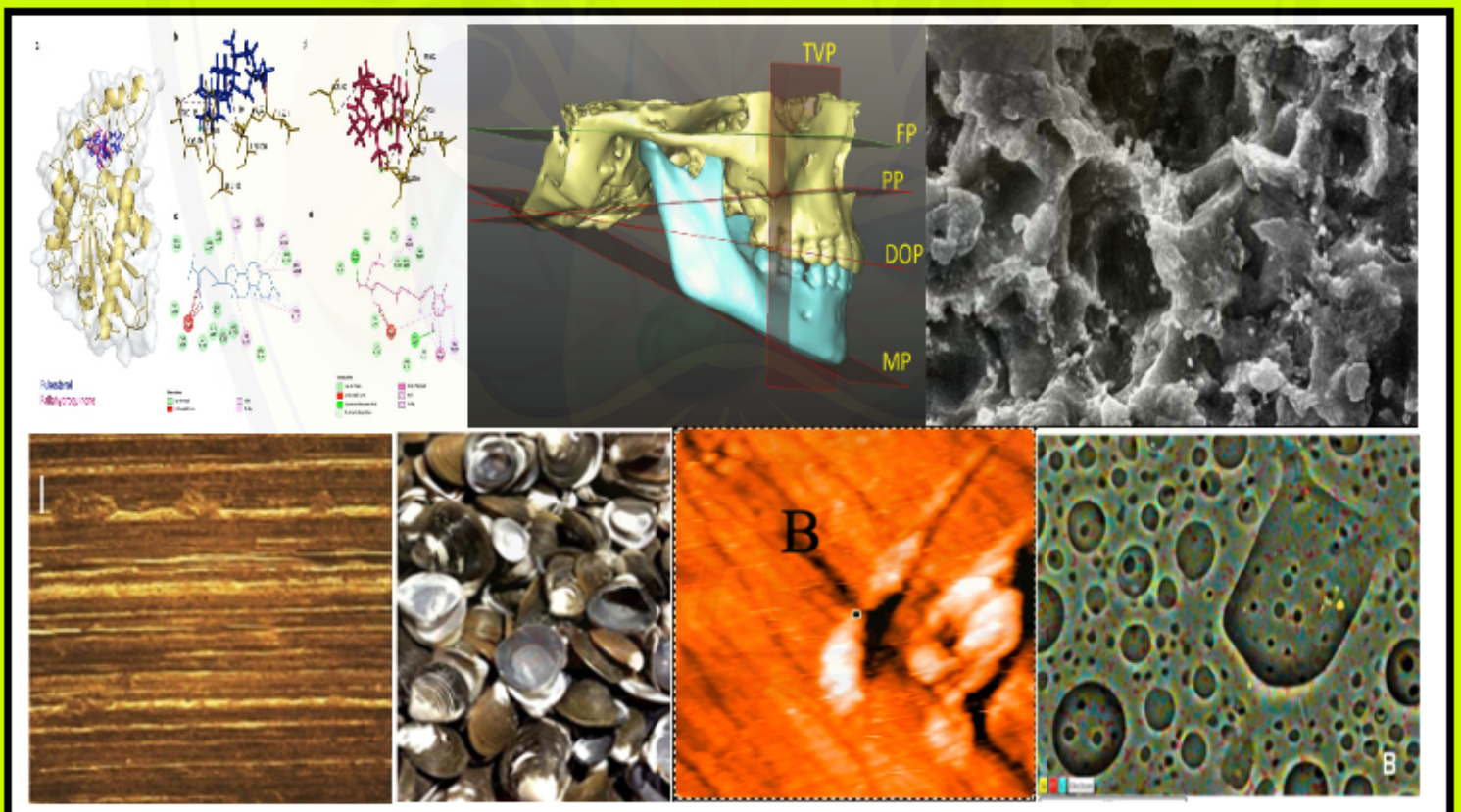


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2023 - Vol. 16 – No. 4

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1-16 of 16

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**TABLE OF CONTENTS / 2023; 16 (4)**

**DENTISTRY**

- EXPERIMENTAL ARTICLE**

**1. Repair Shear Bond Strength of Aged Provisional 3D-Printed Resin: Role of Repair Materials**  
Nuthawat Taokhampu, Hathairat Lekatana, Jadesada Palasuk  
Pages 1389-1394
- EXPERIMENTAL ARTICLE**

**2. The Effect of Calcium Hydroxide and Chitosan Combination as A Pulp Capping Material on The Increased Expression of Osteopontin and Runt-Related Transcription Factor 2 in Dentine Reparative Formation in Osteoporosis**  
Dian Agustin Wahjuningrum, Ravishinta Efty Arwinda, Veronica Regina Rosselle, Setyabudi, Tamara Yuanita, Anuj Bhardwaj, Novaldy Wahjudianto, Michelle Callea, Cruz González Alberto Carlos  
Pages 1395-1399
- EXPERIMENTAL ARTICLE**

**3. Fluoride Levels in Different Dental Pastes Marketed in Morocco**  
Abdelouhahed El Alaoui, Abdellah Moustaghfir, Chadia Ouazzani, Issam Essebbahi, Abdallah Dami, Lhoussine Balouch  
Pages 1400-1407
- EXPERIMENTAL ARTICLE**

**4. The Effect of 96% Ethanol Extract of Bidara Leaf (Ziziphus Mauritiana) on the Hemostatic Activity in Sprague-Dawley Rats**  
Irvan Septrian Syah Putra Rasad, Wiwiek Poedjiastoeti, Moehamad Orliando Roeslan  
Pages 1408-1413
- EXPERIMENTAL ARTICLE**

**5. Evaluation of the Effect of Nano-Hydroxyapatite Versus Nano-Hydroxyapatite 3D Scaffold Membrane on Osseointegration's Quality of Dental Implants (Radiographic and Histopathological Experimental Study)**  
Heba Mohmed Fayed, Sherif Eid, Hala Yassin, Shereen Fathy, Yasmine Alaa El-din, Yasser Mohamed Helmy Elkamary, Waheed Abdelhamid Ahmed  
Pages 1414-1424
- EXPERIMENTAL ARTICLE**

**6. OPG and RANKL Expression on Orthodontic Tooth Movement after Cacao Bean Extract Administration**  
Rina Sutjiati, Leliana S. Devi, Herniyati, Rudy Joelijanto, Vanda Ramadhani, Dwi Prijatmoko, Shierin V. Fiolita, Syafika N. Fadiyah, Millenieo Martin  
Pages 1425-1430
- EXPERIMENTAL ARTICLE**

