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# Inhibition of NF-κB signalling pathway by methanol extract of fruit pericarp of *Garcinia gummi-gutta* in inflammatory breast cancer with triple negative phenotype

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In humans, triple negative breast cancer (TNBC) is a highly truculent form of mammary gland cancer that connexions clinical, pathological and molecular patterns of disease with canine inflammatory mammary carcinoma (IMC). Inflammatory breast cancer with TNBC phenotype has shoddier prognosis and mediocre outcome when juxtaposed with non-inflammatory breast cancer (IBC) with TNBC phenotype. This demands the need for effective therapeutics and targets for its treatment which is currently lacking. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a major regulator of inflammation is a potential therapeutic target in TNBC. The present study was instigated to appraise the anticancer properties of methanol extract of *Garcinia gummi-gutta* (MGG) in MDA-MB-231, a triple negative breast carcinoma cell line. The extract was gaged for its cytotoxic property in tumour necrotic factor- alpha (TNF-α) stimulated MDA-MB-231 cell line, where we obtained a half maximal inhibitory concentration ($IC_{50}$) value of 50.3 µg/mL. On acridine orange/ ethidium bromide staining, MDA-MB-231 cells treated with the extract unveiled marked morphological and nuclear alterations which were characteristics of apoptosis. In the gene expression study, NF-κB expression was down-regulated in the MGG treated TNF-α stimulated MDA-MB-231 cell line. In conclusion, our present study divulged that the MGG exhibited a concentration dependent cytotoxic activity in TNF-α stimulated MDA-MB-231 breast carcinoma cell lines. Furthermore, our study revealed that the extract was effective in inhibiting the NF-κB signalling pathway which makes it a pledging source for isolating therapeutic molecules for the treatment of inflammatory breast cancer with TNBC phenotype.

**Keywords**: Malabar tamarind, MDA-MB-231, Nuclear factor kappa-light-chain-enhancer of activated B cells

Triple negative breast cancer in humans is the most pugnacious form of mammary gland cancer characterised by brisk progression, local and outlying metastasis, early age of onset and lower overall survival juxtaposed with other breast cancers[1]. It has been reported that 15 to 25 percent of the breast cancer cases diagnosed to be of triple negative breast cancer [2]. Dearth of specific therapeutic molecular targets has been compounded as an obstacle in the treatment of TNBC. Inflammatory breast cancer (IBC) is reckoned to account for one to five percent of breast cancer annually[3]. Despite its low incidence, IBC interposes to 10 percent of mortality caused by breast cancer[4]. Inflammatory breast cancer with TNBC phenotype has poorer prognosis and inferior outcome when compared with non-IBC with TNBC phenotype. It has been reported that the prevalence of IBC with TNBC phenotype is 25 to 30 percent[5] whereas in non-IBC with TNBC phenotype, 15 to 20 percent prevalence have been observed[6]. Thus, inflammatory infiltrates play a role in the clinical outcome of TNBC. Nuclear factor kappa light chain enhancer of activated B cells has emerged as a major regulator of inflammation, cellular transformation, tumour cell survival, proliferation, invasion, angiogenesis and metastasis[7]. Evidences reveal that the digressive activation of NF-κB signalling as one of the characteristic features of TNBC[8]. Anti-apoptotic protein, Bcl-2 and cysteine aspartic acid protease-8 (caspase 8) regulation by NF-κB differentially affected mitochondrial function and apoptosis in breast cancer cells[9]. Consequently, agents that can inhibit NF-κB activation pathway could provide a therapeutic molecular target for the treatment of IBC with TNBC phenotype.

Herbs remain a mainstay in the development of anticancer drugs. They have also been used as an adjunctive or supportive therapy along with conventional anticancer drugs to reduce the adverse

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effects[10]. Since most of the drugs are inefficient against triple negative breast cancer due to the lack of receptors of human epidermal growth factor receptor 2 (HER 2), oestrogen and progesterone, targeting NF-κB might be useful in the development of anticancer drugs for TNBC[11]. *Garcinia gummi-gutta* previously known as *G. cambogia* is a tropical species of *Garcinia*. It is commonly known as brindle berry, Malabar tamarind and kudam puli. *G. gummi-gutta* is traditionally used for the treatment of oedema, fever, delayed menstruation, rheumatism, ulcers, hemorrhoids, open sores, and also against intestinal parasites[12].

The fruit pericarp of *G. gummi-gutta* has been reported to have anticancer properties in CaCo2 and HeLa carcinoma cells[13]. Garcinol obtained from other species of *Garcinia viz*., *G. indica* induced caspase mediated apoptosis through downregulation of NF-κB signalling in MDA-MB-231 cells[14]. Apart from its anticancer properties, hypolipidaemic[15], antiulcer[16], anti-inflammatory, antioxidant and antimicrobial[17] and hepatoprotective activities[18] of *G. gummi-gutta* were reported. *G. gummi-gutta* was commercially vital as their fruit extracts were used for numerous treatments such as rheumatism, astringent, purgative, bowel complaints and demulcent[19]. This article deliberates the anticancer activity of methanol extract of fruit pericarp of *G. gummi-gutta* and assessment of mode of induction of apoptosis by the extract targeting NF-κB signalling pathway in MDA-MB-231 breast cancer cell line.

## Materials and Methods

### Plant materials

The fruit of *Garcinia gummi-gutta* was collected from Irinjalakuda, Thrissur, Kerala in the month of May. The pericarp of fruit was shade dried and it was used for the study. The pericarp of *G. gummi-gutta* was authenticated by Raw Material Herbarium and Museum Department (RHMD), National Institute of Science Communication and Policy Research (NIScPR) (erstwhile CSIR-NISCAIR), New Delhi. Herbarium for *G. gummi-gutta* was prepared and the voucher specimen HERB/VPT/CVASMTY/2/2020 was deposited in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala.

### Drugs/chemicals

Acridine orange, agarose-low melting, Dulbecco's phosphate buffered saline, 0.25 % trypsin-EDTA, thiazolyl blue tetrazolium diphenyl-tetrazolium bromide (MTT), ethylenediaminetetraacetic acid (EDTA), ammonium persulphate, gentamicin sulphate, doxorubicin hydrochloride, dimethyl sulphoxide (DMSO), antibiotic antimycotic solution (100x), Roswell Park Memorial Institute (RPMI) 1640 and foetal bovine serum (FBS) were procured from M/s Himedia laboratories Pvt Ltd Mumbai, India.

### Cell lines

Authenticated adherent human breast adenocarcinoma cell line, MDA-MB- 231(1566/2019-20) with passage number P48, procured from National Centre for Cell Science (NCCS), Pune, Maharashtra was used for *in vitro* anticancer studies. The MDA-MB-231 cell line was oestrogen receptor (ER) -ve, progesterone receptor (PR) -ve and human epidermal growth factor receptor (HER) -ve. The cells were cultured in RPMI-1640 supplemented with 10 percent foetal bovine serum, 1 percent antibiotic antimycotic solution containing penicillin-streptomycin and amphotericin B. The cells were maintained in a humidified incubator at 37°C with five percent carbon dioxide ($CO_2$). Cell lines were subcultured by enzymatic digestion with one percent trypsin/1mM EDTA solution when they reached approximately 70 to 80 percent confluency and these trypsinized cells were used for the further studies.

