In Silico Molecular Docking and in Vitro Analysis of Eugenol as Free Radical Scavenger in Patients with Dengue Infection

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Abstract Infection by the Dengue virus (DENV) threatens global public health due to its high prevalence and the lack of effective treatments. Oxidative and cytotoxic damage plays an important role in Dengue pathogenesis and may serve as an important target for treatment. DENV infection activates Keap1/Nrf2 signaling pathway that leads to transcription of downstream antioxidant and detoxification genes such as HMOX-1, SOD2, NQO1, etc. In this study, both the molecular docking technique and In-Vitro experiments were performed to show potentiality of Eugenol as an activator of Keap1/Nrf2 signaling pathway. The molecular docking work concludes that Eugenol can actually induce Keap1/Nrf2 signaling pathway with a significant change in negative Firedock Global-Energy value, AScore value and as well as experimentally, Eugenol demonstrated promising antioxidant potential and free radical (RNS) scavenging activity.

Keywords Keap1/Nrf2 · RNS · DENV · Eugenol · DPPH · PatchDock · FireDock · BioVia discovery studio · Global-energy value · ArgusLab · AScore value

1 Introduction

Dengue-infection is a mosquito-borne viral infection spreading rapidly throughout the world, particularly in tropical or subtropical countries (Mapalagamage et al. 2018). Dengue-virus belongs to the family-Flaviviridae and genus-Flavivirus and might cause dengue-infection. According to WHO guidelines in 2009, Dengue infection has been classified based on their symptoms: Dengue-without warning-sign

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(DWOWS) (Nausea, Vomiting, Aches, and pain leucopenia), Dengue-with warning-signs (DWWS) (Abdominal pain or tenderness, persistent vomiting, clinical-fluid-accumulation, Mucosal-bleeding, Restlessness) and Severe-dengue (SD) (Severe plasma-leakage, Severe bleeding). A current model calculates 390 million belongings each year, with ninety-six million cases manifesting with a minimum of some clinical displays. As per WHO, the South-East Asia and Western Pacific regions square measure extremely endemic for this sickness (Ahmad et al. 2018). In India, Kolkata is a hyperendemic region and has witnessed many Dengue epidemics in current years. So far, dengue infection remains the number one pathological state, in Asian nations and around the world (World Health Organization 2011).

Viral infections usually cause the enhanced expression of proinflammatory cytokines. Reactive-Oxygen and Nitrogen-species (i.e. ROS, RNS) are generated in the monocytes, macrophages, and many other resistant cells in viral infections. However excessive secretion of ROS, RNS makes the inequity between these peroxidant and antioxidants leading oxidative-stress which may cause many deleterious effects on the host. Damages induced by oxidative-stresses and changes into redox status are being identified in some patients with Dengue infection which suggests the crucial role of oxidative-stress in Dengue pathogenesis (Chaturvedi and Nagar 2009). NO is one such extremely reactive molecule and considered as major peroxidant in the body which can spread through cells. The enzyme NO synthase produces NO. Peroxynitrite is also harmful when it is there in high concentration, oxidizing genetic material, lipids, and oxidizing and nitrating proteins thus peroxynitrite enhances oxidative stresses (Chaturvedi and Nagar 2009). Defense against such excessive free radical accumulation and reticence of the RNS is important cytoprotective mechanisms that are regulated and controlled by the activation of Keap1/Nrf2 signaling pathway (Olagnier et al. 2014). Keap1-Nrf2 signaling cascade is the key controller pathway against prolonged oxidative stresses. Nrf2 (nuclear factor E2-related factor 2) belongs to family of leucine zipper transcription protein factors. It binds with Maf-Proteins to the regulatory promoter region of downstream Nrf2 targeted genes and enhances expression of >200 oxidative stress-related genes to protect the cell from oxidative stress-induced damages (Barrera-Rodríguez 2018; Kansanen et al. 2013; Leung et al. 2019). The transactivation of Nrf2 targeted genes within the cell is strictly regulated and maintained at its basal level by Keap1, a cytoplasmic adaptor protein molecule of the Cullin3 based E3- ligase complex (David et al. 2017). The 605-residue human Nrf2 protein is composed of seven Nrf2-ECH homology (Neh1–7) domains all that have distinct functions. The N-terminal Neh2 domain mediates interaction with C-terminal portion of Keap1 that tightly regulates the permanency of Nrf2 (Tonelli et al. 2018). Keap1 (Kelch-like ECH-associated protein 1) contains five domains of which the C terminal portion of Keap1or the Kelch domain interacts with Neh2 domain. The other domains of Keap1 namely BTB domain and IVR help in homo dimerization of Keap1 and interacting with Cullin3 (Taguchi et al. 2011). Neh2 domain is bound to Kelch domain in homeostasis. Keap1 protein moderates Cul3 E3 complex for ubiquitination, which leads to continuous ubiquitination and destruction of Nrf2 during non-stressed conditions (Tong et al. 2006). This kind of