**7. X-Ray Diffraction of Tetragonal Phase Zirconia Dioxide after Arteficial Aging and Loads**  
Oleg Mordanov, Zurab Khabadze, Roman Meremkulov, Mohammad Sheibanian, Alim Bakov, Ahmed Tagirov, Saeid Saeidyan, Karen Karapetov, Shainyan Ashot, Anar Iskenderov, Viktoriia Golovina  
Pages 1431-1435
- EXPERIMENTAL ARTICLE**

**8. The effect of Sarang Semut (Myrmecodia pendens and Myrmecodia tuberosa jack) as Antibacterial for Periodontal Pocket Therapy**  
Lilies Anggarwati Astuti, Ika Fikriah, Sinar Yani  
Pages 1436-1442

TABLE OF CONTENTS / 2023; 16 (4)

- EXPERIMENTAL ARTICLE**
- 9. The Effect of Addition Cacao Pod Shell and Green Tea for Pulp Capping Materials on Reducing Malondialdehyde and Apoptosis Expressions**  
Tamara Yuanita, Dian Agustin Wahjuningrum, Rosa Amalia Iqony, Setyabudi, Felina Lucia Charyadie, Shindyloken Juni Artha Tarigan, Michelle Callea, Niraj Kinariwala  
Pages 1443-1448
- EXPERIMENTAL ARTICLE**
- 10. Surface Roughness of Polished Translucent Zirconia after Immersion in Different Beverages and Temperatures**  
Wasan Vatanasak, Taksid Charasseangpaisarn, Wanchalerm Preedapratayagul, Natchanon Chantasriviroj, Pitchapa Kongsakorn, Nattida Likitsuwanakool, Thankit Kittaweevitak  
Pages 1449-1453
- EXPERIMENTAL ARTICLE**
- 11. The Potential of Rosella Floss (*Hibiscus Sabdariffa* L.) as a Dental Plaque Disclosing Agent**  
Vitri Nurilawaty, Tedi Purnama, Ai Emalia Sukmawati, Silvester Maximus Tulandi  
Pages 1454-1461
- EXPERIMENTAL ARTICLE**
- 12. Comparison of Quality of Obturation, Instrumentation Time and Post Operative Pain in the Primary Mandibular Molar Teeth using Three Different Manual Instrumentation system - a Randomized Clinical Trial**  
Ahsana Asif, Ganesh Jeevanandan, Vishnu Priya Veeraraghavan, Mohamed El-Sherbiny, Jenan Moussa Alnamly, Nawaf Salah Ayad, Noorhan Alsaleebi, Ateya Megahed Ibrahim  
Pages 1462-1468
- EXPERIMENTAL ARTICLE**
- 13. Computational Mechanism of Brown Seaweed (*Sargassum duplicatum*) on Non-Alcoholic Fatty Liver Disease (NAFLD)**  
Herin Setianingsih, Peppy Nawangsasi  
Pages 1469-1473
- EXPERIMENTAL ARTICLE**
- 14. The Effects of Acid Etching Time on Shear Bond Strength of 3Y-TZP and 5Y-TZP Zirconia to Composite Resin**  
Nopchanok Kaewwichian, Piriya Yavirach, Pisaisit Chaijareenont, Apichai Yavirach  
Pages 1474-1482
- EXPERIMENTAL ARTICLE**
- 15. Effect of Combination of Red Pine and Ca(OH)<sub>2</sub> as Root Canal Sealer on Fibroblast Cell Apoptosis Through Caspase-3 Expression**  
Tamara Yuanita, Sylvia Paulina Panggono, Devi Eka Juniarti, Setyabudi, Dian Agustin Wahjuningrum, Iin Indah Aris Wati, Adinda Fazzahra Salma, Michele Callea, Niraj Kinariwala  
Pages 1483-1488
- EXPERIMENTAL ARTICLE**
- 16. Investigation of Cytotoxicity of Dental Light-Curing Composite Materials**  
Zurab Khabadze, Saida Abdulkerimova, Anastasia Likhonina, Yulia Generalova, Irina Eremina, Mariana Borlakova, Marina Dashtieva, Alena Kulikova, Khalimat Magomedova, Anastasia Mordanova, Timur Melkumyan  
Pages 1489-1493
- EXPERIMENTAL ARTICLE**
- 17. Effect of *Clinacanthus nutans* Leaf Extract on the Production of TGF- $\beta$ 1 and bFGF in Human Dermal Fibroblasts**  
Rezky Anggraeni, Moehamad Orliando Roeslan, Melanie Sadono Djamil, Boedi Oetomo Roeslan  
Pages 1494-1500

**TABLE OF CONTENTS / 2023; 16 (4)**

- EXPERIMENTAL ARTICLE**
- 18. Comparative Evaluation of Physical and Antimicrobial Properties of Doxycycline Incorporated Formulation of Mineral Trioxide Aggregate - An In-Vitro Study**  
Vignesh Ravindran, Ganesh Jeevanandan, Vishnu Priya Veeraraghavan, Mohamed El-Sherbiny, Jenan Moussa Alnamly, Nawaf Salah Ayad, Noorhan Alsaleebi, Ateya Megahed Ibrahim  
Pages 1501-1509
- EXPERIMENTAL ARTICLE**
- 19. Observer Agreement in Temporomandibular Joint Space Measurement using Open-Source Software and Commercial Software**  
Alhidayati Asymal, Menik Priaminiarti, Heru Suryonegoro, Brama Kiswanjaya, Hanna H Bachtiar-Iskandar  
Pages 1510-1515
- EXPERIMENTAL ARTICLE**
- 20. Effects of Surface Treatments on Shear Bond Strength of Metal Bracket Bonded to Ultra-Translucent Zirconia**  
Kunpreeya Wiriyamornchai, Paiboon Techalertrpaisarn, Tanan Jaruprakorn, Preeya Suwanwitid  
Pages 1516-1522
- EXPERIMENTAL ARTICLE**
- 21. Increased Expression of Runx2 and Osteocalcin in Freeze Dried Bovine Bone Scaffold-Secretome Mesenchymal Stem Cell (In Vitro Laboratory Experimental Research on MC3T3-E1 Pre-Osteoblast Cell Culture)**  
Danang Priyo Utomo, David Buntoro Kamadjaja, Ni Putu Mira Sumarta, Coen Pramono, Andra Rizqiawan, Andang Miatmoko, Eryk Hendrianto, Mohammad Zeshaan Rahman  
Pages 1523-1529
- EXPERIMENTAL ARTICLE**
- 22. Digital Dental Patient Study Concept: Improved Approach for High Quality Dental Education Within the Conditions of War or Sanitary Restrictions**  
Myroslav Goncharuk-Khomyn, Svitlana Kostenko, Volodymyr Melnyk, Liubov Bilyshchuk, Anatoliy Foros, Stepan Sheveria, Yuriy Bun, Ivan Bohdan, Oleksandr Bilynskyi  
Pages 1530-1538
- EXPERIMENTAL ARTICLE**
- 23. Patient Satisfaction with Orthodontic Treatment**  
Krisnawati, Miesje K Purwanegara, Retno Widayati, Lindawati S Kusdhany  
Pages 1539-1545
- EXPERIMENTAL ARTICLE**
- 24. Incidental Findings in Digital Panoramic Radiographs among Dental School's Patients**  
Hazar S. AlHarbi, Shaikha Aldukhail, Sara M. Elkhateeb  
Pages 1546-1554
- EXPERIMENTAL ARTICLE**
- 25. Increased Number of Osteoblasts and New Bone Formation in Rat's Tooth Socket Implanted with Nanocrystalline Hydroxyapatite from Pensi Shells**  
Andries Pascawinata, Arniati Labanni, Gusti Revilla, Roni Eka Sahputra, Syukri Arief  
Pages 1555-1561
- EXPERIMENTAL ARTICLE**
- 26. User Experience and Perceived Benefits of Oral Health Impact Profile Mobile Application (OHIPMA)**  
Ili Mazlina Mukhtar, Natalia Mohd Dani Goh, Nuruljannah Nor Azmi, Mahyunah Masud  
Pages 1562-1566
- EXPERIMENTAL ARTICLE**
- 27. Lip Print Profiling of Tribes in Aceh Indonesia using the Suzuki-Tsuchiuchi Method**  
Abdillah Imron Nasution, Raihan Saffitri, Rossa Oktaviani, Yunni Kardisa, Khuntum Khaira Ummah  
Pages 1567-1572