### Preparation of methanol extract of pericarp of *G. gummi-gutta* (MGG)

The pericarp of *G. gummi-gutta* was dried at room temperature (37°C) and coarsely powdered using an electric pulveriser. The powder obtained was extracted using a Soxhlet apparatus with methanol at 67°C. The methanol extract was then concentrated using a rotary vacuum evaporator under reduced pressure and temperature (40°C). The yield of the extract was calculated and the prepared extract was kept under refrigeration in an airtight container after complete evaporation of the solvent until further use.

### *In vitro* anticancer activity of methanol extracts of pericarp of *Garcinia gummi-gutta*

#### *Sample preparation*

The MGG was solubilised in DMSO at a concentration of 1 mg/mL; this stock solution was further diluted with RPMI 1640 media to required concentrations.

#### *Cytotoxicity studies: 3-(4,5- dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay*

*Standardisation of model of inflammatory breast cancer with TNBC phenotype*

The concentration of TNF-α that stimulated inflammation without affecting cell viability of MDA-

MB-231 cell was determined using MTT assay[20]. The concentrations selected for the study were 10, 20, 40, 80 ng/mL[21]. The selected concentrations were added to $5x10^3$ MDA-MB-231 cells per well in 200 µL medium and were allowed to attach overnight in a $CO_2$ incubator. The media was removed and 20 µL of MTT (5 mg/mL) in 150 µL medium was added and incubated at 37°C for 4 h. Then the media with MTT was removed and the formed purple formazan crystals were dissolved in 200 µL of DMSO and read at 570 nm in an ELISA plate reader (Varioskan Flash, Thermo Fischer Scientific, Finland).

The percent cell viability was calculated using the formula:

Percent cell viability = (Average absorbance of treated cells /Average absorbance of untreated cells) × 100

*Cytotoxicity studies of methanol extracts of pericarp of G. gummi-gutta*

Cytotoxic evaluation of MGG was assessed using MTT assay in MDA-MB-231 human breast carcinoma cells[20]. A pilot study was conducted to select the concentration at which cytotoxicity occurred. The concentrations used for pilot study were 5, 10, 20, 40, 80, 160, 320 and 640 µg/mL. The results of the pilot study proved MGG to be cytotoxic. Hence the same concentrations were used for the main study. The MDA-MB-231 breast cancer cells was seeded at a density of $5 \times 10^3$ cells per well in 200 µL medium in a 96 well plate and were allowed to attach for overnight in a $CO_2$ incubator. Cells were first treated with 10 ng/mL concentration of TNF-α for 24 h. Then the cells were treated with MGG separately at concentrations of 5, 10, 20, 40, 80, 160, 320 and 640 µg/mL for a period of 24 h. After treatment with plant extracts, the media was removed and 20 µL of MTT (5 mg/mL) in 150 µL medium was added and incubated at 37°C for 4 h. Then the media with MTT was removed and the formed purple formazan crystals were dissolved in 200 µL of DMSO and read at 570 nm in an ELISA plate reader (Varioskan Flash, Thermo Fischer Scientific, Finland).

The percentage of cell viability and percentage of cell inhibition were calculated using the following formula:

Percent cell viability = (Average absorbance of treated cells /Average absorbance of untreated cells) × 100 Percent cell inhibition = 100 - percent cell viability

The net absorbance from the control wells was taken as 100 percent viable. The $IC_{50}$ values of extracts were calculated using Graphpad prism version 5.

**Apoptotic changes**

***Acridine orange/ethidium bromide (AO/EB) staining***

Trypsinized MDA-MB-231 cells at a concentration of $1 \times 10^5$ cells were seeded into a six-well plate and allowed to grow for 24 h. The attached cells were first treated with 10 ng/mL of TNF-α for 24 h. The TNF-α stimulated cells were then treated with $IC_{50}$ concentrations of the plant extract and 0.58 µg/mL of standard drug, doxorubicin. After discarding the media from the wells of treated and control cells, the cells were trypsinized and stained with five microlitres of acridine orange (10µg/mL) and ethidium bromide (10µg/mL) and analysed under Trinocular Research fluorescence Microscope (Axio Vert. A1 FL-LED, Carl Zeiss) with blue excitation (488 nm) and emission (550 nm) filters at 20X and 40X magnification[22].

**Nuclear factor kappa light chain enhancer of activated B cells gene expression study**

Real time – quantitative polymerase chain reaction (RT-qPCR) was employed for studying the gene expression of NF-κB gene in cell culture samples. Total RNA was isolated using TRI Reagent® as per the manufacturer's protocol (Sigma Aldrich, USA). Total RNA was isolated from TNF-α stimulated MDA-MB-231 cells with $IC_{50}$ of MGG, control cells without treatment and doxorubicin treated cells. Complementary DNA synthesis (cDNA) was carried out from total RNA using verso cDNA synthesis kit (Thermo scientific, USA) as per manufacturer's protocol. Gene specific primers for *NF-κB1* were designed using online "Primer three" primer design software (Primer three, http://bioinfo.ut.ee/primer3/) and the reaction mixture was then subjected to PCR for amplication of *NF-κB1* (Forward: 5′-GTGGTGCCTCACTGCTAACT-3′, Reverse: 5′-GGATGCACTTCAGCTTCTGT-3′). Co-amplication of the house keeping gene GAPDH (Forward: GTGTCCTCTGACTTCAACAGCG, Reverse: ACCA-CCCTGTTGCTGTAGCCAA) as internal control was carried out simultaneously. The expressions of *NF-κB1* and housekeeping gene GAPDH was studied using SYBR green chemistry (Maxima SYBR green qPCR master mix (Thermo scientific, USA)[23]. The quantitative real time PCR was carried out in a final volume of 6 µL containing 1 µL of template cDNA, Maxima SYBR green qPCR mastermix (3 µL) and 10 pM/ µL of each

primer. In negative control, template cDNA was replaced by nuclease free water. The reaction was performed in triplicates. The cycling conditions were 5 min for initial incubation at 95°C and 40 cycles of amplification cycle with denaturation at 95°C for 15 s, annealing at 52°C for both *NF-κB1* and GAPDH for 1 min and extension at 72°C for 20 s. The relative change in expression of *NF-κB1* gene was evaluated by comparative CT (Cycle threshold) method and was quantified as 'n' fold change up/ downregulation of the transcripted gene in relation to untreated control group.

**Qualitative phytochemical analysis**

The extracts were tested for the presence of bioactive compounds using methods described as given in (Table 1)[24].