quenching interaction keeps up lower basal expression of Nrf2 mediated cytoprotective gene transcription. However, when cell experiences oxidative stress, Keap1 gets inactivated and the poly-ubiquitination of Nrf2 is halted and newly synthesized Nrf2 proteins bypass Keap1 mediated degradation resulting in accumulation of the Nrf2 in cytoplasm. Consequently, Nrf2 gets accessed into nucleus, transcribes downstream Nrf2 targeted genes like SOD2, HMOX1, etc. (Theodore et al. 2008).

Thus the administration of antioxidant molecules to Dengue infected patients may limit virus-mediated cell damage and restrict the patient to go into severe conditions. Any phytochemical that has potent antioxidant activity and proved to be less toxic to human body can be employed in Dengue pathogenesis. Eugenol, a very common natural phytochemical suffice all our requirements to study its beneficial role in Dengue-induced oxidative stress and in future it may open up novel treatment methods for Dengue-associated diseases. Eugenol (4-allyl-2-methoxy phenol (EUG)) a hydrocarbon is present as yellow viscous oil (de Araújo Lopes et al. 2018). This is an associate with aromatic and phenolic compound from the category of phenylpropanoids (Barboza et al. 2018). It is a key component of cloves and found in bay leaves and all spices (Barboza et al. 2018; Ghofran et al. 2019). This is utilized in food industry (Nagababu et al. 2010)) as a preservative compound, appreciated due to its inhibitor property (Zhang et al. 2009). Eugenol shows numerous biological activities like bactericide, (Xu et al. 2016) antifungal (Chami et al. 2005; Gayoso et al. 2005) antiallergic (Kim et al. 1998; Corrêa et al. 2008) properties. Eugenol conjointly has medication, chemoprotective effects furthermore it has antioxidant-activity (Yogalakshmi et al. 2010) credited due to the existence of the phenolic cluster in its structure. For that reason, Eugenol has attracted several researchers. Numerous studies also opine that Eugenol has bioactive terpenes that inhibit ROS production in human neutrophil. Eugenol is a helpful pain reliever, and has antioxidant-activity. Taken together the current study aims to investigate the effectiveness of Eugenol (if any) in Dengue infection.

2 Materials and Methods

2.1 In-Silico Analysis

Protein preparation: The X-ray Crystallographic structure of Keap1-Neh2 complex (PDB ID 3ZGC) was obtained from the Protein Data Bank (PDB) at a resolution of 2.2 Å. Water molecules, ligands, and other hetero atoms were removed from protein complex and obtained the C-terminal Kelch domain (A and B chain) of Keap1 and the Neh domain (C chain) of Nrf2 separately by using Biovia Discovery Studio client software.

Ligand Preparation: The 3D structure of Eugenol was obtained and downloaded from the PUBCHEM database. The proteins and ligand were saved as PDB format for further analysis.

