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# Antiproliferative Potential of Isolated Phytoconstituents from Fruit Extract of Trapa natans

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**KEYWORDS**

TNBC

Trapa natans

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Phytoconstituent

**ABSTRACT:**

**Background:** Triple Negative Breast Cancer (TNBC) is an aggressive subtype lacking estrogen, progesterone, and HER2 receptors, leading to poor prognosis and limited treatment options. Natural products offer a promising source of anticancer agents. *Trapa natans*, known for its antioxidant and anti-inflammatory properties, is rich in polyphenolics with potential therapeutic value.

**Objectives:** To isolate and evaluate phytoconstituents from the methanolic fruit extract of *T. natans* (TNME) for their antioxidant activity and antiproliferative effects against the MDA-MB-231 TNBC cell line.

**Methods:** A successive extraction of *T. natans* fruits was conducted using solvents of increasing polarity. The methanolic extract was selected for further study based on its superior cytotoxic activity among the five tested extracts. Phytoconstituents were isolated using column chromatography, yielding two major compounds: TNME101 and TNME102. Antioxidant potential was evaluated using DPPH, ABTS, and reducing power assays. Antiproliferative effects against MDA-MB-231 cells were assessed via cytotoxicity assays.

**Results:** Both TNME101 and TNME102 exhibited significant antioxidant activity. TNME101 showed superior performance in the ABTS assay with an $IC_{50}$ of 10.30 ± 1.32 µg/mL, surpassing the standard ascorbic acid ($IC_{50}$ = 51.25 ± 0.49 µg/mL). TNME102 had an $IC_{50}$ of 46.70 ± 2.80 µg/mL. Cytotoxicity assays demonstrated dose-dependent antiproliferative effects. TNME101 exhibited a lower $IC_{50}$ value (244.98 ± 1.23 µg/mL) compared to TNME102 (287.09 ± 2.03 µg/mL), indicating a higher cytotoxic potential.

**Conclusion:** The findings support the therapeutic potential of *T. natans*, particularly the phytoconstituent TNME101, as a promising candidate for anticancer drug development targeting resistant TNBC. The results warrant further investigation into its mechanism of action and in vivo efficacy.

## 1. Introduction

The term "triple-negative breast cancer" (TNBC) was first recognized in the mid-2000s to describe a subgroup of breast cancers characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression [1, 2]. Originally, TNBC was thought to represent a clinical categorycorelated with the basal-like subtype, identified through groundbreaking gene expression microarray analyses conducted in the early 2000s.Based on the currently available evidence, TNBC is best characterized as a broad term encompassing a diverse group of entities that exhibit significant variations in genetic, transcriptional, histological, and clinical profiles [3]. Comprehensive analysis of TNBCs has identified specific histologic subtypes predominantly associated with the triple-negative phenotype, such as metaplastic breast cancers, carcinomas with medullary features, and carcinomas with apocrine features. These high-grade TNBCs are characterized by significant genetic instability, complex

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genomic profiles, and recurrent TP53 mutations, although distinct patterns of somatic alterations vary by tumor type. The pioneering work by Perou et al. established a microarray-based classification of breast cancers into five intrinsic subtypes: luminal A, luminal B, HER2-enriched, normal-like, and basal-like. While TNBCs were initially equated with basal-like cancers, it is now evident that TNBCs exhibit substantial heterogeneity in gene expression profiles [3-5].

In 2018, approximately 2,088,849 cases of TNBC were reported, highlighting its prevalence as a significant cancer type among women [6]. The disease has an average survival duration of around 10.2 months with current therapeutic options. The 5-year survival rate is approximately 65% for cases with regional tumor involvement and drops to 11% for cases with distant metastases [7]. TNBC represents 15–25% of all breast cancer cases. A 2009 case study involving 187 TNBC patients observed a 2.5% increased risk of developing TNBC among individuals who used oral contraceptives for more than one year, with the risk rising to 4.2% in women under the age of 40. Furthermore, a positive correlation was noted between the duration of oral contraceptive use and the associated risk. In the United States, TNBC accounts for approximately 12% of breast cancer cases, with a reported 5-year survival rate ranging from 8% to 16% [8].