**Gas chromatography-mass spectrometry (GC-MS) analysis of methanol extract of pericarp of *Garcinia gummi-gutta***

The active phytochemical principles of MGG were analysed using GC-MS system of Centre for Analytical Instrumentation- Kerala (CAI-K), Kerala Forest Research Institute (KFRI), Peechi, Kerala. Gas chromatography Mass Spectrometer (Shimadzu GC-MS, Japan, QP2010S) with a mass range of 1.5-1000 *m/z* was used. The carrier gas was helium used at flow rate of 1 mL/ min. Oven temperature was sustained at 80°C for 4 min and then amplified to 280°C in 6 min. The injector temperature was 260°C and total analysis time was 50 min. Aliquots of extracts (0.4 µL) were injected into the chromatographic column after a clear baseline was obtained[25]. Major constituents were identified by using mass spectrum library (NIST 11 and WILEY 8).

Table 1 — Qualitative tests for phytochemical analysis

| Tests | Procedure | Expected Inference |
|---|---|---|
| *Tests for detection of steroids*<br>*Salkowski's test* | Fifty milligrams of the extract were dissolved in 3 mL of chloroform. Few drops of concentrated sulphuric acid were added and the solution was allowed to stand. | Formation of red colour directed the presence of steroids |
| *Liebermann Burchardt test* | Fifty milligrams of the extract were mixed with 3 mL of chloroform. To this, five drops of acetic anhydride and 1 mL concentrated sulphuric acid was added along the sides of the test tube. | Development of a reddish ring at the junction of two layers confirmed the presence of steroids |
| *Tests for detection of alkaloids* | One gram of the extract was mixed with 5 mL of ammonia and then extracted with an equal volume of chloroform. To this extract, 5 mL of dilute hydrochloric acid was added. The acid layer obtained was further tested with the following reagents for the presence of alkaloids. | |
| *Dragendorff's test* | Eight drops of Dragendorff's reagent were mixed with 1 mL of acid extract. | Development of a reddish brown precipitate indicated the presence of alkaloids |
| *Mayer's test* | To 1 mL of the acid layer, eight drops of Mayer's reagent were added. | Development of a cream coloured precipitate indicated the presence of alkaloids |
| *Wagner's test* | One millilitre of Wagner's reagent was added to 1 mL of the extract. | Development of reddish brown colour precipitate indicated the presence of alkaloids |
| *Hager's test* | To 1 mL of the acid extract, eight drops of Hager's reagent were mixed. | Formation of yellow precipitate specified the presence of alkaloids |

(*Contd*.)

Table 1 — Qualitative tests for phytochemical analysis (*Contd*.)

| Tests | Procedure | Expected Inference |
|---|---|---|
| *Tests for detection of glycosides*<br>*Sodium hydroxide test* | Approximately 50 mg of the extract was mixed with 1 mL water and six drops of 10 percent sodium hydroxide solution was added to it. | Development of yellow colour indicated the presence of glycosides. |
| *Benedict's test* | Approximately 50 mg of the extract was mixed with 1 mL of water and then 5 mL of Benedict's reagent was added to it. | Formation of brown or red precipitate indicated the presence of reducing sugars. |
| *Test for detection of phenolic compounds*<br>*Ferric Chloride Test* | Five milligrams of the extract was dissolved in 1 mL of water and five drops of 10 percent ferric chloride were added to it. | Development of bluish black colour specified the presence of phenols. |
| *Tests for detection of tannins*<br>*Ferric chloride test* | Treated two milligrams of the extract with 3 mL of one percent ferric chloride solution. | Development of a blue-black or a brownish green colouration showed the presence of tannins |
| *Gelatin test* | One gram of the extract was mixed with a few drops of one percent solution of gelatin containing 10 percent sodium chloride. | Development of a white precipitate indicated the presence of tannins |
| *Tests for detection of flavonoids*<br>*Ferric chloride test* | Treated 2 mL of the methanol extract (0.5 gram extract in 10 mL methanol) with four drops of neutral ferric chloride solution. | Formation of green colour indicated the presence of flavonoids |
| *Lead acetate test* | Treated 2 mL of the alcohol extract (0.5 gram extract in 10 mL methanol) with 3 mL of 10 percent lead acetate solution. | Development of yellow precipitate indicated the presence of flavonoids |
| *Tests for detection of triterpenes*<br>*Salkowski test* | Mixed 3 mL of chloroform to three milligrams of extract and it was shaken with 3 mL concentrated sulphuric acid. | Development of yellow colour in the lower layer on standing indicated the presence of triterpenes |
| *Tests for detection of saponins*<br>*Froth test* | Approximately 200 mg of the extract was shaken with 5 mL of water. | Continuation of foam produced for ten minutes designated the occurrence of saponins |

**_In silico_ screening of phytoconstituents of methanol extract of pericarp of _Garcinia gummi-gutta_**

The compounds identified by GC-MS of MGG were subjected to *in silico* analysis. 1H-pyrazole-3-carboxylic acid, 2-[(2-aminoethyl)amino]-ethanol, 2-hydr-oxypropane-1,2,3-tricarboxylic acid, dimethyl ester, 2-methyl-hexanoic acid, 5-hydroxymethylfurfural, 5-methoxy-2,4-dimethyl-furan-3-one, cis-non-3-enyl propyl ester of fumaric acid, dimethyl 2-butenedioate, dimethyl dl-malate, dimethyl mesaconate, methyl ester of hexadecanoic acid, monomethyl ester of butanedioic acid, propanoic acid and trimethyl ester of citric acid were used as ligands for docking studies. Chemical structure of ligands (SDF files) was obtained from the PubChem Compound Database and MarvinView 17.25.0 (www.chemaxon.com) were used for modifying the ligands (SDF files to .mol2 format). The ligands were analysed for their lipophilicity and violation of Lipinski rule of five using the SwissADME online web tool. The antineoplastic activity of the ligands was predicted using PASS (Prediction of activity spectra for substances) online software. The ligand(s) which does not violate the Lipinski rule of five was further selected, and the SDF file was converted to PDB format using Open Babel.

Important proteins such as NF-κB, Bcl-2 and Caspase-8 which are involved in inflammation and apoptosis of inflammatory breast cancer with TNBC phenotype were used as the receptors for *in silico* docking. The crystal structure of receptors such as NF-κB (PDB ID: 1SVC), Bcl-2 (PDB ID: 6QGG) and Caspase-8 (PDB ID: 3KJQ) was downloaded from the RCSB protein data bank (http://www.rcsb.org). Using auto-dock tools (version 4.2.6), the receptor and selected ligand PDB files were converted to PDBQT files. Grid box was set on the macromolecules and using the grid parameters, a conf.txt file was prepared. Binding affinity was calculated using VINA (1_1_2_win32.msi) and the results were interpreted using Discovery Studio Visualizer version 16.1.0.15350[26].

**Statistical analysis**

All results were expressed as Mean ± SE with 'n' equal to the number of replicates. The results of standardisation of TNF-α and percent cell inhibition were analysed using one way analysis of variance (ANOVA) followed by Tukey's as post hoc test. The $IC_{50}$ values of the extract was calculated using the Graphpad Prism Version 5. The results of gene expression study were analysed using one sample t test.

## Results

**Preparation of methanol extract of pericarp of *Garcinia gummi-gutta* (MGG)**

The yield obtained from methanol extract of MGG was 6.623 percent with reference to starting dry material.

