to the available literature as it demonstrates that 1% *Myristica fragrans* can be utilised to enhance the proliferation of human gingival fibroblasts without any cell toxicity.

5. Conclusion

This discovery offers up the possibility of efficiently treating oral wounds, particularly those brought on by periodontal surgical treatments, with 1% ethanolic extract of *Myristica fragrans* while reducing patient morbidity and inconvenience. Further investigations must be carried out to assess the angiogenic property and cell migration using different concentration and different solvents. Future vivo studies are necessary to validate the results obtained.

6. References

1. Velnar T, Bailey T, Smrkolj V. The wound healing process: an overview of the cellular and molecular mechanisms. Journal of International Medical Research. 2009;37:1528-1542.
2. Darby IA, Laverdet B, Bonté F, Desmoulière A. Fibroblasts and myofibroblasts in wound healing. Clinical,Cosmetic and Investigational Dermatology. 2014;7:301.
3. Kirsner RS, Eaglstein WH. The wound healing process. Dermatologic Clinics. 1993;11:629-640.
4. Ayyanar M, Ignacimuthu S. Herbal medicines for wound healing among tribal people in Southern India: Ethnobotanical and Scientific evidences. International Journal of Applied Research in Natural Products. 2009;2:29-42.
5. Thangaselvabai T, Sudha KR, Selvakumar T, Balakumbahan R. Nutmeg (*Myristica fragrans* Houtt)-the twin spice-a review. Agricultural Reviews. 2011;32:283-93.
6. Naeem N, Rehman R, Mushtaq A, Ghania JB. Nutmeg: A review on uses and biological properties. International Journal of Chemical and Biological Sciences. 2016;9:107-10.
7. Raina R, Prawez S, Verma PK, Pankaj NK. Medicinal plants and their role in wound healing. Vet Scan. 2008;3:1-7.
8. Nair AJ, Soman P, George A, Surendran SA. Formulation of *Myristica fragrans* (Nutmeg) topical gel and it's *in vitro* evaluation for anti-inflammatory activity. International Journal of Pharmacy and Technology. 2016;8:11065-76.
9. Mandroli PS, Prabhakar AR, Bhat K, Krishnamurthy S, Bogar C. An *in vitro* evaluation of cytotoxicity of curcumin against human periodontal ligament fibroblasts. Ayu. 2019;40:192-195.
10. Bulbule AM, Mandroli PS, Bhat KG, Bogar CM. *In vitro* evaluation of cytotoxicity of *Emblica officinalis* (Amla)

on cultured human primary dental pulp fibroblasts. Journal of Indian Society of Pedodontics and Preventive Dentistry. 2019;37:251-257.
11. Douglas M, Alan L. Nutritional support for wound healing. Alternative Medicine Review. 2003;8:359-377.
12. Ayyanar M, Ignacimuthu S. Herbal medicines for wound healing among tribal people in Southern India: Ethnobotanical and Scientific evidences. International journal of applied research in natural products. 2009;2:29-42.
13. Dr Varghese Suresh, Dr Sapna B. Comparative evaluation of antibacterial efficacy of *Myristica fragrans* (1%), a novel mouth wash with chlorhexidine (0.2%) mouth wash on salivary streptococcus mutans counts: an *in vivo*, parallel group pilot study. International Journal of Current Advanced Research. 2019;8:18381-18386.
14. Tan KP, Khoo HE, Azrina A. Comparison of antioxidant components and antioxidant capacity in different parts of nutmeg (*Myristica fragrans*). International Food Research Journal. 2013;20:1049.
15. Gomes A, Fernandes E, Lima JL, Mira L, Corvo ML. Molecular mechanisms of anti-inflammatory activity mediated by flavonoids. Current Medicinal Chemistry. 2008;15:1586-605.
16. Banerjee D, Bauri AK, Guha RK, Bandyopadhyay SK, Chattopadhyay S. Healing properties of malabaricone B and malabaricone C, against indomethacin-induced gastric ulceration and mechanism of action. European Journal of Pharmacology. 2008;578:300-12.
17. Yussof SJ, Omar E, Pai DR, Sood S. Cellular events and biomarkers of wound healing. Indian Journal of Plastic Surgery. 2012;45:220-8.
18. Aslantürk ÖS. *In vitro* cytotoxicity and cell viability assays: principles, advantages, and disadvantages. Genotoxicity-A predictable risk to our actual world. 2018;11:64-80.
19. Janik-Karpinska E, Ceremuga M, Wieckowska M, Szyposzynska M, Niemcewicz M, Synowiec E, *et al*. Direct T-2 Toxicity on Human Skin-Fibroblast Hs68 Cell Line-*In vitro* Study. International Journal of Molecular Sciences. 2022;23:4929.
20. Rengasamy G, Venkataraman A, Veeraraghavan VP, Jainu M. Cytotoxic and apoptotic potential of *Myristica fragrans* Houtt. (mace) extract on human oral epidermal carcinoma KB cell lines. Brazilian Journal of Pharmaceutical Sciences. 2018;29:54.