Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Study of resveratrol against bone loss by using in-silico and in-vitro methods

Estudo do resveratrol contra a perda óssea usando métodos in silico e in vitro

S. R. Abbas[a]*, R. T. Khan[b], S. Shafique[c], S. Mumtaz[a], A. A. Khan[d], A. M. Khan[e], Z. Hassan[d], S. A. Hussain[a], S. Abbas[a], M. R. Abbas[f], A. Batool[g] and M. A. Safder[a]

[a]Karakoram International University, Department of Biological Sciences, Gilgit, Pakistan.
[b]University of Azad Jammu and Kashmir, Department of Botany, Azad Jammu and Kashmir, Pakistan.
[c]University of Poonch, Department of Plant Breeding and Molecular Genetics, Rawalakot, Azad Jammu and Kashmir, Pakistan.
[d]Bahauddin Zakariya University, College of Agriculture Bahadur Campus, Layyah, Pakistan.
[e]University of Sargodha, Department of Biotechnology, Sargodha, Pakistan.
[f]University of Azad Jammu and Kashmir, Department of Computer Sciences and IT, Muzaffarabad, Pakistan.
[g]Virtual University Lahore, Department of Biotechnology, Lahore, Pakistan.

Abstract

By applying the in-silico method, resveratrol was docked on those proteins which are responsible for bone loss. The Molecular docking data between the resveratrol and Receptor activator of nuclear factor-kappa-B ligand [RANKL] receptors proved that resveratrol binds tightly to the receptors, showed the highest binding affinities of −6.9, −7.6, −7.1, −6.9, −6.7, and −7.1 kcal/mol. According to in-vitro data, Resveratrol reduced the osteoclasts after treating Marrow-Derived Macrophages [BMM] with Macrophage colony-stimulating factor [MCSF] 20ng / ml and RANKL 50ng / ml, with different concentrations of resveratrol (2.5, 10 µg / ml) For 7 days, the cells were treated with MCSF (20 ng / ml) and RANKL (40 ng / ml) together with concentrated trimethyl ether and resveratrol (2.5, 10 µg / ml) within 12 hours. Which, not affect cell survival. After fixing osteoclast cells with formaldehyde fixative on glass coverslip followed by incubation with 0.1% Triton X-100 in PBS for 5 min and after that stain with rhodamine phalloidin staining for actin and Hoechst for nuclei. Fluorescence microscopy was performed to see the distribution of filaments actin [F.actin]. Finally, resveratrol reduced the actin ring formation. Resveratrol is the best bioactive compound for drug preparation against bone loss.

Keywords: resveratrol, protein docking, in-vitro, receptor activator of nuclear factor-kappa-B ligand [RANKL], fluorescence microscopy.

Resumo

Com a aplicação do método in-silico, o resveratrol foi ancorado nas proteínas responsáveis pela perda óssea. Os dados de docking molecular entre o resveratrol e o ligante do receptor ativador do fator nuclear kappa-B [*Receptor Activator of Nuclear Factor kappa-B Ligant* (RANKL)] provaram que o resveratrol se liga fortemente aos receptores, mostraram as afinidades de ligação mais altas de −6,9, −7,6, −7,1, −6,9, - 6,7 e -7,1 kcal / mol. De acordo com dados *in-vitro*, o resveratrol reduziu os osteoclastos após o tratamento de macrófagos derivados da medula óssea [*Bone Marrow-derived Macrophage* (BMM)] com fator estimulador de colônias de macrófagos [*Macrophage Colony-Stimulating Factor* (MCSF)] 20ng / ml e RANKL 50ng / ml, com diferentes concentrações de resveratrol (2,5, 10 µg / ml). Durante sete dias, as células foram tratadas com MCSF (20 ng / ml) e RANKL (40 ng / ml) juntamente com éter trimetílico concentrado e resveratrol (2,5, 10 µg / ml) em 12 horas, processo que não afeta a sobrevivência celular. Após a fixação de células de osteoclastos com fixador de formaldeído em lamela de vidro seguido de incubação com 0,1% Triton X-100 em PBS por 5 min, foi realizado posteriormente o procedimento para corar com rodamina faloidina a actina e Hoechst os núcleos. A microscopia de fluorescência foi realizada para ver a distribuição dos filamentos de actina [F.actina]. Finalmente, o resveratrol reduziu a formação do anel de actina. O resveratrol é o melhor composto bioativo para o preparo de medicamentos contra a perda óssea.