***In vitro* anticancer activity of methanol extract of pericarp of *Garcinia gummi-gutta***

***Cytotoxicity studies: 3-(4,5- dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay***

*Standardisation of model of inflammatory breast cancer with TNBC phenotype*

The results of concentration of TNF-α that stimulated inflammation without affecting cell viability of MDA-MB-231 cells were presented in (Fig. 1). The results revealed that the TNF-α concentration of 10 ng/mL and 40 ng/mL showed more than 100 percent cell viability. The low concentration of TNF-α (10 ng/mL) that showed 100 percent cell viability was selected for further studies.

The percent cell inhibition as studied using MTT assay post 24 h of treatment with MGG in MDA-MB-231 cell line pretreated with 10 ng/mL TNF-α is presented in (Table 2). The concentrations which showed cytotoxicity in the pilot study was taken for the main study. The concentrations selected were 5, 10, 20, 40, 80, 160, 320 and 640 µg/mL. The results of the study revealed that MGG showed concentration dependent cytotoxicity.

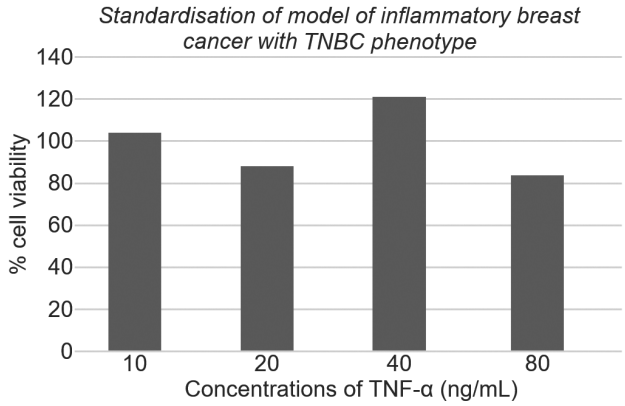


Fig 1 — Standardisation of concentration of TNF-α in MDA-MB-231 cells using MTT assay – 96 well plate incubated with 10, 20, 40 and 80 ng/mL of TNF-α

Table 2 — The percent cell viability of MDA-MB-231 cells after 24 h treatment with MGG

| Concentrations (µg/mL) | % cell inhibition |
|---|---|
| 5 | 2.08±0.342[a] |
| 10 | 5.8±1.107[a] |
| 20 | 8.39±1.415[ab] |
| 40 | 16.77±2.737[ab] |
| 80 | 42.31±4.233[c] |
| 160 | 49.37±2.818[cd] |
| 320 | 50.13±2.811[cd] |
| 640 | 51.07±2.739[cd] |
| IC50 (µg/mL) | 50.03±0.019 |

$IC_{50}$- Half maximal inhibitory concentration

Values are expressed as Mean±SE (n=6). Means bearing the different superscript (a-e) vary significantly at $P< 0.05$

**Apoptotic changes**

***Acridine orange/ethidium bromide (AO/EB) staining***

The results of apoptotic stages analysed by AO/EB staining upon treatment of cells with MGG and doxorubicin pretreated with TNF-α are depicted in the (Fig. 2). Control cells emitted uniform green fluorescence with circular nucleus in the centre whereas MGG treated cells at $IC_{50}$ concentration of 50.03±0.019 µg/mL showed late apoptosis featured by nuclear fragmentation and apoptotic bodies. The doxorubicin treated cells at concentration of 0.58 µg/mL exhibited late apoptosis characterised by cytoplasmic vacuolation emitting orange to red fluorescence. The results of the present study indicated that MGG induced apoptosis.

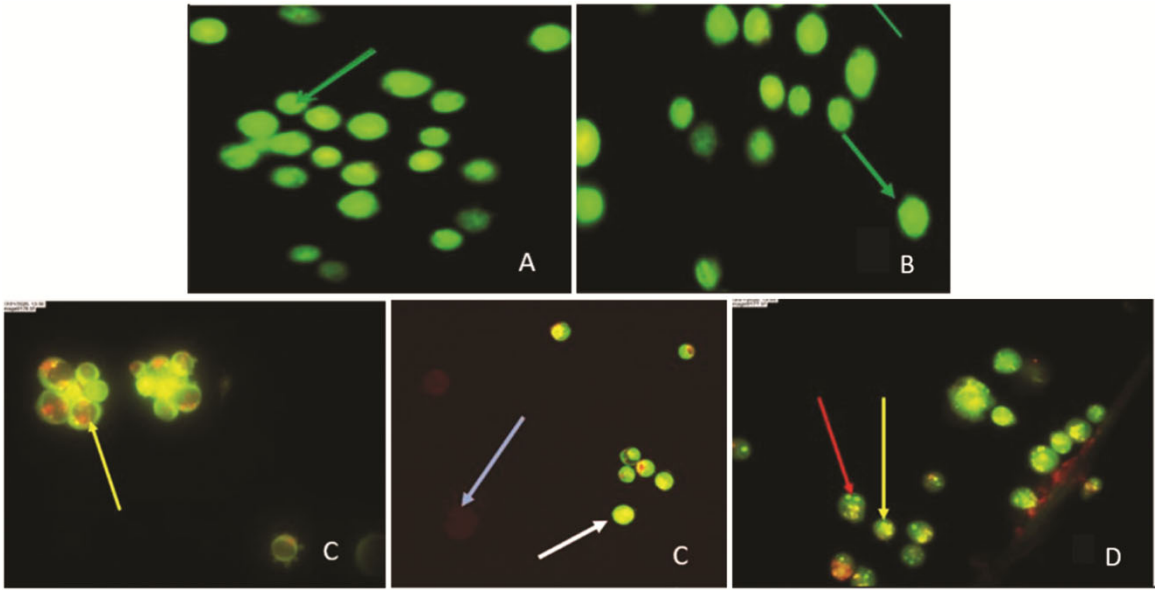


Fig 2 — Acridine orange and ethidium bromide staining: A- control; B- TNF-α stimulated MDA-MB 231 cells; C and D- stimulated cells treated with doxorubicin 0.58 µg/mL and $IC_{50}$ value of MGG respectively. Green arrow- normal cells; white arrow- early apoptotic cells; yellow arrow- late apoptotic cells; blue arrow- necrotised cells; red arrow- nuclear fragmentation

Table 3 — PCR efficiency of *NF-κB1*

| Dilution | cDNA amount | ln cDNA | Ct | PCR efficiency |
|---|---|---|---|---|
| 1x | 1 | 0 | 10.90 | |
| 3x | 0.333333333 | -1.098612289 | 14.65 | 1.1522 |
| 9x | 0.111111111 | -2.197224577 | 26.41 | |

ln- natural logarithms

Table 4 — PCR efficiency of GAPDH

| Dilution | cDNA amount | ln cDNA | Ct | PCR efficiency |
|---|---|---|---|---|
| 1x | 1 | 0 | 15.75 | |
| 3x | 0.333333333 | -1.098612289 | 18.62 | 1.3273 |
| 9x | 0.111111111 | -2.197224577 | 23.51 | |

ln- natural logarithms

Table 5 — The relative *NFκB1* gene expression in TNF-α stimulated MDA-MB-231 cells in response to treatment with MGG

| Cells | | Fold change in *NFκB1* RNA expression |
|---|---|---|
| MDA-MB-231 cells | Control cells | 1 |
| | TNF-α | $0.762\pm0.0012^{***}$ |
| | TNF-α + Doxorubicin | $0.533\pm0.0022^{***}$ |
| | TNF-α + $IC_{50}$ MGG | $0.545\pm0.015^{***}$ |

