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Biocompatibility Assessment of Chamomile Volatile Oil, Double Antibiotic Paste in Subcutaneous Tissue of Rats

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ABSTRACT

This study aimed to assess the biocompatibility of Matricaria *Recutita* volatile oil versus 1% double antibiotic paste on subcutaneous tissue in rats through implantation testing. Thirty-six rats were selected for In *Vivo* assay. The prepared polyethylene tubes were sealed at one end with cyanoacrylate gel carrying the tested medicament and divided into 3 groups (I, II, and III) (36 tubes each) representing the tested material. Group I is the control group (Empty tube), group II comprises 1% DAP (5µl), whereas group III comprises Chamomile volatile oil (2.5µl) according to effective antimicrobial concentration against E. faecalis (ATCC29212), each group was subdivided according to the post-implantation evaluation period at 7, 14, and 21 days. Animals were euthanized and tissue sections were prepared for histopathological and microscopic assessments of inflammation and fibrous capsule thickness & length of each group at different time intervals. The values of fibrous capsule thickness & length (µm) were of the highest value in 1% DAP associated with the highest inflammation intensity followed by the control group, while the lowest value of fibrous capsule thickness & length was in the chamomile group associated with less inflammation, respectively at all-time intervals with statistically significant difference between them. Therefore, chamomile volatile oil exhibits superior biocompatibility over 1% DAP with promising application in endodontics as a biocompatible intracanal disinfectant.

KEYWORDS: Biocompatibility, Chamomile volatile oil, Double antibiotic paste, Implantation test.

1. INTRODUCTION

Dental pulp is a highly vascularized and innervated tissue that contains a diverse population of pluripotent stem cells. Current endodontic therapy approaches emphasize pulp tissue preservation and tooth pulp regeneration following pathological assaults. Human dental pulp stem cells (DPSCs) are being studied as a stem cell-based pulp regeneration therapy [1].

Regenerative endodontic therapy is a biologically based procedure that replaces damaged tissues like dentin, root structures, and pulp-dentin complex cells [2]. The use of stem cells, growth factors, and scaffolds are the main components of regenerative endodontics. These components are put in close proximity to the pulp tissue and must be easy to manipulate, do not cause tooth discoloration, form an antibacterial seal, and be biocompatible [3].

In regenerative endodontic operations, disinfecting chemicals should strike a balance between disinfection and biocompatibility. Although proper disinfection can provide a sterile environment, pulp tissue regeneration necessitates the use of biocompatible molecules to govern the release of growth factors as well as the survival, proliferation, and differentiation of stem cells [4].

Based on the understanding of the varied bacterial communities seen in juvenile teeth, it was suggested that using a combination of antibiotics would more successfully sterilize the canal while simultaneously lowering the risk of antibiotic resistance [5].

The triple antibiotic paste, which contains minocycline, ciprofloxacin, and metronidazole, has

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regained popularity as an intracanal antibiotic for root canal disinfection during regenerative endodontic therapy. According to studies, the paste can kill germs and provide a sterilized environment inside the root canal system, allowing new tissue to infiltrate and develop into the radicular area, allowing for healing [6]. However, crown darkening was the most common side effect of using triple antibiotic paste (TAP), according to various researches. As a result, some studies have suggested replacing TAP with double antibiotic paste (DAP) (metronidazole and ciprofloxacin) [7].

Because adequate disinfection of the root canal through intracanal administration of medicaments is one of the most critical phases in regenerative endodontics, the development of a novel biocompatible intracanal medicament is required [8].

For decades, chamomile (*Matricaria Recutita*) has been used for therapeutic purposes and has remained one of the most popular herbal medicines. Species from the Matricaria genus are widely used in Europe, Latin America, Asia and Africa to treat signs and symptoms related to bacterial infections. Generally, the aerial parts are used, especially the flowers, although the use of the whole plant has also been mentioned. For the treatment of gastrointestinal and urinary disorders the main form of administration is oral, where teas are prepared in the form of decoction or infusion. However, for problems related to the respiratory system, in addition to the oral use, inhalation is also indicated, while topical applications are cited to treat skin disorders. Its antibacterial properties have been investigated, and they are primarily attributed to the presence of α-bisabolol, luteolin, quercetin, and apigenin against a wide range of bacteria, including *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus salivarius*, and fungi such as *Candida albicans*. Its antifungal action is thought to be due to bisabolol's particular suppression of ergosterol production [9,10,11].

An investigation was conducted in order to examine the antibacterial activity of two dosages of vetiver and chamomile essential oils against *Enterococcus faecalis*, compared to either chlorhexidine or calcium hydroxide, chamomile oils showed good efficacy in eliminating *E. faecalis* in root canals throughout a variety of time periods, although vetiver oil's action did not last as long [12].

This study was conducted to evaluate the biocompatibility of chamomile volatile oil used for root canal disinfection. The evaluation was performed through an implantation test.

The null hypothesis of this study was that there is no difference in biocompatibility between chamomile volatile oil and 1% double antibiotic paste in the subcutaneous connective tissue of rats.

2.MATERIALS & METHODS:

This study was conducted in National Research Centre as a randomized controlled preclinical trial analyzing the In *Vivo* reaction of subcutaneous connective tissue of rats and its ability to induce inflammation assessed by hematoxylin and eosin dye staining under the light microscope. Ethical approval for the use of experimental rats was obtained in accordance with guidelines from the Research Ethics Committee (REC), Faculty of Dental Medicine for Girls, AL-Azhar University, Code (REC-EN-22-03). The study was performed according to the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments).

The question in this study was addressed in terms of PICO question which involves 4 elements: [problem (P), intervention (I), comparison (C), and outcome (O)] as follows:

P. Cytotoxicity of intracanal medicaments upon stem cells (Problem).

I. Introduction of new biocompatible intracanal medicament (chamomile volatile oil) (Intervention).

C. 1% double antibiotic paste as an intracanal medicament (Comparison).

O. Biocompatibility results of chamomile volatile oil, 1% double antibiotic paste in relation to the control group (Outcome).

2.a.Sample Calculation:

A power analysis was carried out to ensure that enough power was available to perform a statistical test of the null hypothesis that no difference exists between the groups being examined. An alpha level of (0.05), a beta of (0.2), i.e., power=80%, and effect size (f) of (0.55) were used based on the results of a previous study. A total of 36 samples (n) were expected, with 12 samples in each category. The sample size was calculated using G*Power version 3.1.9.7. [13,14].

2.b.Preparation of the intracanal medicaments:

1-Preparation of 1% double antibiotic paste: Ciprofloxacin (250 mg. Egyptian INT. pharmaceutical industries Co.) and Metronidazole (500 mg. Sanofi-aventis Egypt S.A.E) were mixed in a 1:1 proportion along with 2.5 ml distilled water & 2.5 ml propylene glycol till a creamy homogenous consistency is obtained at room temperature 5 µl of DAP was loaded into the polyethylene tube guided by the micropipette as the previous study indicated minimum inhibitory concentration against *Enterococcus faecalis* (ATCC 29212) [8].

