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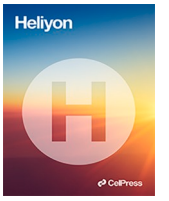

Research article

Uncovering the anti-breast cancer activity potential of east Kalimantan propolis by In vitro and bioinformatics analysis

Paula Mariana Kustiawan [a], Khalish Arsy Al Khairy Siregar [a,b], Putri Hawa Syaifie [b,*], Fauzan Zein Muttaqin [c], Delfritama Ibadillah [b], Muhammad Miftah Jauhar [b], Nailulkamal Djamas [d], Etik Mardliyati [e,**], Nurul Taufiqu Rochman [f]

[a] *Faculty of Pharmacy, Universitas Muhammadiyah Kalimantan Timur, Samarinda, East Kalimantan, 75124, Indonesia*
[b] *Center of Excellence Life Sciences, Nano Center Indonesia, South Tangerang, Indonesia*
[c] *Department of Pharmaceutical Analysis and Medicinal Chemistry, Faculty of Pharmacy, Universitas Bhakti Kencana, Bandung, Indonesia*
[d] *Research Center for Horticultural and Estate Crops, National Research and Innovation Agency (BRIN), Bogor, 16915, Indonesia*
[e] *Research Center for Vaccine and Drugs, National Research and Innovation Agency (BRIN), Bogor, 16911, Indonesia*
[f] *Research Center for Advanced Material, National Research and Innovation Agency (BRIN), PUSPIPTEK, South Tangerang, Banten, 15314, Indonesia*

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ABSTRACT

Numerous side effects of breast cancer drugs have prompted researchers to explore more into new therapeutic approaches derived from natural substances. In this context, our study focused on uncovering the potential of East Kalimantan propolis from *Trigona apicalis* for breast cancer treatment including the underlying mechanisms through bioinformatics approached. We conducted integrated in vitro and bioinformatics analysis of network pharmacology, molecular docking, molecular dynamics and MM-GBSA analysis. Initially, in vitro cytotoxic assay demonstrated the anti-breast cancer activity potential of ethanol extract of East Kalimantan propolis, particularly its ethyl acetate fraction, which exhibited similar activity to doxorubicin, as indicated by their IC_{50} value. This study revealed eight propolis compounds, consisting of flavonoids and phenolic acids, in East Kalimantan propolis. By integrating microarray datasets (GSE29431, GSE36295, and GSE42568) analysis with potential targets derived from propolis compounds, 39 shared target genes were identified. Subsequently, GO and KEGG pathway, protein–protein interaction (PPI) network, core hub genes and gene expression analysis revealed three major targets, namely, PTGS2, CXCL2, and MMP9. Among them, only MMP9 was highly expressed in breast cancer than normal. Moreover, molecular docking revealed the six of propolis compounds which exhibited pronounced binding affinity towards MMP-9, better than marimastat as control drug. Dynamic simulation confirmed the stability of chrysin and quercetin as best compounds. Additionally, MM-GBSA analysis revealed a relative binding energy for chrysin (−25.6403 kcal/mol) that was comparable to marimastat (−27.3827 kcal/mol). In conclusion, this study reveals how East Kalimantan Propolis affect breast cancer and emphasizes MMP9 as a key target for future therapeutics.

* Corresponding author.
** Corresponding author.
E-mail addresses: putri@nano.or.id (P.H. Syaifie), etik002@brin.go.id (E. Mardliyati).

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1. Background

Breast cancer is widely acknowledged as the most often diagnosed ailment among women globally, including approximately 24 % of newly reported cancer cases [1,2]. Moreover, the prevalence of breast cancer is continue increasing [3]. In 2018, this disease became the second most common cause of death among women with cancer, accounting for nearly 15 % of cancer-related deaths [4]. Despite modern medical advancements in cancer research, breast cancer continues to pose a substantial health concern [5,6]. Breast cancer is a prevalent malignancy that significantly impacts women's well-being on a global scale [7,8]. Projections suggest a substantial increase in the occurrence and death rates of breast cancer in the future [9]. Current clinical interventions for breast cancer encompass surgical resection, chemoradiotherapy, and endocrine therapy. Nevertheless, these therapeutics are associated with discernible adverse effects [10].

Research findings indicate that administering radiotherapy and chemotherapy to individuals diagnosed with breast cancer can elevate the development of myelodysplastic diseases and acute myeloid leukemia [11,12]. Consequently, patients with compromised body function and low tolerance may experience heightened pain. On the other hand, natural substances, such as herbal medicines, exhibit little adverse effects and are progressively being utilized by researchers to generate therapeutic interventions targeting breast cancer [13].

Propolis is a promising natural substance with widely reported studies on its efficacy against breast cancer [14], and it has been a common remedy in herbal medicine for humans [15]. Propolis contains various active chemicals, including polyphenols, flavonoids, terpenes, aromatic acids, and esters. These components contribute to its diverse biological activities, which include antioxidant, anticancer, antitumor, antifungal, antibacterial, anti-inflammatory, and anti-diabetic effects [16–22]. The chemical content of propolis varies widely, depending on the species of bee, the flora surrounding bee's hive, and the geographic zone [23]. Thus, the pharmacological action of propolis varied depending on its source and chemical compositions. In previous research, we have found the anti-breast cancer potential of East Kalimantan Propolis through in vitro cytotoxic assay using BT474 cell lines [24–26]. However, ethanol extract of propolis from *Trigona apicalis* exhibited weak activity against breast cancer cell. A study by Ana Sofia Freitas and team (2022) revealed that ethyl acetate fractionation of Portuguese propolis showed a greater cytotoxic activity on cancer cell than its ethanol extract [27]. Another study reported the anti-breast cancer potential of hexane fraction from Lebanese propolis better than its ethanol crude extract [28]. However, underlying mechanisms of propolis' anti-breast cancer was limited explained in depth. The variety of active compounds in Propolis and their potential to act synergistically on various targets pose limitations in explaining their mechanisms of action.

Network pharmacology is a field within systems biology theory that encompasses methodologies, such as tissue database, virtual computation, and high-throughput omics data analysis [29,30]. This approach can predict drug targets from a comprehensive standpoint, enhancing the efficiency of drug development processes [31,32]. The field of network pharmacology has overcome the previous constraints of drug-target research and has gained significant traction in screening active ingredients and understanding effective strategies [33]. Moreover, the differentially expressed genes (DEGs) analysis using the omnibus gene expression database (GEO) over a clinical based approached [34]. Molecular docking with the fundamental principles of ligand-receptor interactions, can be used to elucidate the binding of potential compounds with molecular targets [35–38]. Meanwhile, molecular dynamics (MD)

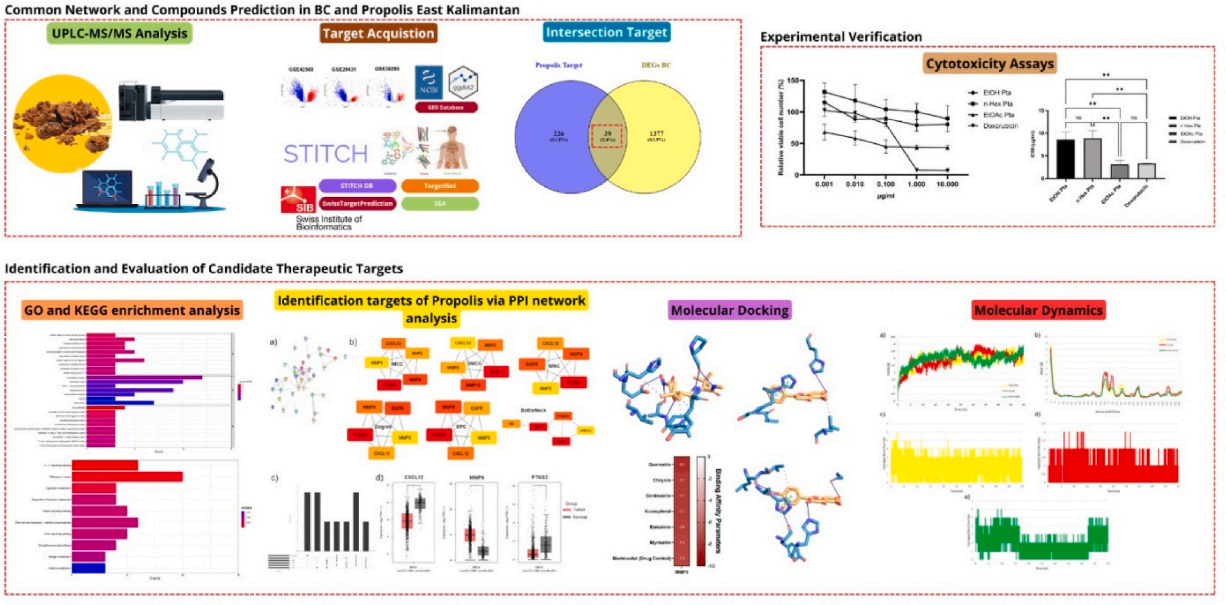

Fig. 1. Research diagram of uncovering the anti-breast cancer activity potential of east Kalimantan propolis by In vitro and bioinformatics analysis.

simulations elucidate behavior structure of complex in dynamic system of biomolecules at the atomic scale, providing alterations of binding conformation [39].

