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THE ANTICANCER EFFICACY OF INDONESIAN NUTMEG HYDROSOL EVALUATED THROUGH IN SILICO AND IN VITRO APPROACHES ON MCF7 AND 3T3 CELLS

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ABSTRACT

Traditionally, the nutmeg plant (*Myristica fragrans* Hooutt) has been used to treat a variety of ailments in Indonesia. This study aimed to determine the anticancer efficacy of nutmeg hydrosol using in silico and in vitro methods. Nutmeg hydrosol refers to distilled water produced during the refining process of nutmeg oil, containing dissolved essential components. The LCMS/MS analysis result showed that the main components of hydrosol were Biondinin A, Edultin, Methyl-3-hydroxy-4-methoxybenzoate, Pluviatilol, Aviprin, Latifolone, and 2,4-Dihydroxyaceto-phenone. Based on the in silico analysis using the Autodock 4.2 method on 4WZV protein, the compounds had high binding energy, suggesting great potential as anticancers. Free radicals were shown to be actively inhibited by the hydrosol's polyphenols. Hydrosol was also classified as a very active antioxidant by the DPPH assay, which yielded an IC_{50} value of 45.3 µg/ml. Using the MTT method, an in vitro cytotoxicity investigation revealed that the substance was cytotoxic to MCF7 cells but harmless to normal cells (3T3), with IC_{50} values of 79.45 µg/ml and >1000 µg/ml, respectively. These findings indicated a favorable association between antioxidants and anticancer characteristics, indicating the possibility of using nutmeg hydrosol as a breast cancer anticancer drug.

Keywords: Antioxidant, Anticancer, Cytotoxicity, Hydrosol Nutmeg, In Silico, In Vitro.

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INTRODUCTION

One of the leading causes of death in Indonesia is cancer, ranked third behind heart disease and stroke[1]. Free radicals could be responsible for the disease, as they are highly polluting the environment. The free radicals react with DNA and cell membranes, both of which play vital roles in the functioning of the cell, causing dysfunction and even death. However, compounds known as antioxidants can counteract the damaging effect of free radicals.[2,3,4] Traditional medicine has used natural resources, particularly Indonesian medicinal plants, to prevent and treat several degenerative and chronic diseases, including cancer.[5] Nutmeg is one of the medicinal plants known to have great potential as an anticancer. This plant, also called *pala* in Indonesia belongs to the Myristicaceae family[6] and has a distinctive aroma with a slightly warm taste, leading to its application as a spice to flavor a wide variety of foods, beverages, perfumery, and soaps. It is used traditionally due to its antioxidant and antimicrobial properties.[7] According to several studies, the phytochemical content of nutmeg includes flavonoids, phenolics, terpenoids, and alkaloids with bioactivities as antioxidants[8,9], anti-inflammatory[10,11], anti-obesity, anti-diabetic, anticarcinogenic, antimicrobial, anti-angiogenetic, hepatoprotective, and aphrodisiac.[12,13] An investigation into the potential anticancer properties of nutmeg hydrosol from Sukabumi, West Java, Indonesia, was conducted in this study. Hydrosol is distilled water containing dissolved volatile oil components originating from the distillation process of aromatic plants.[14] Antioxidant analysis equipped with cytotoxicity tests is the first step in the biological screening of plant extracts to discover natural compounds with potential as anticancer agents.[15,16] Therefore, antioxidant and cytotoxic activity analyses of hydrosol would be conducted in this study. The compounds contained in hydrosol were analyzed using LCMS/MS[17], while the anticancer

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potential was investigated by an in silico study with the Autodock 4.2 method.[18],[19] Antioxidant activity was examined using 1,1-diphenyl-2-pikrilhidrazil (DPPH) as a source of free radicals.[20,21] The initial cytotoxicity analysis was carried out using BSLT method against shrimp larvae *Artemia salina* Leach[22], and continued with an in vitro test against human breast cancer (MCF7) and normal (3T3) cells using tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) through MTT method.[23,24]

EXPERIMENTAL

Material and Methods

The Nutmeg sample was collected from Sukabumi farmers, then the mace and seeds with a moisture content of 6-12% were grounded and sieved into 10, 20, and 40 mesh sizes. About 100 grams was distilled by a steam distillation unit using 2L of distilled water at pressure variations of 1, 2, and 2.5 bar for 3, 4.5, and 6 hours of time variation. During the distillation process, two product fractions were produced, namely the nutmeg oil fraction containing water, and the distilled water fraction known as hydrosol.[14] Subsequently, Merck analytical grade chemicals were used to analyze the hydrosol product in order to determine its chemical composition, antioxidant activity, and cytotoxicity.