2-Preparation of Chamomile (*Matricaria Recutita*) essential oil:

Matricaria Recutita, L. flower heads were collected from cultivated chamomile plants growing in National Research Centre Farm for Research and Production at Al-Nubaria region, Al Bahira

Governorate its location is latitude 30º 30' 1.4" N, and longitude 30º 19' 10.9" E, Egypt Chamomile herbarium spacemen was preserved in the National Research Centre herbarium under No.M143. The air-dried flower heads were collected from the harvest in March 2021 for the essential oil extraction with hydro-distillation (water distillation) at the Laboratory of Medicinal and Aromatic Plants Research Department, National Research Centre, Giza, Egypt.

Extraction and characterization (chromatography) of the volatile oil of Chamomile (*Matricaria Recutita*): Two kgs of air-dried flower heads of Chamomile (*Matricaria Recutita*) were subjected for essential oil extraction with hydro-distillation using Clevenger-type apparatus for 3h according to the Egyptian Pharmacopoeia (1984) and Günter (1995).The resulted essential oil was collected to reach about 5.0 ml, was dehydrated with anhydrous sodium sulfate, and kept in the refrigerator until GC-MS and biological activities analyses. The identification of the main constituents of the volatile oil of Chamomile and their relative percentages were determined using GC-MS [15,16].

2.c.GC-MS analysis:

The GC-MS analysis of the volatile oil was carried out using gas chromatography-mass spectrometry instrument stands at the Department of Medicinal and Aromatic Plants Research, National Research Center with the following specifications. Instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer) as mentioned by Omer *et al*., (2016) and Eldeghedy *et al*., (2022). The Mass spectra of each compound was obtained by electron ionization (EI) at 70 eV; using a spectral range of m/z 50-550 and solvent delay 3 min were. Identification and confirmation of separated compounds were determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data along with published data [17,18].

The antibacterial concentration of chamomile volatile oil is determined using *Enterococcus faecalis* (ATCC 29212) as the reference bacterial strain for determining antibiotic efficacy, 2.5 µl of chamomile oil was utilized for the measurement of the size of the zone of growth inhibition (ZOGI) measured which indicated the strength of antibacterial activity [12].

2.d.Implantation test:

Thirty-six rats weighing between 200 and 250 grams were utilized. The animals were kept in plastic boxes (40 x 32 x 17 cm) in a temperature-controlled environment (12 hours light / 12 hours dark; 21 - 25°C), getting balanced food and water. The animals were anesthetized and prepared for receiving the tested materials. A 1 cm incision was made in each quadrant of the dorsal region in a head-tail direction with a No. 15 scalpel blade to create three scapular and caudal pockets equidistant from the spine. To receive the implants, blunt-tipped scissors were used to dissect the subcutaneous cellular tissue bluntly, generating surgical recesses with a mean depth of 20 mm parallel to the spine. Test materials were prepared under aseptic circumstances and inserted into empty polyethylene tubes with a micropipette and measuring indication right before implantation (2.5 µL for Chamomile volatile Oil, and 5 µL for double antibiotic paste). Each rat had three subcutaneous polyethylene tubes inserted in its surgical recess: an empty polyethylene tube (control), a polyethylene tube containing chamomile volatile oil, and a polyethylene tube containing 1% DAP. The animals were kept in individual boxes with regular food and water balanced diet, and the incisions were closed using polyglycolic acid sutures (International Sutures Manufacturing Co., Egypt). The animals were kept in separate compartments and fed a balanced diet of food and water. According to the allocated follow-up term, the animals were euthanized at the termination of the experimental periods (7,14,21 days after implantation).

2.e.Samples Classification

The tubes were divided into 3 groups (I, II, and III) (total n=108) (36 tubes each) representing the tested material.

Group I: In which empty polyethylene tubes were implanted. **(**Control group)

Group II: In which polyethylene tubes containing 1% Double antibiotic paste were implanted (5 µl).

Group III: In which polyethylene tubes containing Chamomile (Matricaria *Recutita*) were implanted (2.5 µl).

Each group was subdivided according to the post-treatment evaluation period at 7, 14, and 21 days (subgroups A, B, and C) (12 rats each).

2.f.Methods of evaluation:

Histopathological evaluation: After all animals were euthanized by anesthetic overdose, autopsy samples from the subcutaneous tissues of the skin of rats in separate groups containing the tubes and surrounding tissues (along 1-cm safety margins) were taken and prepared for histological examination and stained with hematoxylin and eosin [13,19]. Using Leica Qwin Software 500, Germany at the oral and dental pathology department, Faculty of Dental Medicine for Girls, Al- Azhar University, and fibrous capsule length & thickness surrounding the polyethylene tubes (distance &width) was assessed in µm. The device consists of a color video camera, color monitor, and hard disc of the hp personal

computer connected to the microscope. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer.

Semi-quantitative evaluation methodology:
A blind observer utilized scores to determine the thickness of the fibrous capsule by taking the mean of 10 measurements (5 on each side) near the open end of the tube on the tissue section, whereas the length (distance) of the fibrous capsule was assessed by taking the mean of 2 measurements (on the right and the left sides) of the fibrous capsule on the tissue section.

Fibrous capsules were considered:
(a)thin when < 150 µm
(b)thick when >150 µm,

2.g.Statistical analysis:
The mean and standard deviation (SD) values were used to represent numerical data. The Kruskal Wallis test was used to examine intergroup comparisons. In all tests, the significance level was set at p0.05. IBM® SPSS (® IBM Corporation, NY, USA.) ® Statistics Version 26 for Windows was used for statistical analysis.

3.RESULTS:

A-Results of characterization (GC-MS) of chamomile (*Matricaria Recutita*) volatile oil:
The main constituents of the extracted and used volatile oil as identified with GC-MS are shown in Table (1).
The compound names, chemical formula and Mass/charge ratio (m/z) for the main 19 compounds as resulted from GC-MS analyses are shown in Table (1). The main constituents in the volatile oil of chamomile were identified as Bisabolol oxide A (44.6%) and followed by cis- β –Farnesene (30.0%), then α-Bisabolol oxide B (4.0%). The total of oxygenated compounds accounted for 57.48%, while the non-oxygenated compounds reached 42.5% of the total identified compounds. It is clear that most of the essential oil constituents are oxygenated compounds.