Palavras-chave: resveratrol, docking de proteína, in-vitro, ligante do receptor ativador do fator nuclear kappa-B [RANKL], microscopia de fluorescência.

*e-mail: dr.syedrizwan@kiu.edu.pk
Received: January 27, 2021 – Accepted: May 29, 2021

1. Introduction

Medicinal plants are very rich in phenolics and many bioactive compounds and have the potential for the synthesis of natural drug (Arcanjo et al., 2012; Sabir et al., 2021). Resveratrol is a substance known as Stilbenoid phytoacetin, which is found in plants such as red grapes, red wine, peanuts and other plant sources. The study of cardiovascular models, neurological systems, immunotherapy, and antiretroviral and therapeutic reactions demonstrated vital biological activity in in vitro and in vivo resveratrol and highlighted the potential benefits of this agent for many diseases (Baur and Sinclair, 2006; Pervaiz and Holme, 2009). There is a rich family of complementary nutrients, especially in the case of monomeric trihydroxystylbenzene resveratrol (3 5 4'-trihydroxy-trans-stilbene), which has major components that affect the effects of cardiovascular protection in red wine (Baur and Sinclair, 2006; Sousa et al., 2006). The subsequent studies have shown that resveratrol has more action that promotes health, including anti-cancer, antioxidant and anti-inflammatory properties (Fulda, 2010; Pervaiz and Holme, 2009). The most promising derivative of dimethoxylated, pterostilbene (3,5-dimethoxy-cross-hydroxy stilbene) is located in the palm of the valleys (santalinus pterocarpus), Indian film (marsupium Pterocarpus) spp Vaccinium. Grapes and fruits are low (vinifera Vitis), leaves and fruits. An important part of the body has shown that pterostilbena's anti-cancer evidence has similar antioxidant, anti-inflammatory and on-the-same action shown for its base compound Resveratrol (Joseph et al., 2008; Suh et al., 2007).

All changes that happen in LPS-stimulated cell could be inhibited by Resveratrol. Furthermore, resveratrol suppressed LPS-mediated decreases in HO-1 and Nrf2 levels in the inflamed periodontal tissues. According to our findings, resveratrol protects rats from periodontitic tissue damage by inhibiting inflammatory responses (Bhattarai et al., 2016).

Molecular Docking is an effective and qualitative tool for *in-silico* examinations. It plays an important role and has increased proportions of reasonable drugs (Drews, 2000). Docking is a calculation procedure for finding a suitable ligand that fits very well and geometrically in areas of binding proteins. In other words, it is a study of how two or more molecules, eg. ligands and proteins, stick together. The docking procedure is a combination of search algorithms and reporting functions. The maximum number of search algorithms and scanning features are available. Look for predictive algorithms, ligand-binding regression, and interpretations that are generally referring to the presentation (Sousa et al., 2006).

The software analysis of the binding analogue may be used as a predictive tool for the design of novel therapeutic compounds concerning the blocking of CYP3A4 and to facilitate the detection of biochemical nature of the interaction of dietary components, Plant and pharmaceutical compounds that are facilitated by the enzyme (Basheer et al., 2015).

2. Methods and Material

2.1. Data and databases

Structure of resveratrol obtained from PubChem database in 3D structure and saved in SDF file. For docking, the SDF file is changed into a PDB file by using online software. The X-ray crystallographic structure of the Receptor activator of nuclear factor-kappa-B ligand [RANKL] receptors was obtained from the Protein Data Bank (PDB). The file was downloaded in PDB format.

2.2. Docking tools

PyRx free software was used for Ligand to Protein docking. For result visualization, Discovery studio was used.

2.3. Protein preparation

The crystal structures of RANKL Proteins were obtained from the protein data bank. The active sites of the proteins were identified using reference ligands already in the target site and proteins were prepared by using the receptor preparing tool PyRx.

