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Comparative Study on Cytotoxic Potency, Chemical Composition, and Antioxidant Characteristics of Clove Buds Essential Oil (*Syzygium aromaticum*) Originating from Indonesia, Comoros, and India

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Abstract
To evaluate the anti-tumoral and antioxidant capacities of clove buds essential oils from Indonesia, Comoros Islands and India, we conducted the MTT test, along with various antioxidant capacity assessments, including evaluations of ferrous ions (Fe2+) chelating activity, ammonium molybdate test (TAC), reducing power assay (FRAP), TBARS assay, DPPH, ABTS+ tests, and β-Carotene Bleaching Assay. All examined essential oils displayed significant antioxidant activity in various conducted tests. Moreover, these essential oils have demonstrated strong in vitro cytotoxic effects against two breast cancer cell lines (MDA-MB-468 and MCF-7) and a mouse mastocytoma cell line (P815). Importantly, they have shown the ability to preserve the integrity of healthy cells; specifically peripheral blood mononuclear cells. The chemical composition of the studied essential oils showed that Eugenol, Caryophyllene, and Humulene percentages were notably higher in the Indonesian and Indian samples compared to the Comorian sample. However, the Comorian sample uniquely contained isoeugenol (55.45%), levomenthol (13.39%), eucalyptol (13.30%), and Thymol, which were absent in the Indonesian and Indian samples. The variations in chemical profiles among essential oils from different geographical origins are responsible for the observed differences in antioxidant and cytotoxic activities.

Keywords: Breast cancer; chemical composition; cytotoxicity; essential oil; *syzygium aromaticum;* antioxidant capacity.

1. Introduction

Excluding nonmelanoma skin cancer, breast cancer is the most commonly diagnosed cancer among women in the United States. Notably, it represents the leading cause of cancer-related fatalities for Black and Hispanic women. However, in the broader context, it is the second most prevalent cause of cancer-related deaths in women, trailing only behind lung cancer [1]. Despite the controversies surrounding the effectiveness of complementary and alternative medicine (CAM), its usage is notably increasing among cancer survivors. Breast cancer survivors, in particular, are more likely to utilize CAM compared to survivors of other types of cancer [2].

The Myrtaceae family includes the clove, Syzygium aromaticum (L.) Merrill et Perry. The species is native to a few volcanic islands in Indonesia's eastern region known as the North Molucca [3]. It is abundant in antioxidants and volatile chemicals like β-humulene, β-caryophyllene, and eugenol. Much attention has been drawn to clove essential oil because of its broad use in the culinary, flavoring, health, cosmetic, and perfume sectors. Clove essential oil has biological properties that are important to human health, such as antioxidant and antibacterial properties as well as insecticidal action [4].

The market revolves around six main production areas: Indonesia (comprising the Moluccas, Java, and Sulawesi islands), the eastern coast of Madagascar (specifically the Analanjirofo and Atsinanana regions), the Mozambique Channel islands (including Zanzibar, Pemba in Tanzania, Anjouan, and Moheli in Comoros), central Sri Lanka, southern India, and the southwest region of Bahia state in Brazil [5]. Therefore, it is anticipated that this study will offer insights into the constituents of clove bud oil sourced from Indonesia, Comoros, and India, along with their anticancer and antioxidant properties. Additionally, this research aims to present a comparative analysis of the essential oils extracted from clove buds originating in Indonesia, Comoros, and India.

2. Experimental

Plant material

We bought Indonesian and other Indian cloves from the Italian market, as well as Comorian cloves from Comoros Islands, in order to carry out this investigation.

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Isolation of essential oil by hydro distillation

The hydro-distillation of the three clove bud samples was conducted using Clevenger-type apparatus [6]. Triplicate samples weighing 50g each from their respective origins were subjected to extraction for three hours in 300mL of distilled water at 100°C.

Analysis of volatile constituents

The obtained essential oil samples were subjected to analysis using a gas chromatograph, specifically the Trace GC Ultra, which featured a Flame Ionization Detector (FID) and a capillary column (30 m × 0.25 mm, 0.25µm film thickness) DB-5 (methyl polysiloxane with 5% phenyl). The injection volume for each sample was set at 1µL. The gas chromatograph was coupled with a mass spectrometer, namely the Q Polaris MS, facilitating mass spectrometry analysis (70 eV with an ion trap). The temperature program involved an initial 2-minute period at 40°C, followed by a gradual increase to 180°C at a rate of 4°C/min. Helium was employed as the carrier gas at a flow rate of 1.4 mL/min.

Antitumor activity

Cell culture

Murine mastocytoma P815 cells, human breast cancer MDA-MB-468, and human breast cancer MCF-7 cells, used in the study originated from the stock cultures at the Laboratory of Biological Engineering, Faculty of Science & Technology, Sultan Moulay Slimane University, Morocco, were cultured in RPMI 1640 media supplemented with 1% antibiotic (penicillin G-streptomycin) and 5% heat-inactivated fetal bovine serum. The incubation was at 37°C in a humid atmosphere with 5% CO2.