**TABLE OF CONTENTS / 2023; 16 (4)**

**EXPERIMENTAL ARTICLE**

- 28. Comparison of Intraoral Sites Reached by Different Mouthwash Administration Methods**  
Chalatip Chompunud Na Ayudhya, Chanyanuch Teerawong, Ruchadaporn Kaomongkolgit, Weeraya Tantanapornkul, Peerapong Wamasing, Chutamas Deepho, Sirilawan Tohnak  
Pages 1573-1577

**CLINICAL ARTICLE**

- 29. Tootpaste Formulation of Telang Flower (*Clitoria Ternatea* L.) Ethanol Extract as an Antibacterial for *Streptococcus Mutans* and Antifungal for *Candida Albicans* in the Oral Cavity**  
Dendy Murdiyanto, Sepriyani Kaswindiarti, Diyah Fatmasari, Fauzia Nur Maulida, Naura Hasna Yuanihsan  
Pages 1578-1584

**CLINICAL ARTICLE**

- 30. The Effect of Fluoride Acid Etching Times on the Adhesive Behaviours of Porcelain Restorative Materials on the Cohesive Fractures of Porcelain Fused to Metal Restoration**  
Femy Rilinda, Syafrinani, Saharman Gea, Putri Welda Utami Ritonga  
Pages 1585-1594

**CLINICAL ARTICLE**

- 31. The Stability and Antibacterial Properties of Citronella and Lemon Oil Mouthwashes on Oral Biofilm**  
Friska Ani Rahman, Ahmad Syaify, Ronny Martien, Siti Sunarintyas  
Pages 1595-1601

**CLINICAL ARTICLE**

- 32. Effectiveness of "SIAP" Infection Control Methods to Reduce Microorganism Contamination in Dental Practice at Primary Healthcare Center**  
Nur Khamilatusy Sholekhah, Chriswardani Suryawati, Henry Setyawan, Mohammad Zen Rahfiludin  
Pages 1602-1609

**CLINICAL ARTICLE**

- 33. Intradermal Irritation Assessment of Ethyl Acetate Extract of Sarang Semut (*Myrmecordia pendans*) on Rat Skin: An In-Depth In Vivo Study**  
Lusi Epsilawati, Azhari, Mieke Hermiawati Satari, Bambang Pontjo Priosoeryanto, Tri Isyani Tungga Dewi, Merry Annisa Damayanti, Aga Satria Nurrachman  
Pages 1610-1615

**CLINICAL ARTICLE**

- 34. Genetic Variation using the 21 Str Codis Loci for Forensic Identification Examinations among Siblings of Madurese Living in Surabaya**  
Ahmad Yudianto, Arofi Kurniawan, Beta Novia Rizky, M. Kholil Ikhsan, Shella Morina, William Daniel Napitupulu, Fery Setiawan, Indah Nuraini Masjkur, Qurrotun A'yunil Huda  
Pages 1616-1619

**CLINICAL ARTICLE**

- 35. Production and Characterization of Membranes from Collagen of Carp Scales (*Cyprinus carpio*)**  
Dyah Nindita Carolina, Mieke Hemiawati Satari, Bambang Pontjo Priosoeryanto, Agus Susanto, Cortino Sukotjo, Rahmana Emran Kartasasmita  
Pages 1620-1626

**CLINICAL ARTICLE**

- 36. Salivary Total Protein Level in Elderly Patients with Periodontitis Related to Type 2 Diabetes Mellitus: A Community Based Study**  
Sri Utami, Hari Kusananto, Dibyo Pramono, Nova Oktavia, Novitasari Ratna Astuti, Arya Adiningrat  
Pages 1627-1631

