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CHRONOTROPIC, INOTROPIC, AND RADICAL SCAVENGING ACTIVITY OF *Curcuma heyneana* EXTRACT BY MOLECULAR DOCKING SIMULATION AND *In-vitro* STUDY ON THE HEART OF *Fejervarya cancrivora* FROG

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ABSTRACT

The β-1 adrenergic receptor belongs to GPCRs which communicates via the Gs-α subunit, thus initiating the cAMP-dependent pathway through the upregulation of adenylyl cyclase. The activation of the β-1 adrenergic receptor in the heart triggers the activation of the sinoatrial node and atrioventricular nodes, hence escalating heart rate (positive chronotropic) and contractility (positive inotropic). Flavonoids and terpenes have been reported for their inotropic effect in animal models. In this work, we examined the chronotropic and inotropic activity of *Curcuma heyneana* Valeton and Zijp rhizome by determining the molecular interaction between the bioactive compounds, namely, α-sitosterol, stigmasterol, quercetin, and curcumenol, with human G-protein-coupled receptor kinase 2 (hGRK2), and *in vitro* study of *C. heyneana* extract on the heart of crab-eating *Fejervarya cancrivora* frog. Moreover, the total phenol content in the extract and the radical scavenging activity were also determined. The results indicated that *C. heyneana* positively contains phenolic compounds and weakly inhibits the radicals of the DPPH reagent (IC_{50} = 320.0 µg/mL). The molecular docking study revealed that α-sitosterol and quercetin have the best interaction with important residues in hGRK2, similar to that of the enzyme's inhibitor. *C. heyneana* decreases the heart rate and increases the heart contractility of crab-eating *F. cancrivora* frogs. Based on these findings, *C. heyneana* is the potential in helping the heart pump more blood to fulfill the oxygen demands, but with lesser heartbeats, thus it is useful for patients with cardiovascular disorders.

Keywords: β-1 Adrenergic Receptor, *Curcuma heyneana*, G-protein-Coupled Receptor Kinase 2, Radical Scavenging Activity.

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INTRODUCTION

Human G-protein-coupled receptor kinase 2 (hGRK2) was first found to be intricated in GPCR desensitization, particularly the β-adrenergic receptors. hGRK2 maintains the cardiac structure and function; hence, it is necessitated in various cardiovascular dysfunctions. Its increased level occurs during the initial phases of cardiovascular disorders and in patients with high blood pressure. GRK2 is a node in the cytosol of cardiac muscle cells, that affects cardiac contractile function.[1,2] GRK2 expression elevates forthwith post-cardiac impairment and leads to the desensitization of β-adrenergic receptors.[3,4] The β1-adrenergic receptor is mainly in control for signaling in the sympathetic nervous system. β-agonists attach to this receptor on various tissues, particularly located in the heart. This receptor communicates via the Gs-α subunit, thus initiating the cAMP-dependent pathway through the upregulation of adenylyl cyclase. The activation of the β1-adrenergic receptor in the heart triggers the sinoatrial node and atrioventricular node, hence escalating heart rate (positive chronotropic) and contractility (positive inotropic). Many drugs have been used to manipulate the β1-adrenergic receptor, either by blocking or potentiating the effects to achieve certain conditions.[5] Secondary metabolites in plants, such as flavonoids (genistein, naringenin, quercetin, rutin, etc.), have been reported in possessing a biphasic inotropic effect in an animal model.[6,7] Moreover, monoterpenes and monoterpenoids have been disclosed for their inotropic activity, both positive and

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negative.[8] *Curcuma heyneana* (Zingiberaceae family) (Fig.-1), local name *temugiring*, contained sesquiterpenes, diterpenes, monoterpenes, phenolic compounds, and phytosterols,[9] which contribute to its anti-inflammatory activity by suppressing LPS-stimulated cytokines in RAW264.7 macrophage.[10] In this work, we examined the inotropic and chronotropic activity of *Curcuma heyneana* Valeton and Zijp rhizome (Fig.-1), by determining the molecular interaction between the phytosterols (e.g., α-sitosterol and stigmasterol), flavonols (e.g., quercetin), and terpenes (e.g., curcumenol) with hGRK2, followed with an *in vitro* study on the isolated heart of crab-eating frog (*Fejervarya cancrivora*). Moreover, the total phenol content in the extract and DPPH radical scavenging activity were also determined.