LCMS/MS Analysis

LCMS/MS analysis was conducted using the XevoG2-XS QT mass spectrometer provided by Waters MS Technologies, Milford, USA as reported by Damayanti *et al*. (2021).[17] The electrospray ionization was employed with a scanning range from 50 to 1,200 m/z, and the retention time (RT) parameter was used in the range of 1-16 minutes.

In Silico Study

The 3D structure of the compounds contained in hydrosol was drawn using ChemDraw 12.0, while the crystal structure of the protein used as an enzyme model was downloaded from PDB ID 4wzv. The molecular docking was then performed with AutoDock 4.2. The hydrogen bonding was enhanced through the optimization options. The grid box was 40 x 40 x 40, with a *Root Mean Square Deviation* (RMSD) of 0.375 Å. The center was placed at x, y, and z coordinates of -2.906, -6.983, and 34.468. The method is considered valid when the RMSD value is ≤ 2.[18]

Antioxidant Activity Examination

Antioxidant activity was examined by the DPPH method and compared to positive controls of quercetin and vitamin C as standards, while methanol was used for the negative control. Hydrosol series solutions of 5-100 µg/ml and positive controls of 1-20 µg/ml were prepared. Both solutions were subjected to incubation with 500 µl of DPPH for 30 minutes in a dark setting. Subsequently, the absorbance was assessed using an Agilent Cary 60 UV-Vis spectrophotometer at a wavelength of 517 nm. The percentage inhibition and IC_{50} value, defined as the concentration required to achieve 50% inhibition of DPPH, were calculated.[20,21]

Toxicity Analysis

The preliminary assay of BSLT toxicity analysis was performed with the Meyer method using brine shrimp. Over a period of 48 hours, approximately 50-100 mg of brine shrimp eggs were incubated in a container filled with seawater to facilitate their development into shrimp larvae.[22,25] Subsequently, 10-11 larvae contained in the 100 µl of seawater were transferred into the 96-well microplates. Sample solutions with concentrations ranging from 10 to 1000 µg/ml were introduced into each well until the total volume reached 100 µl. This concentration was replicated three times prior to incubation at room temperature under light conditions for a duration of 24 hours. The counts of viable and non-viable shrimp larvae were subsequently determined in each well. The negative control of dimethyl sulfoxide (DMSO) was used in each experiment. The 50% lethal concentration (LC_{50}) value, which is the sample concentration that causes 50% of shrimp larva mortality was determined based on probit data analysis.

Cytotoxicity Analysis

Cytotoxic analysis was continued by evaluating the safety and efficiency of the sample against the viability of breast cancer MCF7 and normal (3T3) cells using the MTT method.[23,24] The cytotoxic test of the sample

was compared to antimycin as a standard. Cells were maintained at a temperature of 37°C in Dulbecco's Modified Eagle Medium, which was enriched with 10% (v/v) fetal bovine serum. This was conducted in a CO_2 incubator that provided 95% humidity for a duration of three days, allowing the cell cultures to achieve a confluency of 60-70%. Following this period, the existing medium was discarded and substituted with fresh medium, after which the cells were incubated for an additional 24 hours. The culture cells were rinsed once or twice with a phosphoric buffer solution. The trypsin-EDTA solution was suspended in cultivated cells, and additional media was added. Cells were measured using a hemocytometer, then seeded into 96-microwell plates at 1×10^4 cell density per well and cultivated in a CO_2 incubator at 37°C for 24 hours. The concentration of hydrosol solutions in DMSO was placed into well plates and stored in another CO_2 incubator for 24 hours. The test was performed three times, and after a 24-hour treatment period, the supernatant was removed from the well. Each well was treated with 90 µL of serum-free media combined with 10 µL of a 5 mg/ml MTT solution. The plates were then incubated for three hours in a humidified environment containing 5% CO2 at a temperature of 37°C. Following the incubation period, the medium and MTT solution were removed, and 100 µl of DMSO was introduced to each well. Cell viability was assessed by measuring the optical density (OD) at 570 nm using a Varioskan Flash Thermo Fisher Scientific multiwell spectrophotometer. The % viability formula is as follows:

$$\%\ viability = \frac{OD\ (cell + sample) - OD(negative\ control)}{OD\ (cells) - OD\ (negative\ control)}$$

The sample's efficacy was evaluated using a linear regression between the percentage of survival and the log concentration of the sample. Meanwhile, the IC_{50} value is the sample concentration that reduces viability by 50%.