Triple-negative breast cancer (TNBC) presents significant clinical challenges due to its aggressive nature, lack of hormone receptors (ER, PR, and HER2), and limited therapeutic targets. Patients with TNBC often experience high recurrence rates and poor prognosis, particularly those with metastatic disease [2]. Current treatment strategies rely on chemotherapy, but resistance and severe side effects remain obstacles [9]. Moreover, the heterogeneity of TNBC complicates the development of universal treatment approaches [10]. The absence of targeted therapies for triple-negative breast cancer (TNBC) is further exacerbated by its molecular and clinical heterogeneity. TNBC is stratified into various molecular subtypes, such as basal-like, mesenchymal, and immune-related subtypes, which pose significant challenges to the development of standardized therapeutic strategies [11]. Among these, the basal-like subtype—representing the majority of TNBC cases—is characterized by elevated proliferation rates and pronounced genomic instability, which contribute to its highly aggressive behaviour [10]. The prognosis of triple-negative breast cancer (TNBC) is notably poorer compared to other breast cancer subtypes. Patients with TNBC experience reduced progression-free survival (PFS) and overall survival (OS), accompanied by a higher propensity for visceral metastases, particularly to the lungs and brain [12]. This unfavorable outcome is especially pronounced in metastatic cases, where therapeutic options are primarily palliative. Although advancements in immunotherapy and investigational clinical treatments have shown promise, sustained therapeutic responses remain limited to a small subset of patients [13].

Despite the widespread use of synthetic drugs, plant-derived secondary metabolites remain critical in drug design, serving as structural templates for synthesizing novel therapeutics [14, 15]. While the safety and efficacy of synthetic drugs are often debated, natural compounds continue to dominate in treating human diseases due to their better tolerance and acceptance. Conventional chemotherapeutics, such as methotrexate and cisplatin, are linked to severe side effects like gastrointestinal damage, bone marrow suppression, and drug resistance. In contrast, phytocompounds demonstrate higher efficacy and lower toxicity. Natural molecules still constitute a significant portion of modern pharmaceuticals, especially in antibiotics and cancer therapies [16].

Plant-derived natural compounds are pivotal in drug discovery, particularly in oncology, where over half of cancer therapeutics originate from these sources. These compounds have demonstrated potential to alleviate chemotherapy side effects, enhance immunity, and reduce cancer recurrence and metastasis. The intricate chemistry of natural products presents significant challenges for drug development. For instance, out of 35,000 species screened by the National Cancer Institute, paclitaxel and camptothecinyielded viable drug structures. Advances in research have increasingly focused on identifying target plants, isolating bioactive compounds, and elucidating molecular pathways, leveraging multidisciplinary approaches. Numerous phytochemicals, including vinblastine, curcumin, and cannabinoids, have been validated for their anticancer properties, significantly advancing cancer therapy. As a result, anticancer phytochemicals remain a critical focus

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in biomedical research, contributing to substantial scientific progress [17].

Polyphenols, exhibit a wide range of bioactive properties, including antioxidant, anti-inflammatory, anticancer, and antidiabetic effects, alongside their protective roles in cardiovascular and nervous system health. Polyphenols have garnered significant attention as potential agents in cancer prevention and therapy. These dietary compounds can influence and modulate diverse molecular pathways and biological processes integral to carcinogenesis. Moreover, polyphenols function as biological response modifiers, enhancing immune responses and safeguarding cells from oxidative damage caused by free radicals. Their anticarcinogenic effects encompass the regulation of critical processes such as cell proliferation, tumor growth, angiogenesis, metastasis, inflammation, and apoptosis [18].