Automatic Docking: The computational molecular docking was accomplished by PatchDock server. Protein-small ligand platform of PatchDock was employed for docking by using clustering Root-Mean-Square-Deviation (RMSD) value of 4.0 (Chaturvedi et al. 2016). Neh domain of Nrf2 was docked against Kelch domain of Keap1 and Eugenol was docked against Kelch domain followed by docking of Neh domain against Kelch-Eugenol complex domain in separate pair of docking analysis. In both analyses, the complexes were sorted based on their PatchDock scores produced by the server. Further, PatchDock score refinement was accomplished by using FireDock server. The most stable conformations of desired protein–protein and the protein–ligand complexes were selected based on highest negative Global Energy (GE) value given by the FireDock server. Further validation was done by implementing flexible algorithm with ArgusLab 4.0.1 Docking Engine. The grid box was generated for assortment and formation of the dynamic binding pocket where the ligand could actually bind using grid resolution of 0.40 Å. Docking calculation was performed using AScore scoring function and the complexes that were best docked were chosen depending on the least AScore calculated by ArgusLab (Chikhi and Bensegueni 2008). The conformations of the complexes were envisaged by Discovery Studio software for further analysis.

2.2 In-Vitro Experiments

Chemicals: The subsequent compounds used for inhibitor activities, obtained from Sigma-Aldrich: Eugenol (4-allyl-2-methoxyphenol), Ascorbic acid. DPPH was purchased from Himedia. Ethanol was purchased from Merck. Griess chemical agent was purchased from Sigma. NO colorimetric assay kit from Cayman, subsequent experimental procedures were applied to guage the radical scavenging activity of Eugenol.

Study Population: Enrolled 37 Dengue patients at Calcutta School of Tropical Medicine from July 2019 to October 2019 after obtaining their consent. They were confirmed by both Dengue-NS1/IgM ELISA, and RT-PCR. They are classified into Dengue-without warning-sign (DWOWS), Dengue-with warning-signs (DWWS) according to WHO 2009 criteria through their symptoms. We had also enrolled 15 Healthy Donors (HD) with no history of illness in the past 3 months.

Serum separation and processing: Venous blood was collected from all patients and healthy. Five ml of blood were collected by venipuncture into a germ-free clot-activated tube and blood was separated by centrifugation process at 2000 RPM for ten minutes, stored in -20 °C temperature and clear serum were used for experiments.

DPPH assay: To assess the antioxidant potential of Eugenol, DPPH assay based on the methods of Brand-Williams et al. with small modification (Szerlauth et al. 2019) was used. Diphenyl-1-picrylhydrazyl (DPPH) has a radical-scavenger effect and has the ability to donate hydrogen, especially those with a phenolic cluster in their structure. This method is based on electron transport. It produces a purple ethanolic solution. Free radical molecules are decreased by the antioxidant molecules, producing yellowish ethanolic solution. Different concentrations (1.5–5.5 μg/ml) of Eugenol and Ascorbic acid (standard) were used, mixed with equivolume of DPPH solution. Optical density was measured at 492 nm after 30 min incubation at room temperature. The radical scavenging activity was calculated in percentage from the following formula: % scavenging [DPPH] = [(A0 − A1)/A0] × 100**.**Where A0 was the absorbance of the control and A1 was the absorbance of the samples. IC50 value was interpolated from the standard graph.

Serum Nitrite and Nitrate measurement: Reactive-nitrogen-species were determined by estimating the stable merchandise of nitrite and nitrate. Total nitrite + nitrate was considered by utilizing a nitrate and nitrite colorimetric-assay-kit (Cayman, USA) in the serum sample, following the manufacturer's directions. This assay determine nitrite + nitrate depending on the enzymatic translation of nitrate to nitrite by nitrate reductase enzyme. The reaction following a quantitative chemical analysis detection of nitrite by Griess reaction supported by the diazotization-reaction within which acidified NO_2^- produces a nitrosating agent that reacts with sulphanilic acid to yield the anion particle. This particle is then combined to N-(1-naphthyl) ethylenediamine making deep purple chromophoric chemical group spin off that absorbs light-wavelength at 540 nm.