RESULTS AND DISCUSSION

LCMS/MS Analysis

During the distillation process, two product fractions were produced, namely nutmeg oil containing water, and the distilled water known as hydrosol.[14] The highest yield of 5.21% was produced at 2 bar for 6 hours. The analysis was subsequently conducted on the chemical compounds, along with their antioxidant and cytotoxic activity. The chemical content analyzed using LCMS/MS showed seven compounds as shown in Table-1.

Table-1: Compounds of hydrosol

RT (min)	Compounds	Formula	Mass (Da)
5.60	2,4-Dihydroxyaceto-phenone (1)	$C_8H_8O_3$	152.047
5.74	Methyl-3-hydroxy-4-methoxy benzoate (2)	$C_9H_{10}O_4$	182.058
6.96	Aviprin (3)	$C_{16}H_{16}O_6$	304.095
8.78	Latifolone (4)	$C_{11}H_{12}O_4$	208.074
9.00	Edultin (5)	$C_{21}H_{22}O_7$	386.137
9.13	Pluviatilol (6)	$C_{20}H_{20}O_6$	356.126
9.31	Biondinin A (7)	$C_{12}H_{26}O_6$	374.173

Figure-1 shows that the seven compounds of hydrosol are polyphenols, including 2', 4'-dihidroxy acetophenone (1), and acetophenone, containing hydroxy group at positions 2' and 4'. Methyl-3-hydroxy-4-methoxybenzoate (2) is a resorcinol and one of the three isomers of benzenadiol, while Aviprin (3) is considered a linear furanocoumarin where substitution by an oxygenated prenyl residue occurs at C_8. Edultin (5), a furanocoumarin characterized by the molecular formula C6-C3, is classified as a phenolic compound due to the presence of one or more hydroxyl groups bonded to its aromatic ring. Furanocoumarin plays a crucial role as a free radicals scavenger, lowering blood sugar, anti-aging, anti-inflammatory, and anticancer.[26] Furthermore, pluviatilol (6) is a lignan commonly found in plant walls and is a group of phytoestrogens, beneficial for reproductive, heart, bones, and skin health, as well as the immune system. It also has anti-allergic activity and is a potential COVID-19 drug candidate. Lignans are derived from the shikimic acid biosynthetic pathway and structurally contain 2 or more phenylpropanoid units originating from oxidative dimerization.[27] Latifolone (4) and Biodinin A (7) are also phenylpropanoids

biosynthesized from amino acids, phenylalanine and tyrosine. Both have anti-allergic, cytotoxic, and breast cancer antiproliferative activities.[28,29]