Therefore, in this study, we explored anti-breast cancer activity of East Kalimantan propolis from *Trigona apicalis* by using fractionation and validated it by in vitro cytotoxic assay. Subsequently, we elucidate the underlying mechanisms of its anti-breast cancer through bioinformatics study. By using integrated experiment and bioinformatics approached, the possible pathway, biological process and major gene target that contribute to anti-breast cancer mechanism of propolis could be evaluated. Thus, a comprehensive understanding of how East Kalimantan Propolis compounds affect breast cancer can be explained. The comprehensive workflow of this study depicted in Fig. 1.

2. Materials and methods

2.1. Sample preparation

Raw propolis samples were obtained from stingless bee (*Trigona apicalis* species), collected in Lempake District, Samarinda, East Kalimantan. Before used, all samples dried and kept at −20 °C and then crushed it into smaller pieces. It was then placed in a maceration vessel and covered with 70 % ethanol for 72 h at room temperature. This procedure was repeated until the extract color was clear, approximately for 7 days. The extracts were then pooled. An ethanolic extract of East Kalimantan propolis (EEKP) was obtained by filtering the macerate through Whatman filter paper and evaporating it over a water bath at a temperature of 60 °C. The EEKP was then fractionated using two solvents: non-polar n-hexane and relatively polar ethyl acetate.

Twenty grams of EEKP and 400 ml of hot water were used for fractionation to make 50 mg/mL of propolis solution. After stirring the mixture, it was transferred to a separating funnel. 400 ml of solvent (either n-hexane or ethyl acetate) was added in a ratio of 1:1 and mixed. The mixture was left to stand until it separated or formed two phases. After adding ethyl acetate and n-hexane twice through fractionation, a separate ethyl acetate or n-hexane fraction was obtained. It was then concentrated to yield ethyl acetate fraction (EEKP-EAFr) or hexane fraction (EEKP-HFr).

2.2. Cytotoxic determination by MTT assay

The BT474 cell line, ATCC No. HTB 21, derived from ductal carcinoma, was used in this study. The cells were grown in RPMI 1640 medium supplemented with 5 % (v/v) foetal bovine serum. To assess the cytotoxic potential of East Kalimantan propolis extract, the 3-(4,5-dimethyl-thiazol2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cell viability test was employed. The cells were plated (5000 cells per well) in 200 μL of control medium in 96-well plates and incubated at 37 °C for 24 h. After being incubated for a night, the propolis extract was added at varying concentrations (1, 0.1, 0.01, 0.001 μg/mL) with triplicate wells for each condition. After 24 h of propolis treatment, the supernatant was removed. Each well received 10 μL of 5 mg/mL MTT solution with 4 h incubation. To dissolve the formazan crystals and lyse the cells, a solution containing 25 μL of 0.1 mol/L glycine and 150 μL of dimethyl sulfoxide added to each well and carefully mixed. Absorbance at 540 nm was measured to determine cell viability. The percentage of viable cells was calculated by comparing the absorbance of each sample to that of the control, which was set at 100 % viability.

2.3. Analysis of east Kalimantan propolis using UPLC-MS/MS technique

East Kalimantan propolis compounds were identified by employed previous method by Putra et al. (2023) with slightly different [40]. The propolis solution was filtered through a 0.22 μm Millipore membrane filter. The chromatographic analysis used in this study was UPLC H-Class System XEVO-TQD MS with a binary fluid manager, sample manager, triple quadrupole, and a column of Waters Acquity UPLC BEH C18 1.7 μm. The Masslynx analysis program was used to study the data and monitor the instrument. The flow rate through the column is set to 0.3 ml/min with a temperature of 40 °C. Phase A consisted of equates and 0.1 % formic acid (FA), and Phase B of acetonitrile and 0.1 % FA. Elution gradient program applied with the following setting: 0–1 min at 5 % B; 1–1.5 min at 5–10

Table 1
Eleven references standard injected in UPLC-MS/MS.

No	Reference compound	Parent Ion (*m/z*)	MRM transition		Cone Voltage (V)	Collision Energy (V)	
			Quantifier (*m/z*)	Qualifier (*m/z*)		Quantifier (V)	Qualifier (V)
1.	Hesperetin	301.15	163.96	286.03	45	25	17
2.	Chrysin	253.08	62.99	143.08	63	30	25
3.	Myricetin	317.16	151.03	137.00	50	25	28
4.	Naringin	579.46	271.03	151.02	60	35	45
5.	P-Coumaric Acid	163.03	162.91	150.97	10	6	13
6.	Genkwanin	283.13	267.97	116.90	20	23	35
7.	Oleanolic Acid	455.59	407.41	45.160	60	45	50
8.	Baicalein	269.12	139.04	169.10	60	35	27
9.	Caffeic acid	179.00	135.08	107.02	55	15	25
10.	Quercetin	301.08	150.84	178.83	45	20	20
11.	Kaempferol	284.78	92.83	116.86	65	40	45

% B; 1.5–2.0 min at 10–20 % B; 2.0–3.5 min at 20–28 % B; 3.0–5.0 min at 28–30.3 % B; 5.0–5.5 min at 30.3–50 % B; 5.5–6.5 min at 50–70 % B; 6.5–7.0 min at 70–80 % B; 7.0–8.5 min at 80–100 % B; 8.5–15 min at Hold 100 % B. Afterwards, the settings were set back to the beginning for 4 min to re-center the column. The multiple reaction monitoring (MRM) method applied to identify chemical compounds. Electrospray ionization (ESI) is set in the negative mode (ESI -). Additionally, each standard's collision energies, capillary voltages, and cone voltages were adjusted to get the best precision and sensitivity. A total of 11 USP standard compounds used as injected references to identify propolis compounds by UPLC MS/MS.

2.4. Screening of gene targets for breast cancer

The gene expression profiles were obtained from the GEO database based (http://www.ncbi.nlm.nih.gov/geo/) [37,41]. To verify differentially expressed genes between breast cancer samples and normal samples, three GEO datasets were utilized (Table 2). Table 2 provides details on the three GSE profiles (GSE29431, GSE36295, and GSE42568) and the datasets they include. The GEO2R platform was used to screen the breast cancer mechanism dataset. Additionally, DEGs between breast cancer patients and healthy individuals were identified using the 'limma' package in R software with criteria of $P < 0.05$ and $|\log2 \text{ fold change (FC)}| > 2$. Subsequently, volcano plots of DEGs from the three datasets were generated using the 'ggplot' package in R [42].

2.5. Screening of therapeutic targets of east Kalimantan propolis typical of east Kalimantan for breast cancer

Propolis compounds were used as subject to predict their target genes related to breast cancer. The Canonical SMILES for each compound obtained from Pubchem webserver (https://pubchem.ncbi.nlm.nih.gov/) [47] and subsequently utilized in various target predictors webservers, including STITCH (http://stitch.embl.de/) [48], SwissTargetPrediction (http://www.swisstargetprediction.ch/) [49,50], SEA (https://sea.bkslab.org/) [51], and TargetNet (http://targetnet.scbdd.com/) [52]. To maintain uniformity and standardization of genes nomenclature, the UniProt Webserver (http://www.uniprot.org/id-mapping) applied to compare and align gene name data for each obtained gene [53]. It is imperative to remove all duplicate genes. To visualize the overlap between gene targets by East Kalimantan Propolis and the breast cancer genes, we constructed a Venn diagram using Venny2.1 (https://bioinfogp.cnb.csic.es/tools/venny/) [54].

2.6. GO and KEGG pathway analysis

DAVID (http://david.ncifcrf.gov) is a publicly accessible database that incorporates analytical and biological data include GO and KEGG analysis. In DAVID, GO and KEGG enrichment analyses are performed by inputting gene codes using "OFFICIAL GENE SYMBOLS". The gene codes are listed using "GENE LIST," and the species is set to "Homo sapiens." A p-value of less than 0.05 is applied to identify significant targets. This process allows for the identification of statistically significant differences and potential targets along with their molecular function (MF), cellular component (CC), and biological process (BP). Additionally, the Bioinformatics website platform (https://www.bioinformatics.com.cn/en) is used to present the GO barplot and KEGG pathway results.

