

THE EFFECT OF KAEMPFEROL, ETHYL-*p*-METHOXYCINNAMATE, AND THE ETHANOL EXTRACT OF *Kaempferia galanga* RHIZOME ON THE PRODUCTION OF PROSTAGLANDIN BY *In-vitro* AND *In-silico* STUDY

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ABSTRACT

Cyclooxygenases (COX) catalyze the biosynthesis of prostaglandins from arachidonic acid, the substrate. COX has two isoforms which are targets of non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs exhibit their anti-inflammatory activity by blocking the conversion of arachidonic acid to prostaglandins. Overall, NSAIDs are well tolerated, especially when used short-term, nevertheless due to their global use, they are frequently involved in adverse drug reactions. *Kaempferia galanga* has been traditionally used to cure various inflammatory-related disorders by consuming the boiled rhizome tea or applying the rhizome powder paste topically on the inflamed site. This plant has been proven to contain flavonoids and polyphenolic compounds that are considered to have pharmacological activities. This work studied the effect of kaempferol (KAE), ethyl-*p*-methoxycinnamate (EPMC), and *K. galanga* rhizome ethanolic extract (EKG) on prostaglandin production and compared the results with that of celecoxib and triamcinolone acetonide by in vitro and in silico technique. Results revealed that both KAE and EPMC could occupy the catalytic site of COX-2 by building hydrogen bonds with important amino acid residues. Moreover, in vitro study confirmed that lower concentrations of EKG could reduce the production of prostaglandin PGG₂, while higher concentration does not. It is proven that KAE inhibits the production of prostaglandin PGG₂ in both concentration and time-dependent manners. Taken together, the EKG can be further developed as a COX-2 inhibitor drug.

Keywords: Anti-inflammatory, Cyclooxygenase, Ethyl-*p*-methoxycinnamate, *Kaempferia galanga*, Kaempferol
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INTRODUCTION

Cyclooxygenase-2 (COX-2) catalyzes the biosynthesis of prostaglandins by oxygenating arachidonic acid (AA) and endocannabinoid substrates.^{1,2} This catalytic reaction allocates this enzyme at a particular junction between the eicosanoid and endocannabinoid signaling pathways. COX-2 is a homodimer with a half-of-site reactivity, thus only one monomer is active at a given time.² The catalytic activity of COX-2 can be inhibited by nonsteroid anti-inflammatory drugs (NSAIDs), e.g. ibuprofen and mefenamic acid. These two NSAIDs are weak and competitively inhibit the oxygenation of AA by COX-2.³ Per contra, inhibition of both COX isoforms to the AA conversion by NSAIDs will affect the production of prostaglandins, which protects and coats the gastric wall against hydrochloric acid corrosive.⁴ Overall, NSAIDs are well tolerated, especially when used short-term, nevertheless due to their global use, they are

frequently involved in adverse drug reactions. COX-2 catalytic site holds three principal domains: (1) a hydrophilic site at the entrance of the catalytic channel that consists of residues Arg120 and Tyr355; (2) a hydrophobic pocket that consists of residues Ala201, Tyr248, Tyr385, Trp387, Leu352, and Phe518; and (3) a side pocket containing His90, Arg513, and Val523.⁵ *Kaempferia galanga* or aromatic ginger (Indonesian name: kencur, cikur) has been traditionally used to cure various inflammatory-related disorders. Treatment is carried out by consuming the boiled rhizome tea or applying the rhizome powder paste topically on the inflamed site. This plant has been proven to contain flavonoids and polyphenolic compounds,⁶ moreover, ethyl-*p*-methoxycinnamate (EPMC)⁷⁻¹⁰ and ethyl-cinnamate,⁹ have been reported as the major bioactive secondary metabolite of the rhizome. Therefore, it is interesting to exploit the anti-inflammatory effect of EPMC, kaempferol, and ethanolic extract of *K. galanga* rhizome (EKG) by studying their interaction with the residues in the catalytic site of COX-2 as well as in vitro technique using COX-2 enzyme.