B-Intergroup comparisons of length (distance) of fibrous capsule length:
Mean, Standard deviation (SD) of the distance of fibrous capsule length (distance) scores for different groups were presented in figure (1). Results of 1st week showed no significant difference between the different groups (P=0.067), while 2nd week& 3rd week results' showed significant difference between the different groups (P=0.045), (P<0.01), respectively. The highest value was found in double antibiotic paste (50.81±10.97), (89.8±20.31), (113.27±14.11) followed by control (36.47±13.81), (85.43±21.67), (86.15±13.43) while the lowest value was found in the chamomile group (33.855±6.11), (57.79±14.24), (65.01±18.29), respectively.

C-Intergroup comparisons of the width of fibrous capsule thickness:
Mean, Standard deviation (SD) of fibrous capsule thickness (width) scores for different groups were presented in figure (2). 1st, 2nd& 3rd week results revealed significant difference between the different groups (P=0.004), (P=0.003), (P=0.001) respectively. 1st ,2nd & 3rd week results showed the highest value in double antibiotic paste (47.29±12.75), (70.38±21.69), (77.27±22.06) followed by control (29.04±10.54), (62.37±25.88), (71.68±9.47), while the lowest value was found in the chamomile group (15.64±5.28), (20.48±7.45), (22.9±13.61), respectively.

D-Histopathological results:
Histopathological assessment of the control group revealed initial inflammatory reaction at 1st week interval, which subsided at 2nd & 3rd week intervals, while the 1% double antibiotic paste group showed increased inflammation intensity in a time-dependent manner over the 3-week intervals being mild at 1st week, increasing to moderate at 2nd week & exhibiting the maximum inflammation criteria (edema, B.V.s congestion, inflammatory cell infiltration) at 3rd weeks, respectively, whereas chamomile volatile oil group at 1st showed only mild inflammation over the 3 week experimental time interval.

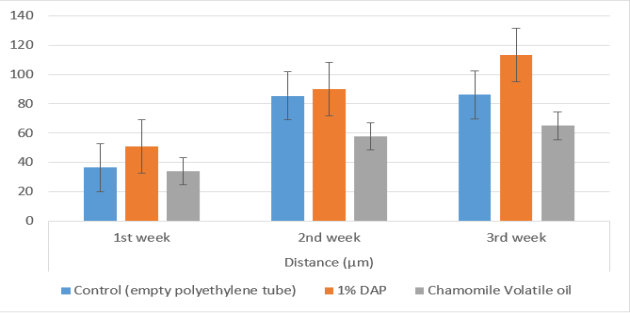

Figure (1): Bar chart showing average fibrous capsule thickness (µm) for different groups (A)

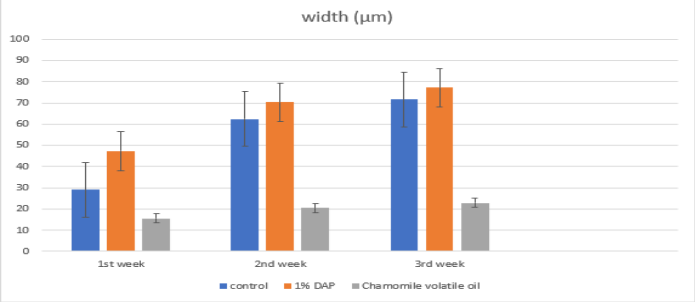

Figure (2): Bar chart showing average fibrous capsule thickness (µm) for different groups (A)

Table 1: Characterization (GC-MS) gas chromatography representing active constituents of chamomile volatile oil and relative area % of each active constituent (Rt: retention time)

R.t.(min)	Compounds	Chemical Formula	Mass/charge ratio (m/z)	Area %
3.87	Butanoic acid, 2-methyl-, propyl ester	$C_8H_{16}O_2$	57,74, 85, 103, 116,	0.17
5.90	p-Cymene	$C_{10}H_{14}$	51, 77, 91, 119, 134	0.41
6.80	β-Ocimene	$C_{10}H_{16}$	41, 53, 67, 79, 93, 105, 121	0.80
6.90	Artemisia ketone	$C_{10}H16O$	55, 69, 93, 136, 203	3.22
7.61	1,5-Heptadien-4-ol, 3,3,6-trimethyl (Artemisia alcohol)	$C_{10}H_{18}O$	41, 55, 67, 85, 93, 105, 121	0.59
11.23	endo-Borneol	$C_{10}H_{18}O$	67, 79, 95, 110, 139, 154	0.22
22.50	cis- β –Farnesene	$C_{15}H_{24}$	41, 53, 69, 93, 107, 120,133, 161	30.0
23.45	Germacrene D	$C_{15}H_{24}$	41, 55, 79, 91, 105, 119, 133, 161, 204	2.80
24.03	Elimene	$C_{15}H_{24}$	55, 67, 79, 93, 107, 121, 136, 161, 189, 204	1.54
24.60	α-Farnesene	$C_{15}H_{24}$	41, 55, 69, 93, 107, 119, 133, 161, 1189	1.00
24.77	ç-Cadinene	$C_{15}H_{24}$	79, 91, 105, 119, 133, 161, 204	0.16
24.83	Benzene, 1-methyl-4-[(1-methylethylidene)cyclopropyl]	$C_{14}H_{18}$	39, 77, 91, 105, 115, 129, 142, 159, 172	0.60
24.92	tau. -Cadinol	$C_{15}H_{26}O$	41, 81, 91, 93, 105, 161	1.64
30.01	ç-Muurolene	$C_{15}H_{24}$	93, 105,119,133,161, 204	1.02
30.39	α-Bisabolol oxide B	$C_{15}H_{26}O_2$	43, 81, 85, 105, 143	4.0
31.46	Bisabolone oxide	$C_{15}H_{24}O_2$	43, 67, 93, 109, 119, 134, 161	4.12
31.70	α-Bisabolol	$C_{15}H_{26}O$	43, 69, 109, 119	1.15
33.29	Naphthalene, 1,2,3,4-tetramethyl (Chamazulene)	$C_{14}H_{16}$	67, 128, 153, 169, 184	1.9
34.02	Bisabolol oxide A	$C_{15}H_{26}O_2$	93, 107, 121, 125, 143	44.60
	Total of oxygenated compounds			57.48
	Total of non-oxygenated compounds			42.50