2.7. 2. 7 Construction of a protein–protein interaction (PPI) network, identification of core hub genes and gene expression analysis

The STRING online database (https://string-db.org/) generated the protein-protein interaction (PPI) network. A thorough regulatory network for crossover genes was established using a medium confidence level of >0.4 [55]. Once all the crucial genes were entered, the PPI network was loaded into Cytoscape software version 3.10 [56]. The CytoHubba plug-in was utilized to identify the primary gene target in the network. This plug-in employs six algorithms, namely MCC, DMNC, MNC, DC, EPC, and BottleNeck [57]. Furthermore, the analysis of gene expression in breast cancer samples was performed using GEPIA (http://gepia.cancer-pku.cn) [58], with a significance threshold of $p\text{-value}<0.05$.

2.8. Molecular docking

The docking procedure was conducted using Autodock Vina, integrated within the PyRx program version 0.9.9 [59]. The crystal structures of the MMP-9 (PDB ID: 1GKC) [60], were retrieved from the RCSB Protein Data Bank (https://www.rcsb.org/) [61]. Chemical compounds from East Kalimantan Propolis were generated from the PubChem (https://pubchem.ncbi.nlm.nih.gov/) [47]. Marimastat served as the reference drug for comparing the molecular docking and dynamics results of the propolis compounds. In Autodock tools, the protein and ligand structures were prepared by water and heteroatoms removal, adding hydrogens, merging non-polar hydrogen, and incorporating Gasteiger charges. First, the protein structure was validated by repositioning the crystallized

Table 2
Description of three sets of gene expression profiles for breast cancer analyzed in this study.

Dataset	Sample Size (Normal vs Affected)	Sequencing Platform	Locations	Ref.
GSE29431	66 (12/54)	GPL570 Affymetrix Human Genome U133 Plus 2.0 Array	Spain	[43]
GSE36295	50 (5/45)	GPL6244 Affymetrix Human Gene 1.0 ST Array	Saudi Arabia	[44,45]
GSE42568	121 (17/104)	GPL570 Affymetrix Human Genome U133 Plus 2.0 Array	Ireland	[46]

ligand of 1GKC (N2-[(2R)-2-{[formyl(hydroxy)amino]methyl}-4-methylpentanoyl]-N,3-dimethyl-L-valinamide) back into its designated binding site. The binding site was identified using a grid box with XYZ coordinates of 65.622, 31.04, and 117.77, and dimensions of 12x12 × 12 Å.

The DockRMSD tool (https://zhanggroup.org/DockRMSD/) was used to calculate the root mean square deviation (RMSD) between ligand structures after re-docking. An RMSD value below 2 Å indicates that the docking method is validated [62]. Additionally, 51 decoy ligands derived from Marimastat were utilized from the DUDE web server (http://dude.docking.org/). Decoy ligands provide a reference point for comparison in molecular docking studies, with more than 50 % of the compounds in the decoy set showing reduced binding affinity to MMP-9 compared to the reference molecule. The docking results were visualized using BIOVIA Discovery Studio Visualizer and PyMOL software. The protein-ligand interaction profiler (PLIP) web was used to analyze the interactions of ligand-protein in the docking data PDB file [63,64].

2.9. Molecular dynamics simulation and MM-GBSA analysis

Molecular dynamics (MD) simulations were performed based on references from our previous research. The optimal docking complex was chosen for MD simulations, and three experiments were conducted using the AMBER 18 software package, involving two ligand compounds and a drug control. The protein simulation employed the AMBER FF14SB force field, while inhibitor charges were determined using RESP fitting methodologies. Ligand topology files were prepared with the ANTECHAMBER module, utilizing the general amber force field (GAFF). The complexes were placed in a truncated octahedral box filled with TIP3P water molecules, with a 10 Å buffer region. Additional Na+ and Cl-ions were included to neutralize the systems' charge. The pmemd.cuda method was used to constrain hydrogen atom bonding during the 50-ns (ns) simulations, each with a 2-fs (fs) step size and a Single-precision Floating-Point (SPFP) accuracy model. Post-dynamic analysis was conducted using the CPPTRAJ module in the AMBER 18 suite, providing RMSD, RMSF, and hydrogen bond analysis. The mmpbsa.py software was also used to estimate the free binding energy of each ligand-protein complex using the MM-GBSA method. Parameter and system topology files for solvated and nonsolvated complexes, receptors, and ligands were obtained from the MD simulation preparation steps with AMBER. All other settings were configured to the default options of mmpbsa.py in the AMBER 18 package [65,66].

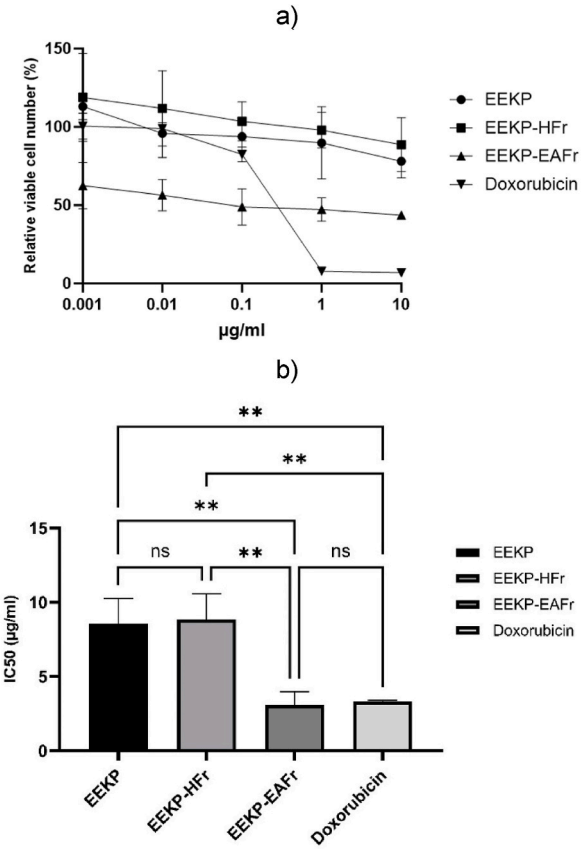

Fig. 2. The cytotoxic activity of East Kalimantan Propolis in various solvents. Relative viable cell number of BT474 cell lines after 48 h treatment with propolis samples and doxorubicin as control (a); IC_{50} values for cytotoxic effect of each.

3. Results

3.1. Cytotoxic activity of east Kalimantan propolis

The impact of different fractions of East Kalimantan propolis was assessed on BT474 cells using MTT cytotoxicity assay. While, doxorubicin was used as a positive control of breast cancer drug. To encompass the range of values ranging from below to above the IC_{50}, the concentrations of each sample were determined between 0.001 and 10 μg/ml. The decreasement in cell viability of BT474 cells after 48 h treatment with ethanol extract of East Kalimantan propolis (EEKP), ethanol extract of east Kalimantan propolis-hexane fraction (EEKP-HFr), ethanol extract of east Kalimantan propolis-ethyl acetate fraction (EEKP-EAFr), and doxorubicin were present in Fig. 2a.

The results of cytotoxicity assay showed that after 48 h treatment with ethanol extract (EEKP) and hexane fraction (EEKP-HFr), there were still 78.02 % and 88.66 % viability of BT474 cell at maximum dose of 10 μg/ml, respectively (Fig. 2a). Meanwhile, ethyl acetate (EEKP-EAFr) exhibits potential cytotoxic activity with result of 44.68 % viable cell at dose of 10 μg/ml (Fig. 2a). Statistical analysis revealed that no significant different of IC50 EEKP-EAFr (3.1 ± 0.89 μg/ml) with doxorubicin (3.32 ± 0.09 μg/ml) (Fig. 2b). Therefore, EEKP-EAFr exhibits strong cytotoxic activity which is comparable to doxorubicin, as a common anti-cancer drug. This activity might be the results of synergistic effect of compounds contained in East Kalimantan propolis that contribute to breast cancer cell inhibition.

3.2. UPLC-MS/MS analysis of east Kalimantan propolis

The UPLC-MS/MS analysis was executed by Multiple Reaction Monitoring (MRM). After injection the reference compounds in UPLC-MS/MS (Table 1), each of retention time and mass spectrum of fragmentations results were recorded. The chromatogram overlay of East Kalimantan propolis extract compared to reference compound is shown in Supplementary Fig. S1. The results revealed eight bioactive compounds in East Kalimantan propolis (Table 3). By using this technique, identifications of propolis compounds can be conducted and validated [40].