Cytotoxicity test

In 96-well flat-bottom microplates containing 100 µl of complete media, human breast cancer cells MDA-MB-468 and MCF7, along with P815 mastocytoma cells, were seeded at a density of 2.10^5 cells/mL for MDA-MB-468 and MCF7, and 10^5 cells/mL for P815. Prior to any treatment, the cells were incubated for a minimum of 12 hours. Various concentrations of the tested essential oils, ranging from 500µg/mL to 3.9µg/mL, were prepared in 100µL of complete media. Control cells were treated solely with DMSO. In all cases, the final DMSO concentration remained below 0.1%. Following an incubation period at 37°C with 5% CO_2 humidity for 48 hours, a volume of 20 µL of MTT solution (5 mg/mL in PBS) was added to each well, followed by incubation in the same conditions. After 4 hours, the plates were exposed to a solution of 0.04N HCl in isopropanol in order to dissolve the blue intracellular formazan product [7]. Subsequently, the optical density was measured at 540 nm using a MultisKan EX (Labsystem) microplate reader. The relative percentage of cell viability was determined using the following formula:

$$\% \text{ Viability} = 100 \times (A/A_0),$$

Where A_0 and A represent the absorbance of the test culture and the negative control, respectively. The IC_{50} values, representing the concentration of examined compounds resulting in a 50% decrease in cell viability, were utilized to compare the cytotoxic effects of oils against the cell lines.

Antioxidant activity study

DPPH radical scavenging assay

Samples were evaluated for their antioxidant activity using the DPPH assay, as outlined in the study by Minarti M, et al.[8], in this procedure, 50 µL of each sample at various concentrations, ranging from 500µg/mL to 3.9µg/mL, was combined with 150 µL of a 0.004% methanolic DPPH solution in a 96-well plate. Afterwards, the plate was shielded from light for thirty minutes, and the absorbance was recorded at 517 nm. The Scavenging activity percentage was determined utilizing the following formula:

$$\text{Scavenging activity (\%)} = [(A_{DPPH} - As)/A_{DPPH}] \times 100$$

Where, A_{DPPH} represents the absorbance of the DPPH solution, and As denotes the absorbance values of the sample.

Antioxidant Potential in Neutralizing ABTS+ Radicals

The evaluation of radical scavenging activity against the ABTS+ radical we utilized the method outlined by Yang, S et al [9]. ABTS was oxidized with potassium persulfate to generate ABTS+. Before the experiment, the ABTS+ stock solution was diluted with methanol to attain an absorbance of 0.700 ± 0.020 at 734 nm. After a 10-minute incubation period, during which 925 µL of the diluted ABTS+ solution was combined with 75 µL of different concentrations ranging from 500µg/mL to 15.62µg/mL in methanol of each essential oil sample, the absorbance at 734 nm was recorded.

The percentage of scavenging activity for each essential oil on ABTS+ was determined using the following formula:

$$\text{Inhibition (\%)} = [(A_0 - As)/A_0] \times 100$$

Where, A_0 represents the absorption of the control, and As represents the absorption of the tested essential oil.

Metal chelating activity

Ersoy et al. [10] with slight adjustments. In a 96-well microplate, 185 µL of various concentrations ranging from 500µg/mL to 3.9µg/mL of each oil in methanol, were added in each well, than mixed with 5 µL of $FeCl_2$ (2mM) solution. The microplate was then shaken and left at room temperature for 10 minutes. Subsequently, 10 µL of ferrozine (5 mM) was added

to all wells of the microplate, and the absorbance was measured at 540 nm. The metal chelating potential was determined using the following formula:

$$Inhibition\ \% = [(A\ control - A\ sample)/A\ control] \times 100$$

Ferric ion reducing antioxidant power (FRAP)

The reducing ability assay operates based on the principle that substances possessing reduction potential engage in a reaction with potassium ferricyanide ($K_3Fe_3 + (CN)_6$), resulting in the formation of potassium ferrocyanide ($K_4Fe_2+(CN)_6$). Subsequently, this ferrocyanide reacts with ferric chloride to produce a ferric-ferrous complex characterized by an absorption peak at 700 nm [11]. The ferric-reducing power was determined according to the method of Anis, S.et al. [12] In brief, 250µl of each sample at various concentrations ranging from 500µg/mL to 15,62 µg/mL were combined with 250µL of phosphate buffer (200 mM, pH 6.6) and 250µL of potassium ferricyanide (1%). Subsequently, the mixture underwent 20-minute incubation at 50 °C in a water bath. The reaction was terminated by adding 250µLof 10% trichloroacetic acid (w/v) followed by centrifugation at 3000 rpm for 10 minutes. Lastly, 400µL of distilled water and 100µL of ferric chloride (0.1%) were added to 500µL of the supernatant. The mixture was thoroughly blended and left undisturbed for 10 minutes at room temperature. Subsequently, the absorbance of the mixture was measured at a wavelength of 700 nm. A standard curve was created using vitamin C, and the results were quantified in terms of vitamin C equivalents.

Total phosphomolybdenum antioxidant capacity assay (TAC)

The Total Phosphomolybdenum Antioxidant Capacity assay (TAC) was employed to assess antioxidant activity, with slight modifications to the procedure outlined by Encarnaç, S. et al. [13]. In test tubes, 500 µL of each oil (concentrations ranging from 500µg/ml to 31,25µg/ml), were individually mixed with 500 µL of the phosphomolybdenum reagent (comprising 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate), along with 500 µL of distilled water. The tubes were enveloped in aluminum foil and subjected to heating at 90 °C for 90 minutes, followed by cooling to room temperature. Absorbances of the resulting greenish solutions were measured at 695 nm against an appropriate blank. Each assay was replicated three times. A standard curve was created using vitamin C, and the results were quantified in terms of vitamin C equivalents.