Statistical Analysis: All analysis was performed using Graph-Pad Prism statistics software (Graph-Pad-Software-Inc., San-Diego, CA, USA). As the numeric variables had nonparametric distribution, One-way ANOVA with Kruskal–Wallis tests was used to differentiate more groups, respectively. Mann–Whitney test was used to compare two groups. For Mean ± SEM values, we used descriptive statistics. Differences with p values smaller than 0.05 were considered to be statistically significant.

3 Results

In-Silico Results: To find the potentiality of Eugenol as an antioxidant molecule and whether it could enhance the Keap1/Nrf2 pathway, two different sets of analysis on molecular docking were done. The first set of docking analysis showed Neh domain of Nrf2 docked against Kelch domain of Keap1 protein via PatchDock server. The docking result shows FireDock GE value of −48.90 with 10 conventional Hydrogen-bonding (SER363, ASN382, SER508, GLN530, TYR572, SER602, ASN387, GLY574, GLY386 of Keap1), 3 salt bridges (ARG380, ARG415, ARG483

of Keap1), 3 electrostatic interactions (ARG415 of Keap1, GLU78, GLU79 of Nrf2) and 2 hydrophobic interactions (GLU82, THR80, GLY81 of Nrf2)—a sum total of 18 Non-bonding interactions (Table 1). In the other set of docking analysis, Eugenol was docked against Kelch domain of Keap1 and the most stable Keap1-Eugenol complex structure was chosen on the basis of highest negative Firedock GE value. Then, Neh domain of Nrf2 was docked against Keap1-Eugenol complex via PatchDock server. The docking result shows decreased FireDock GE value of -38.04 with 2 conventional Hydrogen bonding (ASN382, TYR572 of Keap1), 1 Salt bridge (ARG380 of Keap1) and 4 electrostatic interactions (ARG380, ARG483 of Keap1, and GLU78 of Nrf2)—a total of 7 Non-bonding interactions (Table 2) demonstrating Eugenol prevents Keap1 to bind Nrf2 and thus bypassing proteasomal destruction of Nrf2. ArgusLab flexible docking analysis also shows similar kind of results. Nrf2 when docked against Keap1 best Ligand Pose energy of −5.80586 kcal/mol was obtained and when Nrf2 docked against Keap1-Eugenol complex best Ligand Pose energy of −5.53895 kcal/mol was obtained.

Study Population: Dengue-patients were classified into DWOWS, DWWS as per 2009 WHO guidelines. They were categorized according to their symptoms and the result of the biochemical test.

Table 1 Amino acids involved in non-bonding interactions in Nrf2-Keap1 docked complex (obtained from Discovery Studo software)

Neh2 Domain of Nrf2 interacting with Kelch Domain of Keap1	
Bond Donor to bond acceptor	Type of bond
1. keap1:ARG380:NH1—nrf2:GLU82:OE1	Salt bridge
2. keap1:ARG415:NH2—nrf2:GLU79:OE2	Salt bridge
3. keap1:ARG483:NH1—nrf2:GLU79:OE1	Salt bridge
4. keap1:ARG415:NH1—nrf2:ASP77:OD2	Electrostatic
5. keap1:ARG415:NH1—nrf2:GLU79:OE1	Electrostatic
6. keap1:SER363:OG—nrf2:GLU82:OE2	Conventional hydrogen bond
7. keap1:ASN382:ND2—nrf2:GLU82:OE1	Conventional hydrogen bond
8. keap1:SER508:OG—nrf2:GLU79:OE2	Conventional hydrogen bond
9. keap1:GLN530:NE2—nrf2:GLU78:O	Conventional hydrogen bond
10. keap1:GLN530:NE2—nrf2:GLU78:OE1	Conventional hydrogen bond
11. keap1:TYR572:OH—nrf2:GLU78:OE2	Conventional hydrogen bond
12. keap1:SER602:OG—nrf2:THR80:O	Conventional hydrogen bond
13. keap1:ASN387:N—nrf2:GLY76:O	Conventional hydrogen bond
14. keap1:GLY574:CA—nrf2:GLU78:OE1	Conventional hydrogen bond
15. keap1:GLY386:CA—nrf2:GLY76:O	Conventional hydrogen bond
16. nrf2:GLU78:C,O;GLU79:N—keap1:TYR525	Electrostatic
17. nrf2:GLU82:OE2—keap1:TYR334	Hydrophobic
18. nrf2:THR80:C,O;GLY81:N—keap1:TYR572	Hydrophobic