2.4. Osteoclast cell formation

The bone marrow cells are far from the old C57BL / 6J mice that are 4-5 weeks old (Kyung et al., 2008). Femora and tibiae were made ineffective and edges of the skeleton are reduced to the average of a small means (one microscope) using the 21-gauge needle, where the bone hang is immediately blended with plastic pipes to form single cells.

These cells are twice cleansed and injected into Bardet-Biedl Syndrome [BBS] (10%) serum and the suspension was placed on a plate together with Macrophage colony-stimulating factor [MCSF] (20 ng / ml) (R & D Systems, Minneapolis, MN) for 16 hours. Then, the non-bone cells are collected and concentrated with M-CSF for another two days, when many cells with cells such as molecules/ viruses are removed under a culture cycle.

A few non-adherent cells have been removed by PBS by clean up the dishes. The adherent bone marrow-derived macrophages (BMM) bone marrow is collected and fed. An additional device with M-CSF and RANKL (40 ng / ml) (R & D Systems, Inc.) was added and the container was moved on the 3rd day. After the intake for a specified period, the cells are stable in 10% official form for 10 minutes and the stain for TRAP as described (Kyung et al., 2008). Positive TRAP polygons (MNC) (three or more nuclei) are marked at the same time.

2.5. Bone resorbtion assay

Maturing Osteoclast Cell formation [OC] cells was developed by the M-CSF and RANKL fertilization for 5 days. A sample containing 1,000 cells is painted on a dental edge and is placed within 1 day using M-CSF and RANKL. The chain is sonicated in 1 M NH4OH to remove sticky tissue and stains with chemical Sigma cheese to see the wells for free.

OC survivors and a similar assessment for viability. After these incubations, the samples were stable in the form of 10% official form for 10 minutes and were reacted to tartaric acid-resistant phosphatase (TRAP) (34). The number of positive TRAP polygons of cells (MNC means three nuclei) is evaluated to determine survival.

3. Result and Discussions

3.1. Docking results

Docking was performed in a rigid mood. The docked molecule was examined and the score was considered for better interpretation. Docking of resveratrol ligands against 3ME2 and 1S55 showed positive results. Comparatively, resveratrol showed good results against all proteins and docked at the different energy level, which showed the strength of resveratrol as a drug for bone resorption. Figure 1.

The Molecular docking studies between the resveratrol and RANKL receptors proved that resveratrol binds tightly to the receptors, showed the highest binding affinities of −6.9, −7.6, −7.1, −6.9, −6.7, and −7.1 kcal/mol. resveratrol shall be excellent inhibitors against RANKL receptors (Table 1).

3.2. Docking results of resveratrol against RANKL proteins

Resveratrol is targeted for RANKL proteins and having biological activity as an Anti-bone loss. The previous