*NFκB1*- Nuclear factor kappa-light-chain-enhancer of activated B cells; TNF-α- Tumour necrotic factor-alpha; MGG- methanol extract of *Garcinia gummi-gutta*

Values are expressed as Mean ± SE (n=3); *** denotes significant ($P< 0.05$) difference compared with control

**Nuclear factor kappa light chain enhancer of activated B cells (NF-κB) gene expression study**

Total RNA was secluded from TNF-α stimulated MDA-MB-231 after administration of MGG at its respective $IC_{50}$ concentrations. Ribonucleic acid (RNA) isolated from cells were revealed to be of good quality and free of DNA contamination. The RT-qPCR efficiency of *NF-κB1* and GAPDH was evaluated by finding out the cycle threshold (Ct) using 1x, 3x and 9x dilutions of the corresponding cDNA. The results are presented in (Tables 3 & 4). The Figures 3 and 4 depicted natural logarithms (ln) of cDNA concentration versus Ct graph of *NF-κB1* and GAPDH.

The relative *NF-κB1* gene expression in TNF-α stimulated MDA-MB-231 cells in response to addition of MGG at their respective $IC_{50}$ concentration as compared to control cells is presented in (Table 5). The Figure 5A-C depicted representative amplification plot-

cum- dissociation curves of GAPDH and *NF-κB1* genes respectively in real time PCR of *in vitro* cell culture samples. In TNF-α stimulated MDA-MB-231 cells, *NFκB1* gene expression was significantly ($P< 0.05$) down-regulated for MGG with 0.545±0.015-fold change in the expression when compared with the control. Hence, it was concluded that *NFκB1* gene expression was down-regulated in MDA-MB-231 cells after treatment with MGG.

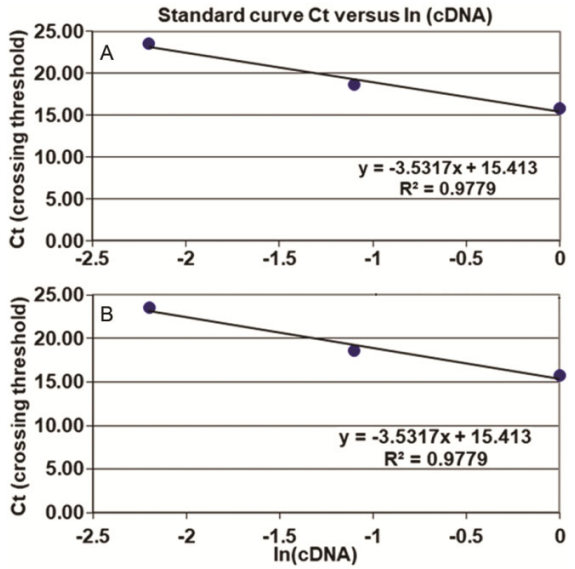


Fig 3 — Standard curve Ct versus ln cDNA of (A) *NF-κB1*; and (B) GAPDH

**Phytochemical analysis of methanol extracts pericarp of *Garcinia gummi-gutta***

The phytochemical analysis of MGG yielded alkaloids, steroids, triterpenes, tannins and saponins (Table 6).

**Gas chromatography mass spectrometry analysis of methanol extract of pericarp of *Garcinia gummi-gutta***

Chromatogram obtained on phytochemical analysis of MGG using GC-MS is given in Figure 6. Phytoconstituents obtained on GC-MS analysis of MGG are listed in (Table 7).

2-methyl-hexanoic acid, 2-[(2-aminoethyl)amino]-ethanol, propanoic acid, methyl 2-formyl-4-pentenoate, dimethyl dl-malate, dimethyl 2-butenedioate, 5-methoxy-2,4-dimethyl-furan-3-one, dimethyl ester of butanedioic acid, 5-hydroxymethylfurfural, 1H-pyrazole-3-carboxylic acid, 2,5-dihydro-5-oxo-, dimethyl mesaconate, monomethyl ester of butanedioic acid, trimethyl ester of citric acid, cis-non-3-enyl propyl ester of fumaric acid, dimethyl ester of 2-hydroxypropane-1,2,3-tricarboxylic acid and methyl ester of hexadecanoic acid were detected in methanol *G. gummi-gutta* fruit pericarp extract.

***In silico* screening of phytoconstituents of methanol extractof pericarp of *Garcinia gummi-gutta***

The *in silico* screening using PASS online software predicted that 14 compounds identified via GC-MS of methanol extract of pericarp of *Garcinia gummi-gutta* displayed antineoplastic activity. Analysis using the SwissADME online web tool revealed that out of

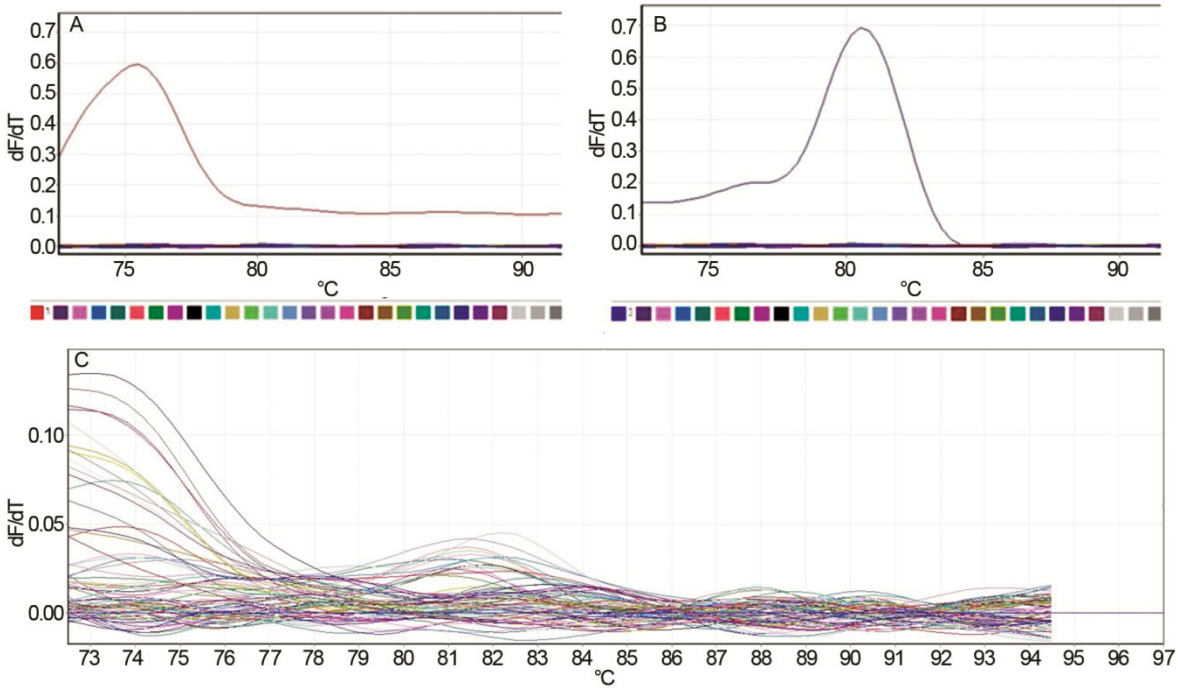


Fig 4 — Amplification plot- A. GAPDH, B. *NFκB1* and C. Melt curve of GADPH and *NFκB1* gene