Table 2 Amino acids involved in non-bonding interactions when Nrf2 docked against Keap1-Eugenol complex (obtained from Discovery Studio software)

Neh2 Domain interacting with Kelch Domain in presence of Eugenol	
Bond Donor to Bond acceptor	Type of bond
1. keap1:ARG380:NH2—nrf2:GLU82:OE2	Salt bridge
2. keap1:ARG380:NH1—nrf2:GLU82:OE1	Electrostatic
3. keap1:ARG483:NH1—nrf2:GLU78:OE2	Electrostatic
4. keap1:ARG483:NH2—nrf2:GLU78:OE1	Electrostatic
5. keap1:ASN382:ND2—nrf2:GLU82:OE1	Conventional hydrogen bond
6. keap1:TYR572:OH—nrf2:GLU79:OE1	Conventional hydrogen bond
7. nrf2:GLU78:OE1—keap1:TYR525	Electrostatic

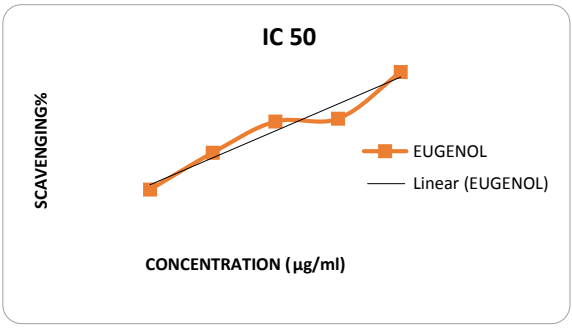

Fig. 1 Determination of IC 50 of Eugenol

DPPH Assay: The antioxidant property of Eugenol was determined by using DPPH scavenging assay followed by gross IC50 value determination. The IC50 of Eugenol was 3.02 µg/ml. A standard curve was prepared using ascorbic acid in different concentrations. The DPPH—scavenging capacity, was calculated from the graph through linear regression (R2 = 0.941). Thus Eugenol's ability to sequester free radicals within the DPPH solution was obtained (Fig. 1).

3.1 Determination of Nitrite and Nitrate Level

High concentration of nitrite and nitrate was observed in the patients with DWWS (57.25 ± 5.9 µM) compared with patients with DWOWS (42.71 ± 5.5 µM) and HD (23.56 ± 2.6 µM) (Fig. 2). Though there was no significant difference between patients with DWWS and DWOWS ($p = 0.3077$). The level of nitrite + nitrate level is significantly higher in DWWS than HD ($p = 0.0340$).