*Trapa* species, particularly *Trapa natans* (water chestnut), have been traditionally used in India for their diverse therapeutic properties, including nutritive, astringent, aphrodisiac, anti-diarrheal, anti-inflammatory, and antileprotic effects. They have also been employed to manage urinary disorders, bronchitis, sore throat, and anaemia.Pharmacological studies of *T. natans* highlight its antibacterial, antifungal, antidiabetic, anti-inflammatory, hepatoprotective, and antioxidant activities, suggesting its potential for drug development [19]**.** Studies have reported the rich content of polyphenols in Trapa natans. The leaf extract of *Trapa natans* is known to contain ten phenolic compounds, with gallic acid, ellagic acid, ferulic acid, and quercetin 3-O-galactoside (hyperoside) being the most predominant among them. The aqueous fruit extract of *T. natans* is notably rich in phenolics, which have garnered significant scientific interest for their potent free radical scavenging properties. The high polyphenol content of the aqueous fruit extract provides strong evidence of its substantial in vitro antioxidant activity, effectively neutralizing free radicals and highlighting its potential for therapeutic applications [20].

Polyphenols are well-documented for their ability to modulate key cellular pathways implicated in carcinogenesis, including apoptosis, angiogenesis, and metastasis [21]. A study utilizing a 70% ethanolic extract of *T. natans* shells revealed significant antioxidant properties, coupled with effects on apoptosis induction, cell cycle modulation, and mitochondrial membrane depolarization, all of which are critical processes in cancer progression [22].

Recent advances have focused on the application of *T. natans* in nanotechnology. For instance, Saber et al. (2018) successfully synthesized silver nanoparticles (AgNPs) using *T. natans* leaf extracts and evaluated their antineoplastic activity against A431 human skin cancer cells. The study demonstrated a concentration-dependent reduction in cell viability, with AgNPs at 100 µg/mL decreasing the viability of A431 cells by 24.3%, as evidenced by MTT assays.

Despite this, comprehensive research of isolated polyphenols from Trapa plants targeting specific cancer types, such as triple-negative breast cancer (TNBC), is yet underexplored. Accordingly, the current research focuses on isolating phenolic phytoconstituents from *Trapa natans* fruits and assessing their anticancer potential against the resistant breast cancer cell line MDA-MB-231.

## 2. Materials and Methods

2.1 **Reagents:** All chemicals and reagents used for analytical purposes were procured from Hi-media and Merck, whereas reference standards for extract characterization were obtained from Sigma Aldrich.

2.2 **Plant authentication:** A herbarium specimen was prepared and authentication of the collected plant was done by the Department of Botany, Gauhati University, Assam, India, with reference number Herb./GUBH/2021/010 and accession number 19766.

2.3 **Successive Extraction of the crude drug material**

Ripe whole of *Trapa natans* were procured from the local market of Goalpara, Assam, India. A total of 5 kg of fruit was collected and subjected to size reduction, followed by dehydration in a tray dryer maintained at 45°C. The dried material was subsequently ground into a coarse powder for further processing. Successive extraction of the crude drug was carried out using a Soxhlet apparatus, employing five solvents of increasing polarity index—hexane, chloroform, ethyl acetate, methanol, and water [23-25]. The residual crude material after each extraction step was quantified,

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and the obtained solvent extracts were air-dried, weighed, and their percentage yields were calculated (Table 3.1). The dried extract residues were stored in a desiccator for further analysis. The presence of phenolic phytoconstituents in the extracts was confirmed through thin-layer chromatography (TLC), and phytochemical screening was performed to identify the bioactive constituents in *T. natans* fruit extracts [26].

**2.4 I*n-vitro* cell cytotoxicity screening**

All the five successive extracts of the *T. natans* fruit were subjected to the preliminary in-vitro MTT assayto assess their cell cytotoxicity against MDA-MB-231 Cell line. MDA-MB-231 cells were cultured in DMEM-HG with 10% FBS and incubated at 37°C with 5% $CO_2$. Cells were seeded in a microtiter plate (200 µL/well) and treated with varying concentrations of the extracts after 24 hours. After 24-hour incubation, the medium was replaced with 200 µL of 10% MTT reagent and incubated for 3 hours. Formazan crystals were dissolved in 100 µL DMSO, and absorbance was measured at 570 nm and 630 nm.

