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Original Research Article

Phytochemical characterization and biological properties of *Ocimum sanctum* L. and its active phytocomponents: Eugenol and β-caryophyllene

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ABSTRACT

This study explored the pharmacognostical and biological properties of *Ocimum sanctum* L., focusing on its hydromethanolic (HME) and aqueous (AQE) extracts, as well as the bioactive compounds eugenol (EUG) and β-caryophyllene (BCP). Phytochemical analysis revealed a diverse range of compounds, with HME showing higher levels of phenolics, flavonoids, and tannins compared to AQE. Both extracts exhibited antioxidant activity, while EUG and BCP displayed significant cytotoxicity against MCF-7 cells. Molecular docking studies indicated that EUG has potential binding to catalase. The findings of this study underscore *O. sanctum* as a valuable source of bioactive compounds with potential antioxidant and anticancer properties. Notably, the extraction method played a crucial role in determining the phytochemical profile. However, further investigations are necessary to fully understand the mechanisms and therapeutic applications of EUG and BCP, particularly as potential catalase inhibitors.

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1. Introduction

The Lamiaceae (Labiatae) family, commonly known as the mint family, is a diverse group of flowering plants that includes over 7,000 species and 236 genera (Hashemi-Moghaddam et al., 2015; Mohammadhosseini et al., 2019a; Mohammadhosseini et al., 2019b; Kianasab et al., 2023). This family is characterized by its distinctive four-sided stems, aromatic leaves, and tubular flowers. Many members of the Lamiaceae family are widely cultivated for their culinary and medicinal properties, such as basil, rosemary, lavender, and thyme, and are found all over the world (Anita Margret et al., 2022; Sarkar et al., 2023). Cancer is a growing global health concern. Conservative estimates suggest that liver, breast, lung, cervical, stomach, and colorectal cancers account for approximately 13% of annual deaths worldwide (Organisation mondiale de la santé, 2018; Omara et al., 2020). Oxidative stress resulting from chemotherapy-induced reactive oxygen species (ROS) contributes to or is primarily responsible for many adverse effects of the treatment. Cancer cells compared to normal cells, have higher levels of ROS and more likely to have mitochondrial dysfunction (Acuña et al., 2012). Overproduction of oxygen-centered free radicals or any ROS such as hydroxyl and superoxide free radicals can cause oxidative damage in biomolecules, *e.g.*, lipids, proteins and DNA which is also evident in some kinds of cancers such as cancer of the liver, lung, stomach, colon, breast, and so forth (Poulsen et al., 1998; Bhalla et al., 2013). The most rational approach to prevent carcinogenesis is by interfering with the basic modulation steps such as initiation, promotion, progression and the associated signal transduction pathways (Fresco et al., 2006; Bhalla et al., 2013). Antioxidants such as vitamin C remove damaging oxidizing agents like hydrogen peroxide from living

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organisms and neutralize free radicals that harm our body cells (Pehlivan, 2017). The modern world's socio-cultural life is heavily reliant on allopathic medicine, including chemotherapy. However, much contemporary research has focused on addressing this disease using ayurvedic herbs as a complementary or alternative approach, driven by the need to mitigate side effects such as alopecia, chemotherapy-induced peripheral neuropathy, nausea and vomiting, cardiotoxicity, diarrhea, infertility, and chemo brain (Pandey et al., 2013; Brianna and Lee, 2023). Therefore, it is crucial to develop an effective strategy and novel drugs with minimal side effects or toxicity that can mitigate the sequelae of cancer chemotherapy.

Numerous herbs have been scientifically shown to possess anti-carcinogenic properties, which are used to treat various cancers (Farzaneh and Carvalho, 2015). Among a broad spectrum of medicinal plants, *Ocimum sanctum* L. has been widely used in traditional Indian medicine for centuries to treat a range of ailments and diseases (Kaushal et al., 2018). *O. sanctum* L., "The Queen of herbs" known as 'Tulsi' in Hindi and 'Holy Basil' in English, belongs to the Angiospermae and family Lamiaceae (Prakash and Gupta, 2005). This herbal species . has two assortments, namely dark (Krishna Tulsi) and green (Rama Tulsi), and their chemical constituents are comparable. Basil or Tulsi leaves contain a high percentage of eugenol (EUG) (57.9%) (4-allyl-2-methoxyphenol) and β-caryophyllene (BCP) (15.3%) [(1*R*,9*S*)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0] undec-4-ene] (9.1%) (Mirdha et al., 2007).

Eugenol (EUG) is a methoxyphenol with a short hydrocarbon chain and an aromatic ring, featuring a hydroxyl group that enables it to act as a hydrogen atom donor and inhibit oxidation. This structural-activity relationship contributes to its antioxidant properties (Gülçin, 2011), anti-genotoxic (Rompelberg et al., 1996), anti-carcinogenic (Zheng et al., 1992; Gülçin, 2011), etc. β-Caryophyllene (BCP) is a sesquiterpene, which has interesting chemopreventive properties such as anti-inflammatory, genoprotective and anti-proliferative (Di Giacomo et al., 2016, 2017).

This investigation aims to:

i) Evaluate the pharmacognostic and antioxidant properties of hydromethanolic (HME) and aqueous (AQE) extracts of *O. sanctum* L., as they contain most of the active phytocomponents.

ii) Study the *in vitro* cytotoxicity of HME, AQE, eugenol (EUG), and β-caryophyllene (BCP) against the human breast cancer cell line MCF-7.

iii) Predict the binding affinity of EUG and BCP with the antioxidant enzymes catalase (CAT) and glutathione reductase (GR) using in silico docking studies.

This work underscores the crucial impact of extraction methods on the phytochemical profile and bioactivity of *O. sanctum*, providing new insights into its antioxidant and anticancer properties and paving the way for further research into the therapeutic applications of its bioactive compounds.

2. Experimental

2.1. Pharmacognostic profile

2.1.1. Collection, authentication of plant material and preparation of *O. sanctum* leaves extracts

Fresh leaves of *O. sanctum* L. or holy basil (Fig. 1a) were collected from the plant grown at Samaras girl's hostel, Navrangpura, Ahmedabad (Fig. 1b) and authenticated by Prof. and Head, Dr. Hitesh Solanki, Department of Botany, School of Sciences, Gujarat University, Ahmedabad. The herbarium number was GU/BOT/L/O18.