EXPERIMENTAL

Study Area

This work was conducted at (1) the Pharmacology Laboratory, Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy Universitas Padjadjaran (August 2019-January 2020) and (2) the Center for Computational Research, Faculty of Pharmacy, Universitas Padjadjaran (December 2021-January 2022).

Instruments

Instruments used were digital analytical balance (Mettler Toledo MS 12001), macerator, rotary evaporator (Buchi R-100), aqua bath (Thermo Scientific 18102A-1CEQ), microplate reader (BioTek EpochTM), microplate 96-well (NEST®), micropipette (Socorex Acura® 20-200 µL and Eppendorf® 10-100 µL), micro tips (Gilson), kymograph apparatus (Palmer), kymograph paper, and analytical glassware.

Materials

The *C. heyneana* plant and rhizome (Fig.-1) were obtained from Balai Penelitian Tanaman Rempah dan Obat (BALITTRO), Manoko Plantation, Lembang, West Java, Indonesia. The sample was taxonomically recognized at the Herbarium Bandungense SITH Bandung Institute of Technology, Indonesia (3745/I1.CO2.2/PL/2019).

Fig.-1: *Curcuma heyneana* Valeton and Zijp Rhizome Obtained from Balai Penelitian Tanaman Rempah dan Obat, Manoko Plantation, Lembang, West Java, Indonesia

Extraction

Extraction of 500 g of the dry thin-sliced rhizomes was performed by obeying earlier techniques described elsewhere.[11-13]

Phytochemical Screening

The existence of secondary metabolites in *C. heyneana* extracts was evaluated by employing standard procedures.[14]

Determination of Total Phenol Content

Analytical-grade chemicals were used in this step. The total phenol content of the ethanol extract of *C. heyneana* (CHE) was determined using the Folin-Ciocalteu reagent. Briefly, 100 µL of CHE (200 µg/mL)

were put into the microplate and were added with increased concentrations (0; 0.5; 1.0; 1.5; 2.0; and 2.5 µg/mL) of quercetin standard. 50 µL of the Folin-Ciocalteu 10% reagent was added to each well and the concoction was allowed for 8 min then was added with 40 µL of 7.5% Na_2CO_3. The solution was left to sit for 30 min and its absorbance was measured at 765 nm against blank. The total phenolic content was calculated as mM quercetin equivalent (mM QUE) by using the quercetin calibration curve.

DPPH Radical Scavenging Assay

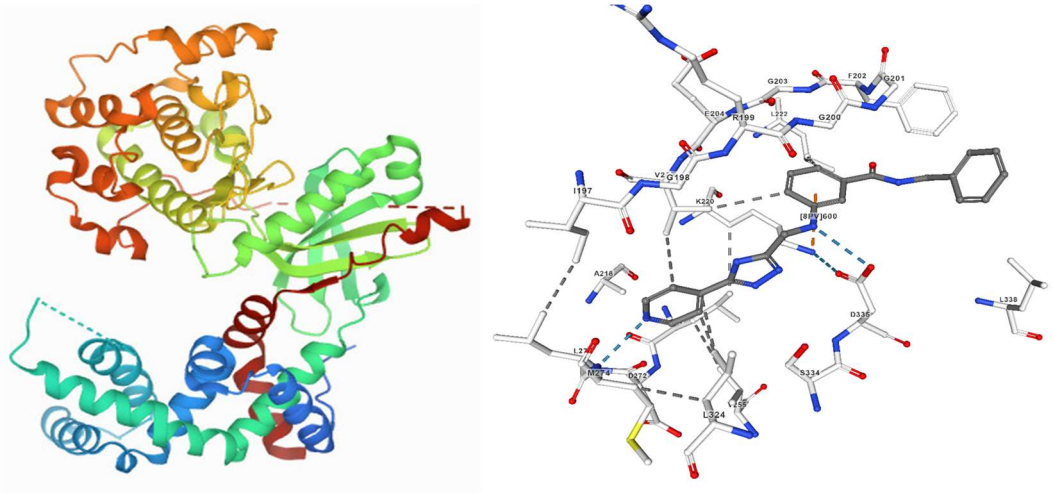
The DPPH radical scavenging assay was determined based on the ability of DPPH radicals to change color in the existence of antioxidants in CHE. The procedure was conducted in triplicates. Quercetin was used as the standard. The IC_{50} was calculated by using GraphPad Prism 8.4.0.[14]

Hardware and Software

The MacBook Pro (13-inch, M1, 2020) embedded with macOS Monterey and an Apple M1 chip processor were utilized in the molecular docking study. Software used was MarvinSketch 17.11.0 (Academic License), LigandScout 4.1.4 (Universitas Padjadjaran License), AutoDock 4.2 (Molecular Graphics Laboratory, The Scripps Research Institute, downloaded from http://autodock.scripps.edu), MacPyMOL: PyMOL 1.7.4.5 Edu.