EXPERIMENTAL

Protein and Ligand Preparation

The X-ray crystallographic 3D structures of COX-2 (PDB code: 5IKR, R = 2.34 Å in complex with mefenamic acid, DOI: <http://doi.org/10.2210/pdb5IKR/pdb>) were downloaded from the online Protein Data Bank (Fig.-1).² Water molecules, ligands, and other heteroatoms were removed from the protein molecule along with the chains B, C, and D, using Swiss-Pdb Viewer v4.0.4 (Swiss Institute of Bioinformatics, downloaded from www.expasy.org). The addition of hydrogen atoms and Kollman charges to the protein was performed using AutoDockVina (Molecular Graphics Laboratory The Scripps Research Institute, downloaded from <http://autodock.scripps.edu>). Kaempferol (KAE), ethyl-*p*-methoxycinnamate (EPMC), and celecoxib (CEL) were generated by using ChemBioDraw Ultra14.0 free trial (downloaded from www.cambridgesoft.com) and were geometry optimized by employing MMFF94 forcefield to produce accurate geometric structures.¹¹ CEL was used as a comparison because it is a non-steroidal anti-inflammatory drug that has been established and is known to selectively inhibit COX-2.⁴

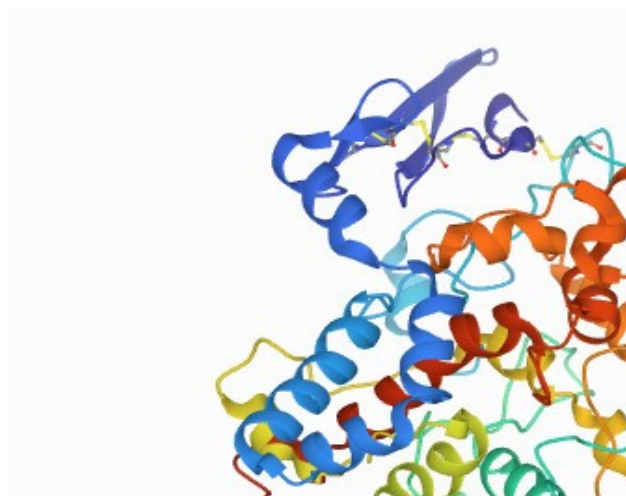


Fig.-1: 3D Structure of Human COX-2 Represented by Chain A (PDB code: 5IKR, R = 2.34 Å in Complex with Mefenamic Acid)

Molecular Docking Simulation

Molecular docking simulation was carried out for KAE, EPMC, and CEL towards the catalytic site of COX-2 at x = -16.0647; y = 41.6941; z = 25.6819 (within 10 Å distance centered to the ligand position). This procedure was repeated 100x using the Linux script command for AutoDockVina embedded in MGL Tools v.1.5.6. The flexibility of COX-2 was adjusted at the scaling factor of 0.8 for the nonpolar atoms. All other parameters were set at the default.

Instruments and Glassware

Instruments used were an electric grinder (Philips®, Cucina HR1741), thermostatic oven (EHRET®), macerator, thermostatic water bath (Huanghua Faithful Instrument Co., Ltd), rotary evaporator (Buchi®),

Rotavapor R-220, set at 40-45°C), and analytical digital balance (Pioneer™, OHAUS) for EKG preparation, as reported previously.⁴ The 96-well plate (Thermo Fisher Scientific-Nunc 96 Flat Bottom White Polystyrene Catalog Number: 136101/136102/236105/236107/236108/436110/436111 [NUN96fw.pdf]), multi-channel pipette (Eppendorf®), multimode plate reader (TECAN®, Infinite 200 PRO, Fluorescence, Kinetic mode), Eppendorf tubes, thermometer, vortex mixer (VM-300), and analytical glassware, also used for in vitro study.