TBARS Assay

The TBARS assay was utilized to assess the influence of clove essential oil on the production of Malondialdehyde (MDA), a notable reactive aldehyde formed in the process of lipid peroxidation. In a succinct procedure, 150 µL of various concentrations of the investigated clove essential oils, ranging from 500µg/mL to 62.5µg/mL, were mixed with 250 µL of 10% egg yolk homogenate (in distilled water, v/v). Subsequently, 100 µL of $FeSO_4$ (17.5 mM) was added. After one-hour incubation at 37 °C, a mixture of 750 µL acetic acid (20%), 750 µL thiobarbituric acid (0.8% in 1.1% sodium dodecyl sulfate), and 50 µL trichloroacetic acid (TCA) (20%) were added to the reaction, followed by heating in a boiling water bath for one hour. The control reaction contained 150 µL distilled water, and the blank mixture lacked $FeSO_4$ After cooling, 1.5 mL of n-butanol was added to the mixture, followed by centrifugation for 10 minutes at 3000 g. The optical density of the organic upper layer was measured at a wavelength of 532 nm. The results were calculated using the provided equation and presented as the percentage of inhibition of lipid peroxidation [14]:

$$Inhibition\ (\%) = (1-(As/A_0))*100$$

Where A_0 represents the optical density of the control reaction, and As represents the optical density in the presence of the tested essential oils.

β-Carotene Bleaching Assay

The lipid peroxidation activity of the samples is evaluated using the β-carotene bleaching assay, following the methodologies outlined by Bryshten, I et al. [15]. Initially, 1mg of β-carotene is dissolved in 2mL of chloroform, followed by the addition of 25µL of linoleic acid and 200mg of Tween 20. After evaporating the chloroform at 37°C, 100mL of distilled water is added to create an emulsion. To enhance convenience, the assay is adapted to a microplate format. Subsequently, 10 µL of various concentrations of the tested essential oils (ranging from 500µg/mL to 3.90µg/mL) are added in the wells. Following this, 150 µL of the linoleic acid-β-carotene solution is swiftly transferred to the reaction plate using a multi-channel pipette. The oxidation of the β-carotene emulsion is monitored by measuring the absorbance at 490 nm using a Multiskan EX (Labsystem) microplate reader after incubation for 2 hours at 50°C. The activity is calculated using the equations provided below:

$$Degradation\ rate\ (DR) = ln\ (A/B) \times 1/t$$

"A" represents the initial absorbance, "B" represents the absorbance at 120 minutes, and "t" denotes the entire duration of the test, which is 120 minutes. The antioxidant activity is presented as percent inhibition using the following formula:

$$\%\ Inhibition = (1 - DR\ of\ sample/DR\ of\ control) \times 100$$

Where DR of the sample is the degradation rate calculated using the provided equation, and DR of the control is the degradation rate of the control.

Statistical analyses

Statistical analyses were performed employing one-way analysis of variance (ANOVA) and Tukey's test, utilizing GraphPad Prism 8.4.2 software to assess the impacts of the investigated oils. The data was expressed as mean ± standard

deviation, derived from three independent experiments, unless stated otherwise. Statistical significance was determined at a p-value of <0.05.

3. Results and Discussion

Oil yield

Through the hydro distillation process, essential oils of clove buds from Comoros, Indonesia, and India were subjected to two replicates for each origin, resulting in average yields of 8.19%, 10.97 %, and 8.48% (w/w), respectively. The clove oil obtained from both origins exhibited a colorless appearance following hydro distillation. Subsequently, the obtained clove oil was stored at 4 °C in a refrigerator. The Indonesian clove buds yielded the highest amount of essential oil in the hydro distillation process. This outcome supports the explanation provided by Alfikri et al, who suggested that the absence of petals and stamens in clove buds could result in a reduction in oil content by 5%–10% [16].

Analysis of volatile constituents

As presented in table 1 and figure 1 Indonesian clove consisted mainly of three key components: eugenol (74.65%), caryophyllene (14.85%), and humulene (2.75%). Additionally, trace amounts of copaene (0.80%), caryophyllene oxide (0.70%), isoeugenol (0.015%), and methyl salicylate (0.05%) were also detected in the clove oil. The predominant chemicals identified in Comoros clove oil include eugenol (55.45%), caryophyllene (3.30%), isoeugenol (55.45%), Levomenthol (13.39%), and eucalyptol (13.30%), with trace amounts of humulene (0.54%). In Indian clove oil, the three primary components are eugenol (70.4%), caryophyllene (19.5%), and humulene (1.9%).

Table1: Chemical composition of Clove Buds Essential Oil from Indonesia, Comoros, and India

Compound name	RT	Indonesia %	Comoros %	India %
Thioacetic acid	4.45	0.016	1.38	-
exo-2,7,7-trimethylbicyclo[2.2.1]heptan-2-ol	7.34	-	0.02	0.1
6-epi-shyobunol	7.34	0.06	0.02	0.1
Limonene	7.54	-	1.01	-
Eucalyptol	7.94	-	13.30	-
Terpinene	8.02	-	0.55	-
Glycerin	8.27	-	0.01	0.01
Methane, nitro	8.27	-	0.01	0.01
Methyl-12-tetradecen-1-ol acetate	9.84	-	0.01	0.01
Levomenthol	10.27	-	13.39	-
Methyl salicylate	10.47	0.051	0.07	0.3
2-Methyl-3,5-dinitrobenzyl alcohol, tert-butyldimethylsilyl ether	11.38	0.016	0.03	-
Benzene, 1-methyl-3,5-bis[(trimethylsilyl)oxy]-	11.38	0.016	0.03	-
Phenol, 4-(2-propenyl)-	11.66	0.165	0.12	0.1
Phenol, 4-(2-propenyl)-, acetate	11.66	0.012	0.12	-

