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## Original Research Article

# *In silico* and *in vivo* anti-inflammatory studies of curcuminoids, turmeric extract with zinc oxide, and eugenol

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### Abstract

**Purpose:** To determine the anti-inflammatory activity of curcuminoids in comparison with that of eugenol *in silico*, and to determine the anti-inflammatory activity of wound dressings made from zinc oxide powder and liquid turmeric extract with a high curcuminoid content.

**Methods:** *In silico* studies were conducted, using Molegro Virtual Docker program, to predict the anti-inflammatory potency of curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin) and eugenol against COX-2 receptors. *In vivo* studies to evaluate anti-inflammatory activity via TNF $\alpha$  expression, were carried out using thirty Wistar rats as subjects, divided into two groups: A (sacrificed on day 3) and B (sacrificed on day 7). Each group contained three subgroups ( $n = 5$ ): A1, B1 were excised without a dressing as control subgroups; A2, B2 were excised followed by the application of zinc oxide with a turmeric extract dressing; and A3, B3 were excised followed by the application of zinc oxide with an eugenol dressing.

**Results:** The *in silico* studies confirmed the anti-inflammatory activity of curcumin (-132.905 kcal/mol), demethoxycurcumin (-130.265 kcal/mol), bisdemethoxycurcumin (-118.827 kcal/mol) in relation to the COX-2 receptor to be greater than that of eugenol (-78.718 kcal/mol). The *in vivo* studies of TNF $\alpha$  expression showed that the levels of activity in the groups without dressings were significantly higher than in those with dressing ( $p < 0.05$ ), while the lowest TNF $\alpha$  expression were for zinc oxide with turmeric extract dressings.

**Conclusion:** The combination of zinc oxide with turmeric liquid extract has a higher anti-inflammatory effect than eugenol as demonstrated by both *in vivo* and *in silico* studies. This combination can, therefore, be used as an alternative to zinc oxide eugenol wound dressings.

**Keywords:** Anti-inflammatory, Curcuminoids, Turmeric, Zinc oxide, Eugenol, Wound dressing, *In silico*, TNF $\alpha$

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## INTRODUCTION

Injuries resulting from trauma or surgery (excision wounds) often occur in the oral cavity.

The application of wound dressings minimizes post-surgical bleeding and infections, while also promoting healing by protecting the surface of the wound when the patient is eating. Moreover,

it reduces the pain caused by contact with the tongue and food. The efficacy of wound dressings which contain zinc oxide and eugenol was first recognized in 1923. However, their use is often avoided because of potential allergic reactions, for instance, a reddening of the area around the application point accompanied by a burning sensation, in some patients. Such symptoms result from a reaction to zinc oxide and eugenol, which produces eugenolate zinc and free eugenol. They will increase due to the decomposition of eugenolate zinc which causes inflammatory reactions, slow healing and tissue necrosis [1].

Alternative dressings that could be applied to wounds include zinc oxide-turmeric rhizome extract that produces an anti-inflammatory effect [2]. Turmeric (*Curcuma longa*) is one herb proven to be safe because of its culinary uses and applications as a treatment for several diseases [3]. The main active ingredients of turmeric rhizome are curcuminoids, flavonoid compounds consisting of curcumin, demethoxycurcumin, and bisdemethoxycurcumin [4].

Before *in vivo* research is conducted, an *in silico* study should be complemented in order to assess the anti-inflammatory properties of curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin), as the active turmeric extract ingredient, compared to those of eugenol. Diclofenac is used as a ligand of the anti-inflammatory drug. *In silico* is a method of predicting the chemical properties of a molecular physics of drug and identifying the nature of compound interaction with receptors, in this case COX-2 receptors. *In vivo* research into the anti-inflammatory properties of zinc oxide combined with turmeric rhizome liquid extract or eugenol was conducted in order to detect the presence of tumor necrosis factor alpha (TNF $\alpha$ ) expression as the anti-inflammatory parameter. This study aimed to analyze anti-inflammatory curcuminoid activity as the main characteristic of turmeric extract compared to eugenol with regard to COX-2 receptors *in silico*, to evaluate the anti-inflammatory activity of zinc oxide with turmeric extract in wound dressing compared to that of eugenol *in vivo* and, finally, to compare *in silico* and *in vivo* results.