Plant Materials

The material used in this study was the ethanol extract prepared from *Kaempferia galanga* rhizome (Fig.-2). The rhizomes were obtained from Subang, West Java, Indonesia. The plant sample was certified taxonomically at the School of Natural Science and Technology (SITH), Bandung, Indonesia.^{6,10}



Fig.-2: *Kaempferia galanga* Rhizome Planted and Obtained from Subang, West Java, Indonesia

Preparation of the *K. galanga* Rhizome Extract (EKG) and Phytochemical Screening

The rhizome was thinly sliced and dried in a thermostatic oven (EHRET) at 50°C. The KG rhizome extract was prepared by soaking the dried rhizome for 3x24 hours in 70% ethanol. The collected liquid was filtered, and the filtrate was evaporated using a rotary evaporator followed by using a water bath (40-45°C) to viscous. The phytochemical screening method was carried out by following the standard color test.^{6,10} The EKG to be tested was prepared by dissolving the viscous extract in 0.1% DMSO until the test concentrations were 500, 250, 125, 62.5, 31.25, and 15.625 ppm. The test solution was vortexed until homogeneous.

Preparation of KAE, EPMC, and Triamcinolone Acetonide (TRA)

The kaempferol (KAE) and EPMC solutions were prepared in advance by dissolving kaempferol and pure EPMC powder (Tokyo chemical industry Co. Ltd) in 0.1% DMSO until concentrations of 20, 10, and 5 ppm were obtained. Meanwhile, TRA was prepared by dissolving triamcinolone acetonide powder (TRILAC®) in 0.1% DMSO to obtain concentrations of 20, 10, and 5 ppm. The test solutions were vortexed until homogeneous.

Inhibitory Activity Study on the Prostaglandin PGH₂ Production by KAE, EPMC, *K. galanga* rhizome extract (EKG), Triamcinolone Acetonide (TRA), and Celecoxib (CEL)

80 µL master mix containing 76 µL COX Assay buffer + 1 µL COX probe + 2 µL diluted COX Cofactor + 1 µL diluted COX-2 enzyme (human recombinant), were added into each inhibitor well, followed by the addition of 10 µL of diluted test solutions (the extracts, KAE, EPMC, TRA, and assay buffer as the enzyme negative control), and 2 µL of CEL + 8 µL COX Assay Buffer as positive inhibitor control.

For substrate preparation, 5 μL of arachidonic acid (AA) was diluted with 5 μL of NaOH and added with 90 μL of ddH₂O. The solution was briefly vortexed to mix homogeneously. 10 μL of diluted AA/NaOH were added simultaneously to each well, using multi-channel pipettes to start the reaction at the same time. The plate was stirred and incubated precisely at 25°C. The absorbance was measured at Ex/Em = 535/587 nm, in 2 minutes intervals for 10 minutes (kinetic). COX-2 Inhibitor Screening Kit (BioVision®, Catalog #K547-100; 100 assays) was used. The assay is based on the fluorometric detection of prostaglandin PGG₂, the intermediate product generated by the COX enzyme.

Statistical Analysis

The fluorescence intensity of prostaglandin PGG₂ productions data was calculated as means $\pm$ SD. The student t-test analysis of the mean differences was used (p-value <0.05 was taken to be significant). Relative inhibition percentage (%) and Growth IC₅₀ (GraphPad Prism 9.10 app software) were measured at the 6th minute which showed the maximum inhibitory activity of all test solutions. The value of the maximum prostaglandin PGG₂ formation intensity and considered 100% was indicated by enzyme control. The inhibition value of the other test solutions was converted to this value in the form of the percentage of prostaglandin formation, so that the effect of kaempferol (KAE), the ethanol extract of *K. galanga* rhizome (EKG), Triamcinolone acetone (TRA), and Celecoxib (CEL) on the production of prostaglandin PGG₂ against time can be observed.

RESULTS AND DISCUSSION

The molecular docking simulation reveals that EPMC builds one hydrogen bond (HB) with Ser530 (distance 2.235 Å) (Fig.-3a). Interestingly, a similar binding mode is observed for KAE. This flavonol builds three HBs with Tyr355 (distance 1.798 Å), Met522 (distance 2.138 Å), and Ser530 (distance 1.906 Å). Moreover, three aromatic pi-pi stackings with Phe381, Trp387, and Phe518 are also detected (Figure-3b). The molecular docking simulation also shows the inhibition constant (K_i) of both compounds. The K_i of EPMC is 18.337 μM while KAE is 16.542 μM , which confirms that KAE is more potent in inhibiting COX-2 than EPMC. A previous study by Selinsky and co-workers reported that reversible noncompetitive COX-2 inhibitors (ibuprofen and methyl flurbiprofen) and slow tight-binding inhibitors (alclofenac and flurbiprofen) bind to the same location in the enzyme catalytic site by forming hydrogen bonds with Arg120 and Tyr355.¹² A more interesting molecular dynamic study reported that rofecoxib and celecoxib, both selective COX-2 inhibitor drugs used to treat osteoarthritis, rheumatoid arthritis, or acute pain, showed that their carbonyl group of furanone ring built an HB with Ser530,¹³ which is similar to the binding mode of our KAE.