After washing thoroughly in distilled water, the green leaves were shade-dried at room temperature, then pulverized into fine powder, and stored in an airtight container for future use (Harborne, 1984). The dried leaf powder of *O. sanctum* L. (5.0 g) was subjected to extraction by soxhlet apparatus using hydromethanol solvent (70:30) to prepare hydromethanolic extract and filtered using charcoal filter paper to remove impurities. For aqueous extract, leaves powder (25 g) was refluxed with 300 mL of double distilled water for 3 h at 60-65 °C on the magnetic stirrer. It was then cooled and filtered using muslin cloth followed by evaporation for desired volume in a hot air oven at 37 to 40 °C.

2.1.2. Qualitative analysis of *Ocimum sanctum* leaf extract

2.1.2.1. Determination of pH and percentage yield

A pH meter (WW-35634-90, Cole-Parmer, USA) was used to determine the pH. HME and AQE were kept in an incubator for 3 days at 37 °C for drying and percentage yield was calculated by the following formula:

Percentage yield (%) = [Weight of product after evaporation in incubator (g)/ Weight of powder used in soxhelt system (g)] × 100 (Eqn. 1)

2.1.2.2. FTIR analysis

O. sanctum L. (Tulsi) leaves extracts (HME and AQE) and their bioactive compounds EUG and BCP were used for FTIR analysis using Fourier transform infrared attenuated total reflection (FTIR-ATR) spectroscopy (Bruker, Germany) to determine functional groups present.

2.1.2.3. Phytochemical analysis of *Ocimum sanctum*

Phytochemicals were analyzed to ascertain the presence or absence of various phytocomponents like saponins, alkaloids, tannins, phenolic compounds, flavonoids, glycosides, fats, steroids, and triterpenoids in *O. sanctum* L. by the method of Harborne (1984). Molisch's, Fehling's, Benedict's, and Barfoed's tests were respectively performed for the absence or presence of carbohydrates. Biuret's, Millon's, and xanthoprotein's tests were performed for proteins. Ninhydrin test was performed for amino acid presence. Salkowaski's and Liebermann-Burchard's tests were performed for steroids and triterpenoids presence. Shinoda and lead acetate tests were performed for flavonoid presence. $FeCl_3$, acetic acid and iodine tests were performed for tannin and phenolic compounds presence.

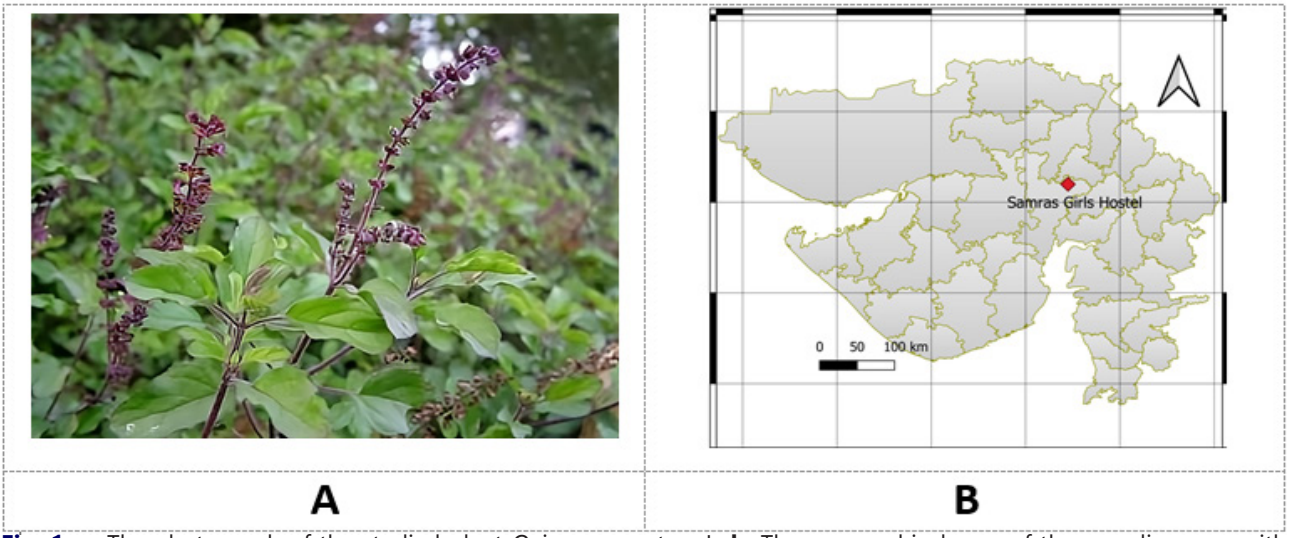


Fig. 1. a: The photograph of the studied plant *Ocimum sanctum* L. **b:** The geographical map of the sampling area with Longitude= 72.540678°, Latitude= 23.037545°.

Dragendorff's, Mayer's, Hager's and Wagner's tests were performed for alkaloids presence. A foam test was performed for fat and saponins presence.

2.1.2.4. Thin layer chromatography (TLC)

Thin layer chromatography was performed as described by Hassan and coworkers (2015) to confirm the presence of EUG and BCP in the extracts of plant leaves using different solvents as mobile phase, namely toluene:ethyl acetate:acetic acid (10:1:0.32) for the presence of EUG and ethyl acetate: *n*-hexane (7:3) for the presence of BCP.

2.1.3. Quantitative analysis of *Ocimum sanctum* leaf extract

2.1.3.1. Estimation of total phenolic content (TPC)

The total phenolic content of *O. sanctum* L. leaf extracts was determined using the method of Singleton and Rossi (1965) with minor modifications. A standard curve was prepared using different concentrations of gallic acid (5-50 µg/mL) to measure the comparative total phenolic level in the samples. The results were expressed in terms of gallic acid equivalent (µg of gallic acid per mg of extract) and calculated using the linear regression equation of the standard curve.

2.1.3.2. Estimation of total flavonoid content (TFC)

With minor changes in the method of Harborne (1984), the total flavonoid content of *O. sanctum* L. leaf extracts was estimated. The concentration of flavonoids in HME and AQE extracts (1 mg/mL) was calculated using a regression equation derived from the graph of quercetin concentration versus absorbance at 470 nm. Quercetin (1-10 µg/mL) was used as a standard, and the results were expressed in terms of quercetin equivalent (µg of quercetin per mg of extract).