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- **Control group:**

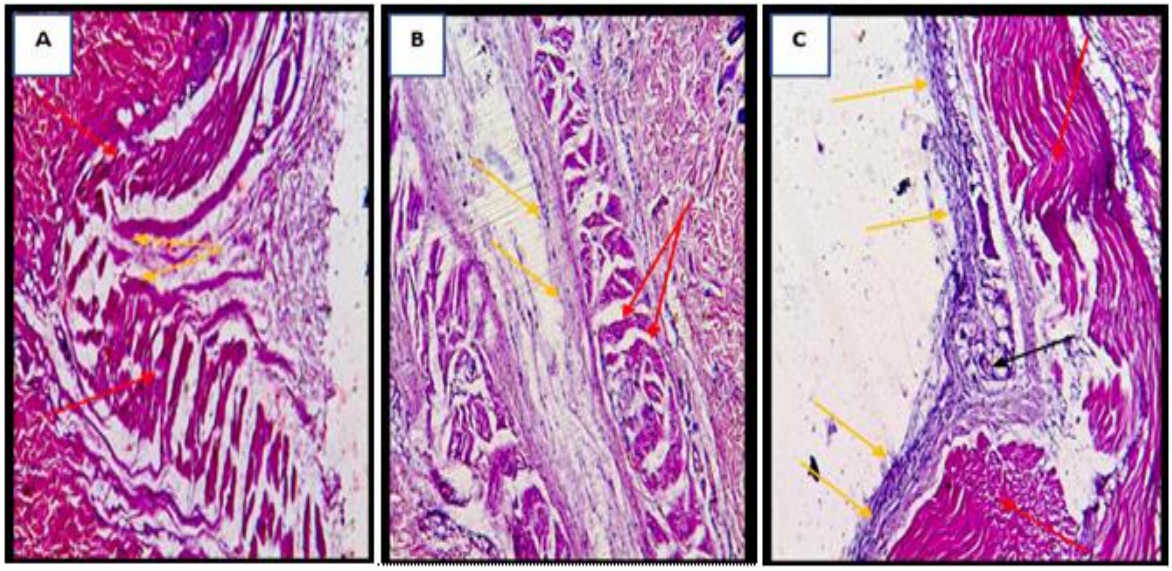

Figure (3): representing skin and subcutaneous tissue of rats of control group (empty polyethylene tube) at 1st, 2nd& 3rd week intervals, respectively. (A); control group shows thin fibrous capsule thickness (yellow arrows) & hyalinization musculature (red arrows), (B)Skin and subcutaneous tissue of rats at 2nd week interval in control group showing thin fibrous capsule thickness (yellow arrows) & hyalinization musculature (red arrows), (C); Skin and subcutaneous tissue of rats at the 3rd-week in control group showing thin fibrous capsule thickness (yellow arrow) & hyalinization musculature (red arrow), B.V. congestion (black arrow) (H&E X40).

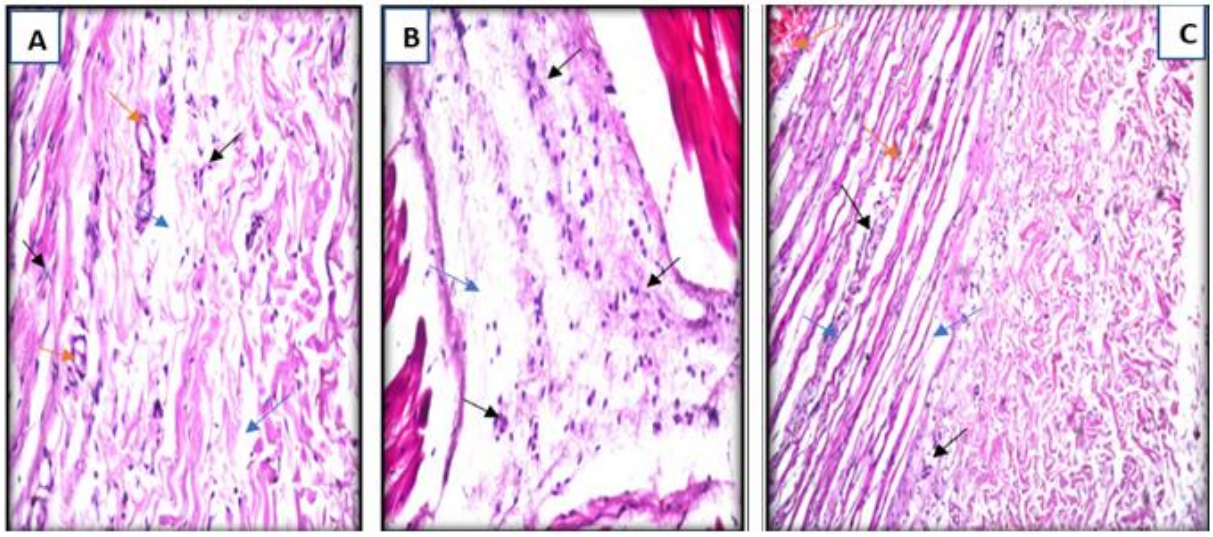

Figure (4): (A, B, C) Histopathological images representing subcutaneous tissue reaction of rats to implanted empty polyethylene tube (control group) at 1st, 2nd & 3rd week intervals, respectively. (A); control group shows mild inflammatory cell infiltrate (black arrows) & B.V. congestion (red arrows) as well as edema (blue arrow), (B) Skin and subcutaneous tissue of rats at 2nd week interval in control group showing mild inflammatory cell infiltrate (black arrows) & edema (blue arrows), (C); Skin and subcutaneous tissue of rats at the 3rd-week in control group showing mild inflammatory cell infiltrate (black arrows) & B.V. congestion (red arrows) as well as edema (blue arrows) (H&E X40).

- **Double antibiotic paste group:**

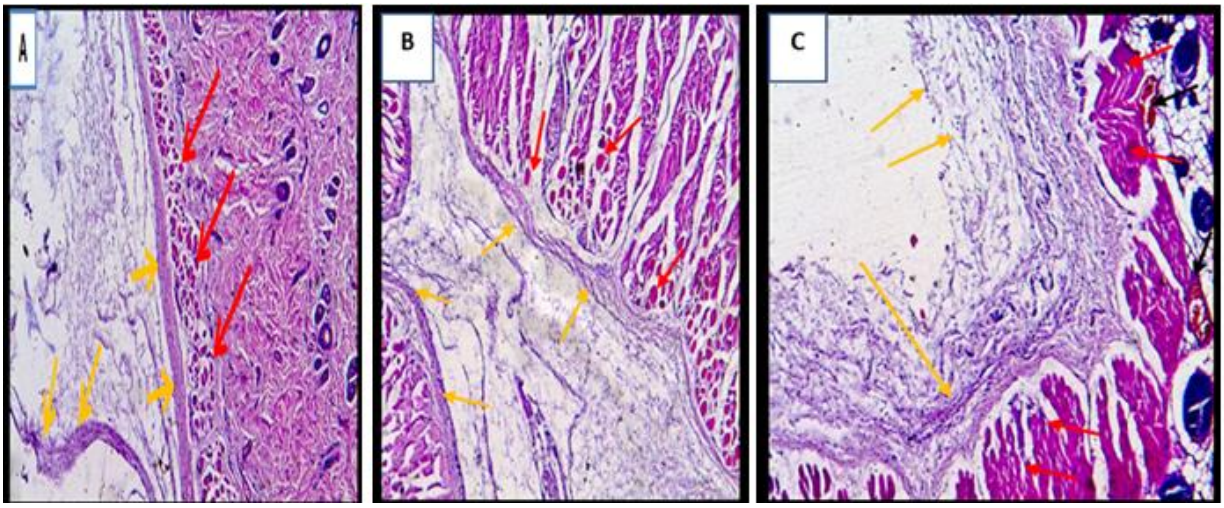

Figure (5): Representing skin and subcutaneous tissue of rats of 1% double antibiotic paste group (10mg/ml) at 1st, 2nd& 3rd week intervals, respectively. (A); 1% DAP group showing thin fibrous capsule thickness (yellow arrows) & hyalinization musculature (red arrows)&B.V. congestion (black arrows), (B)Skin and subcutaneous tissue of rats at 2nd week interval in 1% DAP group showing thin fibrous capsule thickness (yellow arrows) & hyalinization musculature (red arrows), (C);Skin and subcutaneous tissue of rats at the 3rd-week in 1% DAP group showing thin fibrous capsule thickness (yellow arrow) & hyalinization musculature (red arrow), B.V. congestion (black arrow), (H&E X40).