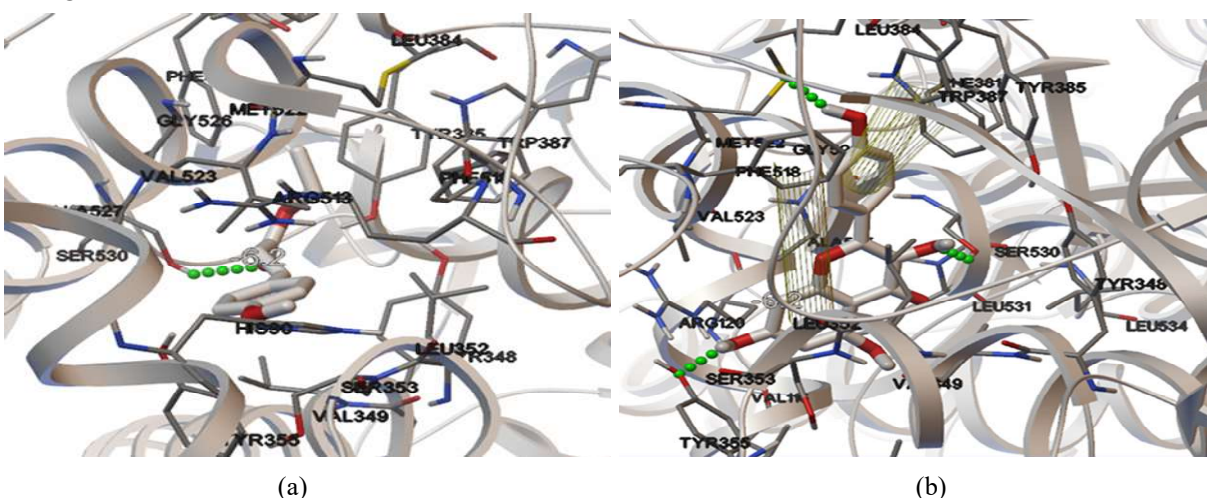


Fig.-3: The Binding Mode of (a) EPMC and (b) KAE, with Catalytic Residues of COX-2. (HB is depicted as green dotted lines; aromatic pi-pi stacking is depicted as light brown cylinders)

The phytochemical screening of EKG originated from Subang, West Java, Indonesia revealed the presence of polyphenols, flavonoids, alkaloids, tannins, triterpenoids, and saponin.⁶ This result is in accordance with the study of Tewtrakul (2005), who reported the presence of terpenoids (31-77%),

polyphenols (9.59%), and other secondary metabolite compounds in the dried rhizome of *K. galanga* planted in Thailand.¹¹ The percent relative inhibition curve for EKG measured at 6th minute when compared to 4th minute, resulting in relative $IC_{50} = 39.85$ ppm ($r = 1$) (Figure-4a), while the Figure-4b shows the growth IC_{50} of EKG = 58.88 ppm ($r^2 = 0.97/r = 0.99$) which was measured at 6th minute. Relative IC_{50} is the concentration that shows the middle value between the top and bottom plateaus of the EKG curve. On the other side, the concentration that results in inhibiting PGG_2 productions by 50% was called growth IC_{50} (GIC_{50}). The intensity of the formation of PGG_2 by the enzyme negative control has a value of 100%.