2.1.3.3. Estimation of total tannin content (TTC)

The total tannin content in *O. sanctum* L. leaf extracts was analyzed using the method of Harborne (1984) with minor modifications. The concentration of total tannins in HME and AQE extracts (1 mg/mL) was calculated using a regression equation derived from the graph of tannic acid concentration versus absorbance at 550 nm. Tannic acid (5-30 µg/mL) was used as a standard, and the results were expressed in terms of tannin equivalent (µg of tannic acid per mg of extract).

2.1.4. Determination of antioxidant activity

2.1.4.1. DPPH scavenging assay

To assess the antioxidant activity of the plant extracts, the method adapted from Blois (1958) was used with slight modifications. A stock solution of DPPH (2,2-diphenyl-1-picrylhydrazyl radical) (0.1 mM) stock solution was prepared in methanol and stored in anamber bottle, kept in the dark at 4 °C between the measurements. For the assay, 50 µL solution of DPPH methanol was added to 50 µL plant extracts solutions at varying concentrations, (3.9 to 500 µg/mL), prepared by serial dilution. The mixture was incubated in the dark at room temperature for 30 min. The decrease in absorbance was measured at 517 nm against methanol as blank. Ascorbic acid, at the same concentrations as the samples, was used as a standard. The IC_{50} value was determined by linear regression analysis of the dose-response curve, plotting percentage inhibition against concentration.

2.1.4.2. ABTS scavenging assay

ABTS (2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) cation radical scavenging activity was assessed as described by Re and coworkers (1999) with slight modifications. The $ABTS^{\cdot+}$ radicals were generated

by mixing ABTS solution (7.0 mM) and potassium persulphate (2.45 mM) and allowing the mixture to stand in the dark overnight. Samples and standards, with concentrations ranging from 4.88 to 625 µg/mL, were then added (100 µL each) to a 12-well plate, followed by the addition of ABTS solution (900 µL) to each well. After 5 min of incubation at room temperature, the decrease in absorbance was quantified spectrophotometrically at 540 nm. Ascorbic acid was used as standard to prepare the standard inhibition curve and the IC_{50} value of *O. sanctum* L. extracts was calculated based on this curve.

2.1.4.3. Superoxide anion scavenging assay

The superoxide radical scavenging activity of *O. sanctum* L. leaf extract was evaluated according to the method of Yen and Hsieh (1995) using nitro blue tetrazolium (NBT) dye with some minor modifications. The reaction mixture contained 0.1 mL of NBT (1 mM), 0.1 mL of NADH (0.1 mM), 0.5 mL of the sample with the concentration range between 100-1000 µg/mL and 0.25 mL of phosphate buffer saline. After 1 min of incubation, 0.1 mL phenyl methosulfate (PMS) (120 µM) was added and absorbance was quantified spectrophotometrically at 560 nm. Ascorbic acid was taken as standard to prepare the standard inhibition curve and the IC_{50} value of leaf extracts was determined based on this curve.

2.1.4.4. Nitric oxide scavenging assay

Nitric oxide radical scavenging capacity was estimated using Griess-Ilosvay's reagent (Green et al., 1982; Badami et al., 2003). The reaction mixture containing 1 mL of sodium nitroprusside (10 mM), 0.25 mL of phosphate buffer saline, and 0.25 mL of sample or standard solution of ascorbic acid at various concentrations ranging between 1-10 µg/mL were incubated at 25 °C for 150 min. After incubation, 0.5 mL of the Griess reagent was added. A pink-colored chromophore was formed in diffused light. The absorbance of these solutions was measured spectrophotometrically at 540 nm. The inhibition curve was plotted and the IC_{50} value of *O. sanctum* L. leaf extracts was determined.

2.2. Cytotoxicity study: MTT assay

2.2.1. Cell Culture

MCF-7, a human breast cancer cell line was purchased from the National Centre for Cell Science (NCCS), Pune, and was cultured in RPMI 1640 media, supplemented with fetal bovine serum (FBS, 10%), 2 mM L-glutamine and 0.1 mM non-essential amino acids. The cells were maintained at 37 °C in a 5% CO_2 incubator (Biocenter, Salvis Lab). Once each cell line became confluent (80%), cells were trypsinized (0.25% trypsin-EDTA), counted, and seeded in a 96-well plate according to the experimental protocol.

2.2.2. Experimental groups

To check the anticancer effects of EUG, BCP, HME and AQE, the study was divided into six different groups as listed in Table 1.

Table 1
Experimental groups for MTT assay.

Group	Compound	Dose
I	Control	-
II	Vehicle control (DMSO)	1.00%
III	Eugenol (EUG)	50-600 nM
IV	β-Caryophyllene (BCP)	100-1200 nM
V	Hydromethanolic extract (HME)	1-20 µg/mL
VI	Aqueous extract (AQE)	1-20 µg/mL

2.2.3. MTT assay

The MTT [3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide] colorimetric assay was used to measure the cytotoxicity and cell proliferation based on the conversion of MTT into formazan crystals by mitochondrial dehydrogenase in living cells (Mosmann, 1983). Cells were harvested and seeded into a 96-well plate (5×10^3 cells/well) and incubated for 24 h. The cells were treated with different doses of compounds as mentioned in Table 1, for 24 h. 20 µL MTT (5 mg/mL in PBS) was added to each well of the 96-well culture plate at the end of various treatments and were incubated for 4 h at 37 °C in a 5% CO_2 incubator. After incubation, 50 µL of dimethyl sulfoxide (DMSO) was added to solubilize formazans. After incubation of at 37 °C for 10 min, the absorbance was recorded at 570 nm using a double beam spectrophotometer (Epoch microplate spectrometer, Biotek, USA). DMSO was used as a vehicle control (1.0%), only media was taken as blank and media along with the cells, without any treatment having maximum cell viability was taken as control.

2.3. Statistical analysis

All the experiments were performed in triplicates and the results were expressed as mean ± SD. The statistical significance was evaluated by two way-analysis of variance (ANOVA) using GraphPad Prism version 8. The value of $p < 0.05$ was considered to indicate significant difference.