## EXPERIMENTAL

### *In silico* study

*In silico* studies were conducted to assess the potency of curcuminoids anti-inflammatory activity (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) and eugenol against

COX-2 receptors. An official drug, diclofenac, was used for the purposes of comparison [5].

### Creation of 2D and 3D molecule curcumin, demethoxycurcumin, bisdemethoxycurcumin, eugenol, and diclofenac structures

2D (two-dimensional) curcuminoid derivative structures (curcumin, demethoxycurcumin, and bisdemethoxycurcumin), eugenol, and diclofenac were drawn using a ChemBioOffice Ultra 12.0 program (Cambridge Soft Co., Cambridge, USA). The 2D structure was subsequently converted to 3D form by means of ChemBio3D 12.0 program (Cambridge Soft Co., Cambridge, USA). The stereochemical form of the compound was observed, with its most stable form being regulated through the minimizing of energy by the MMFF94 method [6]. It was subsequently stored in a SYBYL.mol2 file, enabling it to be read by the Molegro Virtual Docker (MVD) 5.5 program (CLC Bio, Aarhus, Denmark) [7]. The computer used was a PC Intel Core I-7, 4GB RAM, 32bit Operating System.

### Amino acid docking and analysis

Docking drug receptor interaction was conducted by means of an MVD program [8], with all drug and receptor structures used being in the form of 3-D (three-dimensional) image. After the cyclooxygenase-2 receptor binding to the diclofenac ligand (1PXX) had been downloaded from the internet at the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB-PDB) site [9], a cavity on the receptor structure to which the ligand was bound or interacted was detected in the fifth cavity. The 3-D structures of the curcumin/ demethoxycurcumin/bisdemethoxycurcumin/eugenol in the fifth cavity were then connected. The MVD program was implemented by aligning a molecule which attaches three atoms of the compound to three from the same ligand in the receptor. The compounds were docked automatically onto the receptor (1PXX) by the MVD program. The parameters measured in the docking process consisted of the energy values involved in the form of a MolDock Score with three replications.

### *In vivo* studies

The anti-inflammatory activity of a dressing containing zinc oxide powder and curcuminoids from turmeric rhizome liquid extract was compared to one incorporating zinc oxide powder and eugenol, by evaluating the expression of TNF $\alpha$  from *in vivo* studies.

### Experimental animals

Thirty 12-week old Wistar strain *Rattus norvegicus* weighing between 175 and 275 grams were purchased from Wistar Farm Malang, Indonesia. All were kept individually under 12-hour day/12-hour night cycle conditions and had access to standard chow pellets and water *ad libitum*. After one week of adaptation, they were randomly divided into two groups, Group A (sacrificed on day 3) and Group B (sacrificed on day 7). The fifteen rats contained in each group were further divided into three subgroups consisting of five rats: the control subgroups which were excised without dressing (A1, B1), the first experimental subgroups which were excised followed by the application of zinc oxide and turmeric liquid extract dressing (A2, B2), and the second experimental subgroups which were excised followed by the application of zinc oxide and eugenol dressing (A3, B3). The experimental procedure was approved by the Ethical Clearance Section of the Health Research Committee, Faculty of Dental Medicine - Universitas Airlangga, Indonesia (No. 236/KKEPK.FKG/XII/2015).

### Dressing material

Zinc oxide combined with turmeric extract was mixed with a stain less steel spatula on a paper pad. Turmeric rhizome liquid extract, macerated with 96 % ethanol, was purchased from the Department of Health of East Java Province (Materia Medica, Batu, Indonesia). The extract was analyzed by CAMAG Thin Layer Chromatography (TLC) – Densitometry (CAMAG, Muttenz, Switzerland) Testing Service Unit of the Faculty of Pharmacy, Universitas Airlangga, and found to consist of 32.34 % curcuminoids. A 99.8% zinc oxide powder was purchased from Merck-Germany, catalog no. 1.08849.0500, batch no. K43371349 (Merck KGaA, Darmstadt, Germany), while 99.9% eugenol was acquired from Merck-Spain, catalog no. 8.18455.0100, batch no. S6643155 (Merck S.L.U., Madrid, Spain).