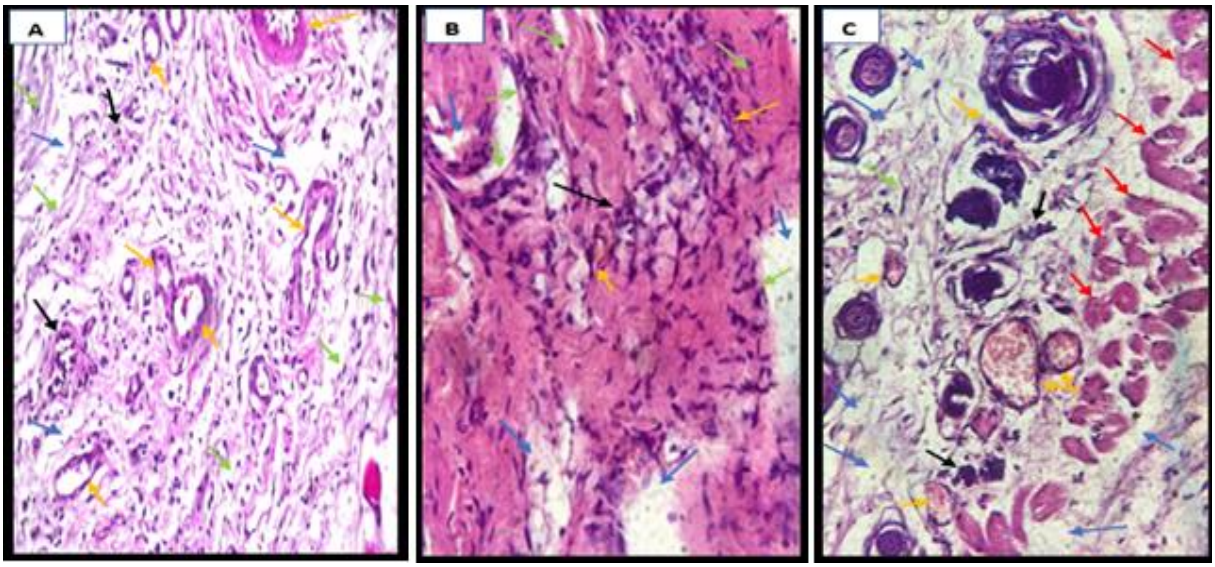

Figure (6): Representing double antibiotic paste (DAP) group, (A); represent subcutaneous tissue reaction to 10 mg/ml DAP at 1st week interval showing congestion in the blood vessels (orange arrows), mild inflammatory cell infiltration (25-50%) (black arrows), edema (blue arrows) with few fibroblastic cell proliferation (green arrows), (B); represent subcutaneous tissue reaction to 10 mg/ml DAP at 2nd week interval showing moderate inflammatory cell infiltration (50-75%) (black arrows) & few fibroblastic cell proliferation (green arrows)& B.V.s congestion (orange arrows)& edema (blue arrows), (C); represent subcutaneous tissue reaction to 10 mg/ml DAP at 3rd week interval, showing inflammatory cells infiltration (black arrows) & fibroblastic cell proliferation (green arrows), hyalinization in the musculature (red arrows), B.V. congestion (yellow arrows)& edema (blue arrows) (H&E X16).

- **Chamomile volatile oil group:**

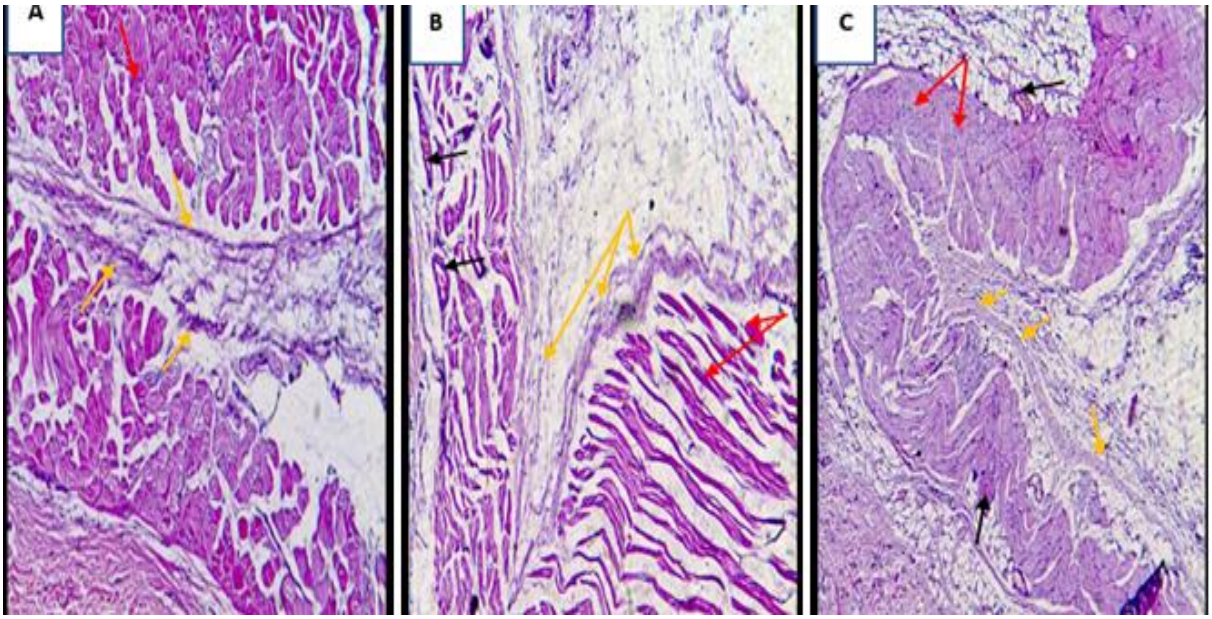

Figure (7): Representing skin and subcutaneous tissue of rats of Chamomile volatile oil (*Matricaria Recutita*) group at 1st, 2nd& 3rd week intervals, respectively. (A); Chamomile volatile oil group showing thin fibrous capsule thickness (yellow arrows) & hyalinization musculature (red arrows), (B); Skin and subcutaneous tissue of rats at 2nd week interval in Chamomile volatile oil group showing thin fibrous capsule thickness (yellow arrows) & hyalinization musculature (red arrows)& B.V.s congestion (black arrows), (C);Skin and subcutaneous tissue of rats at the 3rd-week in Chamomile volatile oil group showing thin fibrous capsule thickness (yellow arrow) & hyalinization musculature (red arrow), B.V. congestion (black arrow) (H&E X40).