2.4. *In silico* study: Molecular docking

EUG and BCP were docked with two enzymatic antioxidants, catalase (CAT) and glutathione reductase (GR). The 3D structure of the CAT (PDB ID: 1DGH), and GR (PDB ID: 1XAN) were downloaded from PDB database (https://www.rcsb.org/) and prepared using *protein preparation wizard*; later Molecular docking was performed using "GLIDE" Software of Schrödinger suite (Schrödinger, LLC, NY, 2023). The structure of EUG and BCP were retrieved from the PubChem database

https://pubchem.ncbi.nlm.nih.gov) and prepared using the *LigPrep* module, inducing potential ionization at pH 7.0. Protein structures were optimized and minimized, then subjected to grid generation using the receptor grid generation module of the software. The 3D structure of target proteins was retrieved in their complex forms with co-crystallized ligands from the PDB. These inhibitor ligands were used as a native ligand and selected during the receptor grid generation to determine the active site of the protein. All the docking calculations were performed using Glide's XP (Extra precision) mode. The visualization of the docked (protein-ligand complex was performed using Discover studio visualizer, Biovia.

3. Results and Discussion

3.1. Qualitative analysis of *O. sanctum* extracts

3.1.1. pH and percentage yield

The pH of *O. sanctum* HME and AQE was 7.0 and 6.0, respectively (Table 2). The HME and AQE extracts were dried in an incubator at 37 °C for 3 days, yielding 16.07% of HME and 16.61% of AQE (Table 2). These results are consistent with the findings of Mandal et al. (1993).

Table 2
pH and percentage yield of hydromethanolic extract and aqueous extract.

Tests	HME	AQE
pH	7	6
Percentage yield	16.07%	16.61%

HME: Hydromethanolic extract, AQE: Aqueous extract.

3.1.2. FTIR analysis

The functional groups in both HME and AQE extracts, as well as EUG and BCP phytocomponents, were analyzed using FTIR spectroscopy. The results are presented in Fig. 2 (a-d), which show the corresponding FTIR spectra. FTIR analysis revealed the presence of various functional groups in all samples, including alcohols, alkanes, aromatic hydrocarbons, and ethers, but alkynes were absent. The analysis also found that carboxylic acids, amines, amides, and halides were absent in EUG and BCP, while aldehydes, ketones, and esters were absent in HME, EUG, and BCP. AQE showed the presence of all functional groups except alkynes. The differences in spectral shape and absorbance intensity indicated variations in composition and quantity among the samples. The results confirmed the presence of EUG and BCP in both HME and AQE extracts. The wavenumbers and corresponding functional groups are presented in Table 3. The presence of various functional groups suggests the existence of multiple active phytochemicals that may interact with biological systems through multiple biochemical pathways, such as enzymatic inhibition, oxidation reactions, and altered biochemical formation processes (Ullah et al., 2020). These functional groups can interact with biological receptors, enzymes, or cellular structures, influencing the compound's effects on the body. For example, the presence of hydroxyl groups (-OH) is commonly found in phenolic compounds like flavonoids and is associated with antioxidant and anti-inflammatory activities (Gomathi et al., 2014). Previous reports indicate that peaks for *Ocimum sp*. are characteristic of EUG, linalool, and terpenes, which are abundant in these plant extracts (Ramteke et al., 2013).

3.1.3. Phytochemical screening of *O. sanctum* L. leaves extracts (HME and AQE)

The phytochemical analysis was carried out for both HME and AQE of the *O. sanctum* leaves to identify the presence of carbohydrates, protein, amino acids, steroid, triterpenoids, flavonoids, tannin, phenol, saponin, fatty acids, cardiac glycosides, and alkaloids. The preliminary phytochemical analysis and characterization of bioactive compounds from plants is an important step in ascertaining their medicinal value (Sasidharan et al., 2011; Madike et al., 2017). The phytochemical analysis and qualitative estimation of HME and AQE of *O. sanctum* L. leaves showed the presence of carbohydrates, proteins, amino acids, steroids, triterpenoids, flavonoids, tannins, phenol, and fatty acids. Saponin and cardiac glycosides were absent in the extracts (Table 4). Similar results were observed by Devendran and Balasubramanian (2011) during qualitative phytochemical screening and GC-MS analysis of extracts of *O. sanctum* L. leaves. The presence of ROS scavenging phytochemical molecules (*e.g*., flavonoids, alkaloids) indicates that the extracts that contain these phytochemicals can be used as an antioxidant to forage free radicals and can also be used as a potent antioxidative agent to treat various diseases.

3.1.4. Thin layer chromatography (TLC)

The presence of EUG and BCP was confirmed in both HME and AQE extracts using TLC. EUG was detected with a solvent system of toluene: ethyl acetate: acetic acid (10:1:0.32) and an Rf value of 0.49 (Fig. 3a) after derivatization with PMA. BCP was detected with a mobile phase of ethyl acetate: *n*-hexane (7:3) and an Rf value of 0.82 (Fig. 3b) after derivatization with $KMnO_4$. The bioactive components EUG and BCP were present in both extracts, as confirmed by TLC with retention factors of 0.49 and 0.82, respectively. This finding is consistent with previous research by Lalla et al. (2007). Additionally, a study by Anandjiwala et al. (2006) reported the presence of EUG in green and black Tulsi using high-performance liquid chromatography (HPLC).

3.1.5. Quantitative analysis of *O. sanctum* L. leaves extracts (HME and AQE)

The quantitative analysis of phytochemicals of HME and AQE was carried out to determine total phenolic (TPC), flavonoid (TFC), and tannin (TTC) content. Results were obtained using a regression formula generated using gallic acid, quercetin, and tannic acid as standard for phenolic, flavonoid, and tannin content, respectively.