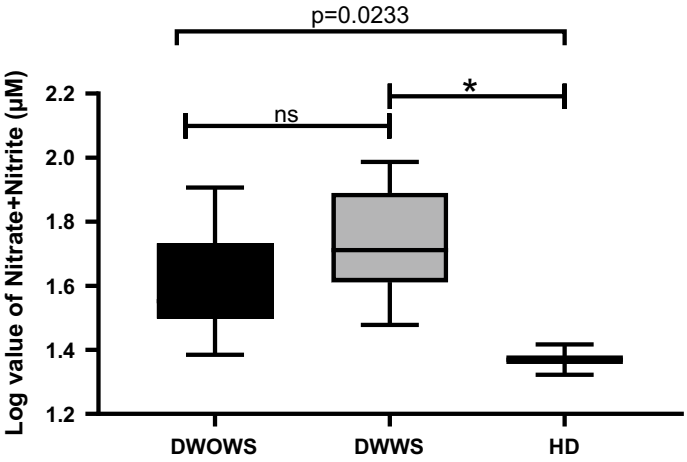

Fig. 2 Nitrite and Nitrate values in serum samples (μM of NO_2^- and NO_3^-) of patients with Dengue without warning sign (DWOWS, $n = 19$), Dengue with Warning sign (DWWS, $n = 18$), and Healthy Donors (HD, $n = 15$) measured by Griess reaction. Results were expressed by the median value using the box plot. Symbols (*) represent statistically significant differences ($p = 0.0340$) between DWWS and HD. Nitrite and Nitrate level is comparatively higher in DWWS ($p = 0.0233$) than DWOWS and HD. One-way ANOVA with Kruskal–Wallis test was done for comparing different study categories and Dunn's multiple comparisons test was done for repeated measures

3.2 Determination of Nitrite and Nitrate level After Eugenol Treatment

From Figs. 1 and 2, the antioxidant property of Eugenol was obtained and additionally high nitrite + nitrate level was found in patients with Dengue with warning signs. Further following Srejayar and Rao.et.al protocol the RNS quenching ability of Eugenol was investigated. Serum samples containing high nitrite + nitrate level were treated with/without Eugenol for 150 min, room temperature, subsequent to which Griess reagent was added and Optical Density was measured at 546 nm after 30 min incubation (Fig. 3).

Interestingly, very low amounts of nitrite and nitrate were obtained in the eugenol treated serum of dengue infected patients (28.37 ± 4.9 μM) as compared to samples without eugenol treatment (59.14 ± 5.9 μM). Thus, 2.1 fold reduced nitrite and nitrate were obtained in the serum of dengue patients when it was treated with eugenol.

4 Discussion

Dengue infection is one of the fastest spreading viral infections, threatening the whole world. Its cure and treatment have become a major concern. In this respect, the current

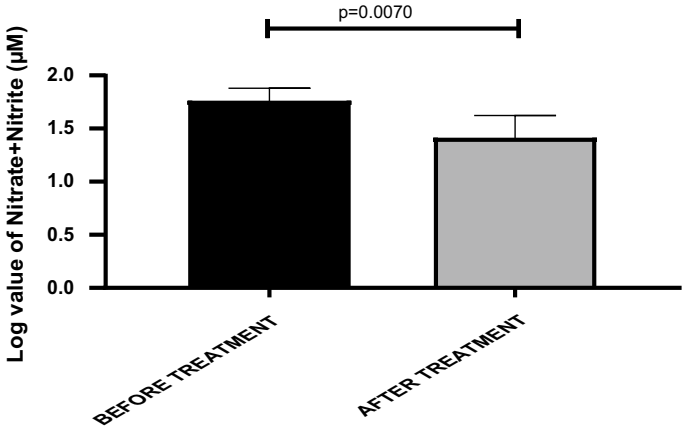

Fig. 3 Nitrite and nitrate value of patients with dengue infection before Eugenol treatment and after Eugenol treatment. Results were expressed, using Mann–Whitney test. Nitrite and Nitrate level is significantly reduced ($p = 0.0070$) after Eugenol treatment