Preparation of Ligands and Molecular Docking Simulation

The X-ray crystallographic 3D structure of hGRK2 (PDB ID: 5UVC; resolution 2.65 Å) (Fig.-2) was downloaded from the online Protein Data Bank https://www.rcsb.org/structure/5UVC.[15] The 2D structure of the ligands (Fig.-3) was generated using MarvinSketch of ChemAxon, then converted to the 3D structure by applying MMFF94 forcefield partial charges in LigandScout. The physicochemical properties (Lipinski Ro5) were predicted using LigandScout.[16]



(a) (b)

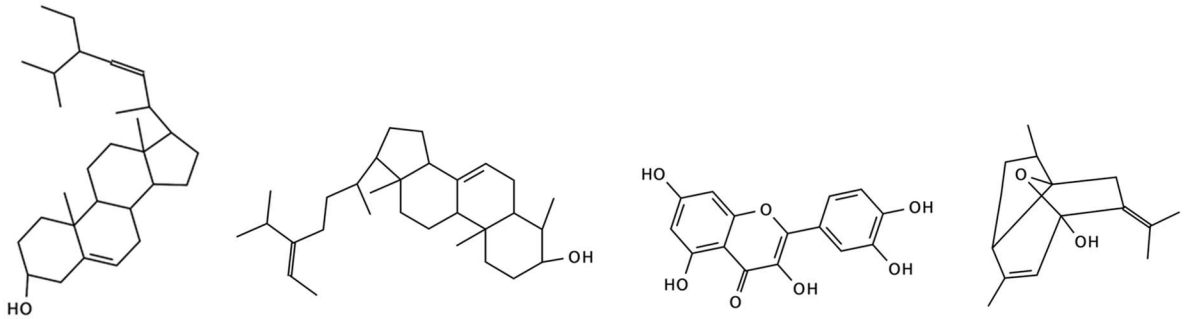
Fig.-2: (a) Human G-Protein-Coupled Receptor Kinase 2 (hGRK2) (PDB ID: 5UVC) Downloaded from https://www.rcsb.org/structure/5UVC. This protein was Deposited in 2017 by I. D. Hoffman, and J. D. Lawson; (b) Molecular Interaction of the First Selective Inhibitor for the Potential Treatment of Heart Failure. Hydrogen Bond is Depicted as Blue Dashed Lines. Hydrophobic Interaction is Depicted as Grey Dashed Lines.

In silico study was carried out for α-sitosterol, stigmasterol, quercetin, and curcumenol, with hGRK2 by molecular docking simulation using AutoDock 4.2 embedded in the LigandScout program. Validation of the molecular docking method was performed by overlaying the natural ligand with the re-docked ligand. At the end of docking, the binding affinity of the receptor/ligand complexes was calculated in terms of docking scores.[16]

Animals

Thirty-five crab-eating *Fejervarya cancrivora* frogs weighing between 40 and 65 g were bought from Subang, Indonesia. The frogs were identified by a certified zoologist at Herbarium Bandungense and

Zoology Museum, Bandung Institute of Technology, Indonesia. Animal adaptation, handling, and euthanasia procedures were approved by the Research Ethics Committee, Universitas Padjadjaran, Indonesia with Document No. 1287/UN6.KEP/EC/2019.