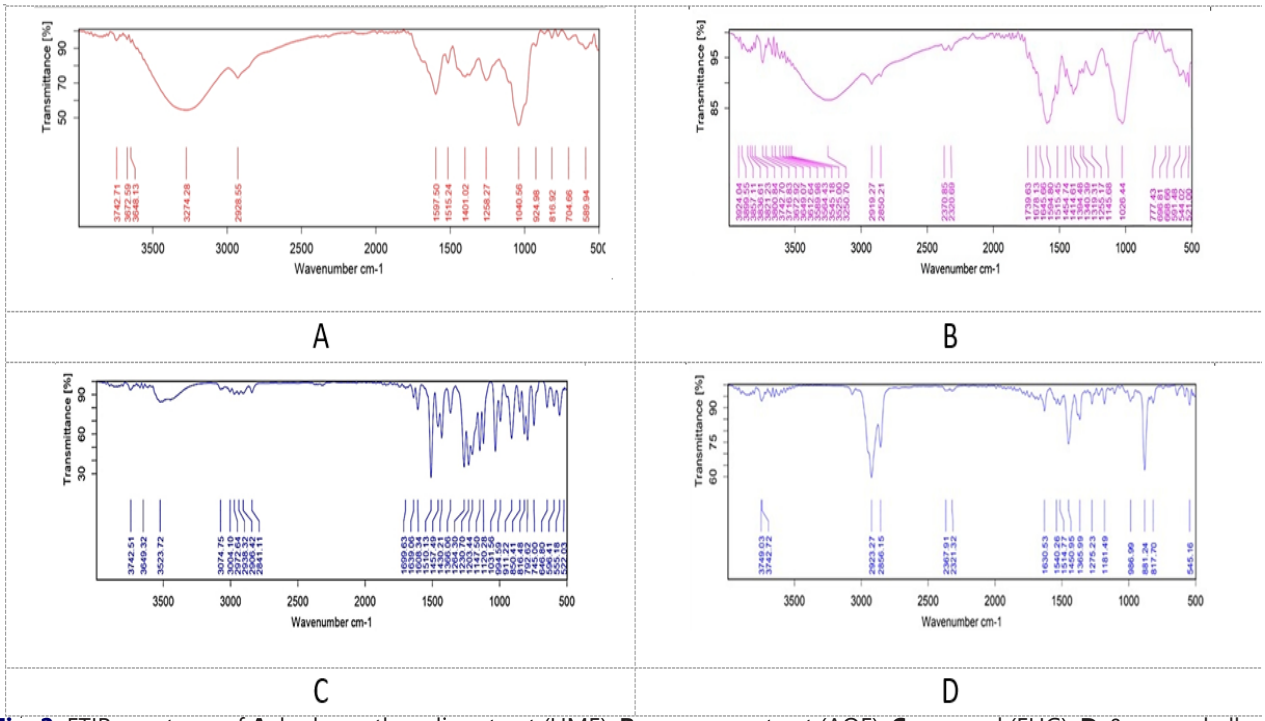


Fig. 2. FTIR spectrum of **A**: hydromethanolic extract (HME), **B**: aqueous extract (AQE), **C**: eugenol (EUG), **D**: β-caryophyllene (BCP).

Fig. no.	3a	3b
Compound	EUG	BCP
Solvent system	Toluene: Ethyl acetate: Acetic acid (10: 1: 0.32)	Ethyl acetate: *n*-hexane (7: 3)
Derivatized with	PMA (propylene glycol monomethyl ether acetate)	$KMnO_4$

When both extracts (1 mg/mL) were compared based on the regression formula generated from the graph for total phenol (Fig. 4a), flavonoid (Fig. 4b), and tannin (Fig. 4c) content, their concentrations were relatively high in hydromethaolic extract compared with aqueous extract (Table 5). It also showed that total phenolic content was relatively higher in both extracts as compared to total flavonoid and tannin content (TPC > TFC > TTC). The quantitative analysis is important due to their potential health benefits as well as for free radical-associated oxidative damages. A previous reports also showed the presence of high phenolic and flavonoid content in *O. sanctum l*eaves (Mondal, 2014). It has also been implied that higher phenolic content is correlated with the relevant antioxidant capacity (Fernandez et al., 2014).

3.1.6. Determination of antioxidant activity of *O. sanctum* L. leaves extracts (HME and AQE)

In this study, four different assays were performed to evaluate the free radical scavenging activity of hydromethanolic (HME) and aqueous (AQE) extracts prepared from *O. sanctum* :
i) DPPH scavenging assay
ii) ABTS scavenging assay
iii) Superoxide anion scavenging assay
iv) Nitric oxide scavenging assay
The results are presented in Fig. 5 and Table 6. Both extracts successfully scavenged free radicals in a dose-dependent manner. Ascorbic acid was used as a standard in all assays. The IC_{50} values were:
i) DPPH: 25.014 ± 0.176 µg/mL for HME and 39.102 ± 0.108 µg/mL for AQE
ii) ABTS: 218.946 ± 3.484 µg/mL for HME (not determined for AQE)
iii) Superoxide anion: 449.8 ± 7.267 µg/mL for HME and 752.31 ± 2.101 µg/mL for AQE
iv)Nitric oxide: 2.480 ± 0.012 µg/mL for HME and 4.478 ± 0.009 µg/mL for AQE
The lower IC_{50} values for HME in scavenging DPPH and NO radicals may be attributed to its higher polyphenolic and flavonoid content compared to AQE (Table 2). Quantitative analysis showed that HME contained more phenols, flavonoids, and tannins than AQE. The higher phenolic content likely contributes to HME's more potent free radical scavenging effects (Pourmorad et al., 2006). This result is consistent with the finding of Gulluce et al. (2007), who reported that methanolic extracts are particularly strong free radical scavengers due to their total phenol and flavonoid content (Lam et al., 2018).

3.1.6. Cytotoxicity study: MTT assay

Dose-dependent cytotoxicity was observed on the

Table 3
Functional groups detected based on FTIR spectra for *Ocimum sanctum* L. leaves extracts (HME and AQE) and phytocomponents (EUG and BCP).

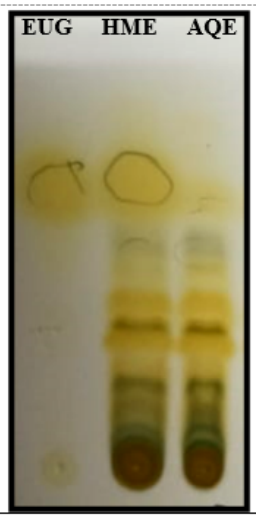
Absorption frequency (cm^{-1})	Characteristic bond	Functional group	HME	AQE	EUG	BCP
3750-3200	O-H stretch	Alcohol	+	+	+	+
1300-1210	C=O stretch					
2960-2850	C-H stretch	Alkanes	+	+	+	+
1300-800	C-C stretch					
3100-3000	C-H stretch	Alkenes	-	+	+	+
1675-1650	C=C stretch					
3500-3300	C-H stretch	Alkynes	-	-	-	-
2150-2100	C≡C stretch					
3050-3000	stretch	Aromatic hydrocarbon	+	+	+	+
1120-1070	R-O-R stretch	Ethers	+	+	+	+
2940-2850	C-H stretch					
1740-1720	CHO stretch	Aldehyde	-	+	-	-
1725-1700	C=O stretch	Ketone	-	+	-	-
1715	COOH stretch	Carboxylic acid	+	+	-	-
1750-1735	C-O-R stretch	Esters	-	+	-	-
3500-3425	N-H stretch	Amines	+	+	-	-
3500-3300	O=C-N-H stretch	Amides	+	+	-	-
500-1000	R-X	Halides	+	+	-	-

Table 4
Phytochemical screening of *Ocimum sanctum* L. leaves extracts (HME and AQE).