### Surgical protocol

The previously reported wound excision model [10] was followed although with a degree of modification. After the administering of a general anesthetic with intramuscular ketamine (0.5 mg/100 g body weight), the vertebral thoracic regions were shaved and the skin subsequently cleaned with 70% ethanol. An excision wound measuring 6 x 6 mm was made with a surgical blade No. 15 (Swann Morton, Sheffield, England, Lot. 5201411) and the wound was cleaned with saline solution (NaCl).

### Wound management

The excision wounds of the A1 and B1 subgroups were left undressed, but covered with hypo-allergenic tape (Hypafix, Hamburg, Germany, Lot. 44120230). The excision wounds of the A2 and B2 subgroup members were dressed with a combination of 0.3g zinc oxide powder and 0.3g turmeric rhizome liquid extract, before being covered with hypo-allergenic tape. The excision wounds of the A3 and B3 subgroup members were dressed with a combination of 0.6g zinc oxide powder and 0.2g eugenol, before being covered with hypo allergenic tape. Turmeric liquid extract contains 32.34 % curcuminoids, giving a dressing zinc oxide to eugenol ratio of 3:1. Post-wound tissue samples were collected by sacrificing the rats on days 3 (A1, A2, A3 subgroups) and 7 (B1, B2, B3 subgroups). The rats were euthanized with a lethal dose of anesthesia before the post-wound areas, consisting of granulation tissues, were excised with an additional 5mm on each margin.

### Immunohistochemistry evaluation

After the tissue sample had been fixed with 10 % neutral buffer formalin (NBF), it was dehydrated using a gradient concentration of ethanol, washed in xylene, impregnated with paraffin wax, embedded in paraffin blocks and cut into 4 µm-thick section, before being fixed on a microscope slide. Subsequent immunohistochemistry evaluation adhered to the previously described method [2]. The procedural steps were completed with minor modifications [11]. A solution of 3% H<sub>2</sub>O<sub>2</sub> was added to the samples for 30 minutes to inhibit endogenous peroxidase activity. The samples were washed in distilled water for 10 minutes, incubated in normal goat serum in order to block the non-specific binding of antibodies. The sections were then incubated with a specific monoclonal antibody TNFα (54B83):sc-52746, (Santa Cruz Biotechnology, Dallas, USA) which was diluted with fetal bovine serum (FBS) at a ratio of 1:100 for 1 hour at room temperature and washed. The sections were subsequently re-incubated with biotinylated anti-mouse IgG, re-washed, and re-incubated with avidin biotin complex before being re-washed.

Peroxidase in the sections was detected with a di-amino benzidine (DAB) substrate kit. After being washed in tap water for 10 minutes and dehydrated, the nuclei in the sections were stained with hematoxylin and the sections mounted with entelan (Merck KGaA, Darmstadt, Germany). The immunohistochemistry kit used

was the Santa Cruz Biotechnology Kit (Santa Cruz Biotechnology, Dallas, USA), while all intra-step dilution and/or washing was performed using phosphate buffer saline (PBS) unless otherwise specified.

### Statistical analysis

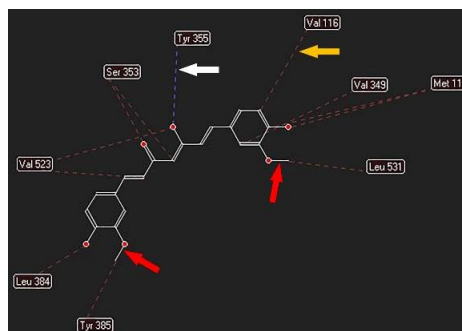
The results were analyzed using SPSS ver. 21 (IBM, New York, USA). A one-way Analysis of Variance (ANOVA) test followed by a least significant difference (LSD) test were applied to assess statistical differences between the study groups at  $p < 0.05$ .

## RESULTS

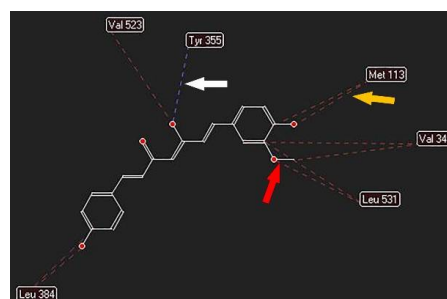
### *In silico* data

An *in silico* study was conducted to quantify the minimum energy of the curcumin, demethoxycurcumin, bisdemethoxycurcumin, eugenol, and diclofenac molecule ligands which were predicted to possess COX-2 inhibiting properties. All steps featured the use of ligands in 3D form.