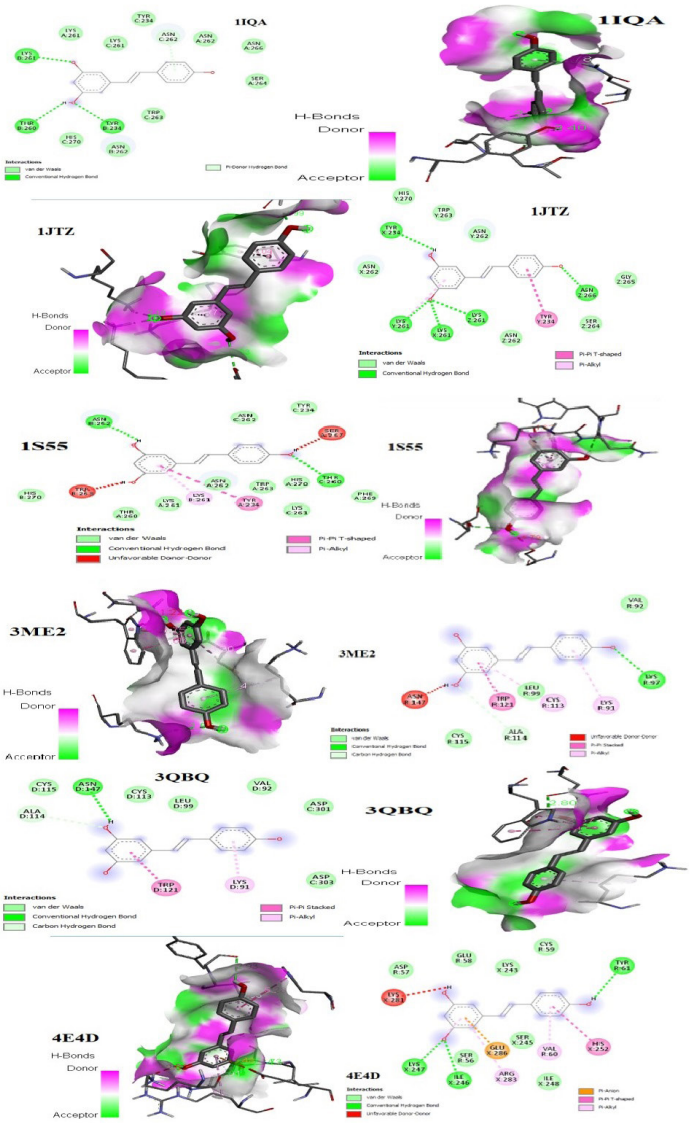

Figure 1. The binding model of Resveratrol against RANKL proteins. A Interactions between. Resveratrol and associated residues in the interface of the homology model for RANKL proteins. B, Binding models of Resveratrol in the RANKL proteins interface pocket. The numbers of lines represent the interaction distance (Å). RANKL proteins is the cause of bone loss and Resveratrol is the best for the inhibition of RANKL proteins.

finding showed Resveratrol is the best compound for bone healings. Our docking results also showed the best results against RANKL proteins (Figure 1). It interacted with a good number of amino acids of RANKL proteins along with the best efficacy. It shows Resveratrol is the best inhibitor for RANKL proteins.

3.3. Resveratrol inhibits OC differentiation

Our findings suggest that resveratrol shows an increase in cAMP in the OC group and prevents bone loss and oxidative stress brought about in mice that show cAMP protection to maintain bone mass. We investigated the Resveratrol results on the development of abnormalities in BMP culture from rats lacking stromal and lymphocytes to check whether it stimulates bone activity. Figure 2.

Bone marrow cells were isolated by flushing the femur bones of 6 week old female mice. Isolated cells were cultured with M.CSF (20ng/ml) overnight. Next day we get the stromal attached cells and floating cells. These floating cells were cultured for extra 3 days with M.CSF

Table 1. Binding energy of RANKL protein with Resveratrol.

	1JTZ	1S55	3ME2	3QBQ	4E4D	4GIQ	1IQA	1TJZ
Resveratrol	-6.9	-7.6	-7.1	-6.9	-6.7	-7.1	-6.9	-6.9