No.	Phytocomponents	HME	AQE
1	Carbohydrates	+	+
2	Protein	+	+
3	Amino acids	+	+
4	Steroids	+	+
5	Triterpenoids	+	+
6	Flavonoids	+	+
7	Tannin	+	+
8	Phenol	+	+
9	Saponin	-	-
10	Fatty acids	+	+
11	Cardio glycosides	-	-
12	Alkaloids	+	+

HME: Hydromethanolic extract, AQE: Aqueous extract.

MCF-7 breast cancer cell line after the treatment of various doses of EUG (0.05-0.6 µM) and BCP (0.1 to 1.2 µM) for 24 h in the culture medium (Fig. 6a and Fig. 6b). It was found that the incubation of MCF-7 cells with EUG at a dose of 387.691 ± 5.99 nM resulted in 50% cell viability (Fig. 6a). Whereas 577.856 ± 14.61 nM dose of BCP on MCF-7 showed 50% cell viability (Fig. 6b). Hydromethanolic and aqueous extracts of *O. sactum* leaves at concentrations of 1-20 µg/mL were checked for cytotoxicity on the MCF-7 cell line (Fig. 6c). These results showed that a significant decrease ($p < 0.01$ to $p < 0.001$) in viability was observed for MCF-7 with both



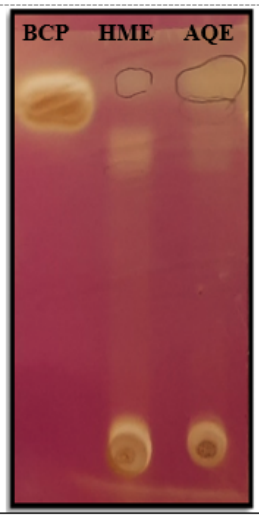


Figure no.	3a	3b
Compound	EUG	BCP
Solvent system	Toluene: Ethyl acetate: Acetic acid (10: 1: 0.32)	Ethyl acetate: *n*-hexane (7: 3)
Derivatized with	PMA (propylene glycol monomethyl ether acetate)	KMnO4

AQE: Aqueous extract, BCP: β Caryophyllene, EUG: Eugenol, HME: Hydromethanolic extract.

Fig. 3. Thin layer chromatography of **a:** Eugenol and **b:** β-Caryophyllene.

the extracts in a dose-dependent manner (5 to 20 µg/mL) as compared with the control group. On MCF-7 cell line, the IC_{50} value of hydromethanolic and aqueous extracts were found to be 23.137 ± 0.965 and 24.963 ± 0.00 µg/mL, respectively, EUG, BCP and both extracts were highly cytotoxic to MCF-7 cells as compared to AQE, the HME showed cell inhibition more effectively and hence, it may have good anticancer property. Vidhya and Devaraj (2011) showed that cells treated with a higher concentration of EUG (1, 2, and 4 µM) exhibited a significant decrease in the number of viable cells. The best cytotoxic activity against MCF-7 cells was obtained for the methanolic extract of *O. sanctum* L. leaves (IC_{50} = 31 mg/mL) (Basak et al., 2014). Moreover, it was observed that HME has a rich source of antioxidant compounds which could be an essential protagonist against excess generation of free radicals which contributes to apoptotic cell death and hence, leads to the death of cancer cells.

3.1.6. Molecular docking study

Molecular docking analysis was used to determine the binding affinity of EUG and BCP with two different enzymatic antioxidants (Fig. 7; Table 7). High binding energy showed good binding affinity between the two molecules. Virtual screening for EUG showed the interaction in higher to lower order as catalase (-3.659 kcal/mol) > glutathione reductase (-4.38 kcal/mol). Whereas BCP showed a relatively high binding affinity for catalase (-2.271 kcal/mol) (Table 7). From both the studied compounds (EUG and BCP), the difference of binding energy between positive inhibitor and EUG was lowest for CAT and GR. An *In silico* study revealed that EUG and BCP showed relatively higher binding affinities for catalase than their binding affinities for GR. Catalase plays an important role in maintaining endogenous ROS production and it shows antioxidant and pro-oxidant activities in response to DNA damage (Kang et al., 2013). The binding of EUG with catalase suggests that it could be enhancing its antioxidant activity. These binding affinities and docking scores suggest that both compounds have strong antioxidant activity. With the possession of free radical scavenging activity, if plant-derived bioactives are non-toxic to normal cell lines and show cytotoxicity in cancer cell lines, such compounds can be advanced to clinical trials for further therapeutic development (Greenwell and Rahman, 2015).

4. Concluding remarks

This study provides insights into the bioactivities of *O. sanctum* L. leaves extracts (HME and AQE) and their bioactive components, EUG and BCP. Medicinal plants have been used for centuries to treat various illnesses worldwide. Phytochemical screening of *O. sanctum* L. leaves extracts revealed a rich presence of major phytochemicals, including phenols, flavonoids, tannins, terpenoids, and alkaloids. Our findings suggest that the hydromethanolic extract could be a valuable source of natural antioxidants with therapeutic potential, surpassing the aqueous extract. TLC results confirmed the presence of EUG, a phenolic compound, and BCP, a plant-derived sesquiterpene,

in both HME and AQE extracts. Notably, EUG, BCP, and both extracts showed strong cytotoxic properties against human breast cancer cells (MCF-7). Molecular docking studies revealed high binding efficacy of EUG and BCP with enzymatic antioxidants. This study contributes to the understanding of *O. sanctum* L. leaves' medicinal properties, anticancer activity, and interaction with antioxidant enzymes, potentially leading to the development of novel therapeutic interventions and natural antioxidant agents. However, further investigation is needed to prove the synergistic effects of EUG, BCP, and both extracts when used incombination with chemotherapeutic drugs, including *in vitro* free radical toxicity testing and *in vivo* experiments.