2-Allylphenol	11.66	0.165	0.12	0.1
Thymol	12.17	-	0.03	-
Phenol, 2-methyl-5-(1-methylethyl)-	12.17	0.017	0.03	0.1
cis-Ocimene, 8-oxo-	12.24	-	0.03	0.1
Benzene, 1-methyl-3,5-bis[(trimethylsilyl)oxy]-	12.62	0.016	0.02	-
alpha-Cubebene	12.85	0.032	0.09	-
Copaene	12.85	0.801	0.09	0.1
isoeugenol	13.25	0.015	55.45	-
Caryophyllene	13.98	14.846	3.30	19.5
Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-	13.98	-	3.30	0.1
cis-à-Bisabolene	14.47	0.017	0.54	0.1
Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-	13.98	0.017	3.30	-
Humulene	14.17	2.756	0.54	1.9
Phenol, 2-methoxy-4-(2-propenyl)-, acetate	15.61	-	3.95	2.1
3-Allyl-6-methoxyphenyl acetate	15.61	-	3.95	2.1
Eugenol	15.61	74.647	55.45	70.4
Caryophyllene oxide	16.46	0.706	0.15	0.4
Alloaromadendrene oxide-(1)	16.46	0.073	0.15	0.3
Diepicedrene-1-oxide	16.46	0.073	0.15	0.1

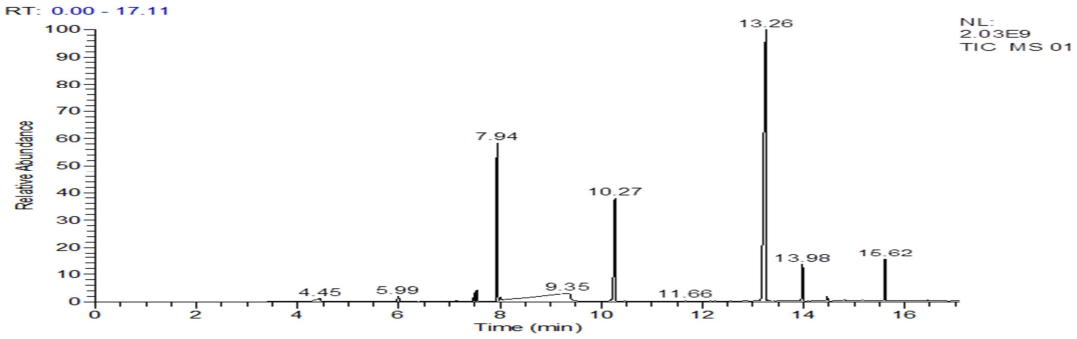

Figure 1: Gas Chromatography-Mass Spectrometry (GC-MS) analysis of Comorian clove buds essential oil.

The essential oils derived from *S. aromaticum* exhibit comparable chemical profiles, consistently identified across various studies, suggesting a shared chemotype characterized by high eugenol content. Eugenol emerged as a significant compound in all examined samples, with percentages of 74.647% in the Indonesian sample, 70.4% in the Indian sample, and 55.45% in the Comorian sample, showcasing consistent results. Caryophyllene was another prevalent component in the studied samples, constituting 14.85% in the Indonesian sample, a higher percentage of 19.5% in the Indian sample, and a lower percentage of 3.30% in the Comorian sample. Humulene exhibited varying concentrations, with the Indonesian sample recording 2.75%, the Indian sample 1.9%, and the Comorian sample 0.54%. the GC-MS analysis showed that some compounds like isoeugenol (55.45%), levomenthol (13.39%), eucalyptol (13.30%) and Thymol (0.03%) are present only in Comorian sample. In our study, certain compounds such as eugenol, caryophyllene oxide, and caryophyllene were found at significantly higher levels compared to the chemical composition of the essential oil tested by Magda A. Ali et al. [38] additionally, they did not identify some compounds like eucalyptol, levomenthol, limonene, and isoeugenol, which are present in the oils we tested. The variability in essential oil content observed in different studies is attributed to factors such as clove bud origin, soil conditions, fertilizer use, and climate influences. Climatic factors like high temperatures, sea levels, rainfall quantity and pattern, wind, and sunlight intensity play pivotal roles. Monitoring changes in soil characteristics and solar radiation becomes crucial, as these variables can activate or deactivate specific enzyme groups, influencing the dominance of particular biosynthetic pathways [17]. This variation can also be attributed to the date of harvest and post-harvest conditions.

Antitumor activity

Cytotoxicity test

After exposing P815, MDA-MB-468 and MCF7 cell lines to different concentrations (ranging from 3.9 to 500µg/mL) of *Syzygium aromaticum* (clove) essential oil for 48 hours, cell viability was assessed by measuring the metabolically converted MTT to a formazan dye. The results indicated a dose-dependent inhibitory effect on all tested cell lines. Table 2 outlines the IC50 values, representing the concentration of the essential oil required to inhibit 50% of cell viability. These values are crucial in understanding the potency of the essential oil in affecting cell viability, with lower IC_{50} values indicating a stronger inhibitory effect.