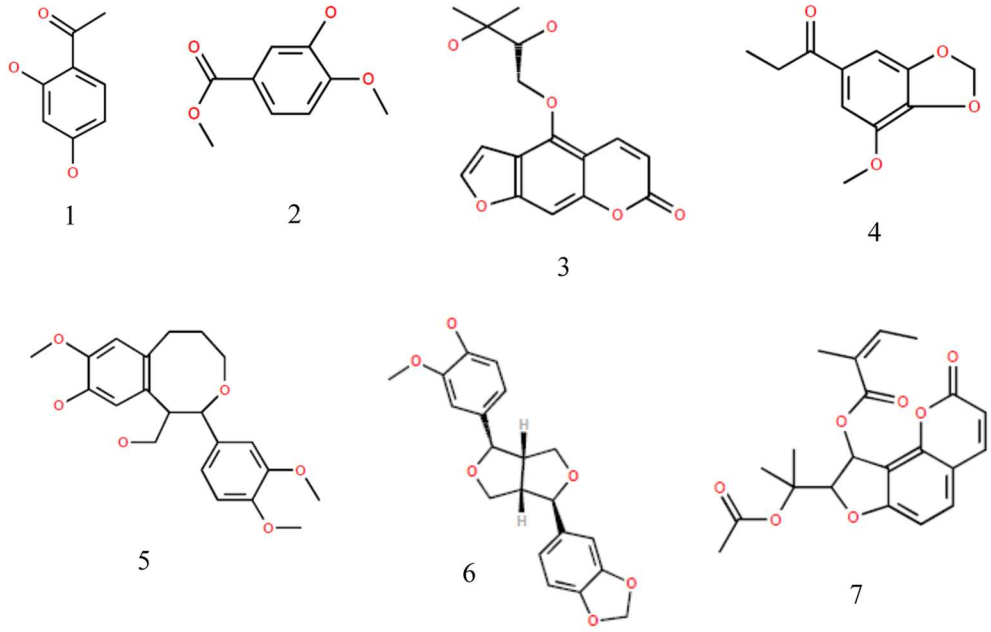


Fig.-1: Molecular Structure of Chemical Compounds Contained in Hydrosol

***In silico* Study**

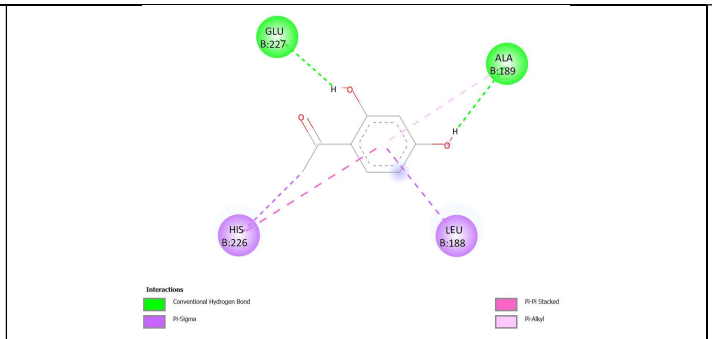
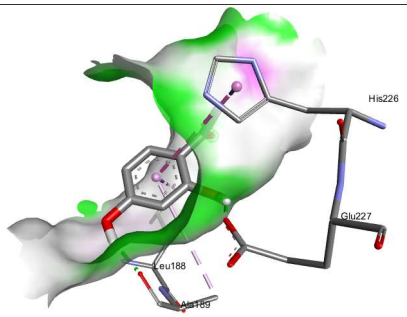
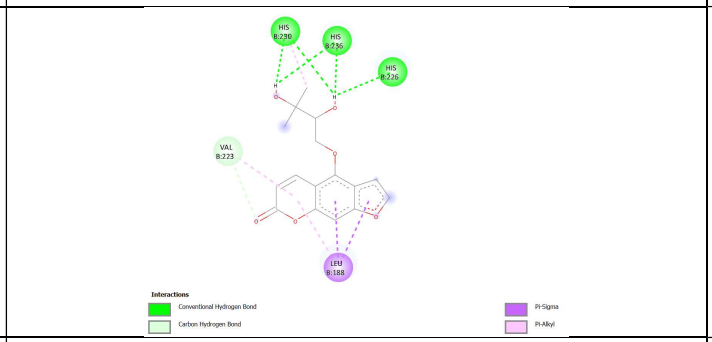
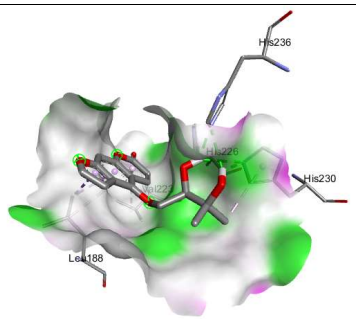
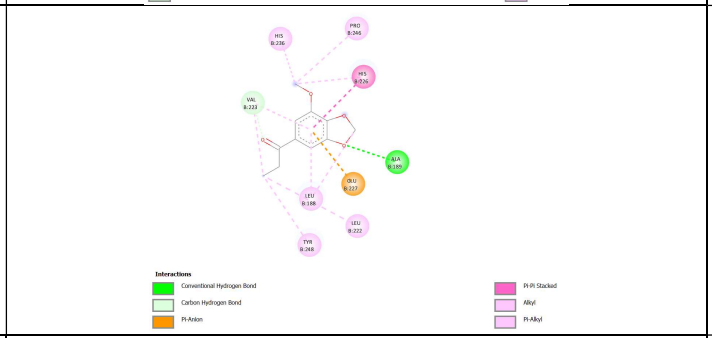
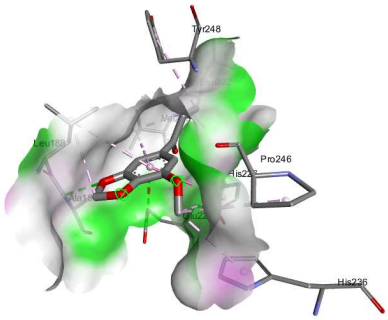
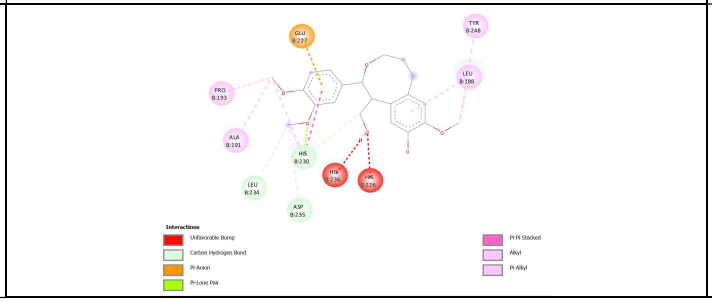
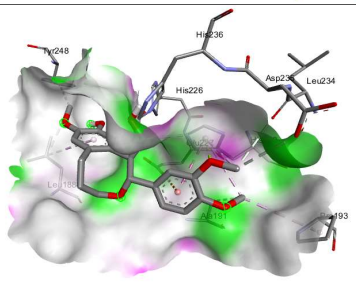
The crystal structure of the 4wzv protein, utilized as a receptor in an in silico investigation, was obtained from the Protein Data Bank (PDB). Meanwhile, the ligands are compounds contained in hydrosol as shown in Table-1 selected based on compliance with Lipinski's five rules and did not violate more than 2 of 5 rules. Bioactive compounds are considered suitable as medicines when the hydrophobicity value is ≤ 5 (log P ≤ 5, with a molecular mass < 500.[30,31] Molecular docking was carried out in the best position, namely grid boxes on x, y, and z coordinates of -2.906, -6.983, and 34.468. Based on the free energy value (ΔG) and inhibition constant (Ki), there was an interaction between the compounds as a ligand and the protein receptor. The implies the compounds can inhibit the performance of the 4wzv protein which is carcinogenic, acting as an anticarcinogenic/anticancer agent. The *in silico* study results are presented in Table-2 and Fig.-2.