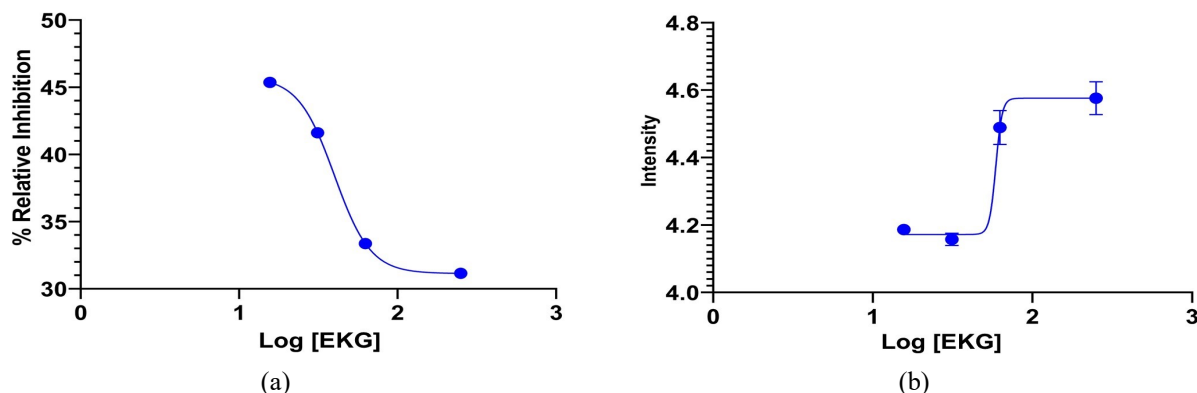


Fig.-4: (a) The Percent Relative Inhibition Curve of EKG and (b) Growth IC_{50} Curve of EKG

Figure-5 shows the effect of kaempferol (KAE), the ethanol extract of *K. galanga* rhizome (EKG), triamcinolone acetonide (TRA), and celecoxib (CEL) on the % production of prostaglandin PGG_2 catalyzed by COX-2. Lower concentrations of EKG (15.625 and 31.25 ppm) reduce the production of prostaglandin PGG_2 in a time-dependent manner, while higher concentration does not. It is proven that KAE inhibits the production of prostaglandin PGG_2 in both concentration and time-dependent manners. CEL indicates the best COX-2 inhibitor, but TRA does not. Triamcinolone (TRA), a glucocorticosteroid anti-inflammatory drug, exhibits its activity not by inhibiting COX-2. Instead, this drug blocks the catalysis function of the phospholipase A2 (PLA_2) on the phospholipid layer of the cell membrane thus forbids the production of arachidonic acid.¹⁵ EPMC tested in this study also did not show the effect of inhibiting the formation of prostaglandins. Umar and co-workers reported that EPMC isolated from the *K. galanga* plant inhibited some pro-inflammatory cytokines (TNF- α and IL-1), as well as the expression of NO and VEGF.⁷ Although EPMC exhibited a role as a non-selective inhibitor of COX-1 and COX-2 activities,¹⁶ our research showed vice versa.

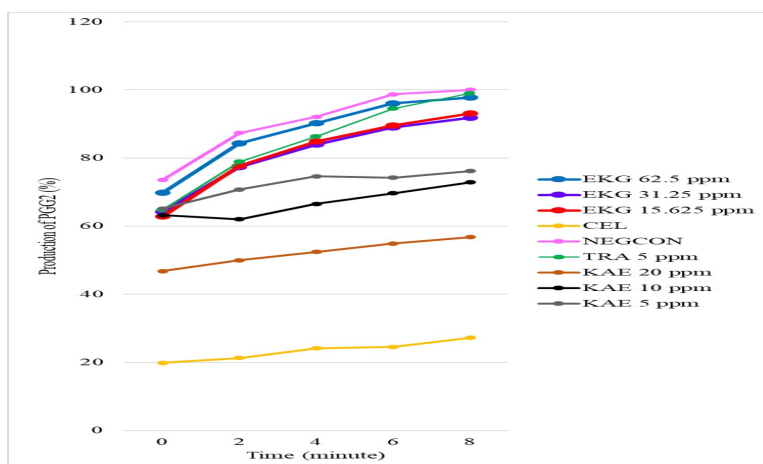


Fig.-5: The Effect of Kaempferol (KAE), the Ethanol Extract of *K. galanga* Rhizome (EKG), Triamcinolone Acetonide (TRA), and Celecoxib (CEL) on the Production of Prostaglandin PGH_2 against Time (y-axis represents the % production of prostaglandin PGH_2 ; the x-axis represents time in minutes)