Our study revealed that the IC_{50} values for all the examined essential oils against P815 were almost identical (16.82±4.93 µg/mL, 28.16±6.64 µg/mL, 13.67±1.19 µg/mL). These IC_{50} values were lower than the IC_{50} (100 µg/mL) reported by Mansi Sharma for an essential oil from a plant of the same Myrtaceae family against colorectal cancer cells [18]. Treatment of MCF7 cells with essential oil extracted from Indonesian clove buds for 48 hours resulted in an identical IC_{50} value of 37.38±0.37 µg/mL, This IC_{50} was significantly higher than the IC_{50} reported in Vinay Kumar Pandey's study, which showed that the IC_{50} of clove bud essential oil against MCF7 cells was 29.7µg/mL [19]. Notably, the remaining tested oils demonstrated IC_{50} values lower than that of the Indonesian essential oil. In particular, Indian and Comorian essential oils, when tested against MDA-MB-468 cells, showed IC_{50} values similar to the 243 µg/mL reported by Valizadeh et al. [20], whereas the Indonesian essential oil exhibited the highest IC_{50} value at 147.3±10.47 µg/mL.

To better understand the cytotoxic effects of *Syzygium aromaticum* essential oil on cancer cells, we selected three cell lines for analysis: murine mastocytoma (p815), as well as human breast cancer cell lines MDA-MB-468 and MCF7. Our cytotoxicity analysis, conducted throughout this research, unveiled substantial effects against the three carcinoma cell lines (MCF-7, P815, and MDA-MB-468) (figure2), demonstrating a dose-dependent relationship. The Indonesian essential oil exhibited the highest cytotoxicity, followed by Indian and Comorian essential oils. This can be attributed to the elevated percentages of eugenol, caryophyllene, and humulene found in these oils. Importantly, regardless of the source of clove buds, *Syzygium aromaticum* essential oils did not demonstrate harm to normal cells (PBMCs). Eugenol demonstrated anticancer activity by inducing apoptosis in Michigan Cancer Foundation 7 (MCF-7) (IC_{50}: 22.75 µM) and MDA-MB-231 (IC_{50}: 15.09 µM) breast cancer cells, leading to increased levels of ROS that inhibited the cell cycle at the G2/M phase, resulting in clastogenesis in vitro. Additionally, it reduced the proliferation of proliferating cell nuclear antigen (PCNA) associated with decreased mitochondrial membrane potential ($\Delta\Psi m$) and upregulation of Bcl-2-associated X protein (Bax)[21] It is well documented that β--caryophyllene oxide influences various critical pathways involved in cancer development, such as the mitogen-activated protein kinase (MAPK), PI3K/AKT/mTOR/S6K1, and STAT3 pathways. Additionally, treatment with this compound decreases the expression of genes/proteins associated with promoting cancer, while simultaneously increasing the levels of those with proapoptotic properties[22]. β-caryophyllene markedly enhanced the effectiveness of a-humulene and isocaryophyllene against MCF-7 cells. It accumulates within the membranes of cancerous cells, thereby augmenting membrane permeability. This alteration in membrane structure might aid in the transportation of bioactive substances across the cytoplasmic membrane of cancer cells. Consequently, β-caryophyllene could boost the intracellular buildup of anti-tumor medications like paclitaxel, enhancing their effectiveness against cancer[23]. In silico studies suggest that α-humulene holds promise as an anti-breast cancer agent, particularly targeting the HER-2 receptor[24]. Semi-synthetic derivatives of isoeugenol inhibited colony formation in MCF-7, reduced ERα concentration, and significantly increased total apoptotic cell death, arresting the cell cycle at the G2/M phase. Furthermore, the molecular mechanism of apoptotic activity was investigated at the gene level, revealing upregulation of pro-apoptotic genes and downregulation of anti-apoptotic genes.

Table 2: Cytotoxic activity of clove buds essential oil from tree origins against P815, MCF7, MDA-MB-486 and PBMC cells

Origin of clove bud	Cytotoxic effect (IC_{50} Values; µg /mL)			
	P815	MCF7	MDA-MB-486	PBMC
Indonesia	16,82±4,93[a]	37,38±0,37[a]	147,3±10,47[a]	NA
Comoros	28,16±6,64[a]	68,91±7,29[b]	265,3±2,19[b]	NA
India	13,67±1,19[a]	78,12±3,91[b]	256,3±17,47[b]	NA
Paclitaxel	0.0012±0.001[c]	0.0013±0.001[c]	0.0027±0.0004[d]	0.0022±0.0003[d]

The presented values represent the mean of three independent experiments, with standard deviations indicated as ±. Distinct lowercase letters indicate significant differences, as determined by one-way ANOVA and Tukey's multiple comparison tests ($p < 0.05$).

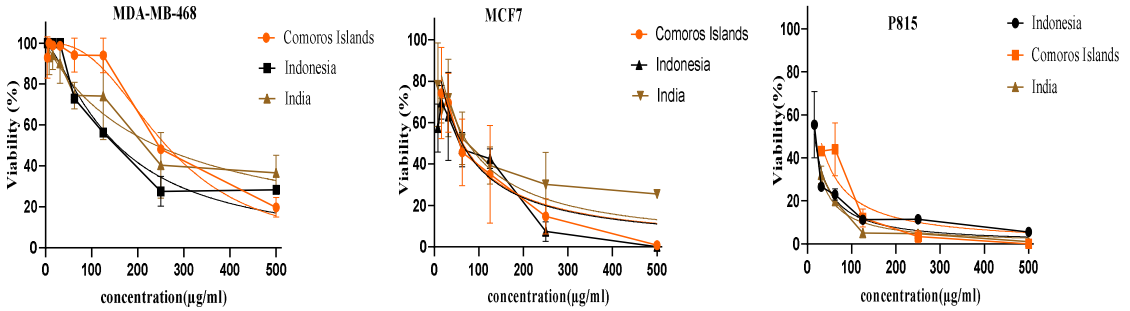

Figure 2: The viability percentages of cancer cell lines (MDA-MB-468, MCF7, and P815) were assessed after a 48-hour treatment with varying concentrations of essential oil from Comorian, Indonesian, and Indian clove buds, using the MTT assay. The provided data represent the means derived from three independent experiments, with corresponding standard deviations indicated as (±).