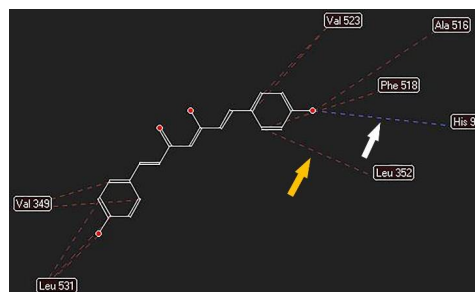
A cavity in the receptor structure (1PXX) was detected, while a diclofenac ligand was found in the fifth cavity, where the curcumin, demethoxycurcumin, bisdemethoxycurcumin, eugenol ligand (in 3D form) interacted. The 2D interaction of curcumin with COX-2 receptor resulting in two active methoxy groups, one hydrogen bond (Tyr 355) and 11 steric interactions (Tyr385, Leu384, 2 Val523, 2 Ser353, Val116, Val349, 2 Met113, Leu531) can be seen in Figure 1. The 2D interaction of demethoxycurcumin with the COX-2 receptor produced one active methoxy group, one hydrogen bond (Tyr355) and nine steric interactions (2 Leu384, Val523, 2 Met113, 2 Val349, 2 Leu531). These can all be seen in Figure 2. The 2D interaction of bisdemethoxycurcumin with COX-2 receptor which produced one hydrogen bond (His90) and 10 steric interactions (2 Val349, 3 Leu531, 2 Val523, Ala516, Phe518, Leu352) can be seen in Figure 3. The 2D interaction of diclofenac with COX-2 receptor had two hydrogen bonds (Tyr385, Ser530) and 4 steric interactions (Tyr385, Ser353, Tyr355, Met522). The 2D interaction of eugenol with the COX-2 receptor had only 1 hydrogen bond (Met522) with no steric interaction (Figure 4).



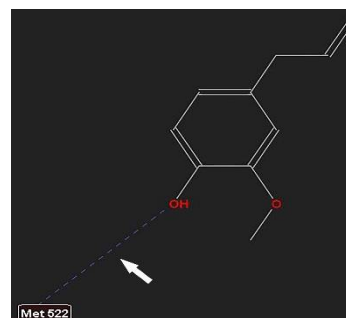
**Figure 1:** The 2D form of curcumin interaction with COX-2 receptors contains two active methoxy groups (red arrow), one hydrogen bond (white arrow) and 11 steric interactions (yellow arrow)



**Figure 2:** The 2D form of demethoxycurcumin interaction with COX-2 receptors contains one active methoxy group (red arrow), one hydrogen bond (white arrow) and nine steric interactions (yellow arrow)



**Figure 3:** The 2D form interaction of bisdemethoxycurcumin with COX-2 receptors contains one hydrogen bond (white arrow) and ten steric interactions (yellow arrow)



**Figure 4:** The 2D form of eugenol interaction with COX-two receptors contains one hydrogen bond (white arrow)

A descriptive analysis of three replications of the docking process, resulting in mean and standard deviation (SD) can be seen in Table 1.

**Table 1:** MolDock score of ligand against COX-2

Ligand	Mean(kcal/mol) ±SD
Curcumin	-132.905 <sup>c</sup> ± 1.989
Demethoxycurcumin	-130.265 <sup>c</sup> ± 5.399
Bisdemethoxycurcumin	-118.827 <sup>d</sup> ± 2.166
Eugenol	-78.718 <sup>a</sup> ± 0,139
Diclofenac	-118.579 <sup>b</sup> ± 0.458

Values are mean ± SD (n=3); significance at  $\alpha = 0.05$ ; <sup>a,b,c</sup> the same superscript denotes no differences between groups

The lower MolDock score showed that drug receptor interaction was more stable and could be used to predict the biological activity of the drug. The results confirmed the following values: curcumin -132,905 kcal/mol, demethoxycurcumin -130,265 kcal/mol, bisdemethoxycurcumin -118,827 kcal/mol, and diclofenac -118,579 kcal/mol. The highest ligand value was eugenol at -78,718 kcal/mol. LSD (superscript) test results, as shown in Table 2, indicated no significant difference in MolDock scores between the curcumin with demethoxycurcumin, and bisdemethoxycurcumin with diclofenac.