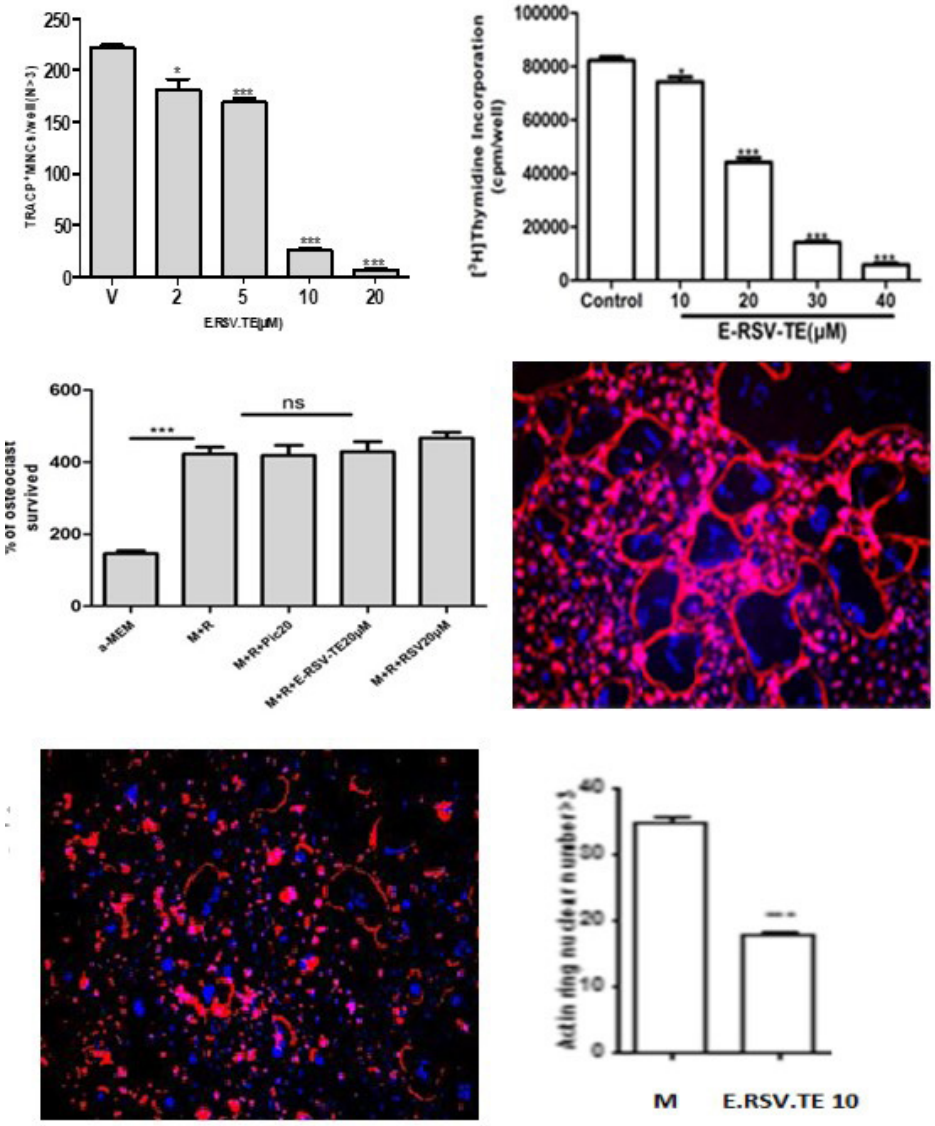

Figure 2. Inhibition of OC differentiation by Resveratrol.

20ng/ml to get Bone marrow-derived macrophages. These BMM were treated with M.CSF 20ng/ml and RANKL 50ng/ml along with different concentrations of Resveratrol (2.5, 10 µg/ml) for 7 days. Mature osteoclast with multiple nuclei was stained with TRAP stain. Our findings showed that Resveratrol dose-dependently reduced the osteoclast differentiation.

BMM are treated with M.CSF (20ng/ml) along with different concentration of Resveratrol (2.5, 10 µg/ml) for a specific time. After that [3H] thymidine was added to each well and incubated for 24 h. Radioactivity was determined by scintillation counting. Which showed Resveratrol Trimethyl ether dose-dependently reduced the BMM proliferation.

3.4. Actin cytoskeleton

Wash the Bone marrow macrophages derived mature osteoclast and treat with Resveratrol (2.5, 10 µg/ml) for 5 hours. Fix the osteoclast cells with formaldehyde fixative on glass coverslip followed by incubation with 0.1% Triton X-100 in PBS for 5 min and after that stain with rhodamine-phalloidin staining for actin and Hoechst for nuclei. Fluorescence microscopy was performed to see the distribution of F.actin. Finally, Resveratrol reduced the actin ring formation (Figure 3).

BMM isolated from Sham and Ovariectomy mice were treated with cytokines like M.CSF and RANKL along with Resveratrol. Trimethyl ether and incubated for 48 hours. After that cells were harvested with Trypsin and EDTA. Suspend the cells in Phosphate buffer saline and load the cells with DCFH-DA. Incubate the cells 37 C for 30 min. ROS measurements was performed using a flow cytometry with a fluorescence-activated cell sorter (FACS) Calibur (Becton Dickinson). Regarding to our findings Resveratrol decreased the ROS level in both Sham and OVX mice as compared to control.