Antioxidant activity study

Numerous in-vitro studies were conducted to evaluate the antioxidant capacity of clove buds essential oil. These assessments encompassed the reducing power assay, total antioxidant capacity (TAC), TBARS assay, ferrous ions (Fe^{2+}) chelating activity, free radical scavenging assays (DPPH and ABTS+ tests) and β-Carotene Bleaching Assay (table 3 & figure 3)

Table 3: Antioxidant activity of essential oils of clove buds from Indonesia, Comoros Island and India

	IC_{50} (µg/ml) of anti-oxidant activity					Total antioxidant capacity (mg AAE/gDW)	Reducing Power(mg AAE/g DW)
	DPPH	β-Carotene Bleaching Assay	ABTS	TBARS	Metal chelating activity		
Indonesian Essential oil	74,62±19,63[a]	37,08±6,93[a]	55,61±4,68[a]	82,38±13,29[a]	39,16±10,54[a]	302,0±2,34[a]	72,28±7,47[a]
Comorian Essential oil	12,50±1,64[b]	7,54±1,83[b]	28,24±0,58[b]	25,49±0,32[b]	36,38±3,57[a]	313,5±2,10[b]	258,5±23,56[b]
Indian Essential oil	66,90±3,57[a]	10,23±3,10[b]	57,65±0,58[a]	38,60±1,62[b]	38,59±3,07[a]	312,8±1,41[b]	202,3±9,8[b]
Controls	5,41±1,66[b]	10,61±4,73[b]	8,17±1,41[c]	40,85±0,00[b]	22,74±1,92[a]	-	-

Control DPPH: Ascorbic acid; control β-Carotene Bleaching Assay: hydroxytoluène butylé, BHT ou 2,6-di-tert-butyl-4-méthylphénol (BHT), controle ABTS assay:BHT, controle TBARS assay: BHT, control Metal chelating activity: acide éthylènediaminetétraacétique (EDTA).

IC_{50}: 50% inhibition concentration, DPPH: 2,2-diphenyl-1-picrylhydrazyl, TAC: Total antioxidant capacity, acide 2,2'-azino-bis(3-éthylbenzothiazoline-6-sulphoniqueN, AAE: Ascorbic acid equivalent. Each value is presented as means ± standard deviation. Different letters in the same column indicate significant difference ($p<0.05$) within conditions according to Tukey's multiple comparison test.