Table-2: The Docking Score of Chemical Substituents from Hydrosol with Protein of 4wzv

Compound	ΔG (Kcal/mol)	Ki (mM)	Interaction with amino acid residue
2,4-Dihydroxyaceto-phenone (1)	-4.90	0.257	Glu 227, Ala 189, His226, Leu118
Methyl-3-hydroxy-4-methoxybenzoate (2)	-4.84	0.282	Tyr248, Val223, His226, Ala189, Leu222, Glu227, Leu188, His236
Aviprin (3)	-6.82	0.010	His226, His230, His236, Val223, Leu188
Latifolone (4)	-5.63	0.075	Pro246, Ala189, Val223, Leu188, Leu222, Tyr248, His226, His236, Glu227
Edultin (5)	+9.69	-	-
Pluviatilol (6)	-7.19	$5.36X10^{--3}$	Leu222, Ala191, His226, His236, Leu188, Val223, Tyr248, His 230, Glu227.

Biodinin A (7)	-0.26	0.646	Pro246, His226, Val223, Leu188, Leu187, His236.
Tamoxifen (standard material)	-11.7	$2.66X10^{-6}$	Ala242, Arg249, Tyr245, Leu243, Met247, Leu188, His226, Glu227.

Note: Ala: alanine, Arg: Argenine, Asp: aspartate, Glu: glutamine, His: histidine, Leu: leucine, Pro: proline, Thr: threonine, Tyr: tyrosine, Val: valine.

1.		
2.		
3.		
4.		