(a) (b) (c) (d)

Fig.-3: 2D Structure of (1) Stigmasterol; (2) α-Sitosterol; (3) Quercetin; (4) Curcumenol

Chronotropic and Inotropic Activity Assay

The frogs were anesthetized using ketamine 0.2 mL via *intramuscular* injection. The isolation of the frogs' hearts was carried out according to the standard procedure.[17] Concisely, the frogs were fixed on a flat surface. The thorax was opened from the abdomen. The *longissimus dorsi* and *latissimus dorsi* were cut and opened until the sternum was displayed. The exposure of the heart was done by removing it from the skin, the thoracic muscle, and the pericardium. A small incision in the venous sinus was made to introduce the cannula filled with batrachian ringer solution. The cannula was gently moved to slide into the ventricle. Subsequently, a ligature was made around the cannula while lifting the tip of the ventricle and the isolated heart was rid of surrounding tissue.[18] The isolated heart was connected to the kymograph apparatus and washed for about 5 min with Ringer's solution until a regular cardiogram was recorded on a kymograph paper wrapped around a rotating drum (the rotation speed was set at 1.25 mm/sec). The hearts of the frogs were divided into 7 groups (n = 5): (1) the normal control group was treated with 2% of Arabic gum (PGA); (2) the positive control group was treated with norepinephrine (NE) 80 µg/mL; (3) was treated with CHE 62.5 mg/kg BW; (4) was treated with CHE125 mg/kg BW; (5) was treated with CHE 250 mg/kg BW; (6) was treated with CHE 500 mg/kg BW; (7) was treated with CHE 1000 mg/kg BW.

RESULTS AND DISCUSSION

The yield of the CHE extract was 105.16 g (21.03 % *w/w*). The phytochemical screening of *C. heyneana* extract confirmed the presence of flavonoids, polyphenols, saponins, and triterpenoids. Additionally, the total phenol compounds in *C. heyneana* extract calculated using the standard addition linear regression equation (Fig.-4) is 83.7 mg of QUE/g CHE.

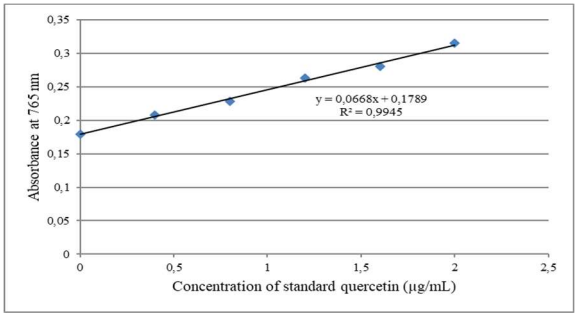


Fig.-4: Standard Addition Curve of Quercetin for the Determination of Total Phenol Compounds in CHE (The slope is 0.0668 and intercepts at 0.1789. The linear regression equation is $y = 0.0668x + 0.1789$; $R^2 = 0.9945$)

Polyphenolic compounds in CHE react with the Folin-Ciocalteu reagent, a specific reduction-oxidation reagent, to produce a blue complex, that can be measured by a visible spectrophotometer. Several reference compounds, e.g., gallic acid, tannic acid, catechin, and pyrogallol, can be employed in this technique.[19] A

recent study by Platzer and co-workers about the reference standards used in the Folin-Ciocalteu method concluded that of the 20 tested reference compounds, among those were quercetin3-D-glucoside, quercetin-7-D-glucoside, and quercetin. Quercetin indicated the highest reducing capacity due to its fulfillment of all three Bors criteria (catechol group on the B-ring; 2,3 double bond and 4-oxo group on the C-ring; hydroxyl groups at positions 3 and 5, the hydroxyl group on the A- and C-rings, and 4-oxo group on the C-ring.[20] Thus, in our work, quercetin was chosen as the reference compound. The DPPH radical scavenging assay of quercetin and CHE was tabulated in Table-1. CHE shows a weak radical scavenging activity with IC_{50} = 320.0 µg/mL, while quercetin strongly attacks the free radicals of DPPH.

Table-1: % Inhibition of DPPH Radical by Quercetin and CHE

Concentration of Quercetin	% Inhibition	Concentration of CHE	% Inhibition
QUE 20.00 µg/mL	93.855	CHE 1000.0 µg/mL	86.124
QUE 8.00 µg/mL	91.572	CHE 400.0 µg/mL	61.946
QUE 3.20 µg/mL	85.039	CHE 160.0 µg/mL	41.114
QUE 1.28 µg/mL	41.789	CHE 62.0 µg/mL	30.124
QUE 0.51 µg/mL	22.359	CHE 25.6 µg/mL	27.765
IC_{50} of QUE = 1.637 µg/mL		IC_{50} of CHE = 320.0 µg/mL	
R^2 = 0.9984		R^2 = 0.9923	

A previous study by Marianne and co-workers using gallic acid as the reference reported the total phenolic content of the *C. heyneana* harvested in North Sumatra, Indonesia, in July 2018, was 400.37 mg of GAE/g extract.[21] Interestingly, another study reported that the methanol extract of *C. heyneana* also resulted in a weak DPPH radical scavenging activity, with the IC_{50} more than 100 µg/mL.[22] Furthermore, our study revealed that the highest hydrophobic compounds are α-sitosterol (cLogP = 8.191) and stigmasterol (cLogP = 7.801), respectively. The molecular redocking simulation proved that the two native inhibitor molecules overlap nicely with an RMSD of 1.273 Å (Fig.-5), which indicates a good validity result.