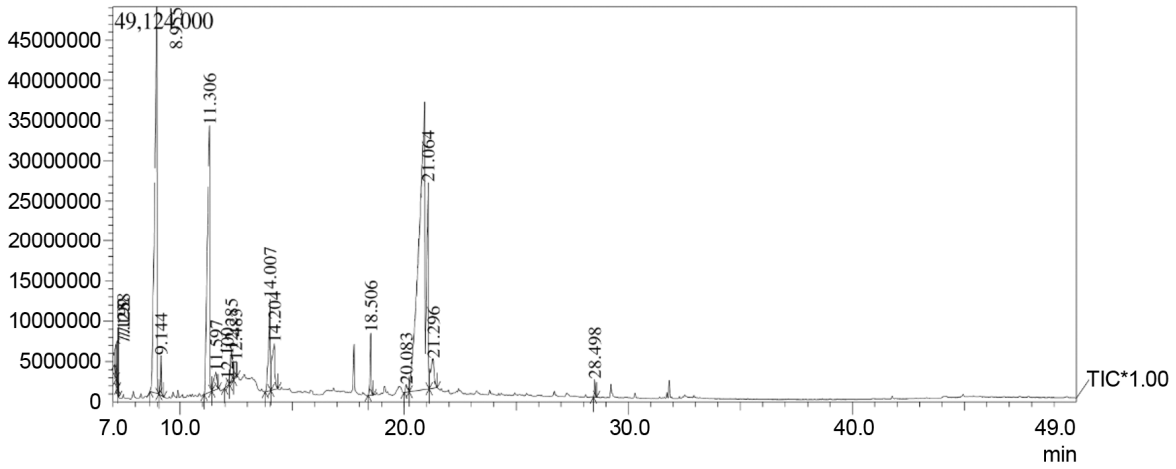


Fig 5 — Gas chromatography- mass spectroscopy (GC-MS) chromatogram of methanol extract of *G. gummi-gutta*

Table 6 — Phytochemical analysis of methanol extract of *G. gummi-gutta*

| Phytochemical tests | Methanol extract of Fruit pericarp of *Garcinia gummi-gutta* |
|---|---|
| *Steroids* | |
| *Salkowski's test* | + |
| *Alkaloids* | |
| *Dragendroff's test* | - |
| *Mayer's test* | - |
| *Wagners's test* | - |
| *Hager's test* | + |
| *Glycosides* | |
| *Sodium hydroxide test* | - |
| *Benedict's test* | - |
| *Tannins* | |
| *Ferric chloride test* | + |
| *Flavonoids* | |
| *Lead acetate test* | - |
| *Ferric chloride test* | - |
| *Phenolic compounds* | - |
| *Saponins* | |
| *Foam test* | + |

14 compounds, 13 compounds adhered to the Lipinski rule and had a LogP value less than 4.15 as given in (Table 8).

The binding affinity studies revealed that most of the selected phytoconstituents had a binding affinity for NF-κB, Bcl-2 and Caspase-8 as given in (Table 9).

## Discussion

Inflammatory breast cancer with TNBC phenotype is a rare, locally aggressive and highly metastatic tumour that is poorly responsive to treatment. Inflammation, cellular transformation, tumour cell survival, proliferation, invasion, angiogenesis and metastasis in IBC is mediated through NF-κB, an important inflammatory signalling molecule[49]. Agents that can inhibit NF-κB activation pathway might have the potential to prevent and treat IBC with TNBC phenotype. The present study evaluated the anticancer activity of methanol extracts of fruit pericarp of *G. gummi-gutta* targeting NF-κB in triple negative breast cancer.

Extraction of pericarp of *Garcinia gummi-gutta* with methanol yielded 6.623 percent in the present study. Similar findings were perceived from aqueous extraction of fruit rinds of *G. gummi-gutta* where six percent yield was obtained[27].

Tumour necrotic factor-alpha is a proinflammatory cytokine known to be significant in physiological and pathological processes, including cell necrosis and apoptosis[51]. Tumour necrotic factor-alpha plays a pivotal role in activation of NF-κB signalling pathway[28]. Studies revealed that TNF-α augmented breast cancer stem like cells through upregulation of TAZ expression,

Table 7 — Gas chromatography- mass spectrometry analysis of phytochemicals in methanol extract of *G. gummi-gutta*

| Selected compounds | Retention time | Probability |
|---|---|---|
| Hexanoic acid, 2-Methyl | 7.125 | 0.90 |
| Ethanol, 2-[(2-aminoethyl)amino]- | 7.192 | 1.08 |
| Propanoic Acid | 7.238 | 0.80 |
| Methyl 2-formyl-4-pentanoate | 8.955 | 20.05 |
| Dimethyl dl-malate | 9.144 | 0.85 |
| Dimethyl 2-butenedioate | 11.306 | 14.44 |
| 5-methoxy-2,4-dimethyl-furan-3-one | 11.597 | 1.19 |
| Butanedioic acid, dimethyl ester | 12.100 | 0.33 |
| 5 Hydroxymethylfurfural | 12.285 | 1.82 |
| 1H-Pyrazole-3-carboxylic acid, 2,5-dihydro-5-oxo | 12.483 | 0.83 |
| Dimethyl mesaconate | 14.007 | 3.67 |
| Butanedioic acid , monomethyl ester | 14.204 | 3.12 |
| Citric acid, trimethyl ester | 18.506 | 1.52 |
| Fumaric acid, cis-non-3-enyl propyl ester | 20.083 | 0.38 |
| 2-Hydroxypropane-1,2,3-tricarboxylic acid, dimethyl ester | 21.296 | 2.48 |
| Hexadecanoic acid, methyl ester | 28.498 | 0.33 |