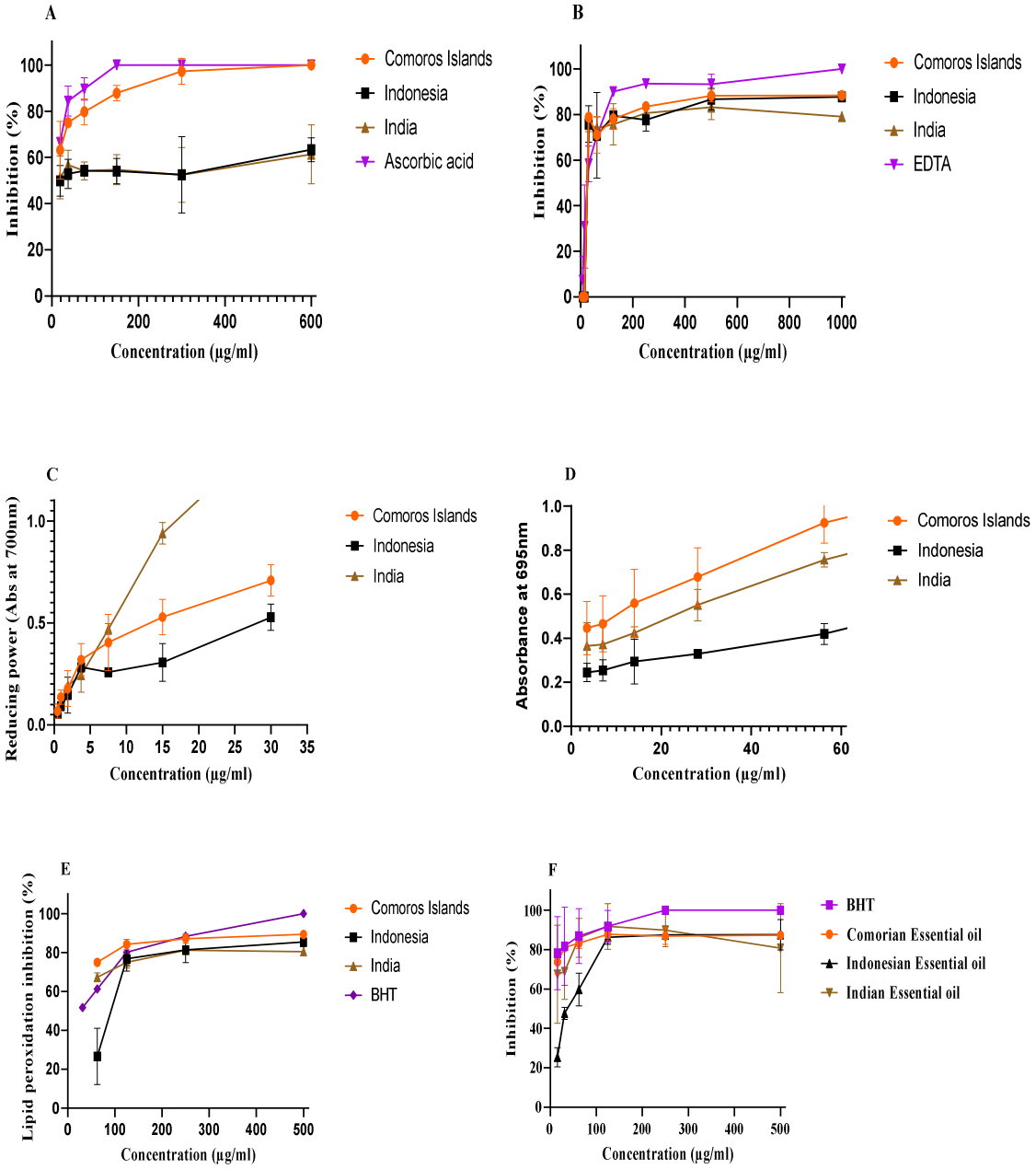

Figure 3: Antioxidant potential of clove essential oil from Comoros Island, Indonesia, and India was evaluated at various concentrations using the following assays: (A) DPPH Assay, (B) Iron Chelating Activity, (C) FRAP, (D) Total Antioxidant Capacity (TAC) assay, (E) TBARS assay and (F) β-Carotene Bleaching Assay. The presented values represent the means of three independent experiments, accompanied by standard deviation.

The essential oil derived from mature buds in Comoros exhibited significantly potent antioxidant properties by DPPH assay (IC_{50}=12.50±1.64 µg/mL) in contrast to the essential oils from Indonesia and India. In a study conducted by Faisal Nur Alfikri, Indonesian mature clove buds demonstrated a remarkably high antioxidant capacity compared to the findings in our

study [16]. The presence of thymol and eugenol may account for the elevated antioxidant activity observed in Comorian clove oil, in contrast to other oils lacking thymol, as identified by Xiaohua C.et al. [25].

The ABTS test demonstrated an IC_{50} of 55.61±4.68 µg/mL for the essential oil of Indonesian clove buds, marking a higher value than the IC_{50} reported by Farida Aryani and colleagues [26]. Furthermore, our investigation indicated that the Comorian essential oil displayed notably higher antioxidant activity when compared to other tested essential oils, corroborating the results obtained from the DPPH assay.

The results obtained through the TBARS assay demonstrated a significant antioxidant capacity in all the tested oils. Among them, the essential oil extracted from Comorian clove buds exhibited particularly potent antioxidant properties, as evidenced by its notably high IC_{50} compared to other essential oils. Additionally, The application of clove essential oil reduced lipid peroxidation levels, as reported in the study by Daniela Kaizer Terto et al. [27].

Ferrozine and Fe^{2+}can form complexes quantitatively. Chelating agents disrupt the formation of these complexes, leading to a decrease in the red color of the complex. Therefore, assessing the chelating activity of a coexisting chelator is possible by measuring the decrease in color [28]. The results of our experiment reveal that all tested essential oils exhibit chelating activity, capturing ferrous ions before ferrozine. Moreover, they interfere with the formation of the ferrous and ferrozine complex. In a study by M. Edyta Kucharska et al., the iron chelation capacity of clove bud essential oil demonstrated an IC_{50} value (0.32 mmol Fe^{2+}/L ± 0.01) [29] higher to our findings (IC_{50}=36.38µg/mL).

The Total Antioxidant Capacity (TAC) of essential oils extracted from three distinct sources was assessed using the phosphomolybdenum test. This technique is based on the reduction of Mo (VI) to Mo (V) facilitated by diverse antioxidants present in the oils. This reduction leads to the formation of a green-colored phosphate/Mo (V) complex [3]. The essential oil with the highest total antioxidant capacity was found to be from Comorian origin (313.5±2.10 mg AAE/g DW), followed by oils from Indian and Indonesian sources. These results were notably higher when compared to the findings of El Hassania Loukili et al. [30].

The FRAP assay assesses the ability of bioactive compounds to donate electrons, enabling the calculation of their reducing power [31]. In this assay, the Comorian clove buds essential oil exhibited similar results regarding antioxidant power (258.5±23.56 mg AAE/g DW). This suggests that the presence of compounds such as isoeugenol, levomethane, eucalyptol, and thymol in this essential oil may contribute to its antioxidant potency compared to the other tested essential oils. However, it's worth noting that the observed reducing power in all tested oils was lower than that reported by Adriana Trifan et al. [32].

The β-Carotene bleaching technique relies on the fading of the yellow hue of β-carotene caused by its interaction with radicals generated during the oxidation of linoleic acid within an emulsion. The pace of β-carotene fading can be decreased when antioxidants are present [33]. In this assay, the most significant antioxidant effect was noted in both Comorian (IC_{50}=7.54±1.83µg/mL) and Indian essential oils (IC_{50}=10.23±3.10µg/mL). However, in contrast to these results, the Indonesian clove essential oil exhibited slightly lower efficacy (IC_{50}=37.08±6.93µg/mL), as demonstrated in other antioxidant tests. Comorian and Indonesian essential oils yielded results consistent with those reported by Sneha Shendeet al..[34].