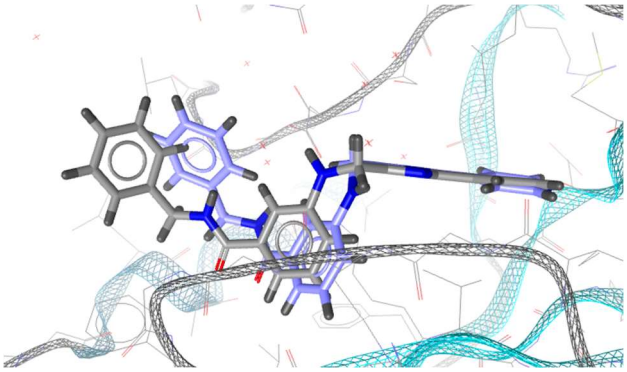


Fig.-5: Validation of the Molecular Docking Simulation by Overlaying the Origin Inhibitor (Grey) and the Redocked Inhibitor (Purple), Resulted in a Valid Value (RMSD = 1.176 Å)

It was reported that the GRK domain comprises an amino-terminal (small) lobe, a carboxylate-terminal (large) lobe, and a C-terminal tail (C-tail). Close-contact residues namely His280, Tyr281, Gln285, Ser284, His394, Lys395, and Lys397 were present in the active domain where the substrate binds.[23] Moreover, a pharmacophore modeling study of GRK2–Gβγ·GSK180736A reported the presence of the hinge region, P-loop, the hydrophobic subsite, and the ribose subsite.[24,25] Our molecular docking simulation indicates that α-sitosterol, stigmasterol, quercetin, and curcumenol could interact with important residues in hGRK2, similar to that of the inhibitor (Table-2). α-sitosterol (Fig.-6a) and quercetin (Fig.-6b) reveal the best affinity (docking score -7.98 kcal/mol and -7.04 kcal/mol; Ki: 1.42 µM and 6.94 µM, respectively) of all studied compounds. α-Sitosterol builds 2 hydrogen bonds (HBs) with Lys319 and Thr353, and exhibits van der Waals interaction with Phe202 (located within the P-loop), Leu222, Ala236 (in αC), and Leu238. Similarly,

quercetin builds 3 HBs with Phe202 and Leu235 and van der Waals interaction with Phe202 and Leu222. A previous study of GRK2 inhibitors reported that the studied inhibitors bound to the macromolecule by building HBs with Asp272 and Met274, with Asp335 and Asn322, or with the backbone nitrogen of Phe202 in the P-loop.[25] Another work focusing on balanol, a kinase inhibitor, revealed that balanol interacts with hGRK2 by binding to Gly201 in the P-loop, Gly232 in αB, Leu235, and Ala236 in αC, and Leu338 in the large lobe, thus inhibiting hGRK2. Moreover, *in vitro* study showed that the IC_{50} of balanol was ~50 nM for GRK2.[26] The building of stable HBs with Phe202 and Lys220 of GRK2 is essential for the selective inhibition of GRK2.[27] Because GRK2 is overexpressed during heart failure,[28] targeting GRK2 in mice had led to improvement in both cardiac function and survival.[29]