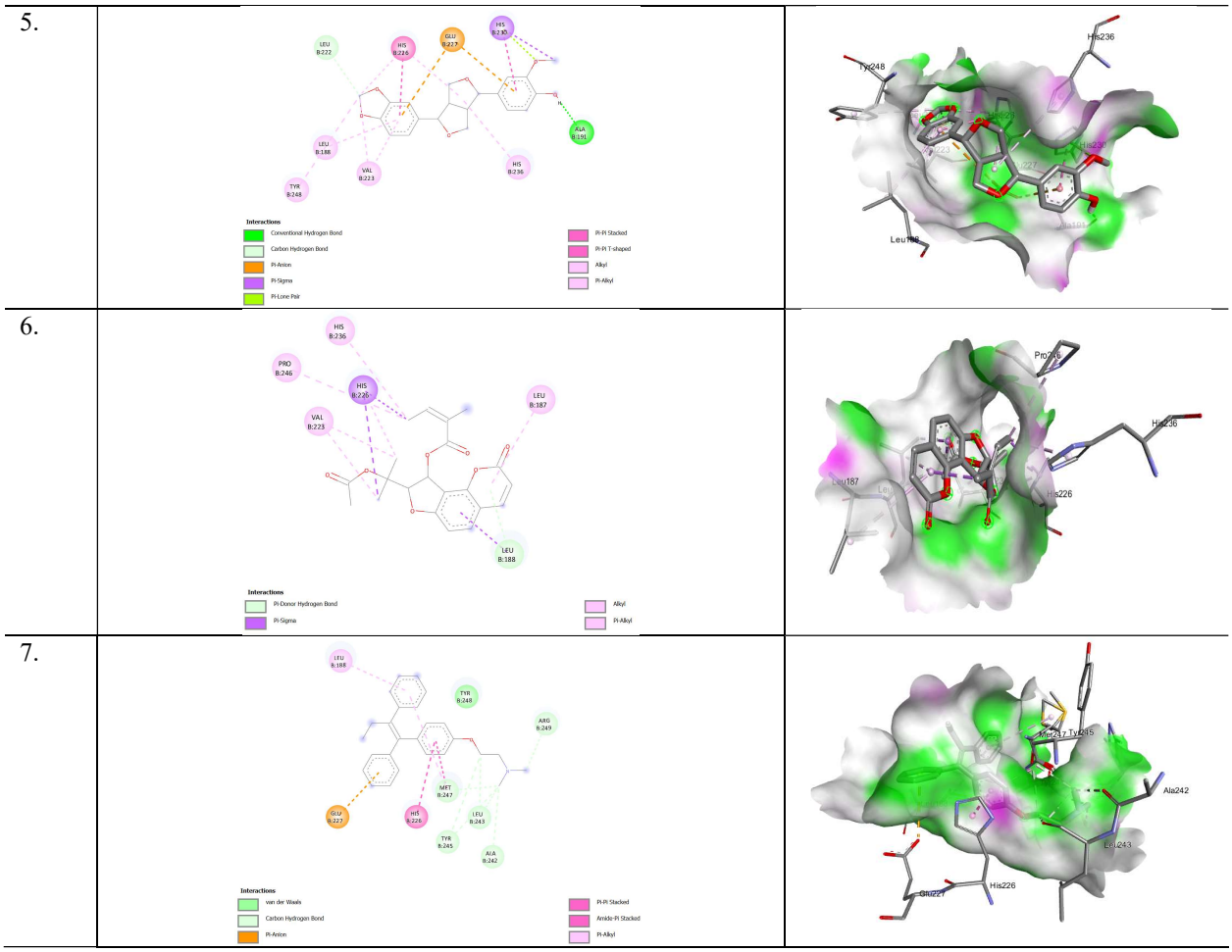


Fig.-2: 2D and 3D Interaction of the Compounds 1-7 (Table-1 and Fig.-1) from Hydrosol with Amino Acids of 4wzv Protein and Tamoxifen (8)

Hydrogen interactions occurred between compounds of hydrosol with amino acid residues of 4wzv (Fig.-2), as well as the standard material from tamoxifen. The docking simulation results showed that hydrosol had consistent active predictions on the 4wzv protein and produced small inhibitory energy (Ki) of the hydrogen bonding. Subsequently, in vitro analysis was conducted to determine cytotoxicity activities.

Antioxidant Activity

The antioxidant property of hydrosol tested using DPPH radical scavenging assay is shown in Table-3 and Fig.-3. A sample is considered an active antioxidant when the IC_{50} is less than 100 µg/mL.[4,21] Hydrosol with an IC_{50} value of 45.3 µg/ml showed a very active antioxidant property, but the activity was weaker than vitamin C and quercetin with IC_{50} values of 4.11 and 9.84 µg/ml respectively. This shows that hydrosol has high radical scavenging activity related to the stronger proton donating ability. The polyphenols present in hydrosol contribute to this activity.