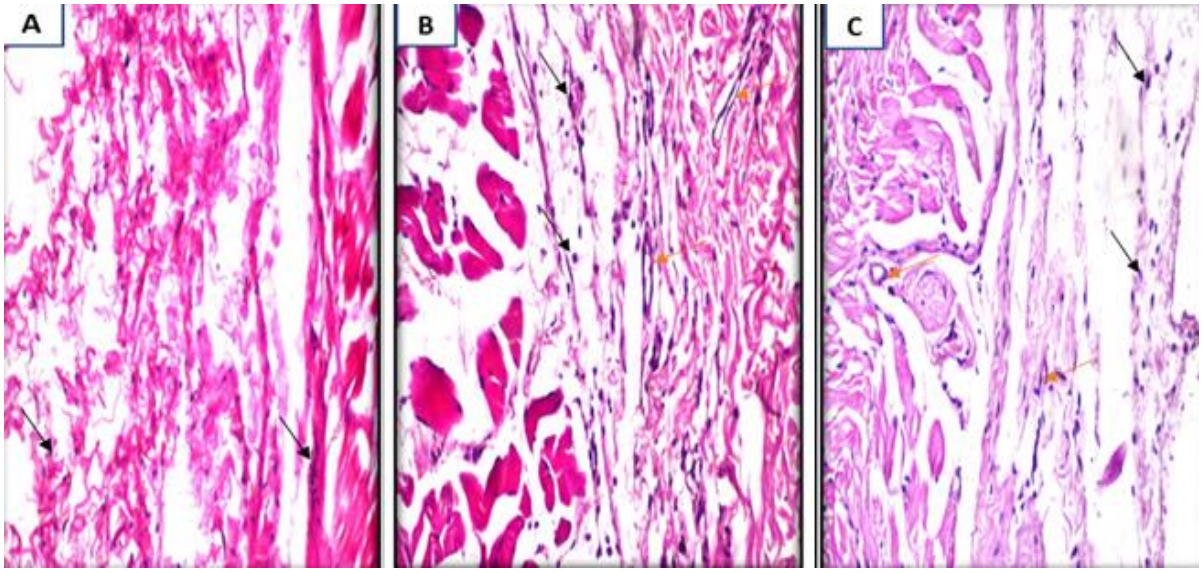

Figure (8): (A, B, C) Histopathological images representing subcutaneous tissue reaction to Chamomile volatile oil group at 1st, 2nd & 3rd weeks, respectively. (A); represent subcutaneous tissue reaction to chamomile volatile oil at 1st week interval showing mild inflammatory cell infiltration (black arrows), (B); represent subcutaneous tissue reaction to chamomile volatile oil at 2nd week interval showing mild inflammatory cell infiltration (black arrows) & B.V. congestion (orange arrows), (C); represent subcutaneous tissue reaction to chamomile volatile oil at 3rd week interval, showing mild inflammatory cell infiltration (black arrows) & B.V. proliferation (orange arrows), (H&E X40).

4.DISCUSSION:

Antiseptic intracanal medicaments that are both biocompatible and efficacious are an important aspect of regenerative endodontics. Traditional intracanal medications cause cytotoxicity in the host stem cells that are not selective. As a result, a novel antimicrobial agent that balances disinfection and biocompatibility must be introduced [4]. In this study, chamomile volatile oil was used as an alternative to synthetic medications for intracanal disinfection since it was found to be more effective against *E. faecalis* at different time spans than chlorhexidine and calcium hydroxide at 1, 7, and 14 days [12].

The direct steam distillation method was used to extract the active constituents of chamomile (*Matricaria Recutita*) volatile oil, which included volatile terpenoids, sesquiterpene lactones such as matricin, and phenolic compounds [9]. Chamomile volatile oil contains active components such as bisabolol oxide A, cis-β-farnesene, and α-Bisabolol oxide B. Alpha-bisabolol is a natural monocyclic sesquiterpene found in essential oils that has piqued the interest of the chemical and pharmaceutical sectors and is now used in a variety of applications, primarily cosmetics. A molecular docking investigation also demonstrated that it has a high affinity for the active sites of pro-inflammatory proteins. These findings suggested that α-bisabolol could be a promising therapeutic candidate for treating skin inflammation. Anti-inflammatory, antibacterial, antibiotic, antioxidant, anticancer, anti-anxiety, anti-depression, and analgesic activities are among the medical effects of bisabolol. It can also boost other molecules' absorption by the skin's dermal layers, making it excellent for topical applications [20]. Chamazulene also inhibits the synthesis of leukotriene B4 and the peroxidation of arachidonic acid and has a dose-dependent anti-inflammatory effect [11].

The double antibiotic paste was used in this investigation because it has emerged as an intracanal medicament with little tooth discoloration potential, less cytotoxicity and comparable antibacterial efficacy to traditional TAP [21,22]. The AAE Regenerative Committee recently advocated using lower concentrations of antibiotics rather than the typically utilized higher concentrations (500mg/ml, 1 gm/ml) to reduce their negative effects on stem cells and root structure [23,24].

In this study, In *Vivo* experiments were chosen to analyze the complicated cellular and molecular mechanisms involved in the immuno-inflammatory response caused by endodontic medicaments, which may aid tissue repair or otherwise sustain a chronic inflammatory response, which in vitro studies failed to do. One of the most effective

techniques for identifying the nature and progression of local reactions generated by experimental materials is subcutaneous implantation within the connective tissue of a rat animal [25].

Polyethylene tubes were utilized in this investigation to assure consistency and clinical realism since they are similar to tooth root canals and are more efficient than injecting the substance straight into the tissue. It has been reported that implanting the materials through a sterile tube hinders their liberation into the exterior tissue except through the tube's opening side. Polyethylene tubes are useful for implantation investigations due to their efficiency. As a result of this technique, we were able to predict the responses that occur in the periapical region after root canal medicament loading [26].

The antibacterial concentration of chamomile volatile oil is determined using *Enterococcus faecalis* (ATCC 29212) as the reference bacterial strain for determining antibiotic efficacy. According to CLSI guidelines, it is the best strain for measuring the MIC of medications using dilution procedures. The reference bacterial strain used to determine the antibacterial effectiveness of medicines was Enterococcus faecalis (ATCC 29212) [27,28].