**TABLE OF CONTENTS / 2023; 16 (4)**

- CLINICAL ARTICLE**
- 37. Physical Health of Males Residents from the Lowland Districts of Ukrainian Transcarpathia During the Post-Pubertal Period Depending on the Component Body Composition**  
Olena Dulo, Nataliia Hema-Bahyna, Rustam Akhmetov, Myroslav Goncharuk-Khomyn, Yevhen Lokota, Viktoriia Onishchuk, Yurii Lokota  
Pages 1632-1637
- CLINICAL ARTICLE**
- 38. Stigma Toward People With HIV/AIDS among Health Science Students in Indonesia**  
Indriasti Indah Wardhany, Lila Fairuz Febriyanty, Yuniardini Septorini Wimardhani  
Pages 1638-1646
- CLINICAL ARTICLE**
- 39. An Association Between Osteoporosis and the Mandibular Cortical Bone Among Post-Menopausal Women as Revealed by Pixel Intensity Imagery in Panoramic Radiograph**  
Sirilawan Tohnak, Weeraya Tantanapornkul, Chanya Wongkasikam, Sirinthip Chansampao, Suwadjana Thongsuk, Taksaporn Thiansuwan, Thosapol Piyapattamin  
Pages 1647-1651
- CLINICAL ARTICLE**
- 40. The Role of Salivary Receptor Activator NF-Kappa B Ligand (RANKL) On Persistent Primary Teeth in Minangkabau Children**  
Fuccy Utami Syafitri, Nila Kasuma, Eti Yerizel, Roni Eka Sahputra, Fildzah Nurul Fajrin  
Pages 1652-1656
- CLINICAL ARTICLE**
- 41. Recurrent Aphthous Stomatitis and Allergy: is it Related?**  
Ariyati Yosi, Nurdiana, Nenni Dwi Aprianti Lubis  
Pages 1657-1661
- CLINICAL ARTICLE**
- 42. Chronic Lip Fissure: Clinical Presentation and Risk Factors**  
P.M. Skrypnykov, I.M. Tkachenko, O.A. Pysarenko, T.A. Khmil, T.P. Skrypnikova, Y.V. Tymoshenko, O.O. Kulai  
Pages 1662-1669
- CLINICAL ARTICLE**
- 43. Measurement of Several Profile Angles in Chinese and Deutro- Malayid Populations in Surabaya to Determine Mean Profile Angles (Anthropometric Study for Surgical Guidance)**  
Indra Mulyawan, Qonita Gurusy, Arien Safira Damayanti, Aloysius Donny Kuncoro Sigit, Coen Pramono Danudiningrat, Ganendra Anugraha, Reza Al Fessi  
Pages 1670-1673
- CLINICAL ARTICLE**
- 44. Examination of Salivary TNF-A and Antioxidant Capacity in Smokers Who Switch to Non-Combustion Product: A Randomized-Controlled Trial**  
Indra Mustika Setia Pribadi, Afianti Sulastri, Amaliya Amaliya, Nina Djustiana, Achmad Syawqie Yazid Muqawwi  
Pages 1674-1680
- CLINICAL ARTICLE**
- 45. The Differences of Patient Satisfaction Level Post Two-Jaw and Single-Jaw Orthognathic Surgery**  
Ida Ayu Evangelina, Avi Laviana, Elih Sayuti, Iwa Rahmat Sunaryo, Endah Mardiaty, Abel Tasman, Melly Sriwijayanti  
Pages 1681-1686

TABLE OF CONTENTS / 2023; 16 (4)

- CLINICAL ARTICLE**
- 46. New Three-Dimensional Cephalometric Analysis Based on Medium Field of View CBCT Scans: Setting of Normality Values for the 7-12 Years Old Children**  
Larysa A. Dakhno, Tamara Vyshemyrska, Pavlo Burlakov, Myroslav Goncharuk-Khomyn, Natalia Rashchenko  
Pages 1687-1700
- CLINICAL ARTICLE**
- 47. Oral Manifestations of Asymptomatic and Mild-Symptomatic COVID-19 Patients**  
Dhona Afriza, Fredia Heppy  
Pages 1701-1705
- CLINICAL ARTICLE**
- 48. Immunoexpression Rate of Human Telomerase Reverse Transcriptase in Rapidly Involuting Congenital Hemangioma Patients**  
Cahyono Yudianto, Endang Sjamsudin, Yulianti Herry, Tantry Maulina  
Pages 1706-1710
- CLINICAL ARTICLE**
- 49. Feasibility Test of the "BAHUTE" Model: Technique for Cleaning Teeth in Deaf Children**  
Emma Kamelia, Lina Rismayani, Hadiyat Miko  
Pages 1711-1716
- CLINICAL ARTICLE**
- 50. Correlation between Tooth Loss with Cognitive Function and Memory Function in the Elderly**  
Rahmi Syafliida Dalimunte, Fasihah Irfani Fitri, Dina Nazriani  
Pages 1717-1720
- CLINICAL ARTICLE**
- 51. Correlation Between Parenting Stress and Parents' Knowledge and Attitude about Maintaining Dental and Oral Health Cleft Lip and Palate Children**  
Nidia Risky Primanda, Willyanti Soewondo, Ratna Indriyanti, Eka Chemiawan, Arlette Suzy Setiawan  
Pages 1721-1725
- CASE REPORT**
- 52. Submandibular Duct Salivary Stone Perforating the Floor of the Mouth: Review of Literature and Case Series**  
Fareedi Mukram Ali, Mohammed Mousa Bakri, Ahmed Shafer Alqahtani, Alwaleed Essam Bakri, Salman Ahmad Maree, Hassan Mohammed Mahnashi, Abrar Mohammad Madkhali, Abdulelah Talal Khawaji, Hajar Saeed Al Dirra  
Pages 1726-1733
- CASE REPORT**
- 53. Maxillofacial Prosthesis: Hollow Customized Ocular Prosthesis Rehabilitation in Patient with Large Orbital Defect - Case report**  
Ivony Fitria, Gunawan, Lisda Damayanti, Valentine Rosadi Sinaga, Calvin Atherton  
Pages 1734-1737
- CASE REPORT**
- 54. Cap Splint For Segmental Dentoalveolar Fractures In 11-Years Old Girl: A Case Report**  
Tania Saskianti, Paramita Devi Oktaviani, Udijanto Tedjosasongko, Luc A.M. Marks, Achmad Nadian Permana, Puspita Ayuningtyas, Tiarisna Hidayatun Nisa  
Pages 1738-1741
- CASE REPORT**
- 55. Orthodontic Treatment of an Impacted Maxillary Central Incisor with Dentigerous Cyst in Male Patient – A Case Report**  
I Gusti Aju Wahyu Ardani, Ari Triwardhani<sup>1</sup>, Alida, Indra Mulyawan, Iyanda Vemala, Nina Agustin Chrystinasari, Dyshafilia Charindra  
Pages 1742-1746



TABLE OF CONTENTS / 2023; 16 (4)