Table-3: Antioxidant Activity

No.	Name of the samples	IC_{50} (µg/ml)
1	Hydrosol	45.30
2	Quercetin	4.11
3	Vitamin C	9.84

The potent antioxidant properties of nutmeg can be ascribed to the presence of lignans, β-caryophyllene, and eugenol, which feature hydrogen atoms located in allylic or benzylic positions. Eugenol further amplifies these antioxidant effects by increasing the activities of several key enzymes, including superoxide

dismutase, catalase, glucose-6-phosphate dehydrogenase, glutathione peroxidase, and glutamine transferase.[6]

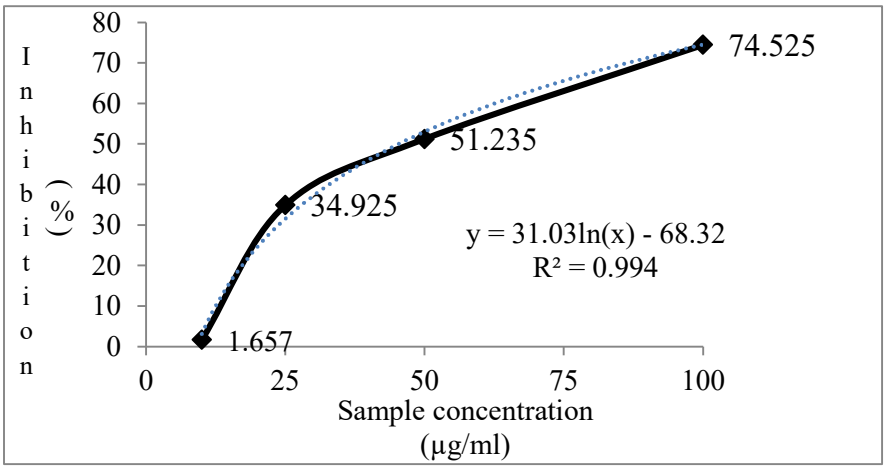


Fig.-3: Antioxidant of Nutmeg Hydrosol

Various in vitro antioxidant tests of methanolic and hydroalcoholic nutmeg extracts reported high antioxidant activity attributed to a significant proportion of phenol and flavonoid content.[8] Nutmeg is rich in essential oils, triterpenes, and various types of phenolic compounds that enable high antioxidant activity, leading to its use in traditional medicine.[3] Hydrosol from nutmeg which is a strong antioxidant can be used to reduce the increasing problem of degenerative diseases including cancer. The diseases could be caused by impaired cellular function due to increased exposure to free radicals. The negative effects can be combated by using exogenous antioxidants with have the ability to capture free radicals. Therefore, the occurrence of free radical reactions can be inhibited and the various associated diseases are prevented. Polyphenolics in hydrosol contribute to antioxidant activity, due to the hydroxyl (OH) group with high radical scavenging activity.[6,8,10]

Toxicity

The BSLT toxicity assay represents a straightforward, rapid, and relatively precise technique employed in the identification of novel pharmaceuticals.[25] Toxicity is indicated by the LC_{50} value which is the concentration capable of causing the death of an organism up to 50%. Hydrosol concentrations used in this test were 10, 200, 500, and 1000 µg/ml. An LC_{50} of ≤ 30 µg/ml is considered very toxic; $30 \leq LC_{50} \leq 100$ is quite toxic; $100 \leq LC_{50} \leq 1{,}000$ µg/ml is toxic, and>1,000 µg/ml is non-toxic.[22,25] The results showed that hydrosol was moderately toxic with LC_{50} of 56.26 µg/ml, suggesting potential as an anticancer agent. Cytotoxicity of hydrosol was in vitro tested using MTT assay, a multipurpose and well-known method. This assay entails the conversion of yellow, water-soluble MTT into insoluble purple formazan by mitochondria bioreductases.[23,24] Hydrosol concentrations used to test the viability of MCF7 and 3T3 cells evaluation ranged from 10-150 µg/ml. At 50 µg/ml, MCF7 cell viability was 69.93%. Cell viability decreased significantly to 8.47-2.63% at 125 µg/ml and 150 µg/ml doses (Fig.-4). The results revealed that the samples had a very high level of 3T3 cell viability. No cytotoxicity was seen against 3T3 cells at concentrations ranging from 10-150 µg/ml, with an IC_{50} value more than 500 µg/ml (Fig.-5). Evaluation results of hydrosol's effect on the survival of MCF7 and normal 3T3 cells are shown in Table-4.