Table 8 — *In silico* analysis of various phytoconstituents obtained in GC-MS analysis for their lipophilicity and violation of Lipinski rule using SwissADME online web tool and antineoplastic activity predicted using PASS online software

| Sl no | Phyto constituents | LogP | Lipinski Rule | Antineoplastic activity |
|---|---|---|---|---|
| 1 | H-pyrazole-3-carboxylic acid | -0.81 | No violation | Yes |
| 2 | 2-[(2-Aminoethyl)amino]-ethanol | -0.89 | No violation | Yes |
| 3 | 2-Hydroxypropane-1,2,3-tricarboxylic acid, dimethyl ester | -0.79 | No violation | Yes |
| 4 | 2-Methyl-hexanoic acid | 1.63 | No violation | Yes |
| 5 | 5-Hydroxymethylfurfural | -1.06 | No violation | Yes |
| 6 | 5-Methoxy-2,4-dimethyl-furan-3-one | -0.16 | No violation | Yes |
| 7 | Cis-non-3-enyl propyl ester of fumaric acid | 2.98 | No violation | Yes |
| 8 | Dimethyl 2-butenedioate | 0.15 | No violation | Yes |
| 9 | Dimethyl dl-malate | -0.58 | No violation | Yes |
| 10 | Dimethyl mesaconate | 0.50 | No violation | Yes |
| 11 | Methyl ester of hexadecanoic acid | 4.44 | Violation | Yes |
| 12 | Monomethyl ester of butanedioic acid | 0.25 | No violation | Yes |
| 13 | Propanoic acid | 0.03 | No violation | Yes |
| 14 | Trimethyl ester of citric acid | -0.48 | No violation | Yes |

Table 9 — Docking scores of selected phytoconstituents from MGG against NF-κB, Bcl-2 and Caspase-8 in kcal/mol

| Sl no | Phyto constituents | Bcl-2 | Caspase 8 | NF-κB |
|---|---|---|---|---|
| 1 | H-pyrazole-3-carboxylic acid | 4.9 | 5.1 | 5.2 |
| 2 | 2-[(2-Aminoethyl)amino]-ethanol | 4.5 | 3.8 | 4.0 |
| 3 | 2-Hydroxypropane-1,2,3-tricarboxylic acid, dimethyl ester | 5.6 | 5.6 | 5.5 |
| 4 | 2-Methyl-hexanoic acid | 5.1 | 5.8 | 5.1 |
| 5 | 5-Hydroxymethylfurfural | 4.8 | 5.4 | 5.7 |
| 6 | 5-Methoxy-2,4-dimethyl-furan-3-one | 5.6 | 6.1 | 5.5 |
| 7 | Cis-non-3-enyl propyl ester of fumaric acid | 6.7 | 6.3 | 6.5 |
| 8 | Dimethyl 2-butenedioate | 5.1 | 5.0 | 4.9 |
| 9 | Dimethyl dl-malate | 4.7 | 4.9 | 5.3 |
| 10 | Dimethyl mesaconate | 4.9 | 5.5 | 5.1 |
| 11 | Methyl ester of hexadecanoic acid | 5.8 | 6.4 | 5.4 |
| 12 | Monomethyl ester of butanedioic acid | 4.6 | 4.7 | 4.9 |
| 13 | Propanoic acid | 3.7 | 3.7 | 4.1 |
| 14 | Trimethyl ester of citric acid | 5.4 | 5.3 | 5.7 |

TNBC- Triple negative breast cancer, TNF-α- Tumour necrotic factor-alpha

a transcriptional co-activator via the non-canonical NF-κB pathway and suggested the crucial part of TAZ in inflammatory factor–promoted breast cancer stemness[29]. Hence, we have used TNF-α for stimulation of inflammation in MDA-MB-231 cell line for standardising the model of IBC with TNBC phenotype in our present study.

The concentration of TNF-α that stimulated constitutively present NF-κB in MDA-MB-231 cell line with 100 percent cell viability was used for standardisation and the results revealed that the TNF-α concentration of 10 ng/mL and 40 ng/mL showed more than 100 percent cell viability suggesting its role in augmentation of breast cancer cells probably due to improved inflammation. In MDA-MB-231 cells, non-lethal working concentration of 40 ng/mL TNF-α stimulation led to increased rise of C-C motif ligand 2 (CCL2), granulocyte-macrophage a colony-stimulating factor (GMCSF), IL-1 and IL-6[21]. The TNF-α promoted migration, growth and invasion of MDA-MB-231 cell lines, partly by inducing the expression of dipeptidylpeptidases and matrix metalloproteinases (MMPs)[30]. Tumour necrotic factor- alpha invigorated tumour cell proliferation, survival, migration and angiogenesis as well as induced cancer cell death, making it a double-edged sword in cancer therapy[31]. Henceforth the low concentration of TNF-α, 10 ng/mL with 100 percent cell viability was chosen for stimulation of inflammation in MDA-MB-231 cell lines.

*In vitro* anticancer activity of MGG was performed using MTT assay. Cytotoxicity studies of methanol extract of pericarp of *G. gummi-gutta* produced concentration-dependent cytotoxicity in MDA-MB-231 cells with $IC_{50}$ value of 50.03 µg/mL in the present study. *In vitro* screening of ethanol *G. gummi-gutta* fruit extract exhibited tumouricidal activity in a murine neuroblastoma cell line (Neuro-2A cells), with an $LC_{50}$ value of 0.235 mg/mL[32] although methanol, water, ethyl acetate and chloroform extracts of the *G. gummi-gutta* fruit pericarp against CaCo2 and HeLa carcinoma cells illustrated $IC_{50}$ values less than 200 µg/mL[13] which was similar to our findings. The MTT assay is a colourimetric assay for determining cell metabolic activity. It is established on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)- dependent oxidoreductase enzymes to reduce the yellow-coloured tetrazolium dye MTT to purple coloured insoluble formazan crystals which is later solubilized by DMSO. Enzymatic conversion of the tetrazolium to water insoluble compound formazan by dehydrogenase present in mitochondria of living cells as well as other organelles such as endoplasmsic reticulum help us to determine the cell viability by virtue of their reductive activity. In case of cytotoxicity, there will be decrease in the development of purple coloured formazan crystal due to decrease in the number of live cells which is estimated using ELISA reader[33]. The findings of the present study revealed that MGG could be grouped as cytotoxic anticancer agents for triple negative breast cancer.

In the present study, MGG and doxorubicin treated cells showed late apoptosis post 24 h of treatment in AO/EB staining of TNF-α stimulated MDA-MB-231 cells. The apoptotic features like apoptotic bodies, nuclear fragmentation, chromatin condensation, cytoplasmic vacuolation and nuclear marginalisation were noticed in the plant extract and doxorubicin treated cells. The hexane extract of *G. quaesita* fruits in MDA-MB-231 cell line produced apoptosis characterised by green fluorescence in viable cells and bright yellow and red in early and late apoptotic cells. Apoptotic changes such as condensed and fragmented chromatin similar to our study were observed in extract treated cell lines[34]. The apoptotic properties such as condensation of chromatin, nuclear fragmentation, nuclear marginalisation and apoptotic bodies were noticed in MDA-MB-231 cells treated with methanol extract of seeds of *Hordeum vulgare*[35], similarly chloroform fraction of methanolic extract of seeds of *Annona muricate* treated MDA-MB-231 cells were in their late apoptotic stage and displayed chromatin condensation, nuclear fragmentation, apoptotic bodies, karyorrhexis[36]. The AO/EB staining is a procedure for identification of stage of programmed cell death. Ethidium bromide solely enters those cells with reduced cell integrity as seen in necrotic and apoptotic cells whereas acridine orange dye diffuses into all the cells and produces green fluorescence[37]. Early apoptotic cell will stain green and have bright green dots within the nuclei as a sequalae of chromatic granule condensation and nuclear fragmentation whereas the late apoptotic cells will incorporate ethidium bromide therefore stains orange but, in distinction with the necrotic cells. Necrotic cells stain orange, however have a nuclear morphology similar to that of viable cells, with no condensed chromatin granule[38]. The results of the present study revealed that MGG induced late apoptosis leading to cytotoxicity.