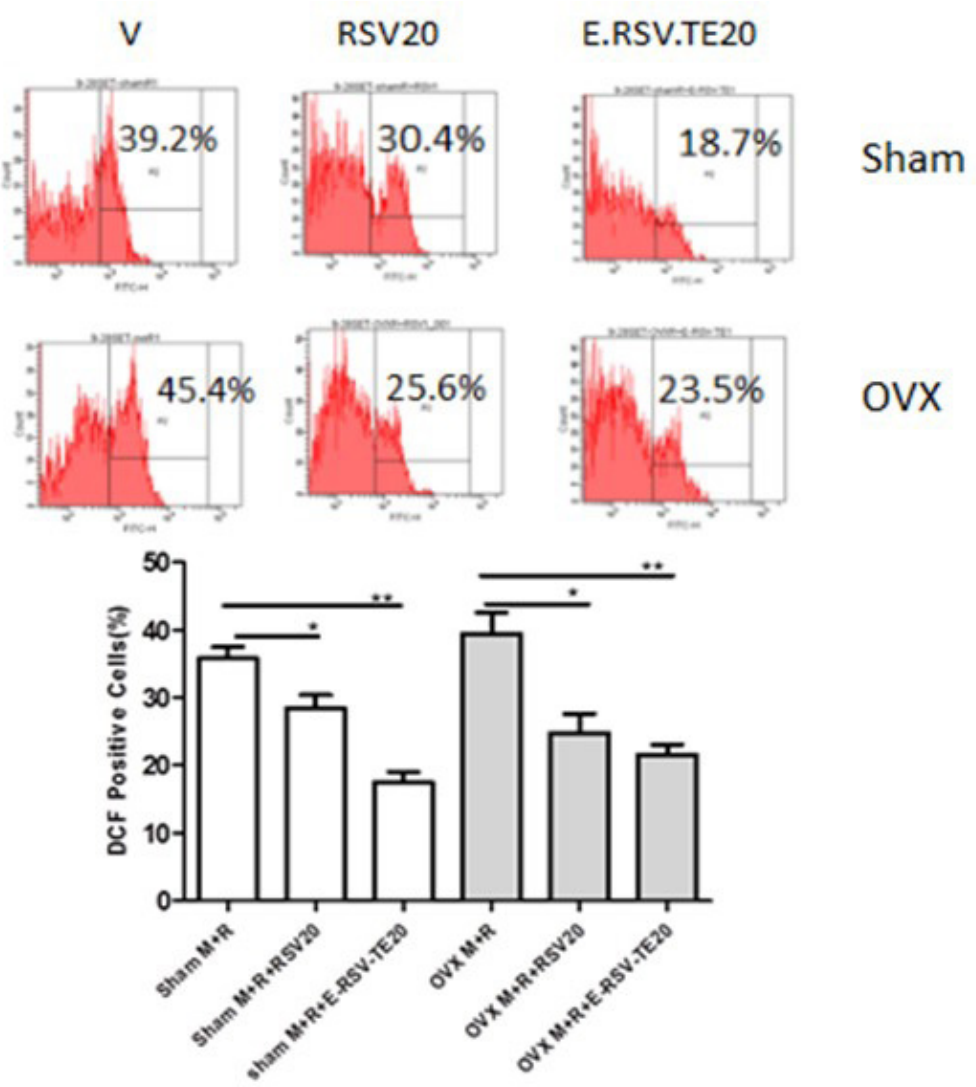

Figure 3. Florescence microscopy of BMM after Resveratrol Treatment.

4. Conclusions

Rheumatoid arthritis is a major disease in human beings. Our findings showed that bone loss is because of RANKAL proteins. Resveratrol is a natural-based bioactive compound was selected for this study. Both in-silico and in-vitro studies were performed. According to an In-silico study, eight RANKAL protein were selected for docking. Resveratrol showed the best efficacy with all selected RANKL proteins. Furthermore, in-vitro studies were made on mice model for more verifications. Resveratrol also performed well regarding bone loss. So, according to our study Resveratrol might be the best drug for bone loss.