2d structures of propolis compounds depicted in Fig. 3. There are six flavonoid compounds and two phenolic acids identified in propolis. Among them, there are flavones and flavanols as main groups of flavonoids. Flavonoids have a basic skeleton of flavan nucleus, which is constructed of 15 carbon atoms organized in three aromatic rings [67]. Chrysin, genkwanin and baicalein have double bond between positions 2 and 3, a ketone in position 4 of C ring, and hydroxyl group in position 5 of A ring. Thus, they were classified as flavones. While, genkwanin has a distinction of methoxy group in position 7 of A ring and baicalein has hydroxyl group in 6 and 7 of A ring. Myricetin, quercetin and kaempferol were classified as flavonols groups as they have hydroxyl group in position 3 of C ring. Moreover, 2 phenolic acids found in East Kalimantan propolis, they are P-coumaric acid and caffeic acid. Flavonoid and phenolis compounds are well known for their health benefits, particularly their anti-oxidant, anti-inflammation, anti-cancer and anti-carcinogenic activities [68].

Chrysin, and *p*-coumaric acid are major constituents found in poplar type-propolis which means the poplar trees as their plant sources. Amirta, R. et al. (2016) have been reported more than 30 species of wood shrubs and tropical trees grown in East Kalimantan, while, poplar is the one of wood shrub plant species [69]. Therefore, these identified compounds of propolis highly correlated with the diversity of plant sources in East Kalimantan, especially for the poplar plants. In addition, quercetin, kaempferol, myricetin and caffeic acid reported as the major flavonoids that responsible for antiviral activity [70].While, *p*-coumaric acid, baicalein, quercetin and caffeic acid, reported as major flavonoids in propolis and have excellent antioxidant activities [71]. Previous researches have been reported the anticancer activities of quercetin, *p*-coumaric acid and caffeic acid through in vitro and in vivo studies [72–74]. Therefore, these compounds might be crucial for biological effect of East Kalimantan propolis, particularly in cancer.

3.3. Selection of the target genes by microarray data analysis

Three data series from GEO database, namely GSE29431, GSE36295, and GSE42568 were used in this study (Fig. 4), DEGs between breast cancer and healthy individuals were screened using the 'limma' package of R software according to $P < 0.05$, and $|Log2$ fold change (FC)$| > 2$. Finally, after combining the DEGs and deleting duplicate values, total of 132, 60, and 578 upregulated genes and 833, 117, 1040 downregulated genes were conducted in GSE29431, GSE36295, and GSE4256, respectively. These differential expressed

Table 3
Compounds identified in East Kalimantan Propolis using UPLC-MS/MS.

Compounds	Molecular Formula	Molecular Weight (g/mol)	Compound Class	Retention Time (min)
Chrysin	$C_{15}H_{10}O_4$	254.24	Flavonoids	7.60
Myricetin	$C_{15}H_{10}O_8$	318.23	Flavonoids	3.60
Quercetin	$C_{15}H_{10}O_7$	302.23	Flavonoids	4.87
Kaempferol	$C_{15}H_{10}O_6$	286.24	Flavonoids	6.19
P-Coumaric Acid	$C_9H_8O_3$	164.16	Phenolic acid	2.74
Genkwanin	$C_{16}H_{12}O_5$	284.26	Flavonoids	7.65
Baicalein	$C_{15}H_{10}O_5$	270.24	Flavonoids	6.95
Caffeic Acid	$C_9H_8O_4$	180.16	Phenolic acid	2.28

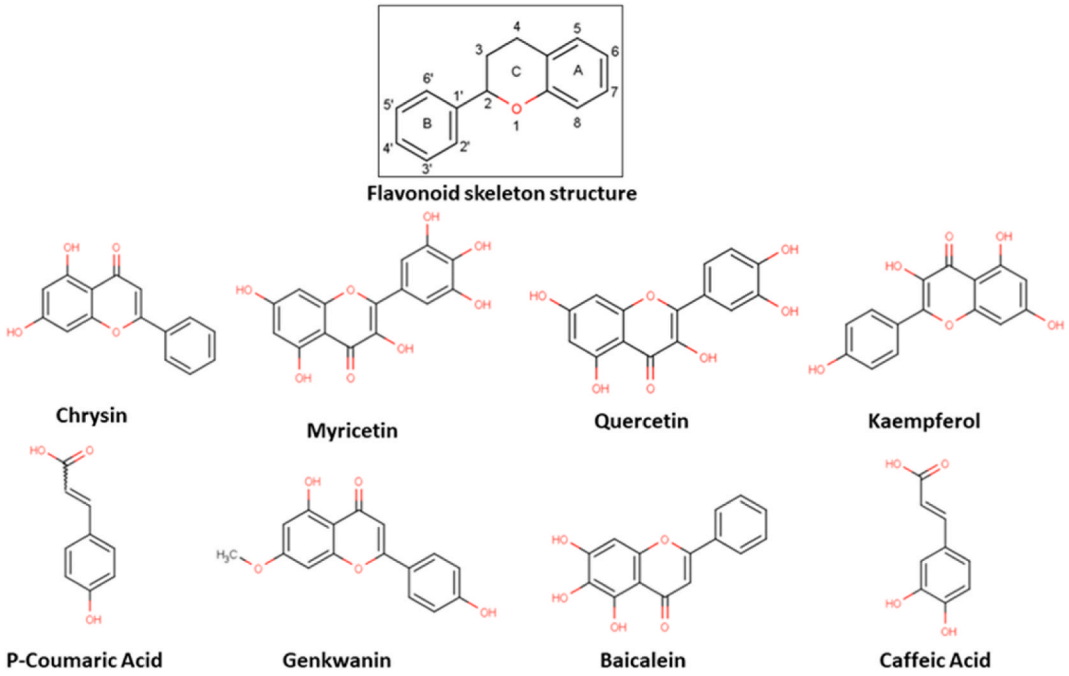

Fig. 3. Molecular structure of eight compounds of East Kalimantan propolis.

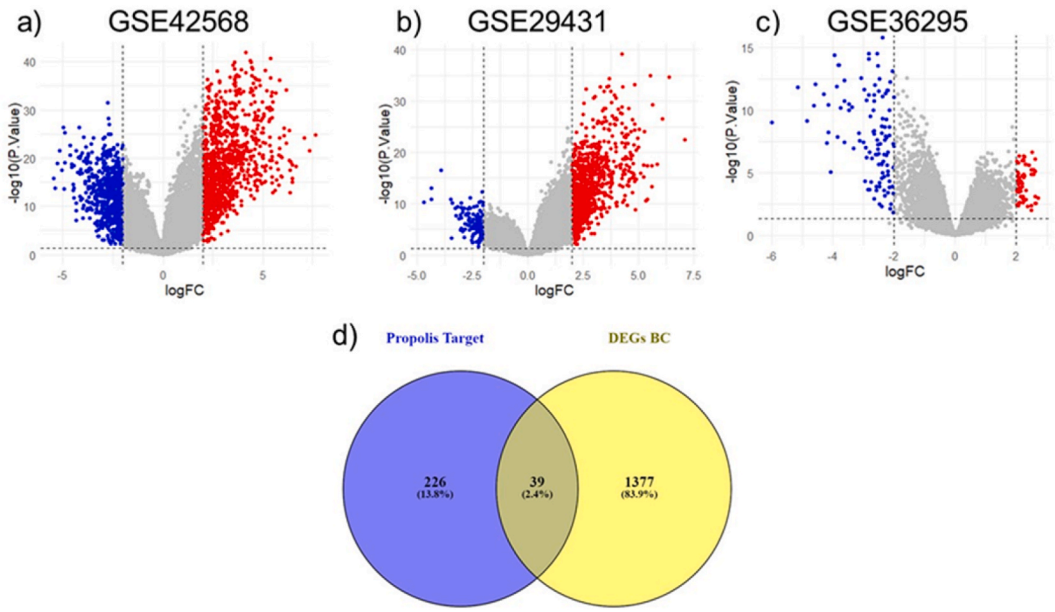

Fig. 4. Volcano plot distribution of low expression of genes in breast cancer. DEGs (a) GSE42568, (b) GSE29431, (c) GSE36295, Red and blue represent as high and low expression of genes in Breast Cancer, respectively. While, (d) intersection between DEG breast cancer (GSE42568, GSE29431, and GSE36295) and Related Protein from East Kalimantan Propolis Compound. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

genes (DEGs) were visualized in volcano bar plot by 'ggplot' package in the R software, whereas blue as down regulated genes, and red as upregulated genes (Fig. 4a–c). In results, by removing duplicates, a total of 1416 genes that consist of 515 up-regulated genes and 901 down-regulated genes detected. Therefore, these 1416 DEGs of breast cancer diseases used in further analysis.