Nuclear factor kappa light chain enhancer of activated B cells is a transcription factor involved in the regulation of multiple physiological and pathological cellular

processes, including inflammation, cell survival, proliferation, and cancer cell metastasis. In the present study, the gene expression of NF-κB was down-regulated in the TNF-α stimulated MDA-MB-231 cell lines treated with MGG. Rugosin E, an ellagitannin, inhibited proliferation of MDA-MB-231 and induced apoptosis by inhibiting NF-κB signalling pathway[39,50]. The NF-κB inhibition prevented tumour resistance to chemotherapeutic agents and hence development of NF-κB inhibitors could increase the efficacy of many anticancer agents[40]. The NF-κB activation is significantly higher in TNBC than in other subtypes[41]. The down-regulation of constitutively active NF-κB in primary effusion lymphoma (PEL) led to the cell growth inhibition and induced apoptosis in PEL cell lines[42]. The present study revealed that the down-regulation of NF-κB by MGG would be responsible for inducing apoptosis.

The phytochemical analysis of MGG divulged the presence of steroids, alkaloids, tannins, triterpenes and saponins in the present study. The qualitative analysis of ethyl acetate and ethanol extracts of *G. gummi-gutta* contained high amounts of flavonoids, phenols, terpenoids and saponins[43] whereas the presence of alkaloids, saponins, tannins, carbohydrates and proteins were detected from the phytochemical screening of the aqueous extract of *G. cambogia* fruit rinds[27]. Phytochemical screening of methanol extract of leaf and fruit of *G. gummi-gutta*, indicated the presence of alkaloids, tannins, phenols, terpenoids, saponins, reducing sugars, steroids and phylobatannins[44].

Majority of the pharmacological effects yielded by plants are due to the existence of phytochemicals in them. Phytochemicals have a vital role in protecting plants from environmental stressors as well as providing desirable health benefits to living beings. Diverse phytochemicals display varying effects on the biochemical reactions occurring in the body. The variations in the results of phytochemical screening obtained from the studies conducted by different authors could be due to various factors such as part of the plant extracted, climatic conditions, geographical area, type of soil, stress factors, weather conditions and seasonal variations.

In GC-MS, phytoconstituents are detected using a combination of gas chromatography and mass spectrum division patterns with a database and relative peak area percent method for its quantitative estimation. Nowadays, GC- MS evolved as a key method for metabolic profiling[45]. Gas chromatography and mass spectrometry analysis of MGG depicted the presence of 2-methyl-hexanoic acid, 2-[(2-aminoethyl)amino]-ethanol, propanoic acid, methyl 2-formyl-4-pentenoate, dimethyl dl-malate, dimethyl 2-butenedioate, 5-methoxy-2,4-dimethyl-furan-3-one, dimethyl ester of butanedioic acid, 5-hydroxymethylfurfural, 1H-pyrazole-3-carboxylic acid, 2,5-dihydro-5-oxo-, dimethyl mesaconate, monomethyl ester of butanedioic acid, trimethyl ester of citric acid, cis-non-3-enyl propyl ester of fumaric acid, dimethyl ester of 2-hydroxypropane-1,2,3-tricarboxylic acid and methyl ester of hexadecanoic acid in the present study. The chemical characterization of methanolic fruit extract of *G. cambogia* using GC-MS revealed the presence of 40 compounds in which the major constituents were cyclohexanecarboxylic acid, hexadecanoic acid, methyl ester, 2ethylcyclohexyl ester-, octadecanesulphonyl chloride, cycloheptasiloxane, tetradecamethyl and chloromethoxy ethane, along with other minor constituents[25]. Out of the identified phytoconstituents, 14 compounds were predicted to have antineoplastic activity analysed using PASS online software. Hence it could be concluded that these phytoconstituents would be responsible for the anticancer activity of the plant extract.

The 'drug-likeness' property of a chemical compound in its discovery and development stages is attributed to lipophilicity. The absorption of the drug molecules into the body is designated by lipophilicity of a compound which is expressed as Log P. Lower Log P value is allied to greater absorption[46], hence 13 compounds identified by GC-MS of MGG illustrated a Log P value less than 4.15 which is reminiscent of its virtuous absorption into the body. Furthermore, the Lipinski's rule of five validates that a successful drug molecule should have properties within the acceptable range of the five Lipinski's rules: lipophilicity (expressed as LogP): ≤4.15, molar refractivity from 40 to 130, molecular weight: ≤500, number of hydrogen bond acceptors: ≤10, and number of hydrogen bond donors: ≤5. Thirteen phytoconstituents had proved to abide by the Lipinski rule of five[47,52].

Lower docking score (binding energy) corresponds to higher binding affinity, hence the ligand molecules that had the lowest binding energy were deliberated to be the best ligand molecules in inhibiting their respective receptors[36]. Apoptosis is arbitrated through mitochondria mediated intrinsic, and extrinsic pathways[53]. The extrinsic pathway of apoptosis is incited by caspase-8 which further cleaves and activates caspase 3, 6 and 7 whereas Bcl-2 family proteins located in the outer mitochondrial membrane are found to be the

antiapoptotic proteins interceding the intrinsic pathway of apoptosis[48]. Our compounds had lower binding energy and hence higher binding affinity with receptors such NF-κB, Bcl-2 and caspase 8 suggesting its probable role in inflammation and apoptosis in inflammatory breast cancer with TNBC phenotype. Affinity of these compounds to the NF-κB receptor validates the downregulation of NF-κB illustrated through gene study.

Hence, it could be concluded that the phyto-constituents identified from MGG could evolve as a prospective drug candidate against TNBC. Further *in vitro* and *in vivo* studies in connection with the anti-cancer effect of these phytoconstituents in TNBC are warranted.

## Conclusion

The present study substantiated that the methanol extract of fruit pericarp of *G. gummi-gutta* exhibited a concentration dependent cytotoxicity in TNF-α stimulated MDA-MB-231 cell lines which was illustrated by nuclear fragmentation and late apoptotic cells in AO/EB staining. The existence of the phytochemical constituents indicated that the fruit pericarp of the plant could be used in a variety of ways which would be beneficial to the population. Gas chromatography mass spectroscopic analysis and *in silico* docking studies with Bcl-2 and caspase-8 revealed the presence many compounds presumed to be responsible for eliciting the apoptotic activity of the plant by targeting the NF-κB signalling pathway exemplified through its downregulation in gene expression study. Therefore, our plant extract could be a promising candidate for therapeutic management of TNBC. Further in-depth study on isolating the bioactive molecules is warranted.

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## Conflict of interest

Both the authors declare no conflict of interest.

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