2.5 **Isolation of phytoconstituents**

The methanolic fruit extract of *T. natans* showed highest cytotoxicity in the in-vitro assay and was selected for isolation of phytoconstituents using column chromatography. The TNME sample was dissolved in methanol and required amount of silica gel was added for better flow properties. It was subjected to column chromatography using a pre-packed silica gel G column (60–120 mesh size). The elution process was carried out with100% chloroform, followed by a gradual increase in methanol concentration to enhance polarity. The pooled fractions showing same TLC profiles were concentrated [27]. Specifically, TNFD 9-15 and TNFH-5-10 fractions were pooled and concentrated using a rotary evaporator. The isolated compounds, TNME101 and TNME102, underwent purification through solvent washing with acetone and chloroform. A secondary TLC analysis was performed to verify their purity.

## 2.6 Estimation of Antioxidant Activity

### 2.6.1 *DPPH Radical Scavenging Assay*

The antioxidant potential of the isolated compounds was assessed using a modified DPPH assay. Ascorbic acid was taken as the standard [28]. A standard stock solution (1 mg/mL) of the isolated compounds and ascorbic acid was prepared. Serial dilutions of ascorbic acid (25–200 µg/mL) were made to generate a calibration curve. For the assay, 0.3 mL of each extract or standard dilution was mixed with 3 mL of reagent mixture (5 mL of 100 µM DPPH solution in methanol) to each sample containing the test compound and standard respectively. The resulting mixture was incubated in the dark for 30 minutes at room temperature to allow the reaction to proceed. After incubation, the absorbance was recorded at 517 nm using a UV-Vis spectrophotometer to evaluate the free radical scavenging activity.

The percentage inhibition was calculated using the formula below:

$$\text{Percentage Inhibition}=\frac{(Abs.of\ sample-Abs.of\ blank)}{Abs.\ of\ control}\text{X}100\%$$

### 2.6.2 *ABTS Free Radical Scavenging Activity*

The assay was carried out as per the method designed by Jimoh et. al., 2019. 2.45 mM potassium persulfate ($K_2S_2O_8$) was mixed with 7 mM ABTS solution in methanol to prepare the ABTS reagent and generate the radicals. The reagent was then incubated in dark for 12-18 hours at room. The solution was then diluted with methanol in a ratio of 1:50. So that an absorbance of 0.700 was obtained at 734 nm. Successive concentrations of both the compounds and standard drug were respectively reacted with equal volume of diluted ABTS radical solution (1:1, v/v). after incubating the solution in the dark for 6 hours, absorbance was measured at 734 nm [29].

The percentage inhibition was calculated as below:

$$\%\ \text{ABTS} = \frac{(Abs.of\ sample-Abs.of\ blank)}{Abs.\ of\ control}\ \text{X}\ 100\%$$

### 2.6.3 *Reducing Power Assay*

Reducing power of the isolated compounds were estimated using modified potassium ferricyanide reduction method. Serial dilutions (25-200 µg/mL) of the Separate stock solutions (1mg/mL) of the samples and standard were prepared. 1 mL aliquot of each dilution was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide solution (10 mg/mL). After incubation (<50°C, 20 minutes), 1.5 ml trichloroacetic acid (100 mg/mL) was added to the solutions and centrifuged at 3000 rpm for

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10 minutes. 2.5 ml distilled water and 0.5 ml $FeCl_3$ solution (1 mg/mL) was added to the supernatant. Absorbance of the samples were observed at 700 nm [28].

### 2.7 **HPTLC Chromatographic Analysis**

The TNME extract was standardized via high-performance thin-layer chromatography (HPTLC). Stock solutions of p-coumaric acid, synaptic acid (0.1 mg/mL) taken as standard and CCME (10 mg/mL) were prepared in methanol. Chromatographic separation was carried out using a mobile phase consisting of toluene, ethyl acetate, and formic acid in a 6:4.5:0.3 (v/v/v) ratio. The HPTLC analysis was performed using a CAMAG® system (Anchrom Enterprises (I) Pvt. Ltd., Mumbai, India). The setup included a Linomat V sample applicator, operating at a dosage speed of 150 nL/s with a pre-dosage volume of 0.2 µL, along with a twin trough chamber (20 × 10 cm) that underwent a 20-minute saturation period. Detection and scanning were carried out using a Camag TLC scanner 4, configured with a scanning speed of 20 mm/s and slit dimensions of 5 × 0.2 mm. A deuterium lamp set at 254 nm was used for detection, while visualization and data analysis were carried out using the Camag TLC visualizer and VisionCATSsoftware.The chromatographic plate was observed under ultraviolet light at 254 nm and the Rf values were calculated.