- |  |               |
|--|---------------|
| <p><b>56. Cone-Beam Computed Tomography in Dental Practice: Literature Review and Own Observations</b><br/>Yuliia L. Korobeinikova, Leonid S. Korobeinikov<br/>Pages 1747-1752</p>   | <p>REVIEW</p> |
| <p><b>57. Systematic Review and Meta Analysis: The Effect of Oral Health Literacy to Increasing Mother's Knowledge</b><br/>Willia Novita Eka Rini, Muhammad Rusdi, Jodion Siburian, Umami Kalsum<br/>Pages 1753-1757</p>   | <p>REVIEW</p> |
| <p><b>58. Predictive Factors and Ideal Timing for Spontaneous Eruption of Displaced Canine after Primary Canine Extraction: A Literature Review</b><br/>Padalino Gabriella, Guarnieri Rosanna, Mariani Andrea, De Giovanni Daniela, Di Giorgio Roberto, Galluccio Gabriella, Barbato Ersilia<br/>Pages 1758-1765</p>   | <p>REVIEW</p> |
| <p><b>59. The Relationship between Neurology Disorder and Impacted Tooth- A Narrative Review</b><br/>Indra Mulyawan, Andra Rizqiawan, Leanita Berliani, Secha Amelia, Muhammad Abdul Mukti<br/>Pages 1766-1769</p>   | <p>REVIEW</p> |
| <p><b>60. Benefits of Different Techniques for the Treatment of Miller Class Iii-Iv Recession: A Systematic Review</b><br/>Zurab Khabadze, Kristina Inozemtseva, Darina Shirokova, Anastasia Zakharova, Yulia Generalova, Omargadzhi Magomedov, Alena Kulikova, Saida Abdulkerimova, Marina Dashtieva, Yusup Bakaev, Zinaida Kozlova, Tatyana Eliseeva<br/>Pages 1770-1773</p> | <p>REVIEW</p> |
| <p><b>61. Capsaicin as a Potential Anticancer Agent: A Literature Review</b><br/>Winnie Yohana, Risti Saptarini Primanti<br/>Pages 1774-1776</p>   | <p>REVIEW</p> |
| <p><b>62. Effect of Initial Periodontal Treatment on Interleukin-1<math>\beta</math> Gingival Crevicular Fluid with Chronic Periodontitis: A Rapid Review</b><br/>Agus Susanto, Siti Sopiati, Adnin Rahma Dini<br/>Pages 1777-1784</p>   | <p>REVIEW</p> |
| <p><b>63. Cases of Obstructive Sleep Apnea in Mouth Breathers: Rapid Review</b><br/>Winnie Yohana, Syahda Salsabila Nursafudin, Kartika Sari Indah<br/>Pages 1785-1790</p>   | <p>REVIEW</p> |
| <p><b>64. The "Platform Switching" Effect in Implant-Supported Prosthetics as a Prevention of Alveolar Ridge Bone Loss</b><br/>Zurab Khabadze, Adam Kubrin, Yulia Generalova, Alena Kulikova, Saida Abdulkerimova, Marina Dashtieva, Yusup Bakaev, Akhmed Tagirov, Viktoriia Golovina, Karen Karapetov, Saied Saaidyan<br/>Pages 1791-1796</p>                                 | <p>REVIEW</p> |
| <p><b>65. An Overview of Titanium Dioxide Effect on Mechanical Properties of PMMA-TiO<sub>2</sub> Nanocomposites</b><br/>Purnomo, Dwi Windu. K. Arti, Ana Hidayati Mukaromah, Muhammad Subri, M. Edi Pujiyanto<br/>Pages 1797-1803</p>   | <p>REVIEW</p> |

TABLE OF CONTENTS / 2023; 16 (4)

REVIEW

- 66. Lesson Learned from Antiseptic Mouthwash in COVID-19 Pandemic Then, Now and Future: A Systematic Review**

Patr Pujarern, Jidapha Taruwunnapun, Boontharika Chuenjitkuntaworn  
Pages 1804-1818

REVIEW

- 67. Clinical Strategies in Prosthetic Rehabilitation and Candidiasis Management in a Cleft Lip/Palate Patient: A Case-Report**

Nicolas Pinto-Pardo, Matias Junge, Javier Montecinos  
Pages 1819-1823

REVIEW

- 68. Astaxanthin is a Promising Therapy for Wound Healing in Diabetic Conditions: A Review**

Dwi Andriani, Retno Indrawati Roestamadji, Retno Pudji Rahayu  
Pages 1824-1829

REVIEW

- 69. The Prevalence of Inflammatory Periodontal Diseases (Gingivitis, Periodontitis) among the Population**

Zurab Khabadze, Alena Kulikova, Yulia Generalova, Saida Abdulkherimova, Marina Dashtieva, Yusup Bakaev, Ashot Shainyan, Malik Yusupov, Viktoriia Golovina, Karen Karapetov, Saeid Saeidyan, Adam Kubrin  
Pages 1830-1835

REVIEW

- 70. The Role of Hyperbaric Oxygen Therapy on the Management of Mandibular Osteoradionecrosis: A Scoping Review**

Yun Mukmin Akbar, Santika Nuroktapiani, Sri Tjahajawati, Suhardjo Sitam, Amaliya Amaliya, Ani Melani Maskoen, Endah Mardiaty, Ganesha Wandawa  
Pages 1836-1845

## OPG and RANKL Expression on Orthodontic Tooth Movement after Cacao Bean Extract Administration

Rina Sutjiati<sup>1\*</sup>, Leliana S. Devi<sup>1</sup>, Herniyati<sup>1</sup>, Rudy Joelijanto<sup>1</sup>, Vanda Ramadhani<sup>1</sup>, Dwi Prijatmoko<sup>1</sup>, Shierin V. Fiolita<sup>2</sup>, Syafika N. Fadiyah<sup>2</sup>, Millenieo Martin<sup>2</sup>

1. Department of Orthodontics, Faculty of Dentistry, University of Jember, Jember, Indonesia.

2. Graduated Student of Dentistry, Faculty of Dentistry, University of Jember, Jember, Indonesia.

### Abstract

Teeth that are given orthodontic forces can relapse because of excessive resorption on the pressure side. Cocoa bean extract increases bone apposition during bone remodeling by enhancing osteoblast proliferation.

This study aimed to determine the role of cacao bean extract (*Theobroma cacao* L.) on the expression of OPG and RANKL on orthodontic tooth movement in the alveolar bone.

This research is a laboratory experiment applying the posttest-only control group design. The NiTi closed coil spring was applied between the right maxillary 1st molar and the maxillary incisor. Strength of 10gF was measured using a tension gauge to move the molars in a mesial direction. Wistar rats were decapitated after 7 days and 14 days of treatment. Immunohistochemistry staining was done to determine the expression of OPG and RANKL by counting the number of expressed osteoclasts. The data analysis used the one-way ANOVA test followed by the LSD test.

The research showed that the expression of OPG and RANKL enhanced significantly in the treatment group compared to the positive control group. Conclusion: The administration of cacao (*Theobroma cacao* L.) bean extract of the lindak variety to the alveolar bone could significantly enhance the expression of OPG and RANKL in the 7 and 14 days of the treatment group than the positive control group.