References

ARCANJO, D.D.R., ALBUQUERQUE, A., MELO NETO, B., SANTANA, L., MEDEIROS, M. and CITÓ, A., 2012. Bioactivity evaluation against Artemia salina Leach of medicinal plants used in Brazilian Northeastern folk medicine. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 72, no. 3, pp. 505-509. http://dx.doi.org/10.1590/S1519-69842012000300013. PMid:22990821.

BASHEER, L., SCHULTZ, K., FICHMAN, M. and KEREM, Z., 2015. Use of in vitro and predictive in silico models to study the inhibition of cytochrome P4503A by stilbenes. *PLoS One*, vol. 10, no. 10, pp. e0141061. http://dx.doi.org/10.1371/journal.pone.0141061. PMid:26485399.

BAUR, J.A. and SINCLAIR, D.A., 2006. Therapeutic potential of resveratrol: the in vivo evidence. *Nature Reviews Drug Discovery*, vol. 5, no. 6, pp. 493-506. http://dx.doi.org/10.1038/nrd2060. PMid:16732220.

BHATTARAI, G., POUDEL, S.B., KOOK, S.H. and LEE, J.C., 2016. Resveratrol prevents alveolar bone loss in an experimental rat model of periodontitis. *Acta Biomaterialia*, vol. 29, pp. 398-408. http://dx.doi.org/10.1016/j.actbio.2015.10.031. PMid:26497626.

DREWS, J., 2000. Drug discovery: a historical perspective. *Science*, vol. 287, no. 5460, pp. 1960-1964. http://dx.doi.org/10.1126/science.287.5460.1960. PMid:10720314.

FULDA, S., 2010. Resveratrol and derivatives for the prevention and treatment of cancer. *Drug Discovery Today*, vol. 15, no. 17-18, pp. 757-765. http://dx.doi.org/10.1016/j.drudis.2010.07.005. PMid:20692359.

JOSEPH, J.A., FISHER, D.R., CHENG, V., RIMANDO, A.M. and SHUKITT-HALE, B., 2008. Cellular and behavioral effects of stilbene resveratrol analogues: implications for reducing the deleterious effects of aging. *Journal of Agricultural and Food Chemistry*, vol. 56, no. 22, pp. 10544-10551. http://dx.doi.org/10.1021/jf802279h. PMid:18954071.

KYUNG, T., LEE, J., SHIN, H. and CHOI, H., 2008. Rutin inhibits osteoclast formation by decreasing reactive oxygen species and TNF-α by inhibiting activation of NF-κB. *Experimental & Molecular Medicine*, vol. 40, pp. 52-58. http://dx.doi.org/10.3858/emm.2008.40.1.52. PMid:18305398.

PERVAIZ, S. and HOLME, A.L., 2009. Resveratrol: its biologic targets and functional activity. *Antioxidants & Redox Signaling*, vol. 11, no. 11, pp. 2851-2897. http://dx.doi.org/10.1089/ars.2008.2412. PMid:19432534.

SABIR, S.M., ZEB, A., MAHMOOD, M., ABBAS, S.R., AHMAD, Z. and IQBAL, N., 2021. Phytochemical analysis and biological activities of ethanolic extract of Curcuma longa rhizome. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 81, no. 3, pp. 737-740. http://dx.doi.org/10.1590/1519-6984.230628.

SOUSA, S.F., FERNANDES, P.A. and RAMOS, M.J., 2006. Protein–ligand docking: current status and future challenges. *Proteins*, vol. 65, no. 1, pp. 15-26. http://dx.doi.org/10.1002/prot.21082. PMid:16862531.

SUH, N., PAUL, S., HAO, X., SIMI, B., XIAO, H., RIMANDO, A.M. and REDDY, B.S., 2007. Pterostilbene, an active constituent of blueberries, suppresses aberrant crypt foci formation in the azoxymethane-induced colon carcinogenesis model in rats. *Clinical Cancer Research*, vol. 13, no. 1, pp. 350-355. http://dx.doi.org/10.1158/1078-0432.CCR-06-1528. PMid:17200374.