### 2.8 **Spectroscopical Analysis**

UV-visible spectrophotometric analysis of the TNME extract was conducted using a Shimadzu UV-1900i spectrophotometer, wherein both the extract and isolated compounds (TNME101 and TNME102) were scanned over a wavelength range of 200–700 nm and compared with the reference standard [30, 31]. Fourier-transform infrared (FTIR) spectroscopy was performed using a Perkin Elmer-UATR Two instrument. The isolated compounds were compressed into a thin film between spectrophotometer discs, and infrared transmittance spectra were recorded across a wavenumber range of 400–4000 $cm^{-1}$. Nuclear magnetic resonance (NMR) spectroscopy, including both $^{1}H$ and $^{13}C$ NMR, was carried out using a Bruker Advance Neo spectrometer at 500 MHz. Additionally, mass spectrometric analysis was performed using a Maldi-TOF Synapt XS HD mass spectrometer, and the obtained spectral data were systematically interpreted.

### 2.9 **MTT Cell Viability Assay of Isolated Compounds**

Isolated compounds TNME101 and TNME 102 were subjected to MTT Assayagainst the resistant breast cancer cell line MDA-MB-231. The cells were cultured in DMEM-HG supplemented with 10% foetal bovine serum. For the assay, 200 µL of cell suspension was seeded into each well of a microtiter plate and incubated at 37°C in a 5% $CO_2$ atmosphere for 24 hours. Various concentrations of the test compounds (200 µL) were then added to the respective wells and incubated for another 24 hours. After incubation, the medium was aspirated, and 200 µL of 0.5 mg/mL MTT reagent in medium was added to each well. The plate was incubated for 3 hours at 37°C, followed by careful removal of the medium to avoid disrupting the formed crystals. Then, 100 µL of DMSO was added to each well to dissolve the formazan crystals, and the plate was gently agitated on a gyratory shaker. Absorbance was measured at 570 nm and 630 nm using a microplate reader.

## 3. Results and discussions

### 3.1 **Successive extraction of crude drug**

Successive extraction of the crude drug of fruits of *T. natans* was carried out using different solvents with increasing order of polarity. The methanolic extract (TNME) showed the highest percentage yield at 49.23%, followed by the aqueous extract (TNAQ) at 24.6%, the chloroform extract (TNCL) at 4.01%, the ethyl acetate extract (TNETA) at 3.07%, and the hexane extract (TNHE) at 2.55%.

### 3.2 **Comparative cytotoxicity profiles of extracts**

All five extracts were subjected to an in-vitro cytotoxicity assay against MCF-7, T47-D and MDA-MB-231 breast cancer cell lines at various concentrations. Table 3.1 summarizes the $IC_{50}$ values of the extracts against the cell lines. At the tested concentrations, TNME extract showed comparatively better results against MCF-7, T-47D and MDA-MB-231 cell lines, followed by TNHE extract against T47-D and TNETA extract against MDA-MB-231 cell lines. TNCL extract didn't show any considerable antiproliferative activity against either cell lines. While the aqueous extract TNAQ showed much higher $IC_{50}$values for

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MCF-7 and T-47D cell lines, it was non toxic against the MDA-MB-231 cell line.

**Table 1:** In-vitro cell cytotoxicity assay of extracts against breast cancer cell lines

| ***Extracts*** | **$IC_{50}$ values (µg/ml) against cell lines** | | |
|---|---|---|---|
| | **MCF-7** | **T47-D** | **MDA-MB-231** |
| *TNHE* | Non cytotoxic | 479.13 ± 1.23 | Non cytotoxic |
| *TNCL* | Non cytotoxic | Non cytotoxic | Non cytotoxic |
| *TNETA* | 848.88 ± 1.14 | 646.229 ± 0.63 | 527.783 ± 0. 37 |
| *TNME* | 463.23 ± 0.74 | 287.16 ± 1.42 | 496.271 ± 0. 32 |
| *TNAQ* | 758.384 ± 1.56 | 911.78 ± 1.25 | Non cytotoxic |