Figure 4 presents a comparison of IC_{50} values for essential oils from three different regions (Indonesia, Comoros, India) across various antioxidant activity tests. These tests include DPPH, ABTS, Bleaching Assay, Chelating Activity, FRAP, TAC, and TBARS. The differences in IC_{50} values illustrated by the heatmap highlight the variation in antioxidant efficacy of the essential oils depending on their geographical origin. For instance, the essential oil from the Comoros shows a significantly lower IC50 in the DPPH test compared to the other samples, indicating a stronger antioxidant activity in this particular case.

The geographical origin of essential oils significantly influences their chemical composition, which in turn can explain the differences observed in antioxidant activity tests. For instance, certain bioactive compounds may be more abundant in oils sourced from the Comoros, potentially contributing to their superior performance in various antioxidant assays. This observation aligns with studies like that of B. Sang et al [35], which demonstrated that essential oils from *Atractylodes lancea* exhibited significant chemical variations depending on their region of origin, thereby affecting their biological activities

In our study, the essential oil from the Comorian sample displayed the highest antioxidant capacity compared to the Indonesian and Indian samples. This suggests that the emergence of new compounds such as isoeugenol, levomenthol, eucalyptol, and thymol, as identified through gas chromatography, contributes to its potent antioxidant capacity. Chemical composition analysis via gas chromatography indicated that the Comorian sample contained isoeugenol (55.45%), levomenthol (13.39%), and eucalyptol (13.30%), which were absent in the Indonesian and Indian samples. Mass spectrometry of the three studied essential oils revealed variations in the percentages of eugenol, caryophyllene, and humulene, with these compounds being more abundant in the Indonesian and Indian samples compared to the Comorian sample. The exceptional antioxidant activity of Comorian essential oil can be attributed to the combination of thymol and eugenol, which enhances the scavenging of free radicals. This explanation is supported by the review conducted by Nagaraj Basavegowda and colleagues [36], which highlights that oregano essential oil (abundant in thymol and carvacrol) has been extensively utilized in combination with cinnamon, rosemary, and thyme for various industrial applications. Similarly, the synergistic effects observed when thyme and clove essential oils (rich in thymol and eugenol, respectively) are combined with cumin and cinnamon further support this notion. In addition the presence of Eucalyptol mitigates oxidative stress by modulating signaling pathways and scavenging radicals[37].

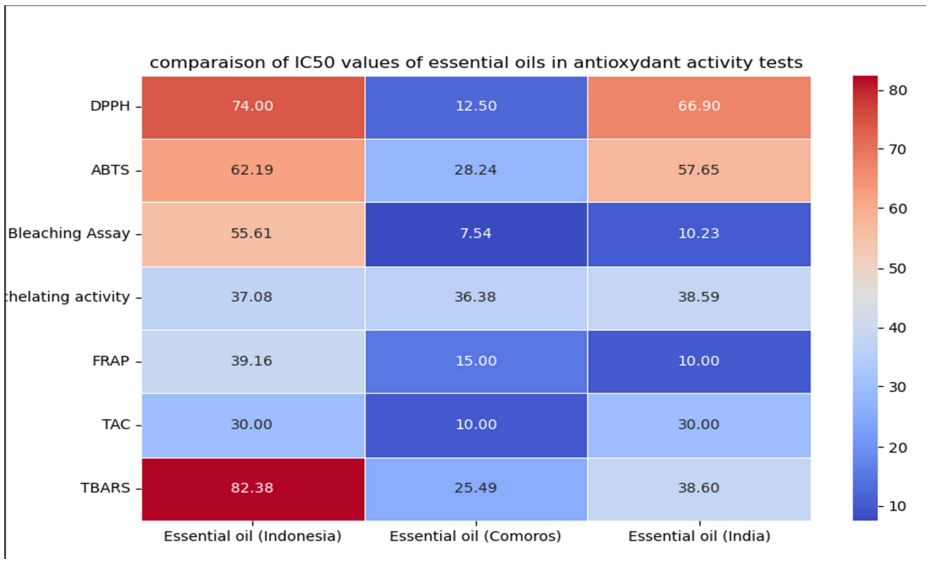

Figure 4: Comparison of the founded IC_{50} values across all antioxidant tests will be presented using a heat map. Lower colour intensity on the heat map will indicate higher IC_{50} values.

4. Conclusions

This study demonstrated that essential oils from Syzygium aromaticum, sourced from Indonesia, India, and Comoros, exhibit significant cytotoxic effects on cancer cell lines MCF-7, P815, and MDA-MB-468, with the highest cytotoxicity observed in the Indonesian essential oil. This efficacy is attributed to the high concentrations of compounds such as eugenol, caryophyllene, and humulene, which play a critical role in inducing apoptosis and modulating cellular pathways involved in cancer development. Notably, the studied essential oil did not show harmful effects on normal cells (PBMCs), highlighting its potential as a selective anticancer agent. Additionally, the Comorian essential oil displayed superior antioxidant capacity, likely due to the presence of compounds like isoeugenol and thymol. These findings underscore the therapeutic potential of clove essential oils in cancer treatment, while also emphasizing the need for further studies to better understand the underlying mechanisms and to evaluate their efficacy in vivo.

5. Conflicts of interest

There are no conflicts to declare.

6. Formatting of funding sources

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7. Acknowledgments

There are no Acknowledgments

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