study tried to find out a natural cure for dengue pathogenesis and found Eugenol to be a potent candidate for our studies. Eugenol was demonstrated to modulate the antioxidative Keap1-Nrf2 pathway through In Silico studies and could results in reduction in the free radical accumulation during Dengue infection through in vitro serum analysis. In this investigation, In Silico studies showed a reduction in free energies as well as diminution in the number of non- bonding interactions. Though the changes obtained in the free energy by the docking engines may seem very little and insignificant but all these changes are moderated by only a single molecule of Eugenol. Decreasing the number of non-bonding interactions inbetween Keap1 and Nrf2 is evident that Eugenol binds with Keap1 in the same interacting domain where Nrf2 binds. The lowering in the negative FireDock GE value as well as AScore pose energy value indicates that Keap1 cannot bind Nrf2 so firmly that it can act as an adaptor protein molecule for the Cul3 E3 ligase as long as Eugenol is bound to it. This propounds that newly synthesized Nrf2 can then bypass Keap1 mediated proteasomal degradation pathway which might lead to summoning up of Nrf2 in the cytosol followed by translocation into nucleus where it can induce transcription of oxidative stress-related genes (Fig. 4).

Eugenol, a compound, containing phenolic resin clusters, exhibits antioxidant-property by ending radical species through the loss of atom. In step with the results of our study, Eugenol had the foremost powerful antioxidant-activity. Nitric oxide is an essential chemical moderator, generated by neurons, macrophages, endothelial cells etc., and is implicated in many physiological processes. NO produced in macrophages and epithelial cells acts as an important molecule in the regulation of the diameter of blood vessels, inhibiting WBC adhesion and platelet aggregation. A balanced quantity of NO in the body is essential to maintain vital metabolic activities, a decreased or increased level, however may be deleterious to health. Figure 2,

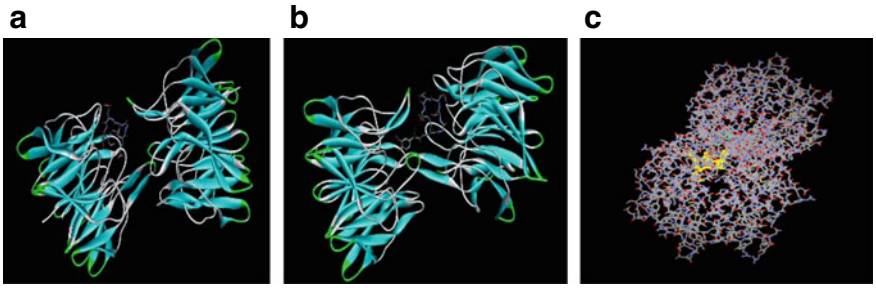

Fig. 4 **a** Neh domain interacting with Kelch domain. **b** Neh domain interacting with Eugenol-Kelch complex (Discovery Studio visualization). **c** Neh domain interacting with Eugenol-Kelch complex (ArgusLab visualization)

demonstrated, higher concentration of Reactive Nitrogen species (nitrite + nitrate) in Dengue with warning sign. In the acute phase of Dengue infection, the amount of Reactive-Nitrogen-Species remains high in infection, causing major pathophysiological effects. Therefore, the RNS quenching ability of Eugenol was next investigated, Fig. 3 demonstrated the amount of nitrite + nitrate, which is drastically reduced after Eugenol treatment. This may be caused by occurrence of antioxidant properties in the Eugenol, which competes with oxygen, doing a reaction with nitric oxide and thereby blocking the formation of nitrite and nitrate. This, therefore, demonstrates the usefulness of Eugenol in Dengue infection.

5 Conclusion

Thus, In-Silico analysis concludes that Keap1 cannot bind Nrf2 to that extend so that it can degrade Nrf2 by Proteasomal pathway as long as Eugenol is bound to Keap1. So, Keap1-Nrf2 pathway can be modulated in presence of Eugenol which ultimately leads to cytoprotection during oxidative stress. We conclude from the above discussion that Eugenol has an antioxidant activity by scavenging the free Reactive-Nitrogen-Species. Eugenol thus could be a possible drug candidate for treating Dengue infections. However, our conclusions must be verified in a larger study population.

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