### 3.3 **Isolation of Phytoconstituents**

Based on i*n-vitro* cell cytotoxicity results and in accordance with previous reports of rich phenolic contents [32], the TNME extract was selected for chromatographic separationusing column chromatography. A gradient of chloroform and methanol in varying ratios was used as the mobile phase to increase polarity. Fractions TNFD 9-15 and TNFH-5-10 were pooled due to their similar $R_f$ values. The TLC conditions were optimized using a mobile phase composed of toluene, ethyl acetate, and formic acid in the ratio of 6.4:5:0.3. Two major phytoconstituents TNME101 and TNME102 were isolated through the process.

### 3.4 **The antioxidant potential of the isolated compounds**

TNME101 and TNME102 was assessed using DPPH, ABTS and FRAP reducing power assay and ascorbic acid as the standard.

#### 3.4.1 **DPPH Assay**

In the DPPH assay, TNME101 showed high antioxidant potential with an $IC_{50}$ 12.22 ± 2.5 µg/mL while TNME102 reported an $IC_{50}$value of 48.15 ± 3.53 µg/mLwhen compared to the standard ascorbic acid which reported an $IC_{50}$ of 67.63 ± 1.2 µg/mL.The crude extract TNME showed a higher $IC_{50}$value of 108.14 ± 1.9 implying weaker antioxidant potential. When compared statistically, both TNME101 and TNME102 reported markedly higher antioxidant activity than the TNME extract($p < 0.001$). Both the isolated compounds showed greater efficiency than standard ascorbic acid while TNME101 reported higher scavenging potential than TNME102 ($p < 0.01$)(Fig.).

#### 3.4.2 **ABTS Assay**

The ABTS assay the highest antioxidant potential was shown by TNME101 with an $IC_{50}$ of 10.30 ± 1.32 µg/ml, followed by TNME102 with an $IC_{50}$ of 46.70 ± 2.80 µg/ml. In comparison, crude extract TNME reported a higher value of $IC_{50}$ 85.89 ± 0.35 µg/mL.When compared to the standard ascorbic acid($IC_{50}$ = 51.25 ± 0.49 µg/mL), both isolated compounds reported significantly higher levels of radical scavenging action. Statistical analysisshowed that the differences in $IC_{50}$values between the crude extract TNME, isolated compounds (TNME101 and TNME102) and ascorbic acid were statistically significant ($p < 0.001$) while TNME101 demonstrated much higher activity than TNME102 ($p < 0.01$).Figure …. provides a visual representation of these findings. The presence of phenolic hydroxyl groups responsible for releasing electrons to neutralize free radicals can be the reason for the high antioxidant potential of TNME101($IC_{50}$= 10.30 ± 1.32 µg/mL in ABTS assay) [18].

#### 3.4.3 **Reducing Power Assay**

The electron-donating capacity of the TNME extract and its isolated compounds, TNME101 and TNME102, was evaluated using the reducing power assay in comparison to, ascorbic acid. At 200 µg/ml, TNME

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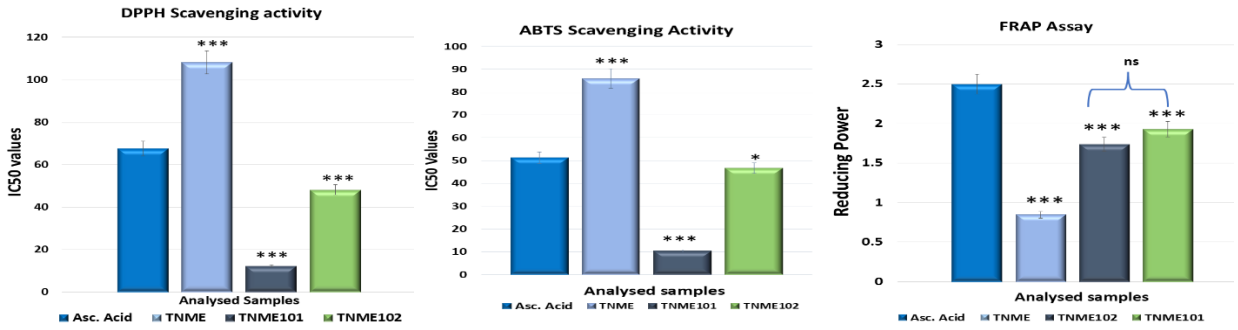
extract showed absorbance of 0.846 ± 0.11 followed by TNME101 at 1.93 ± 0.10 and TNME102 at 1.74 ± 0.86. statistical analysis reported the significant difference ($p < 0.001$) in the reducing power of isolated compounds. The reducing power of TNME101 was substantially higher than that of TNME102 ($p < 0.05$), and both isolated compounds were significantly more active than the TNME extract ($p < 0.001$).