### In vivo results

The conducting of a Kolmogorov-Smirnov normality test produced a TNF $\alpha$  expression of 0.876 ( $p > 0.05$ ) with normal distribution. Levene homogeneity of variance significance was 0.504 ( $p > 0.05$ ) based on homogeneous data. Normal and homogeneous data were required to perform a one-way ANOVA test with a 95 % level of confidence, significance  $p = 0.000$  ( $p < 0.05$ ),

significant difference. This was followed by an LSD test to ascertain the difference between groups, as seen in superscript of Table 2.

**Table 2:** TNF $\alpha$  expression in wound excision (n = 5)

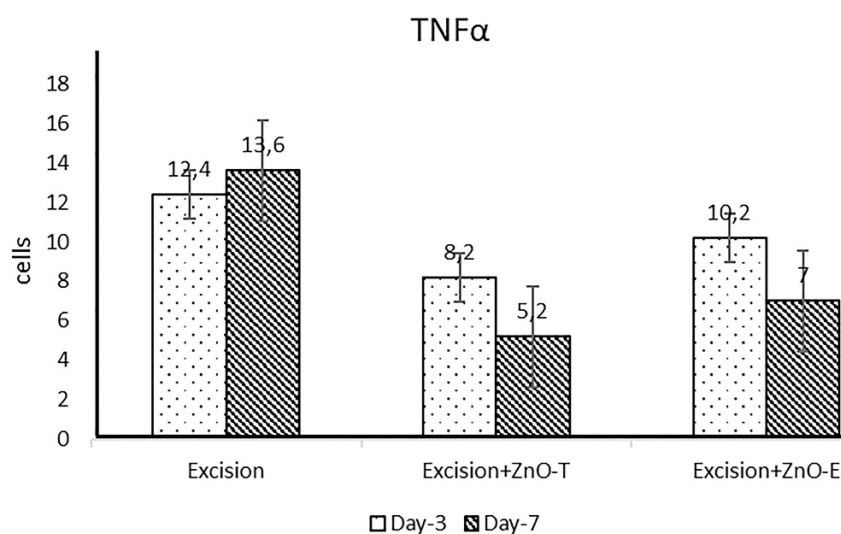
Group	Mean ± SD
A1=Excision day 3 (*)	12.40 <sup>a</sup> ±2.074
A2=Excision + ZnO-T day 3 (*)	8.20 <sup>b</sup> ±1.924
A3=Excision + ZnO-E day 3	10.20 <sup>ab</sup> ±1.095
B1=Excision day 7 (*)	13.60 <sup>a</sup> ±1.817
B2=Excision + ZnO-T day 7 (*)	5.20 <sup>c</sup> ±1.483
B3=Excision + ZnO-E day 7	7.00 <sup>bc</sup> ±2.646

**Note:** \*Meizarini et al [2]. <sup>a,b,ce</sup> Same superscript indicates no significant differences between groups ( $p < 0.05$ ); ZnO-T = zinc oxide mixture with turmeric extract; ZnO-E = Zinc oxide mixture with eugenol

There was a decrease in TNF $\alpha$  expression in the groups whose wounds were dressed with zinc oxide-turmeric liquid extract and zinc oxide-eugenol dressing on days 3 and 7 (A2, A3, B2, B3). However, the lowest expression was found in the B2 group (Figure 5).

### DISCUSSION

MolDock is a molecular docking algorithm with prediction algorithm of a cavity. The docking scoring function of MolDock is an extension of the piecewise linear potential (PLP) including hydrogen bond and electrostatic terms. MolDock was capable to identify the correct binding mode of 87 % of the complexes [12]. The MolDock score to be that of curcuminoids consisting of curcumin (-132.905kcal/mol), demethoxycurcumin (-130.265 kcal/mol) and bisdemethoxycurcumin (-118.827 kcal/mol). The scoring function was intended to evaluate the



**Figure 5:** TNF $\alpha$  expression on days 3 and 7. Excision without dressing (A1, B1), excision with ZnO-T (zinc oxide with turmeric extract) dressing (A2, B2), and excision with ZnO-E (zinc oxide with eugenol) dressing (A3, B3).

minimum energy of the ligand in relation to the protein target.