Table-4: Cytotoxicity Test Result on MCF7 and 3T3 Cells

Name of the samples	IC_{50} (µg/ml)	
	MCF7	3T3
Hydrosol	79.45	>500 µg/ml
Antimyicin	1.24	>500 µg/ml

The National Cancer Institute classifies the cytotoxicity of the substances as follows. IC_{50} ranges from <20 µg/ml (severe cytotoxicity) to >500 µg/ml (no cytotoxicity).[33] The results showed that nutmeg hydrosol was toxic for MCF7 cancer cells and nontoxic against 3T3 normal cells. The IC_{50} value of hydrosol against

the growth of MCF7 cancer cells was 79.45 µg/ml, showing a cytotoxic effect. Meanwhile, the IC_{50} of the positive control, namely antimycin as a standard material was 1.24 µg/ml. Antioxidant activity is closely related to anticancer properties, and free radicals have been implicated in the early onset of various chronic diseases by inducing oxidative stress.

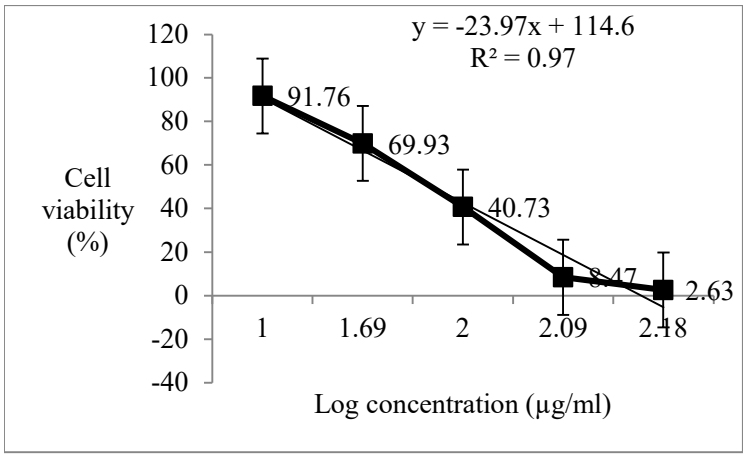


Fig.-4: Cytotoxic Activity of Hydrosol Against MCF7

High levels of free radicals trigger harmful physiological responses capable of damaging cells, which underlies the development of cancer cells. Meanwhile, phytochemicals are known to significantly reduce the risk of chronic disease development, due to antioxidant properties. Phenolics and flavonoids from plant extracts are known to possess antioxidant effects that can prevent and inhibit oxidation by free radicals, as well as reduce oxidative stress.[3,34] This observed high radical scavenging activity of hydrosol is related to the stronger proton donating ability. Polyphenolics in hydrosol contribute to antioxidant activity, with the hydroxyl (OH) group playing a significant role.[6,8,10]

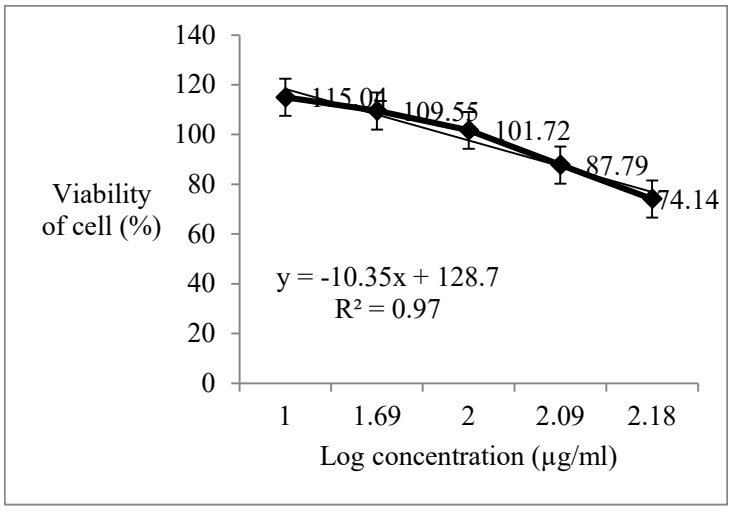


Fig.-5: Cytotoxicity of Hydrosol on Normal 3T3 cells

CONCLUSION

In conclusion, hydrosol from nutmeg consists of polyphenols including Biondinin A, edultin, Methyl-3-hydroxy-4-methoxybenzoate, Pluviatilol, Aviprin, Latifolone, and 2,4-Dihydroxyaceto-phenone. Strong antioxidant activity was observed alongside high toxicity against MCF7 cancer cells but not normal cells. These results suggest that hydrosol could be used as an anticancer agent, specifically for breast cancer. The constituent compounds influence antioxidant properties which correlate with cancer activity.

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CONFLICT OF INTERESTS

All authors affirm that they possess no conflicts of interest and take full responsibility for the design, collection, analysis, and interpretation of the data presented in this publication.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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