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### Introduction

Orthodontic treatment is one type of treatment carried out in dentistry that aims to obtain an aesthetically dentomaxillofacial appearance. The process of bone remodeling, namely alveolar bone resorption in the pressure area and bone formation in the tension area, is triggered when orthodontic mechanical force is applied to the teeth. Both occur in the periodontal ligament<sup>1</sup>.

It is known that orthodontic treatment takes a long time. In fixed orthodontic treatment, the duration of the treatment can last as long as 24.9 months. Even in the case of extraction, it

can reach up to 35 months<sup>2</sup>. The length of orthodontic treatment is related to the bone remodeling process, and good bone remodeling results can prevent excessive bone resorption on the pressure side<sup>3</sup>.

Bone resorption occurs due to the activity of osteoclasts, which are bone cells generated from hematopoietic stem cells (HSCs), also known as monocytes<sup>4,5</sup>. Due to orthodontic mechanical forces, inflammatory cells will be activated, produce proinflammatory cytokines such as interleukin-1, tumor necrosis factor-alpha (TNF- $\alpha$ ), and trigger osteoclast differentiation by receptor activator NF- $\kappa$ B and ligands (RANK and RANKL) and osteoprotegerin (OPG), then stimulates alveolar bone resorption<sup>6,7</sup>. OPG is a RANKL decoy receptor that can inhibit RANKL-RANK binding so that osteoclast differentiation does not occur but osteogenesis does<sup>8,9</sup>.

The occurrence of this inflammatory process is also capable of causing an expansion in the synthesis of free radicals with the

#### \*Corresponding author:

Rina Sutjiati,  
Department of Orthodontics, Faculty of Dentistry, University of Jember, Kalimantan Tegalboto Street No.37, Krajan Timur, Sumbersari 68121, Jember, Indonesia.  
E-mail: rinasutjiati@unej.ac.id

consequence of oxidative stress<sup>10,11</sup>. During the bone remodeling process, the RANKL/OPG ratio is sensitive to an increase in oxidative status that induces upregulation RANKL and decreased OPG<sup>12,13</sup>. This situation can activate the differentiation of preosteoclast thereby increasing bone resorption<sup>12,14</sup>.

An imbalance in activity between osteoclasts and osteoblasts is caused by the inflammatory process and an increase in oxidative stress brought on by orthodontic mechanical forces<sup>2,15</sup>. Increased bone resorption and apoptosis of osteoblasts and osteocytes that support osteoclastogenesis are hallmarks of this condition<sup>2,16</sup>. As a result of excessive resorption, there can be an imbalance in the remodeling process<sup>17</sup>. Therefore, antioxidants are needed in reducing bone resorption activity during orthodontic treatment<sup>18,19</sup>.

One source of natural antioxidants that contains polyphenols/flavonoids is cacao beans (*Theobroma cacao* L.)<sup>20-22</sup>. Polyphenols are a group of different substances found in unfermented cacao beans. Raw cacao beans contain flavanol monomers and procyanidin oligomers, which account for approximately 60% of the total polyphenols<sup>18</sup>.

The purpose of this research is to prove whether cacao bean extract (*Theobroma cacao* L.) can affect the expression of OPG and RANKL on orthodontic tooth movement on the pressure side of the alveolar bone.

## Materials and methods

This research used an experimental laboratory method with a posttest-only control group design. The total samples of male Wistar rats were 36 which were divided into 6 groups. Group A : group of negative control rats was the group that was not given any treatment (K-7, K-14); group B : the positive control rat group was given orthodontic mechanical force (K+7, K+14); and group C : the treatment group was given orthodontic mechanical force and cacao bean extract (P7, P14). This research has received permission in the form of Ethical Clearance No. 1727/UN25.8/KEPK/DL/2022.

The cacao beans were unfermented lindak cacao varieties obtained from the PTPN X Kertosari plantation, Jember, Indonesia, which identified in the form of Plant Identification No. 153/PL17.8/PG/2021. Cacao beans were

extracted using the maceration method. The whole cacao beans used were 1 kg extracted with a ratio of 1:4 to 96% ethanol solvent, carried out for 3 days, and covered with aluminum foil with occasional stirring. The results of the macerate were concentrated using a rotary evaporator for 2 hours at a temperature of 40-50. The final result was 10.3 grams of cacao bean extract. The administration of cacao bean extract at a dose of 250 mg/kg BW to the rats could reduce the level of oxidative stress, which is in line with the optimization of bone remodeling through the increased osteoblast number<sup>23</sup>. The sample of this study was 36 rats weighing 200 g, so the appropriate dose of cacao bean extract was 50 mg. Meanwhile, according to their stomach capacity, the recommended maximum volume of fluid given to rats is 10 ml/kg BW, so 200 g rats will need 2 ml of aqua<sup>24</sup>. As a result, the cacao bean extract given to each rat was 50 mg diluted in 2 ml of aqua once a day orally.

As a mechanical force, NiTi closed coil spring was placed between the rat's maxillary incisor and the right maxillary of the first molar to move the molar mesially. The strength given to this NiTi closed coil spring was 10gF as measured using a tension gauge. Wistar rats were decapitated after 7 days and 14 days of treatment. Furthermore, the sample was taken on the right region of the maxilla and then fixed using 10% BNF for 24 hours before making histological preparations. Tissue blocks made of paraffin were cut using a rotary microtome, carried out from the coronal to the apical direction to show the shape of the teeth and alveolar bone intact in the tension and pressure area until it became 5 µm of thickness. OPG and RANKL expression from the sample on the right region of the maxilla was examined by immunohistochemical staining. After the tissue slice was deparaffinized, it was incubated using primary antibody anti-OPG & anti-RANKL with Universal Horseradish Peroxidase (HRP) as its secondary antibody. These tissue slices in the pressure and tension area of the alveolar bone were then divided into three visual fields: cervical, middle, and apical third to be observed by three observers, respectively, under a light microscope at 400× magnification. The positive number of each OPG and RANKL expression in osteoclasts can be seen from the brown-stained osteoclasts. The data obtained were then analyzed using a one-way ANOVA test followed by an LSD test.