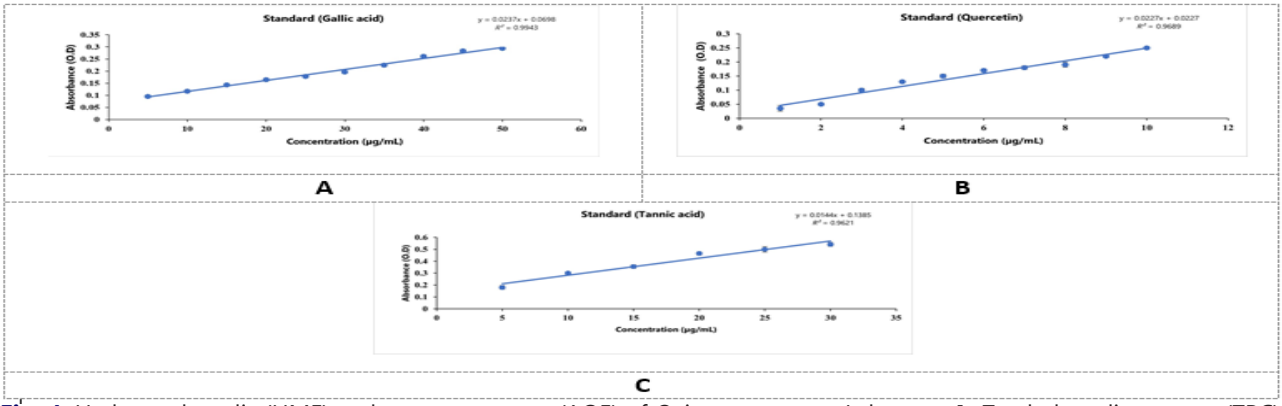


Fig. 4. Hydromethanolic (HME) and aqueous extract (AQE) of *Ocimum sanctum* L. leaves: **A:** Total phenolic content (TPC), **B:** Total flavonoid content (TFC), **C:** Total tannin content (Values are expressed as mean ± SD).

Table 5
Total phenolic, flavonoid and tannin content in in hydromethanolic (HME) and aqueous extract (AQE) of *Ocimum sanctum* L. leaves.

Extracts of Ocimum sanctum L. leaves	TPC (µg GAE/ mg extract)	TFC (µg QE/ mg extract)	TTC (µg TAE/ mg extract)
HME	176.24	145.82	4.84
AQE	103.68	72.34	2.28

AQE: Aqueous extract; GAE: Gallic acid equivalent; HME: Hydromethanolic extract; TAE: Tannic acid equivalent; TFC: Total flavonoid content; TPC: Total phenolic content; TTC: Total tannin content; QE: Quercetin equivalent.

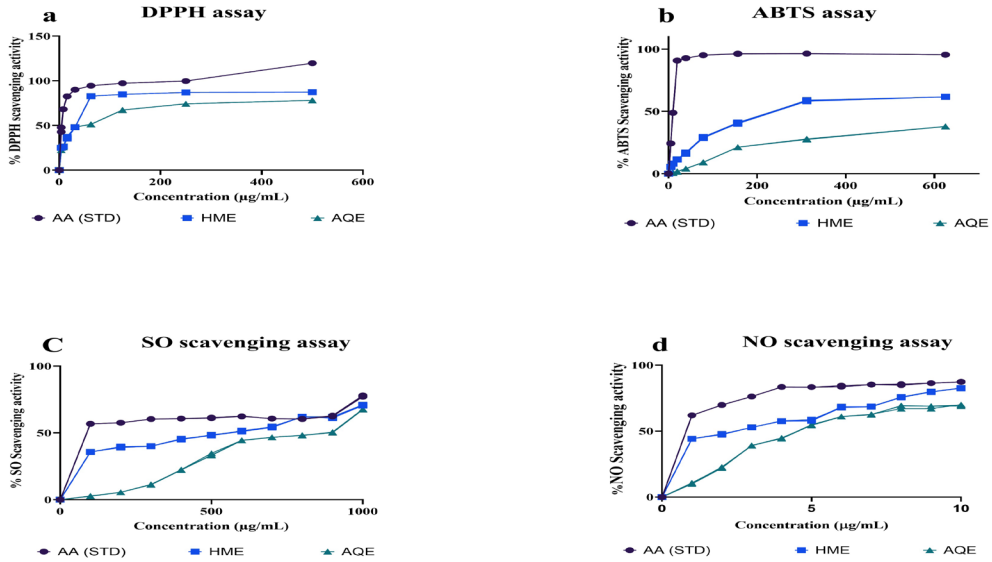


Fig. 5. Scavenging of free radicals at various concentrations of ascorbic acid (AA), hydromethanolic extract (HME) and aqueous extract (AQE); Values are expressed as mean ± SD. **a:** DPPH free radical scavenging activity; **b:** ABTS free radical scavenging activity; **c:** Superoxide (SO) free radical scavenging activity; **d:** Nitricoxide (NO) free radical scavenging activity.

Table 6
IC_{50} values of AA (STD), HME and AQE for DPPH, ABTS, SO and NO free radical scavenging activity.

Test compounds	IC_{50} in DPPH free radical scavenging activity (µg/mL)	IC_{50} in ABTS free radical scavenging activity (µg/mL)	IC_{50} in Superoxide (SO) free radical scavenging activity (µg/mL)	IC_{50} in Nitric oxide (NO) free radical scavenging activity (µg/mL)
AA (STD)	8.193 ± 0.287	13.764 ± 0.003	176.50 ± 0.096	1.337 ± 0.002
HME	25.014 ± 0.176	218.946 ± 3.484	449.8 ± 7.267	2.480 ± 0.012
AQE	39.102 ± 0.108	-	752.31 ± 2.101	4.478 ± 0.009

Values are expressed as mean ± SD; AA (STD): Ascorbic acid (Standard); HME: Hydromethanolic extract; AQE: Aqueous extract; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; ABTS: (2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).