(a) (b) (c)

**Fig:** Histogram depicting the comparative antioxidant activities of standard ascorbic acid, crude extract (TNME), and isolated compounds (TNME101 and TNME102) from the methanolic fruit extract of Trapa natans across **(a)** DPPH, **(b)** ABTS, and **(c)** FRAP assays*(* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)*

3.5 Two distinct compounds, **TNME 101** and **TNME 102**, were identified through chromatographic analysis, characterized by retention factors ($R_f$) values, regression models, and concentration profiles. **TNME 101** displayed an $R_f$ values range of 0.344–0.348, with a linear regression equation of $y = 6.451 \text{ X } 10^{-9}x + 1.606 \text{ X } 10^{-3}$, and with an average concentration of 53.40 µg/ml of the extract (Fig. 3.2). **TNME 102** exhibited a$R_f$ range 0.611-0.618, with quadratic regression equation $y = 1.835 \text{ X } 10^{-7}x + 9.102 \text{ X } 10^{-2}$, and accounted for 171.4µg/mlof the extract (Fig. 3.3). These results demonstrate the compounds' divergent chromatographic behaviors and quantitative distributions, laying the groundwork for subsequent structural elucidation and bioactivity assessments.

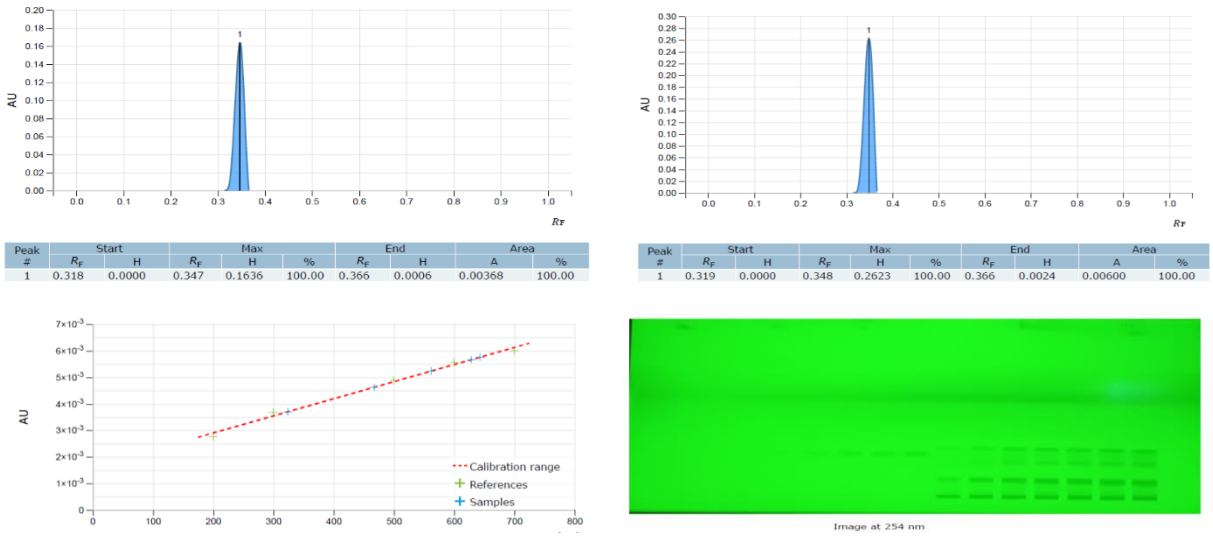



**Figure .** HPTLC standardization of compound TNME101 with sinapic acid as reference. (A) Chromatogram of standard sinapic acid; (B) Chromatogram of TNME101 in TNME extract; (C) Standard calibration curve of sinapic acid; (E) HPTLC analysis of standard sinapic and TNME extract under UV 254 nm.