By using the SwissTargetPrediction, SEA, STITCH, and TargetNet databases, a total of 265 target utilized from 8 propolis compounds. In results, 39 intersection gene targets obtained from overlapped of 265 potential propolis targets with 1.416 DEGs of breast cancer, (Fig. 4d). These overlapped genes were strongly associated to breast cancer and potential targets of East Kalimantan propolis.

3.4. GO and KEGG pathway analysis

To explore the underlying biological processes which linked to 39 selected genes, we employed GO and KEGG enrichment analyses. The GO analysis identified numerous BP, MF, and CC which enriched among the selected genes. In this study, we obtained a total of 56 BP, 7 CC, and 39 MF items from the results of GO analysis in DAVID by applied p-value less than 0.05. Then, we highlighted the top 10th BP, MF and CC for further analysis. As the results, these genes have major roles in various biological process namely, cellular response to jasmonic acid stimulus, response to ethanol. collagen catabolic process. extracellular matrix disassembly. positive regulation of protein kinase B signaling, daunorubicin metabolic process, positive regulation of cell migration, doxorubicin metabolic process, progesterone metabolic process, and cellular response to UV-A. Regarding cellular components, GO analysis revealed that only seven terms that related to the target genes. While for molecular functionalities, the targets exhibited significant enroll in some functionalities, including bile acid binding, phenanthrene 9,10-monooxygenase activity, ketosteroid monooxygenase activity and others (Fig. 5). While, the KEGG analysis showed 15 pathways which linked to 39 target genes by using p-value <0.05. In Table 4, we highlighted top 10 results of KEGG pathways. Among them, IL-17 signaling pathway, pathways in cancer, Tryptophan metabolism, and Regulation of lipolysis in adipocytes for associated genes were recognized for their crucial involvement in the signaling pathways linked to the pathophysiology of breast cancer development and progression (Fig. 6). In summary, GO and KEGG analyses results in valuable insights into the dysregulated biological processes and pathways associated with breast cancer. These findings can potentially guide future investigations and identify promising treatment targets.

3.5. Identification of specific targets of east Kalimantan propolis via PPI network analysis

A protein-protein interactions (PPIs) network was constructed by applying threshold >0.4, that represents a medium confidence level and significant interactions. From the input of 39 genes, the PPI network results constructed in 35 nodes and 77 edges (Fig. 7a). While, the remaining 4 genes has not any edge with other genes, subsequently it was then removed. The overall average node degree, local clustering coefficient, and network PPI enrichment p-value were determined to be 3.95, 0.568, and 1.33e-15, respectively. Afterwards, the network was imported into Cytoscape version 3.10 with CytoHubba plug-in. By filtering top 5 hub genes in six algorithm analysis in Cytohubba, namely MCC, DMNC, MNC, EPC, and Bottleneck (Fig. 7b), as well as analyze the most appeared genes in the results, PTGS2, MMP-9, and CXCL12 revealed as potential primary targets of breast cancer in this study.

To evaluate the potential of these genes as therapeutic targets, their expression levels in both health tissues and breast cancer were analyzed. The results found a notable increase in the expression of MMP-9 in breast cancer tissues compared to normal tissues. At the same time, CXCL12 and PTGS2 were lower expressed in breast cancer than normal (Fig. 7c). Therefore, this observation implies that MMP-9 could be a promising candidate for further exploration of breast cancer targets.

3.6. Molecular docking analysis

Molecular docking was conducted to examine the binding affinity between MMP-9 and East Kalimantan Propolis compounds. Firstly, the validation of MMP-9 involved redocking the native ligand of MMP-9 (PDB ID: 1GKC) to the binding site, resulting in an RMSD lower than 2 Å and a binding affinity of −7.0 kcal/mol. Secondly, the docking of 51 decoy ligands, serving as a set of negative compounds for docking MMP-9, revealed that 33 out of 51 compounds exhibited weaker binding with MMP-9 than Marimastat (−7.3 kcal/mol), with a range of binding affinities between −7.2 kcal/mol to −2.6 kcal/mol. Therefore, both validation methods confirm the efficiency and protocol of the MMP-9 docking.

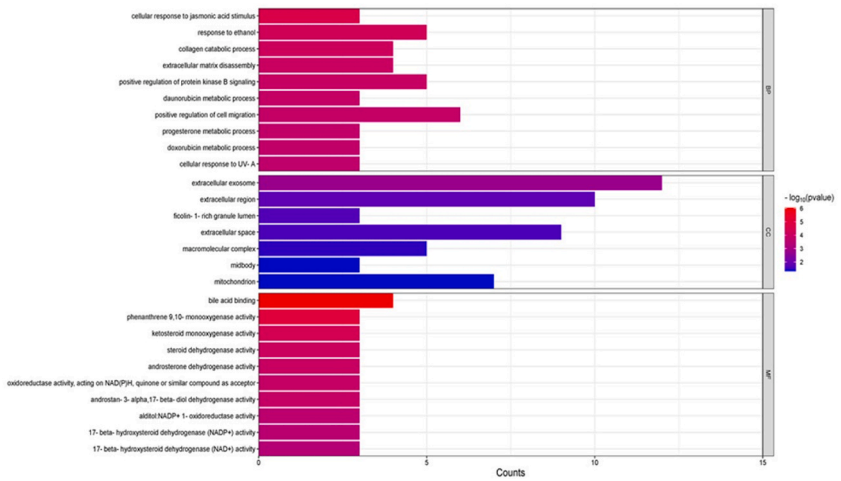

Fig. 5. Top 10 BP, CC, and MF enrichment, P-value is shown by the color of the bar chart. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Top ten results of KEGG enrichment analysis.

	ID	Description	Count	*p-value*
KEGG	hsa04657	IL-17 signaling pathway	6	2.82E-05
KEGG	hsa05200	Pathways in cancer	10	1.39E-04
KEGG	hsa00380	Tryptophan metabolism	4	5.59E-04
KEGG	hsa04923	Regulation of lipolysis in adipocytes	4	0.001439
KEGG	hsa04926	Relaxin signaling pathway	5	0.001496
KEGG	hsa05208	Chemical carcinogenesis - reactive oxygen species	6	0.001576
KEGG	hsa04068	FoxO signaling pathway	5	0.001583
KEGG	hsa00140	Steroid hormone biosynthesis	4	0.001746
KEGG	hsa00910	Nitrogen metabolism	3	0.001932
KEGG	hsa00340	Histidine metabolism	3	0.003243

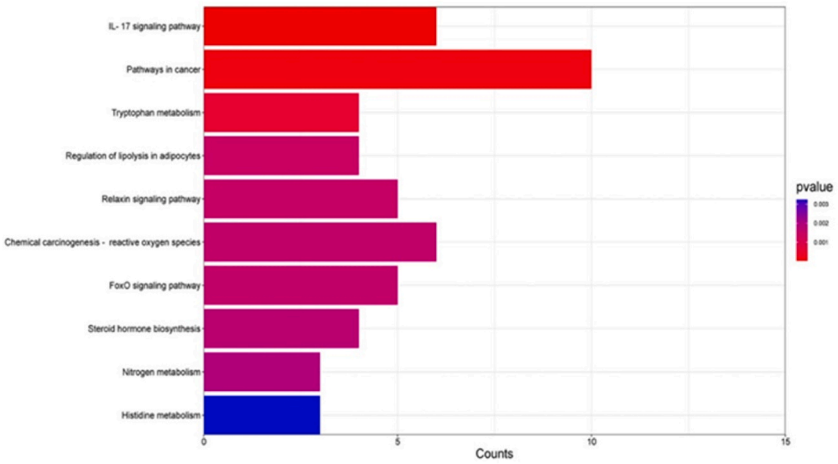

Fig. 6. Top 10 KEGG Pathway enrichment, P-value is shown by the color of the bar chart. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

After docking eight propolis compounds and to MMP-9, the results showed that quercetin, chrysin, genkwanin, kaempferol, baicalein, and myricetin had better binding affinity than marimastat as control drug of MMP-9 (Table 5). Quercetin is the most favorable propolis compound (quercetin) with binding free energy of −8.2 kcal/mol. While, the binding free of Marimastat-MMP9 was −7.3 kcal/mol (Table 5). Molecular Docking results were visualized with PLIP Webserver to analyze their molecular interaction (Fig. 8).