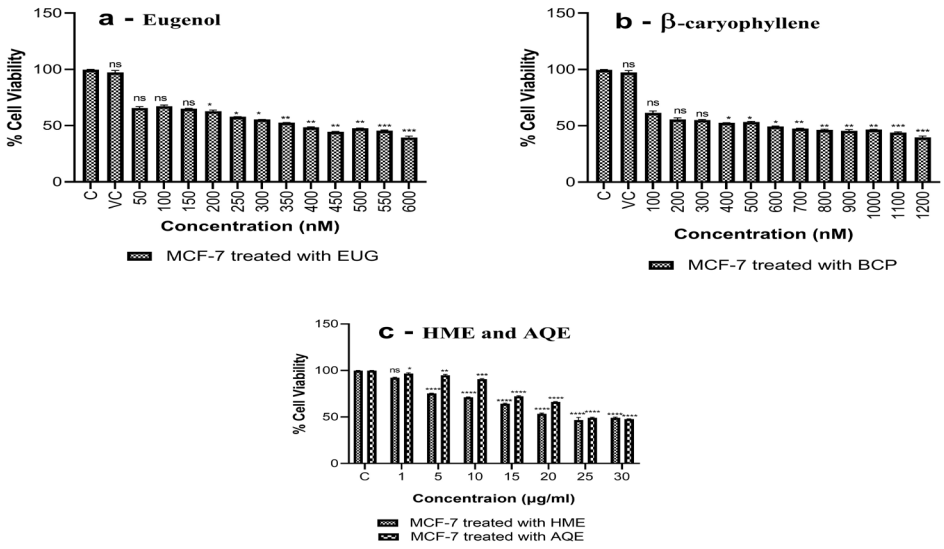


Fig. 6. Cytotoxicity study by MTT assay of **a:** Eugenol at different concentrations (50 nM to 600 nM) **b:** β-caryophyllene at different concentrations (100 nM to 1200 nM) **c:** HME and AQE at different concentrations (1 µg/mL to 20 µg/mL) on the MCF-7 cell line. CON: Control, VC: Vehicle Control (DMSO). Values are expressed as mean ± SD; * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, and ns: Non-significant when the different concentrations of eugenol and vehicle control compared with the control group.

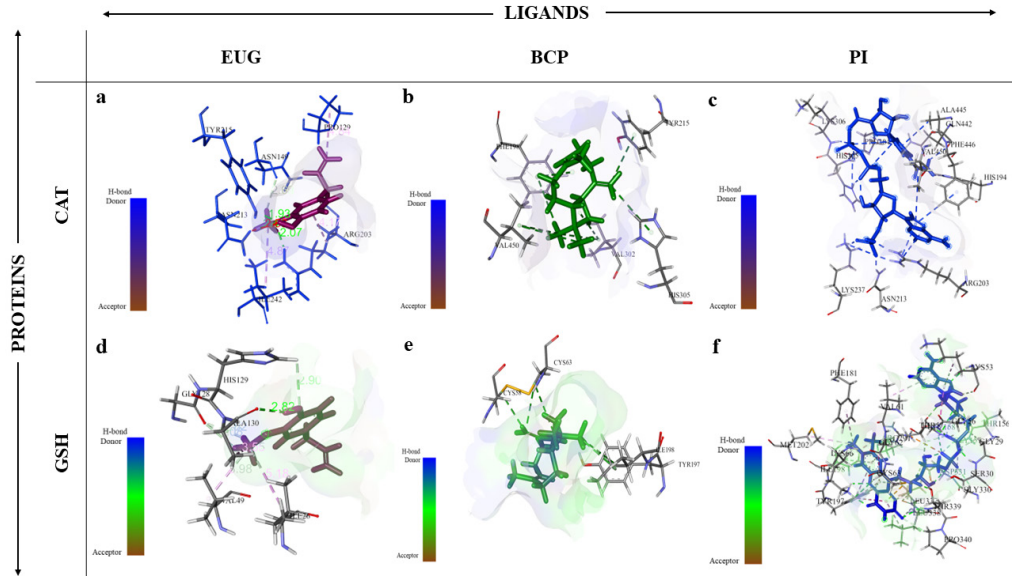


Fig. 7. 3D Docked protein- ligand interaction pose based on hydrogen bond acceptor (HBA) and donor (HBD) of Catalase (CAT) protein with **a:** Eugenol (EUG), **b:** β-Caryophyllene (BCP), and **c:** NADPH (PI-Positive Inhibitor); and Glutathione reductase (GR) proteins with **d:** Eugenol (EUG), **e:** β-Caryophyllene (BCP), and **f:** FAD (PI-Positive Inhibitor).

Table 7
Binding energy and contacting receptor residues in docking study of eugenol and β-caryophyllene with antioxidant enzymes.

Sr no.	Macromolecule	Code	Method	Resolution	Binding energy (kcal/mol)		
					EUG	BCP	PI
1	Catalase (CAT)	1DGH	X-RAY DIFFRACTION	2.00 Å	-3.659	-2.271	-7.791
2	Glutathione reductase (GR)	1XAN	X-RAY DIFFRACTION	2.00 Å	-4.38	-2.509	-9.322

BCP: β-Caryophyllene, EUG: Eugenol, PI: Positive Inhibitor (NADPH for CAT; FAD for GR).

List of abbreviations

ABTS: 2,2-Azino-Bis (3-Ethylbenzothiazoline-6-Sulfonic Acid); **AQE:** Aqueous Extract; **BCP:** β-Caryophyllene; **CAT:** Catalase; **DPPH:** 2,2-Diphenyl-1-Picrylhydrazyl; **EUG:** Eugenol; **FTIR:** Fourier Transform Infrared Spectroscopy; **GR:** Glutathione Reductase; **HME:** Hydromethanolic Extract; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; **TFC:** Total Flavonoid Content; **TPC:** Total Phenolic Content; **TTC:** Total Tannin Content.

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Author contribution statement

Devendrasinh Jhala: Conceptualization, methodology, experimental studies, formal analysis, investigation, resources, manuscript review and editing, funding acquisition; Krupali Trivedi: Conceptualization, software, methodology, experimental studies, data curation, data analysis, writing-original draft preparation, manuscript review and editing, funding acquisition; Nilam Parmar, Khairah Ansari, Nishi Modi and Vaibhavi Srivastava: Experimental studies, formal analysis, manuscript review.

Conflict of interest

The authors declare that there is no conflict of interest.

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