Indeed, the thickness of fibrous capsules of control and DAP & chamomile volatile oil increased over time in the present study. Nevertheless, the interpretation of this finding remains speculative and is up for debate. Khalil et al. [29], Pinheiro et al. [30] Taha et al. [31] stated that the deposition of a fibrous capsule around the implanted material is an indication of tissue tolerance. Khalil et al. suggested that an increase in fibrous capsule thickness may be related to the effects of mast cells on fibroblast proliferation [29]. However, it is inconsistent with the observations of other previous studies in which the authors proposed that the biocompatibility of the material and its sign of inflammation are inversely related to the amount of fibrous capsule that develops around the tested material [32,33].

The control, DAP, and chamomile volatile oil groups all demonstrated thin fibrous capsule thickness (<150µm) consistently increasing in thickness (width) and in distance (length) in all groups throughout the 7, 14, and 21 experimental periods. Thoroughly, during the first week of the study, the maximum value of fibrous capsule length & thickness (distance, width) was discovered in 1% DAP and control groups, while the lowest value of fibrous capsule length & thickness (distance, width) was observed in the chamomile volatile oil group with no statistically significant difference between the different groups in the length (distance) values, whereas a statistically significant difference was found in the thickness (width) values of fibrous capsules. Because the sterile polyethylene tube is an inert substance that does not produce inflammation, the initial inflammatory reaction in control samples was most likely due to surgical trauma & foreign body reaction [34].

It is worth noting that all polyethylene tubes were implanted prior to material setting, which increases the probability of dissolution and biological concerns because the polyethylene tube diameter was 2.5 mm, allowing for the ejection of contents into nearby tissues. The bearing surface was a circle with a minimum diameter of one centimeter, which is less favorable than normal clinical conditions. As a result, intracanal material particles directly interact with tissues surrounding the open end of the tubes, perhaps causing reactions worse than those reported clinically [35]. Other studies using smaller diameter tubes (0.9 mm, 1.3 mm) demonstrated a greater capillary action, maintaining the loaded material for a longer amount of time, with minimum material exudate during the 1st week post-implantation [36,37].

This initial variation in response between DAP and chamomile may be attributed to changes in the chemical composition of these materials, as DAP contains cytotoxic components, in contrast to biocompatible chamomile oil essential oil components [38,39]. This finding is consistent with another study, which discovered above 20% DNA genotoxicity in dental stem cells (DSCs) 24 hours after treatment with DAP and a 15%, 22.5%, and 30% decrease in DSC viability after exposure to 5 mg/ml DAP at 1,2,3 days, respectively [40].

This finding is in accordance with a previous study that looked at the immunomodulatory activity (cytokine up/down-regulation) and biocompatibility (on RAW 264.7 murine macrophage (at 1- & 3-time intervals), L929 fibroblast cell line (at 2- & 3-time intervals)) (of DAP & TAP in an in vitro infection model. Cells were stimulated with heat-killed antigens (HK) generated from E. faecalis and S. aureus (10^6CFU of each strain in the presence of recombinant interferon-γ) (rINF-γ). In comparison to TAP, DAP attained a lower dose for minimum bactericidal concentration against E.faecalis. In the absence of both stimuli, DAP was able to induce the pro-inflammatory cytokines IL-1, IL-6, IL-12, and TNF-α in RAW 264.7 macrophages. Double antibiotic paste increased IL-6 in the absence of antigens and in the presence of HK- S. aureus and rIFN-γ for L929 fibroblast biocompatibility. All of these are linked to increased inflammation.

Furthermore, neither the presence of both antigens with rIFN-γ nor the presence of DAP affected the cytokine TGF-β associated with the repair. These findings may be undesirable, and they may explain DAP's cytotoxic effect at the first-week interval [41].

Furthermore, the 1% DAP initial high inflammation intensity on subcutaneous tissues among test groups could be related to the effect of medicaments when diluted by bodily fluids when used at relatively high concentration, as found in another study that stated that DAP responded equally across the range of doses studied (0.5, 1, 2.5, and 5 mg/ml) upon dental pulp stem cells in terms of cell cytotoxicity. In the lactate dehydrogenase assays, four DAP doses (0.5, 1, 2.5, and 5 mg/ml) were found to be significantly hazardous to the DPCs as compared to the untreated control. In contrast, the maximum concentrations that did not significantly affect the proliferation rate of DAP in the water-soluble tetrazolium-1 assays were 0.3 mg/ml, which was exceeded more than 33 times in this investigation to literature based inevitable concentration of 10 mg/ml aiming at establishing proper disinfection necessary for regeneration to occur [38].

In addition, another study that evaluated the cytotoxicity of double antibiotic paste on stem cell viability using a cell culture assay found that at clinically used concentrations, DAP had detrimental cytotoxic effects on stem cell survival, with less than 25% viability observed when exposed to DAP at 10 mg/ml. This could be due to the "paste" or "slurry"-like consistency utilized in that investigation, which occurs at antibiotic doses of around 1,000 mg/mL USP grade. As a result, it is possible to conclude that the paste or slurry contains a saturating concentration of each antibiotic, which is more likely to be harmful to living cells [42].

Whereas our findings contradict those of another study, which discovered that increasing the concentration of DAP from 0.007mg/ml (minimum inhibitory concentration) to 0.031 mg/ml (minimum bactericidal concentration) to 0.156 mg/ml (minimum biofilm inhibitory concentration) resulted in an increase in DSCs proliferation percentages of 151.27%, 180.53%, and 206.87%, respectively, relative to the control group. This could imply that some medications have a favorable effect when diluted by bodily fluids at 64 times lower than the concentration employed in our study (10mg/ml), which is consistent with the findings of another study [33]. In terms of cell toxicity, DAP responded equally across the range of doses studied. While diluted low concentration antibiotics may have similar cytotoxic effects at higher concentrations, it is worth noting that at the lowest bactericidal concentration, the least DSCs proliferation rate was related to Ca $(OH)_2$, followed by Augmentin, which may explain Ca $(OH)_2$ exclusion from this study [8].

Chamomile, on the other hand, has few adverse effects due to its slow beginning to maximal activity; also, chamomile has anti-inflammatory, antioxidant, and antibacterial characteristics [43,44]. The extraction conditions determine the quality and amount of these bioactive compounds [45,46]. This finding is consistent with another study that examined the cytotoxicity and antibacterial efficacy of chamomile volatile oil and discovered that it is biocompatible and antimicrobial in nature [47]. Another study used a scanning electron microscope to examine the morphology of seeded stem cells on chamomile-loaded patches after 6 days of incubation time. The results showed that the accumulation of stem cells was about 10 times higher, with more effective cell proliferation, on the polycaprolactone/polystyrene nanofibrous patches containing 15% chamomile than in the neat sample [48].