Table 5 showed that almost all of East Kalimantan propolis have favorable binding affinities to MMP-9, except for *p*-coumaric acid and caffeic acid (data not presented). Catalytic residues of MMP-9 were observed in the binding of quercetin, chrysin, genkwanin, kaempferol and baicalein to MMP-9, except for myricetin. Rowsell et al. (2002) have been reported the MMP-9 active residues of His401, Glu402, His405, and His411. Thes residues must be present for MMP-9 inhibitors in their interaction to block MMP9 proteins [75]. These residues have important role in catalytic site of protein. Our docking results show that His411 were interact strongly within hydrogen bond to chrysin, quercetin and kaempferol (Table 5). While, genkwanin and marimastat form strong hydrogen bond with Glu402. Another key residue of MMP-9, His401, was forming hydrophobic bonds with chrysin, genkwanin, kaempferol, and baicalein. To validate the stability of propolis compounds to MMP-9, molecular dynamic was applied for two best compounds, namely quercetin and chrysin. Then, they were compared with marimastat as control drug.

3.7. Molecular dynamic analysis and MM-GBSA calculations

Molecular dynamics (MD) simulations were performed for three complexes of quercetin-MMP9, chrysin-MMP9, and marimastat-MMP9 to provide additional validation for their specific binding and their stability of interactions. RMSD results for complex of quercetin-MMP-9 and chrysin-MMP9 indicate minimal fluctuations with average RMSD values of 2.87A and 2.89A, respectively. Compared with complex of marimastat-MMP9 as control with average RMSD of 2.94 Å, quercetin and chrysin maintain more stable complexes with MMP9. During the simulation, the RMSD of ligand were recorded consistently below 4 Å. Fluctuations in RMSD indicate the notable changes in conformation of complex. In contrast, stable form is characterized by the absence of such fluctuations. The RMSF results indicated that MMP-9 exhibited a stable form while maintaining adequate stability when exposed to Quercetin, Chrysin, and Marimastat. Based on the structural arrangement of MMP-9 - Quercetin complex and its interaction with the binding site residues, specifically Gly186 (1.11 Å), Leu397 (0.46 Å), His411 (0.89 Å), Tyr420 (0.65 Å), and Val398 (0.44 Å), it can be observed that the binding of quercetin to active site of MMP-9 was stable during 50 ns simulations. The MMP-9 - Chrysin complex also show a stable

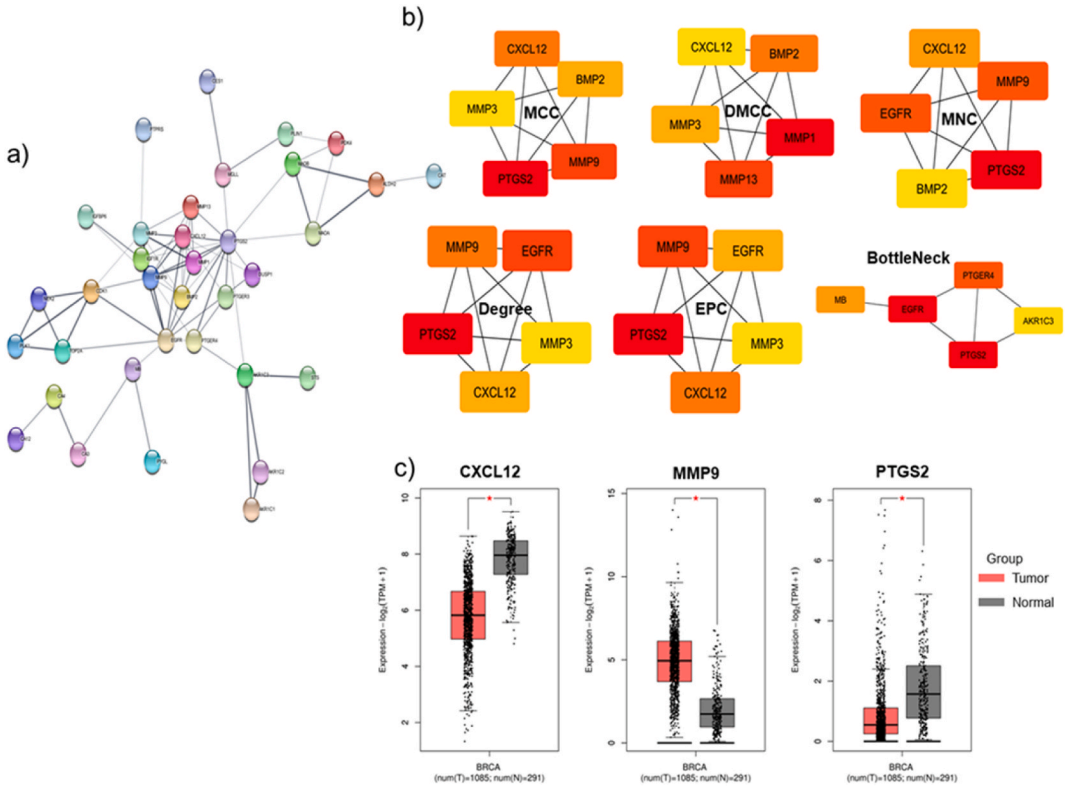

Fig. 7. East Kalimantan'Propolis' STRING-DB analysis of the PPI network of proteins involved in breast cancer (a). The top five targets were screened using the MCC, DMNC, MNC, Degree, EPC, and BottleNeck algorithms in the CytoHubba plug-in (b). The depth of the color represents the importance of the target in each algorithm. Differential expression of core genes in normal tissues and breast cancer (c). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

binding during simulations at the active site residues of His411 (0.90 Å), His401 (0.47 Å), Leu187 (1.14 Å), Val398 (0.52 Å), and Tyr423 (0.83 Å). Similarly, the MMP-9 - Marimastat complex exhibits consistent stability during simulations at the active site residues, which include Leu188 (0.73 Å), Pro421 (0.74 Å), Ala189 (0.54 Å), Glu402 (0.39 Å), Val398 (0.39 Å), His401 (0.39 Å) and Tyr423 (0.67 Å) (Fig. 8a and b). However, the fluctuation on 4 Å had been observed in other amino acid that was not served as the active site for this protein. Based on the comprehensive analysis of the RMSF, it can be concluded that no significant fluctuations are seen in the MMP9 active residues under investigation. The obtained average RMSF values for Quercetin, Chrysin, and Marimastat are 1.07 Å, 1.16 Å, and 0.94 Å, respectively, indicating a relatively stable behavior. Furthermore, the hydrogen bond interactions between Gly186, Leu397, His411, and Tyr420 in the MMP-9-Quercetin Complex, and His411 in the MMP-9-Chyrsin Complex, as well as the hydrogen bonds of Leu188, Ala189 Glu402, Pro421, and Tyr423 in the MMP-9-Marimastat Complex were contributing to the stability of the complex (Fig. 8c–e). The binding free energies and energy components of best ligand complexes are shown in Table 6.

In this study, binding energy was determined by MM-GBSA analysis. The study of MM-GBSA energy calculation pertains the relative binding energy exhibited by each compound in the complex with MMP9. The results indicated that complex of marimstat to MMP-9 have binding energy of (ΔG_{bind}) −27.3827 kcal/mol, whereas the chrysin molecule exhibited a comparable docking score than marimastat with ΔG_{bind} value of −25.6403 kcal/mol (Table 6). While for quercetin, ΔGbind value of −17.7315 kcal/mol might be showed a potential MMP-9 inhibitory activity depending on the concentration. The results of our investigation indicate that propolis compounds showed a potential inhibition activity to MMP-9.

4. Discussion

Breast cancer encompasses a diverse combined of tumors that exhibit differences in potential for recurrence, molecular characteristics, physical appearance, responsiveness to treatment, and overall prognosis [8,76,77]. The formidable nature of breast cancer, coupled with the restricted availability of prognostic and diagnostic techniques, the multifaceted causes of its occurrence, and its propensity for metastasis, poses significant obstacles in advancing efficacious therapeutic interventions for this disease [78,79]. The current therapeutic options for breast cancer differ depending on several criteria, including the patient's overall health, the stage of the disease, and the specific subtype of breast cancer. Surgical intervention, radiation therapy, chemotherapy, hormone therapy, and targeted therapy are the primary treatments for breast cancer [80,81]. While these therapeutic options have demonstrated efficacy in managing breast cancer, they are also associated with undesirable side effects [82]. This emerged condition have prompted researchers

Table 5
The result of molecular docking MMP-9 with Two Best Compound and Drug Control.