Furthermore, the statistical analysis resulting that there is a significant difference ($p < 0.05$) between NEGCON and KAE 20 ppm ($p = 2.27799\text{E-}09$), KAE 10 ppm ($p = 7.79146\text{E-}07$), KAE 5 ppm ($p = 1.04081\text{E-}06$), EKG 31.25 ppm ($p = 0.001325177$), EKG 15.625 ppm ($p = 0.00060641$), and CEL ($p = 4.61029\text{E-}11$) at 6th minute. There is also a significant difference between NEGCON and KAE 20 ppm ($p = 6\text{E-}07$), KAE 10 ppm ($p = 1.56\text{E-}05$), KAE 5 ppm ($p = 5.17\text{E-}05$), EKG 31.25 ppm ($p = 0.037628$), EKG 15.625 ppm ($p = 0.015975$) and CEL ($p = 8.85\text{E-}08$) at 8th minute. In summary, we propose a simple graphical mechanism of the anti-inflammatory activity of *K. galanga* rhizome via the inhibition of the COX-2 enzyme (Fig.-6).

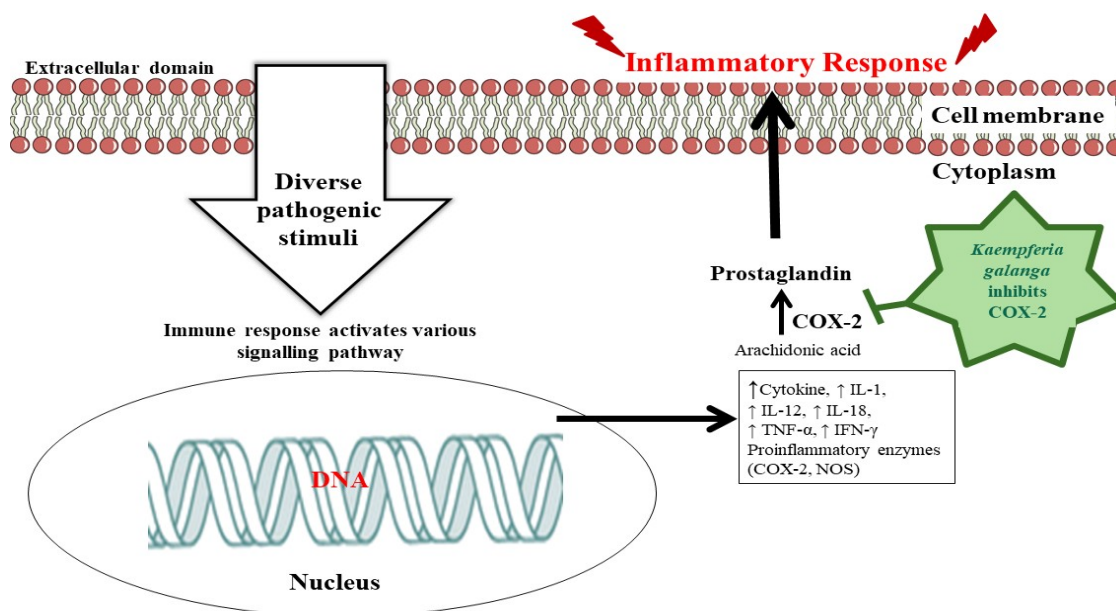


Fig.-6: Proposed Mechanism of Inhibitory Activity of *K. galanga* Extract on COX-2 Enzyme to Prevent Inflammatory Response

CONCLUSION

This study discloses the effect of kaempferol (KAE), ethyl-*p*-methoxycinnamate (EPMC), and the ethanol extract of *Kaempferia galanga* rhizome (EKG) in inhibiting the COX-2 enzyme. EPMC binds with residues in the catalytic site of COX-2 by building one hydrogen bond with Ser530 similar to that of celecoxib and rofecoxib, selective COX-2 inhibitors. KAE builds three hydrogen bonds with Tyr355, Met522, and Ser530. Moreover, three aromatic pi-pi stackings with Phe381, Trp387, and Phe518 are also detected with KAE. Lower concentrations of EKG inhibit the production of prostaglandin PGG₂. KAE exhibits the same activity in both concentration and time-dependent manners. Taken together, the ethanol extract of *K. galanga* can be further developed as a COX-2 inhibitor drug.