**Results**

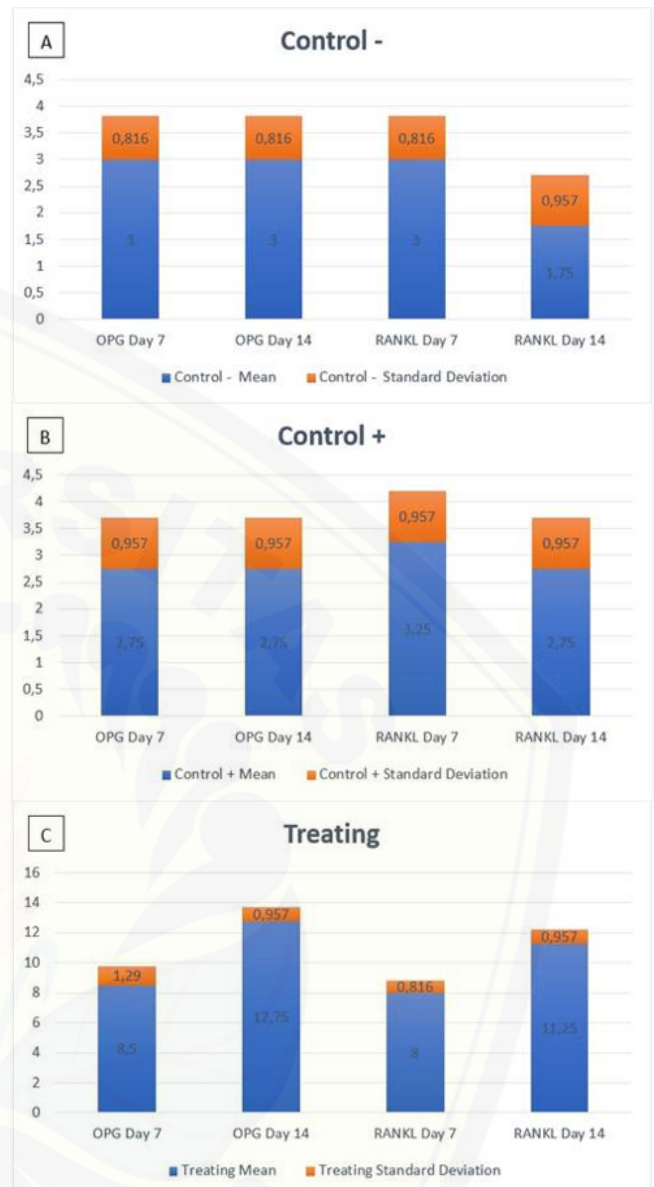
**Description of Research Data**

Thirty male Wistar rats (*Rattus norvegicus*) aged 3 to 4 months were divided into three groups: (K-) without treatment and movement of maxillary incisor teeth; (K+) with the movement of maxillary incisor teeth without treatment; (P) with treatment and movement of maxillary incisor teeth by cacao bean extract. Variables in each group were observed on day 7 and 14. OPG and RANKL were found to have a positive immunohistochemistry response, according to the result. The mean and standard deviation (SD) of the immunohistochemistry staining results for the osteoclast in the expressions of OPG and RANKL are shown in Table 1.

Group Experiment	K-7 Mean ± SD	K-14 Mean ± SD	K+7 Mean ± SD	K+14 Mean ± SD	P7 Mean ± SD	P14 Mean ± SD
Expression of OPG	3±0.816	3±0.816	2.75±0.957	2.75±0.957	8.5±1.290	12.75±0.957
Expression of RANKL	3±0.816	1.75±0.957	3.25±0.957	2.75±0.957	8±0.816	11.25±0.957

**Table 1.** Description of variable data on the number of osteoclasts in the expressions of OPG and RANKL (K-), (K+), and (P) by administration of cacao bean extract.

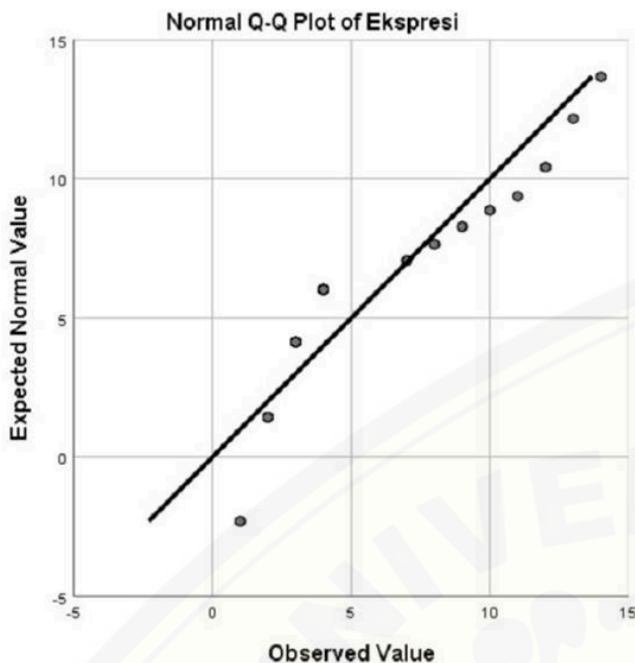
The normal distribution of the data is required for certain statistical analyses carried out in this study. Before additional information examination on the outflow of OPG and RANKL, information on homogeneity was assessed first. The Box's Test analysis was used to determine whether the data were homogeneous. The significant value of 1.000, which is greater than 0.05. This indicates that the expressions of OPG and RANKL have completed the homogeneity assumption. Then, all variables were tested for normal distribution. The test results, presented in Fig.1, show that the results of osteoclasts in the expressions of OPG and RANKL data using a QQ Plot for data analysis have a normal distribution. A straight and diagonal line seems likely to be formed by the scatter plot in Fig.2. The test measurement Z shows a higher value than 0.05, and that implies that the information OPG and RANKL articulation by immunohistochemistry staining has satisfied the normal assumption, so the following examination stage, in particular one-way ANOVA examination, can be proceeded.



**Figure 1.** Average data of osteoclasts on the expressions of OPG and RANKL on days 7 and 14, on (A) negative control groups; (B) positive control group; and (C) treatment groups.

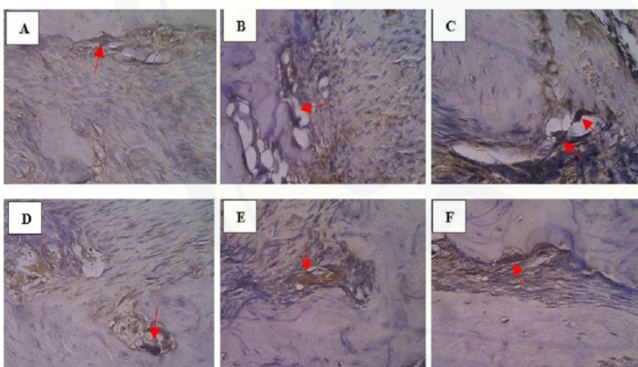
**The Results of Immunohistochemistry Examination of the OPG Expression**

The observation of osteoclasts cells generated positive expression data of OPG by immunohistochemistry methods in the negative control group (K-), positive control (K+), and the treatment group (P) because of the administration of cacao bean extract. Image A to F showing the expression of OPG on osteoclast's alveolar bone on day 7 and 14 is described below.