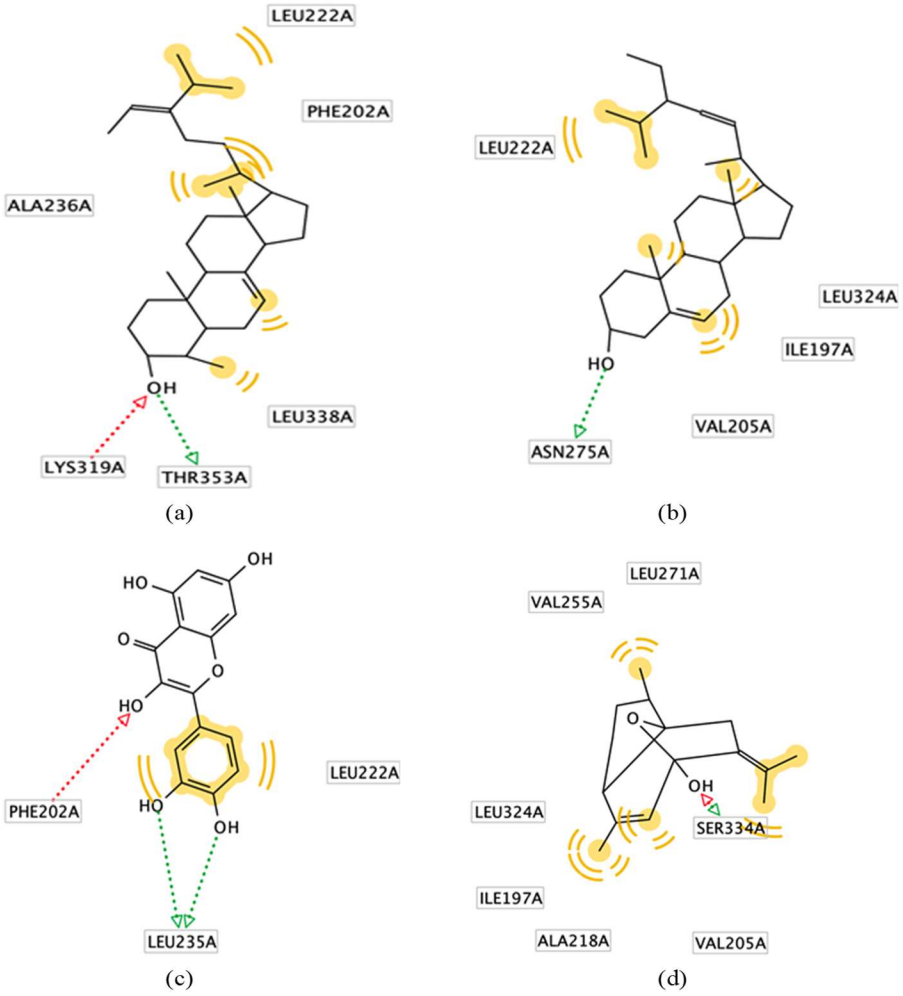


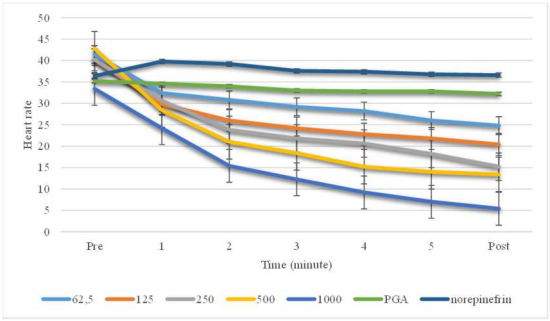
Fig.-6: Molecular Interaction of α-Sitosterol (a); Stigmasterol (b); Quercetin (c); and Curcumenol (d) with Important Amino Acid Residues in hGRK2

The chronotropic activity assay revealed that all concentrations of CHE decrease the heart rate (defined as negative chronotropic) of crab-eating *F. cancrivora* frogs, weaker compared with that of norepinephrine (NE), as depicted in Fig.-7(a). Drugs that work by changing the heart rate and rhythm are defined as chronotropic drugs. These drugs affect the intrinsic conduction system of the cardiac. Positive chronotropic drugs (adrenergic agonists) speed heart rate, while negative chronotropes (e.g., beta-blockers, acetylcholine, digoxin) decrease heart rate. β-blocker drugs reduce the rate of V_{max} of the action potential, and prolong the duration and the effective refractory period, thus, decreasing oxygen requirements in the myocardium. This is beneficial for patients with coronary artery disorders.[30] Interestingly, the inotropic activity assay showed that all concentrations of CHE increase the heart contractility (defined as positive inotropic) of crab-eating *F. cancrivora* frogs as depicted in Fig.-7(b).