Ligand	Binding Affinity (Kcal/Mol)	Interaction			
		Hydrogen Bond	Distance (Å)	Hydrophobic Interaction	Distance (Å)
Quercetin	−8.2	Gly186	2.69	Val398	3.66
		Leu397	3.12		
		His411	2.37		
		Tyr420	2.05		
Chrysin	−7.7	**His411**	3.18	Leu187	3.54
				Val398	3.29
				His401	3.82
				Tyr423	3.62
Genkwanin	−7.7	Leu188	2.80	**His401**	4.42
		Ala189	3.03	Met422	2.86
		Glu402	1.98		
		Tyr423	2.86		
Kaempferol	−7.5	**His411**	2.76	Leu187	3.81
				Val398	5.00
				His401	3.91
Baicalein	−7.6	–	–	Val398	4.78
				His401	4.05
				Tyr423	5.43
myricetin	−7.4	Gly186	2.46	Val398	4.78
		Leu188	2.80	Met422	4.75
		Ala189	2.51		
		Tyr423	2.86		
Marimastat*	−7.3	Leu188	2.30	Leu188	3.40
		Ala189	2.24	Val398	3.78
		Glu402	2.50	**His401**	3.90
		Pro421	1.97	Tyr423	3.68

Note: bold letter means catalytic residues of MMP-9, * reference/control drug.

to explore more into new therapeutic approaches derived from natural substances.

In this study, the potential anti breast cancer activity of propolis was evaluated by cytotoxic assay using BT474 cell line and compared with doxorubicin. Doxorubicin, a standard drug, is widely used as chemotherapy agent for treatment of cancer, especially in breast cancer patients. However, resistance to doxorubicin involves multiple mechanisms, such as alterations in apoptosis, autophagy, ATP-binding transporter overexpression, and cell arrest [82]. Thus, natural components such as propolis might help overcome the limitations.

Results of this study on cytotoxic assay showed that EEKP-EAFr has stronger cytotoxic activity than EEKP and EEKP-HFr. Statistical analysis of our result of cytotoxic study exhibited no significant difference in the IC_{50} of EEKP-EAFr compared to doxorubicin in the range of 0.001–10 μg/ml (Fig. 2). The polar compounds contained in the ethyl acetate fraction of propolis may exhibit a synergistic effect, which could be responsible for its potent cytotoxic activity. This result aligned with a previous study by Ana Sofia Freitas et al. (2022), which discovered Portuguese propolis ethyl acetate fraction exhibited the highest toxicity against renal cancer cells, whereas this finding was strongly correlated with the presence of flavonoid compounds such as quercetin derivates, caffeic acid derivates, *p*-coumaric acid, pinobanksin, and isorhamnetin [83].

In East Kalimantan propolis, eight active compounds were identified using the UPLC-MS/MS screening method by using 11 reference standard compounds. Among them, quercetin, *p*-coumaric acid and caffeic acid were found in East Kalimantan propolis (Table 3). Previous studies have been reported the anticancer activities of quercetin, *p*-coumaric acid and caffeic acid through in vitro and in vivo studies [72–74]. Consequently, these compounds may be essential as primary compounds with potent anti-cancer effects. Although, the mechanism action, therapeutic targets and signaling pathway of anti-breast cancer of East Kalimantan propolis will be further discussed in bioinformatic study.

One of bioinformatics approached is network pharmacology. Studies based on network pharmacology have shown significant potential in identifying novel therapeutic targets and developing more efficient treatment approaches for breast cancer [84]. Research employing network pharmacology has shown great potential in discovering novel therapeutic targets and creating more potent treatment for breast cancer [79,85,86]. Researchers can identify critical signaling pathways and possible targets for pharmacological intervention that contribute to the onset and progression of breast cancer by developing and evaluating molecular networks associated with the disease [87].

By using eight compounds of propolis as an input, a total of 265 breast cancer targets acquired from target predictors servers. While, 1416 genes breast cancer targets retrieved from microarray Datasets GSE29431, GSE36295, and GSE42568. The 'limma' package of R software used in this study to screen the DEGs between breast cancer and healthy individuals. Then, the volcano plot was visualized using 'ggplot' package in R software. Limma is an R package for analyzing gene expression data. It's equipped with robust features for reading, normalizing, and exploring data, and excels in performing gene differential expression analyses [88]. Afterwards, by using Venn diagram between those data targets, 39 shared targets were identified as potential major target of breast cancer and East Kalimantan Propolis.

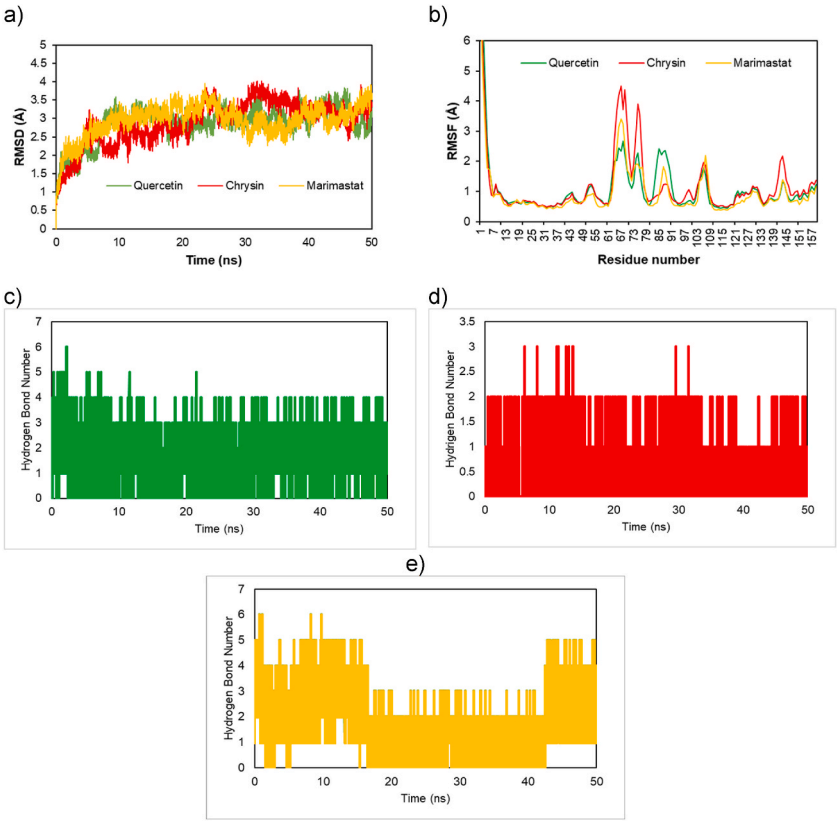

Fig. 8. Molecular dynamics simulations of ligand-protein complexes during 50 ns at 310 K, RMSD results (a), RMSF of residues (b), hydrogen bonds results (c–e). In all panels the color code is-Quercetin (green), Chrysin (red), and Marimastat (yellow). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 6
The relative binding energy and energy components of complex calculated by MM-GBSA.

System	MMP-9 – Quercetin	MMP-9 - Chrysin	MMP-9 – Marimastat (control)
ΔE_{vdw}	−30.0979	−32.5594	−30.7372
ΔE_{elec}	−23.1814	−8.5973	−55.6318
ΔG_{GB}	39.6398	19.3285	63.3264
ΔG_{SA}	−4.0921	−3.8121	−4.3401
ΔG_{bind}	−17.7315	−25.6403	−27.3827

The KEGG enrichment analysis performed on the 39 targets revealed related pathways involved by these targets (Table 4). Among them, the IL-17 signaling pathway, pathway in cancer and chemical carcinogenic reactive oxygen species (ROS) might be major pathways of action to treat breast cancer (Fig. 5). A recent study has found that the IL-17 signaling pathway has major role in promoting the proliferation of breast cancer cells [89]. Furthermore, the IL-17 signaling cascade is responsible for mediating the invasiveness and metastasis of cancer cells through MMP-9, while also stimulating the expression of MMP-9 mRNA. Therefore, it was necessary to employ MMP-9 inhibitors in order to hinder the invasion and metastasis of breast cancer cells that are dependent on IL-17A. Meanwhile, chemical carcinogenic ROS pathway associated with different pathophysiological function in breast cancer and promote tumor microenvironment reprogramming, as well as induces breast cancer metastasis [90].