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REFERENCES

1. U. Harahap, H. Purnomo and D. Satria. *Rasayan Journal of Chemistry*, **14**(3), 1489(2021), <https://doi.org/10.31788/RJC.2021.1436163>
2. B. J. Orlando and M. G. Malkowski, *The Journal of Biological Chemistry*, **291**(29), 15069(2016), <https://doi.org/10.1074/jbc.m116.725713>

3. J. J. Prusakiewicz, K. C. Duggan, C. A. Rouzer and L. J. Marnett, *Biochemistry*, **48**(31), 7353(2009), <https://dx.doi.org/10.1021%2Fbi900999z>
4. S. Damayanti, D. Puspaningrum, H. N. Muhammad, T. Amelia, B. Permana, R. Hartati and I. Wibowo. *Rasayan Journal of Chemistry*, **14**(2), 844(2021), <https://doi.org/10.31788/RJC.2021.1426117>
5. P. D'Mello, M. K. Gadhwali, U. Joshi and P. Shetgiri, *International Journal of Pharmacy and Pharmaceutical Sciences*, **3**(4), 33(2011).
6. I. S. Wahyuni, I. Sufiawati, W. Nittayananta and J. Levita, *Materials Science Forum*, **1028**(4), 371(2021), <https://doi.org/10.4028/www.scientific.net/MSF.1028.371>
7. M. I. Umar, M. Z. Asmawi, A. Sadikun, A. M. S. A. Majid, F. S. R. Al-Suede, L. E. Hassan, R. Altaf, and M. B. K. Ahamed, *Clinics*, **69**(2), 134(2014), [http://dx.doi.org/10.6061/clinics/2014\(02\)10](http://dx.doi.org/10.6061/clinics/2014(02)10)
8. N. Srivastava, Ranjana, S. Singh, A. C. Gupta, K. Shanker, D. U. Bawankule and S. Luqman, *Toxicology Reports*, **6**, 521(2019), <https://doi.org/10.1016/j.toxrep.2019.05.014>
9. R. Othman, H. Ibrahim, M. A. Mohd, M. R. Mustafa and K. Awang, *Phytomedicine*, **13**(1-2), 61(2006), <https://doi.org/10.1016/j.phymed.2004.07.004>
10. I. S. Wahyuni, I. Sufiawati, W. Nittayananta and J. Levita. *International Journal Of Applied Pharmaceutics*, **13**(4), 132(2021), <https://doi.org/10.22159/ijap.2021.V13s4.43841>
11. S. Megantara, J. Levita, S. Ibrahim and B. P. Nguyen, *Rasayan Journal of Chemistry*, **14**(1), 241(2021), <http://dx.doi.org/10.31788/RJC.2021.1416070>
12. B. S. Selinsky, K. Gupta, C. T. Sharkey and P. J. Loll, *Biochemistry*, **40**(17), 5172(2001), <https://doi.org/10.1021/bi010045s>
13. P. K. Deb, R. P. Mailabaram, B. Al-Jaidi and M. J. Saadh, *IntechOpen*, London(2017), <https://doi.org/10.5772/intechopen.68318>
14. S. Tewtrakul, S. Yuenyongsawad, S. Kummee and L. Atsawajaruwan, *Songklanakarin Journal of Science and Technology*, **27**(Suppl.2), 503(2005)
15. G. Sidhu and C. V. Preuss, Triamcinolone, StatPearls [Internet] Publishing, Treasure Island Florida, (2021).
16. M. I. Umar, M. Z. Asmawi, A. Sadikun, I. J. Atangwho, M. F. Yam, R. Altaf and A. Ahmed, *Molecules* (Basel, Switzerland), **17**(7), 8720(2012), [https://doi.org/10.3390/molecules17078720\(2012\)](https://doi.org/10.3390/molecules17078720(2012))

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