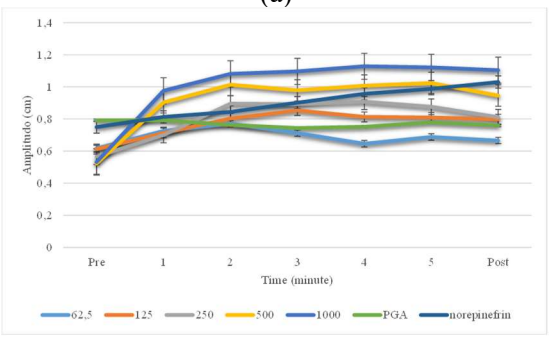
Table-2: The Binding Mode and Affinity of α-sitosterol, Stigmasterol, Quercetin, and Curcumenol with hGRK2

No.	Ligand	Docking Score (kcal/mol), inhibition constant (Ki), and Amino Acid Residues Involved in the Interaction
1.	Inhibitor	Docking score: -7.18 kcal/mol Ki: 5.48 µM HBs with Met274, Asp335 van der Waals interaction with Met274, Leu338
2.	α-sitosterol	Docking score: -7.98 kcal/mol Ki: 1.42 µM HBs with Lys319, Thr353 van der Waals interaction with Phe202, Leu222, Ala236, Leu238
3.	Stigmasterol	Docking score: -6.92 kcal/mol Ki: 8.50 µM HBs with Asn275 van der Waals interaction with Ile197, Phe202, Leu222
4.	Quercetin	Docking score: -7.04 kcal/mol Ki: 6.94 µM HBs with Phe202, Leu235 van der Waals interaction with Phe202, Leu222
5.	Curcumenol	Docking score: -6.52 kcal/mol Ki: 16.7 µM HBs with Ser334 van der Waals interaction with Ile197, Val205, Ala218, Val255, Leu271, Leu324, Ser334 (Curcumenol interacts with hGRK2 by occupying a different site) *

Bold residues indicate similarity with the previous studies[25,26]
* Interaction at a different site



(a)



(b)

Fig.-7: The Chronotropic (a) and Inotropic (b) Activity of Various Concentrations of CHE Against Time, NE was Used as the Positive Control Drug

CHE concentrations of 500 mg/kg BW and 1000 mg/kg BW reveal stronger positive inotropic activity than NE. Positive inotropic drugs help the heart pump more blood to fulfill the oxygen demands, but with lesser

heartbeats. Positive inotropic drugs also increase myocardial contractility by elevating the concentration of intracellular calcium.[31] In this study, norepinephrine (NE) was used as the control drug. NE is an inotrope and a vasopressor.[32] This drug works on both alpha-1 and beta-adrenergic receptors, thus, causing a notable elevation in blood pressure than increasing the heart rate.[33] This sympathomimetic amine is obtained from tyrosine. The structure of NE is identical to epinephrine without a methyl group on its nitrogen atom. This distinction causes its agonistic character at α1- and β1-adrenergic receptors. At smaller doses, the beta-1 effects are stronger thus increasing the cardiac output. However, in higher doses, the α1 effects are predominant.[34]

CONCLUSION

Our study confirmed that flavonoids, polyphenols, saponins, and triterpenoids were present in the ethanol extract of *Curcuma heyneana* (CHE). The total phenol content is 83.7 mg of QUE/g CHE. CHE indicated a weak radical scavenging activity. The molecular docking study confirmed that the best affinity was shown by α-sitosterol and quercetin, both interact with important residues in human G-protein-coupled receptor kinase 2 (GRK2), a protein in the cardiac myocyte, which affects the contractile function of the heart. Moreover, all concentrations of CHE decrease the heart rate (negative chronotropic) and increase the heart contractility (positive inotropic) of crab-eating *Fejervarya cancrivora* frogs. Taken together, *C. heyneana* is the potential in helping the heart pump more blood to fulfill the oxygen demands, but with lesser heartbeats, thus it is useful for patients with cardiovascular disorders.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

AUTHOR CONTRIBUTIONS

Jutti Levita was involved in the conceptualization, methodology, funding acquisition, supervision, and manuscript drafting and revision. Mutakin was involved in methodology, supervision, and data curation. Sri Adi Sumiwi was involved in methodology and supervision. Sandra Megantara was involved in the molecular docking study. Kiki Ikrima determined the total phenol content and radical scavenging activity. Izzatul Khoirunnisa assayed the chronotropic and inotropic activity. All authors have read and approved the final article. The research profile of the faculty authors can be verified from their ORCID ids, given below:

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