To enhance the identification of prevalent targets of breast cancer, a PPI network was established utilizing the 39 selected proteins and then analyzed major hub genes using Cytohubba plugin by Cystoscape. Based on six algorithm analysis, three potential major targets were retrieved, namely, CXCL12, PTGS2, and MMP9. These targets appeared consistently across all six algorithms analysis results in CytoHubba analysis results. Thus, we validate this result by analysis of expressed genes In GEPIA server, which used the clinical data from cancer genome atlas (TCGA) and genotype-tissue expression (GTEx) project [91]. In results, MMP-9 exhibited the most potential target gene and considerable upregulation in breast cancer tissues compared to health tissues. While, CXCL2 and PTGS2 showed lower expressed genes in breast cancer tissues compared to health tissue. This research implies that MMP-9 could be a viable biomarker therapeutic target for East Kalimantan propolis against breast cancer. Subsequently, to validate this finding, molecular

docking was conducted on MMP-9 and propolis as well as to examine the binding affinity between MMP-9 and a set of East Kalimantan Propolis compounds. It was observed that quercetin and chrysin derived from East Kalimantan Propolis exhibit favorable binding affinity with MMP-9, displaying significant binding free energies of −8.2 kcal/mol and −7.7 kcal/mol, respectively. While, the control drug, Marimastat, demonstrates a lower binding affinity of −7.3 kcal/mol (Fig. 9d). Rowsell et al. (2002) reported key active residues that must be appeared in MMP9 inhibitors binding, namely His401, and His411 [75]. While, these residues observed well in chrysin and quercetin interactions to MMP9, moreover, they both pose strong hydrogen and hydrophobic bonds (Fig. 9a and b)

In addition, to elucidate the stability of the protein-ligand interactions and the stability of binding propolis compounds with MMP-9 structures in the complexes, we conducted MD simulations in 50 ns using the AMBER18 software. RMSD results of the propolis ligands complex (chrysin and quercetin) showed similar pattern with marimastat complex in within 25 ns and start to fluctuate until 50 ns. Moreover, RMSD in final step of 50 ns simulation for all complex reached approximately less than 4 Å (Fig. 7a). RMSF results analysis demonstrated that MMP-9 stability was intact while exhibiting sufficient flexibility upon exposure to Quercetin, Chrysin, and Marimastat (Fig. 7b). Especially, the binding of propolis ligands maintain their interaction with active residues in MMP-9 complex within 50 ns. This study reports the stability of MMP-9 Active Sites in all complex, which serves to validate the Docking and Complex Conformation obtained from dynamic simulations. The simulations consistently demonstrate the stability of the propolis ligand-MMP9 complexes throughout the simulation period, with deviations of <1 Å.

Previous research has investigated the expression of MMP-9 in both normal breast cancer tissue and human breast tissue, while the result showed the high expression of MMP-9 in the breast tissue of individuals without any pathological conditions [92–94]. Furthermore, another study has shown that the expression of MMP-9 varies among distinct molecular subtypes of breast cancer. The evident characteristic of triple-negative and HER2-positive breast cancer is the overexpression of MMP-9 [95,96]. In addition, previous research has demonstrated the significant involvement of MMP-9 in breast cancer in laboratory settings (in vitro) and living organisms (in vivo), where it has been shown to contribute to tumor growth, angiogenesis, and invasion of breast cancer cells [97–99]. Hence, MMP-9 has the potential to serve as potential target of breast cancer, thereby potentially facilitating the exploration of novel therapeutic interventions.

This is the first study which explored potential anti breast cancer activity of *Trigona Apicalis* propolis from East Kalimantan, Indonesia with the underlying critical pathways, and target associated with breast cancer. The synergistic studies derived from natural constituents, disease, in vitro assay and bioinformatics analysis give a comprehend understanding of novel potential treatments for accelerate healing of diseases. Based on our findings, MMP-9 served as potential therapeutics and target treatments of breast cancer. Although this research has several limitations, it requires further validation and investigation in future research using transcriptomic analysis including RNA extraction, qPCR, etc. to prove the molecular mechanism of Propolis from East Kalimantan [100]. Therefore, Propolis from East Kalimantan, Indonesia, may be a promising candidate treatment for breast cancer.

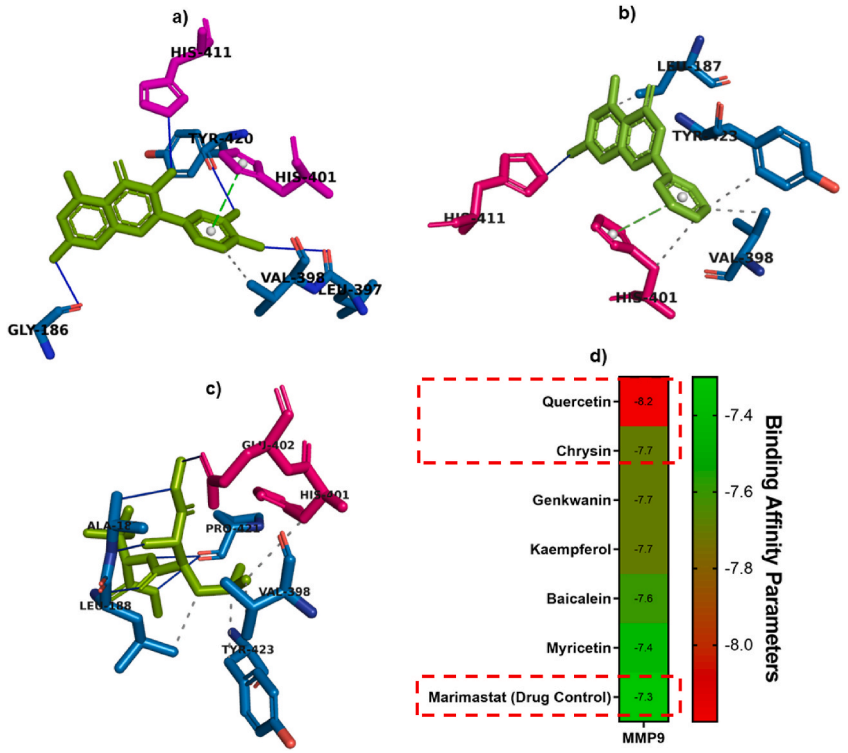

Fig. 9. Molecular interaction of Quercetin (a), Chrysin (b), and Marimastat (c), and Headmap results of docking (d).

5. Conclusions

In conclusion, this study revealed the potential of East Kalimantan propolis from *Trigona apicalis* as anti-breast cancer and their underlying mechanism of action. According to the results, in vitro cytotoxic assay found no significant difference between cytotoxic activity of EEKP-EAFr and doxorubicin (as common anti-cancer drug). Furthermore, bioinformatic study has indicated that East Kalimantan propolis compounds exhibit the potential activity in targeting MMP-9 as novel candidate of breast cancer therapy. In particular, quercetin and chrysin maybe the most favored compounds as MMP-9 inhibitor for the treatment of breast cancer based on molecular docking, molecular dynamics and MM-GBSA analysis. This study also elucidated various biological processes and pathways that potentially participate in the mechanism action of East Kalimantan propolis against breast cancer. Additionally, it employed integrated network pharmacology, molecular docking and molecular dynamic analysis to provide further evidence supporting MMP-9 as a viable therapeutic target. Nevertheless, further investigation is required to validate these findings. In summary, this study may carry the significance of advancement in novel therapeutic approaches in the treatment of breast cancer.

CRediT authorship contribution statement

Paula Mariana Kustiawan: Writing – review & editing, Resources, Methodology, Funding acquisition. **Khalish Arsy Al Khairy Siregar:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Putri Hawa Syaifie:** Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Formal analysis, Conceptualization. **Fauzan Zein Muttaqin:** Software, Resources. **Delfritama Ibadillah:** Software, Methodology. **Muhammad Miftah Jauhar:** Investigation, Formal analysis, Data curation. **Nailulkamal Djamas:** Writing – review & editing. **Etik Mardliyati:** Writing – review & editing, Validation, Supervision, Resources, Investigation. **Nurul Taufiqu Rochman:** Supervision, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviation

BP: Biological Processes
CC: Cellular Components
DEGs: Differentially Expressed Genes
EEKP: Ethanolic Extract Of East Kalimantan Propolis
EEKP-EAFr: Ethanolic Extract Of East Kalimantan Propolis Ethyl Acetate Fraction
EEKP-HFr: Ethanolic Extract Of East Kalimantan Propolis Hexane Fraction
GEO: Gene Expression Omnibus
GO: Gene Ontology
HER2: Human Epidermal Growth Factor Receptor 2
KEGG: Kyoto Encyclopedia of Genes and Genomes
MD: Molecular Dynamics
MF: Molecular Functions
MM-GBSA: Molecular Mechanics Generalized Born Surface Area
MMP9: Metaloproteinase matriks-9
MRM: Multiple Reaction Monitoring
PPI: Protein-Protein Interaction
RMSD: Root Mean Square Deviation
RMSF: Root Mean Square Fluctuation

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33636.

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