**Figure 2.** The results using the normality distribution with Plot QQ test.

The results of one-way ANOVA analysis show a significant difference ( $p < 0.05$ ) between the (K-) and (K+) as well as the (P). The results show that the administration of cacao bean extract on the pressure area can cause an increase in the mean expression of OPG in osteoclasts cells significantly compared to the positive control on day 14 as shown in Fig.1.



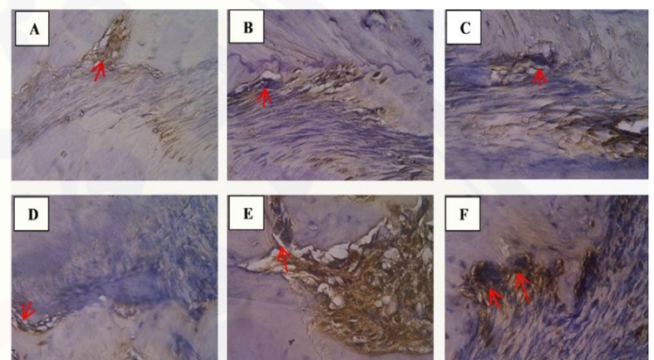
**Figure 3.** The red arrow marks the expression of OPG on osteoclast alveolar bone cells. A is the (K-), B is the (K +), C is the (P) by administration of cacao bean extract on day 7, D is the (K-), E is the (K +), F is the (P) by administration of cacao bean extract at day 14, with 400X magnification.

The Results of Immunohistochemistry Examination of the Expression of RANKL

RANKL positive expression data are derived from observations of osteoclast cells by

immunohistochemistry methods in the (K-), (K+), and (P) by administration of cacao bean extract on the pressure region. Fig. 4 shows the RANKL expression in osteoclasts' alveolar bone on day 7 and day 14.

There is a significant difference ( $p < 0.05$ ) between the (K-), (K+), and (P) groups in the one-way ANOVA analysis, shown on table 1. The results show that the mean expression of RANKL in osteoclasts cells can be enhanced significantly by the administration of cacao bean extract on the pressure area compared to the positive control on day 14 as shown in fig.1.



**Figure 4.** The red arrow indicates the expression of RANKL on osteoclast alveolar bone cells. A is the (K-) group, B is the (K+) group, and C is the (P) group by the administration of cacao bean extract on day 7, D is the (K-) group, E is the (K +) group, F is the (P) group with administration cacao bean extract on day 14, with 400X magnification.

### Discussion

Orthodontic treatment is one of dental treatment procedures that takes a long time, ranging from 25-35 months. Many efforts have been made to accelerate orthodontic treatment because it not only shortens the duration of treatment but also correlates to root resorption and excessive resorption on the pressure side<sup>1,25</sup>. This study aims to examine the role of cacao bean extract (*Theobroma cacao* L.) on the number of osteoclasts expressed OPG and RANKL on the alveolar bone on the tooth pressure side of male Wistar rats on orthodontic tooth movement for 7 days and 14 days. The method is administering diluted cacao bean extract using a gastric probe as much as 2 ml.

The results of the ANOVA test (Table 1) show that group 1 had a significant difference

with a significance value of 0.020 ( $p < 0.05$ ). The increase in the osteoclast number of the positive control group was due to the mechanical force applied to the rat teeth. This mechanical force can trigger an inflammatory process associated with an increase in free radical synthesis with consequences of oxidative stress<sup>26</sup>. Cytokines produced during the inflammatory process can cause osteoblasts to express the protein RANKL which will then bind to RANK. RANKL-RANK binding will stimulate osteoclast formation<sup>6,9</sup>. The increase in osteoclasts is also caused by oxidative stress, which can activate the differentiation of preosteoclasts into osteoclasts by increasing the RANKL expression, in line with the increasing number of osteoclasts and the occurrence of bone resorption<sup>11</sup>.

The number of the OPG and RANKL expressed osteoclast count in the treatment group was higher than that in the positive control group. This is supported by the results of the ANOVA test (Table 1) presenting a significant difference between the K+7 group and the P7 group ( $p = 0.021$ ) and between the K+14 group and the P14 group ( $p = 0.028$ ). This significant difference shows that cacao bean extract can increase the expression of OPG and RANKL during orthodontic treatment.

The increase in OPG and RANKL expression that occurred in the treatment group was due to the potential chemical compounds possessed by cacao beans. Cacao beans are rich in flavonoids with antioxidant activity reaching 80% at a certain concentration<sup>27</sup>. Flavonoids are proven to be able to reduce alveolar bone resorption with a mechanism that can reduce the interaction between RANK and RANKL. Furthermore, it will increase the expression of OPG thereby reducing the process of osteoclast formation. The increase in OPG gives a great impact to the activation of osteoclast. As a decoy receptor for RANKL, OPG block the binding between RANKL and RANK in the cell membrane of osteoclast precursor, therefore inhibiting osteoclastogenesis. The inhibition of osteoclastogenesis can decrease the number of osteoclast, so that bone resorption can be reduced, and reported that cacao bean extract could significantly inhibit the activation of NF- $\kappa$ B<sup>6,28</sup>. NF- $\kappa$ B is a transcription factor which is translocated to the nucleus through RANK-RANKL binding. It plays a crucial role in the initial stages of osteoclast development. There will be

no osteoclast differentiation in mice with a small amount of NF-B.

Overall, the treatment group has a significantly increased expression of OPG and RANKL compared to the positive control group. Cacao bean extract of the lindak variety can potentially increase the expression of OPG and RANKL on the pressure side of Wistar rat's alveolar bone in orthodontic tooth movement<sup>3,6</sup>. This automatically reduces the process of osteoclastogenesis, thus reducing the excessive resorption process on the pressure side when the teeth are moved orthodontically and a balance can be achieved between the resorption and apposition of alveolar bone to speed up the remodeling process of alveolar bone during orthodontic tooth movement.

## Conclusions

The expression of OPG and RANKL on the alveolar bone in the area of tooth pressure in Wistar rats on orthodontic tooth movement was significantly higher in the treatment group than that in the positive control group when cacao bean extract (*Theobroma cacao* L.) was given to day 7 and 14 of the treatment group.

## Acknowledgements

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## Ethical Approval

This experimental study has received ethical approval from the Ethics Committee of the Faculty of Dentistry Jember Universitas Jember No. 1727/UN25.8/KEPK/DL/2022.

## Declaration of Interest

All the authors hereby declare that there is no conflict of interest.

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