As regards to 2nd week results, there was a statistically significant difference in the values of fibrous capsule thickness & length (width & distance) between different groups. Fibrous capsule measurements being of maximum value associated with increased inflammation intensity to moderate level in the double antibiotic paste group whereas the lowest value of fibrous capsule measurements associated with slightly increased mild level of inflammation in the chamomile volatile oil group, moreover, the control group showed increase in the fibrous capsule thickness & length (width & distance) associated with decline in the inflammation intensity in a time dependent manner. With regards to increased inflammation intensity in the double antibiotic paste group, this finding is attributed to the time dependent cytotoxicity of double antibiotic paste [40,49]. Whereas concerning chamomile 2nd week interval slightly increased inflammation, the immunotoxicity aspect of any chemical reflects the association between these chemical and cellular responses. This finding may be attributed to the presence of Farnesene (F), as one of the chamomile essential oil components with the highest immunogenicity and mutagenesis capacity. Moreover, a calculation in a recent study revealed that the unsaturated chamomile component (F) has the strongest immunogenic and mutagenic effects. Furthermore, the structure contains furanosyl and glucosyl moieties, which

results in more oxygen atoms and increases interaction with active molecules [39].

This finding is in disagreement with the results of another investigation which studied the wound healing impact of chamomile by inserting it into a multilayered nanofibrous patch using carboxyethyl chitosan/poly (vinyl alcohol) and polycaprolactone as a potential wound dressing. The rat wound model revealed that samples containing 15% chamomile extract were surprisingly capable of healing the wounds up to 95% after a 14-day treatment period. This could be attributable to methodological differences, as chamomile was placed onto electro-spun multilayer nanofibrous patches, and distinct chamomile compounds (flavonoids) were extracted using different extraction conditions [48].

In terms of the third week time interval, the results showed that the 1% DAP group had the greatest value of fibrous capsule thickness & length (width, distance), indicating decreased biocompatibility over time, followed by the control group, and the chamomile group had the lowest value and highest biocompatibility of all test groups with statistically significant difference between different groups of fibrous capsule thickness & length.

In terms of decreasing biocompatibility in the double antibiotic paste at the third-week interval, it may be attributed to the time-dependent cytotoxic and genotoxic effect of the double antibiotic paste [40]. This finding is consistent with another recent study that found that 10 mg/mL DAP, which exhibited strong antibacterial activity against E. faecalis, reduced the proliferation of dental pulp stem cells (DPSCs) significantly when compared to the untreated negative control dental pulp stem cells. Furthermore, when compared to the 1 mg/mL group, 10 mg/mL of DAP resulted in significant decreases in alkaline phosphatase (ALP) activity and mineralized nodule development [50].

This finding contradicts another study that looked at the effects of double antibiotic paste (DAP) (1mg/ml) on the viability of dental pulp stem cells sown on irrigated radicular dentin (DPCSs) 4 weeks after treatment. The DAP-treated group showed no significant differences in DPSC survival compared to the control group while cultured on irrigated dentin for 4 weeks, which could be related to methodological variations where a lower concentration (1mg/ml) of DAP was used in that study [51].

The reduction in inflammatory intensity at the 3rd week interval in the chamomile group may be related to active ingredients such as α-bisabolol and spiroether, which increase TGF-1 and collagen deposition while decreasing the IL-1 and MMP-9/ tissue inhibitor metalloproteinase-1 ratio [11].

As a result, the material is deemed to be biocompatible if inflammation lessens over time; these findings favor chamomile volatile oil over the double antibiotic paste. This outcome is consistent with another study that assessed the biocompatibility of chamomile volatile oil upon Aliivibrio fischeri luminescence assay and upon L929 fibroblast cell line and discovered it to be biocompatible even when was used in combination with several antimicrobial drugs such as ampicillin sodium, cefuroxime acetyl, tetracycline hydrochloride, fluconazole, and nystatin. The inhibitory concentrations of combinations against the clinical Candida strain can be regarded as selective when compared to their effect on fibroblast cells based on the IC50 values obtained. Furthermore, the essential oil combination of fluconazole and nystatin was found to be non-toxic to Aliivibrio fischeri. [52].

Furthermore, all essential oil fractions were tested in combination for minimum inhibitory concentrations (MIC) and fractional inhibitory concentrations (FIC) against resistant microbiological infections. Antimicrobial activity was assessed using the microdilution method, while antimicrobial interactions were assessed using the checkerboard method. The essential oil combination of fluconazole and nystatin demonstrated "synergistic and additive inhibitory actions" against the clinical Candida strain [53]. Furthermore, the essential oil was fractionated using column chromatography with n-hexane, diethyl ether, dichloromethane, and methanol, respectively. The studies revealed that the primary components were α-bisabolol oxide A (47.7%), (E)—farnesene (21.5%), α-bisabolol oxide B (6.2%), α-bisabolone oxide A (5.8%), chamazulene (4.1%), and α-bisabolol (2.2%), which corresponded to our chromatography (GC-MS) analysis [52].

Concomitantly, an investigation of wounded rats using chamomile oil cream for healing the epidermal layer of the chamomile oil cream treated rats showed histological improvement comparable to the untreated and plain solid lipid nanoparticles groups, and the epidermal layer of the chamomile oil-solid lipid nanoparticles (CM-SLN) treated group showed more or less than the untreated and plain solid lipid nanoparticles groups. Chamomile oil's ability to stimulate faster reepithelization and tissue granulation may explain the findings [53]. This supports the findings of other studies that demonstrated that chamomile volatile oil is a biocompatible medicament [48,52].

In brief, the increase in fibrous capsule thickness & length observed in the present study for Chamomile from 7 to 21 days of observation,

showed the least value both in width and distance & occurred concomitantly with a decrease in the inflammatory reaction towards the loaded tube at the end of the experimental period, whereas fibrous capsule thickness & length values were maximal & occurred concomitantly with an increase in inflammation intensity in 1% DAP group over the 3-week time interval. Therefore, fibroblastic proliferation may be compatible with the progressive resolution of the inflammatory reaction towards chamomile volatile oil. Thus, the null hypothesis is rejected in this investigation as chamomile volatile oil proved to be biocompatible material over 1% DAP at 7,14,21 days with promising use for intracanal disinfection.

5.CONCLUSION:

Chamomile (*Matricaria Recutita*) volatile oil intracanal medicament showed superior biocompatibility over 1% DAP thus overcoming cytotoxicity of DAP with a promising application in endodontics.

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RECOMMENDATION

Further research is needed to determine the synergistic effect of chamomile volatile oil with other disinfectants to eliminate endodontic pathogens & simultaneous biocompatibility of the tested drug combinations.

CONFLICT OF INTEREST:

There was no conflict of interest.

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