A strong predictor of potent anti-inflammatory activity of curcuminoids is curcumin. This is due to its chemical structure which incorporates two methoxy active groups (OCH<sub>3</sub>) capable of binding the receptor via steric interactions (Tyr385, Leu531), besides the other nine steric interactions. While demethoxycurcumin has only one methoxy active group, it can bind two steric interactions (Val349, Leu531) with one hydrogen bond and the seven other steric interactions. Bisdemethoxycurcumin has no methoxy active group, but has one hydrogen bond and ten steric interactions. The presence of methoxy active groups and the number of steric interactions will lead to more extensive interaction with COX-2 receptors. The lower the minimum energy required for ligand-receptor interaction, the stronger the interaction affinity between curcumin and COX-2. Diclofenac, an official anti-inflammatory drug, has two hydrogen bonds and four steric interactions (MolDock score value = -118,579 kcal/mol) which are higher than curcuminoids. Therefore, it has lower anti-inflammatory potential. The MolDock scores for eugenol were highest, due to its only having one hydrogen bond without steric interactions. The results of the *in silico* study confirmed the predictive anti-inflammatory activity of curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin) on the COX-2 receptor, which was greater than that of eugenol.

The results of *in vivo* research showed that TNF $\alpha$  expression increased significantly in the control groups (day 3 (A1) and day 7 (B1)), which were subjected to excision without dressing, when compared to the experimental groups (B2, B3). This indicates the condition of control group on day 7 to still be in the inflammatory phase due to NF- $\kappa$ B releasing TNF $\alpha$  as a pro-inflammatory cytokine [2]. The A3 and B3 groups which were treated with a zinc oxide and eugenol dressing showed a lower expression of TNF $\alpha$  than the control group. The study of the group treated with zinc oxide with turmeric rhizome liquid extract showed a TNF $\alpha$  expression lower than that of the other groups on day 3 (A3) which decreased further on day 7 (B3), indicating that the inflammatory process had subsided. This proved that the curcuminoids from zinc oxide dressing with turmeric extract effectively counteract oxidant and free radicals which are detached from HSP 70 due to excision trauma [13]. These results are consistent with studies showing an increase in macrophage phagocyte activity in animal subjects given curcumin. The action of curcumin on macrophages is explained by the

increase in free oxide capture capacity under non-inflammatory conditions [14]. The effect of using topical curcumin in treating wounds in the inflammatory phase can inhibit the transcription activity of NF- $\kappa$ B. This, decreases the production of TNF $\alpha$  and IL-1 cytokines, which would further reduce the inflammatory activity [15]. Ramsewak *et al* also highlighted that the liquid extract of turmeric rhizomes, produced by macerating them with ethanol, performed a number of biological activities including antibacterial, antioxidant and anti-inflammatory [16].

The *in silico* results predicted the anti-inflammatory activity of curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin) against the COX-2 receptor to be greater than that of eugenol. The results of this *in silico* study were consistent with the results of *in vivo* research which found the expression of proinflammatory TNF $\alpha$ , an inflammatory cytokine, in the group treated with zinc oxide and turmeric liquid extract on day 3 was equivalent with the group treated with zinc oxide and eugenol on day 7. The group treated with zinc oxide and eugenol to be higher than the group treated with zinc oxide and turmeric rhizome liquid extract. Thus, turmeric liquid extract containing curcuminoids produces greater anti-inflammatory activity than eugenol.

## CONCLUSION

A combination of zinc oxide and rhizome extract of turmeric can produce higher anti-inflammation effect and is shown by *in vivo* studies to be more effective than eugenol. These results are supported by *in silico* studies which predict that the potential anti-inflammatory effect of curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin) will be higher than that of eugenol. This suggests that the combination of zinc oxide powder and turmeric liquid extract has the potential to be used as an alternative to zinc oxide eugenol dressing.

## DECLARATIONS

### Acknowledgement

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### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. AM, S designed the experiment; S performed the in silico experiment; AM, WR, RPR performed the vivo experiment and immunohistochemistry; AM, S, WR, RPR prepared the manuscript.

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