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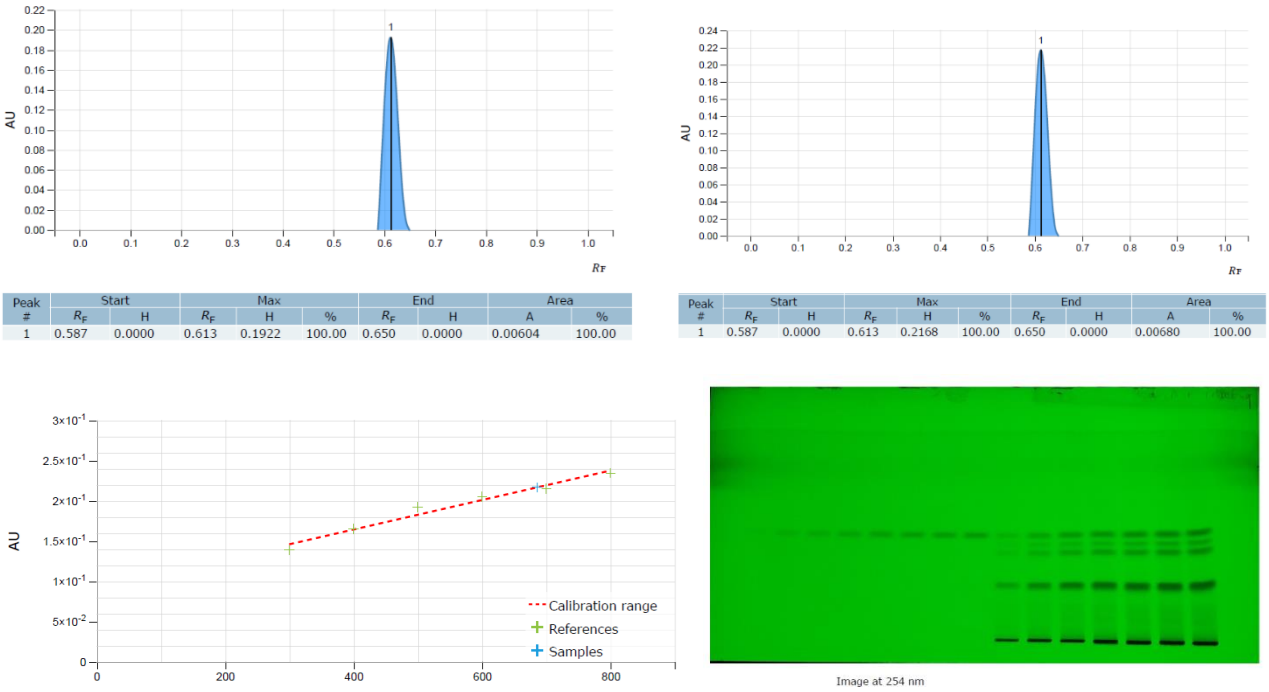


**Figure.** HPTLC standardization of compound TNME102 with p-coumaric acid as reference. (A) Chromatogram of standard p-coumaric acid; (B) Chromatogram of TNME102 in TNME extract; (C) Standard calibration curve of p-coumaric acid; (E) HPTLC analysis of standard caffeic acid and TNME extract under UV 254 nm.

### 3.6 **Spectral Data**

A sharp absorption peak at 324 nm with absorbance 0.068 was shown by the UV- vis spectral data for isolated compound TNME101, closely corresponding to the standard compound sinapic acid, which displayed a peak at 326.5 nm with an absorbance of 0.046. Similarly, the spectral profile of compound TNME102 revealed an absorption maximum at 308.5 nm with an absorbance of 0.176, which closely matched the peak observed for the standard p-coumaric acid at 307 nm with an absorbance of 0.165.

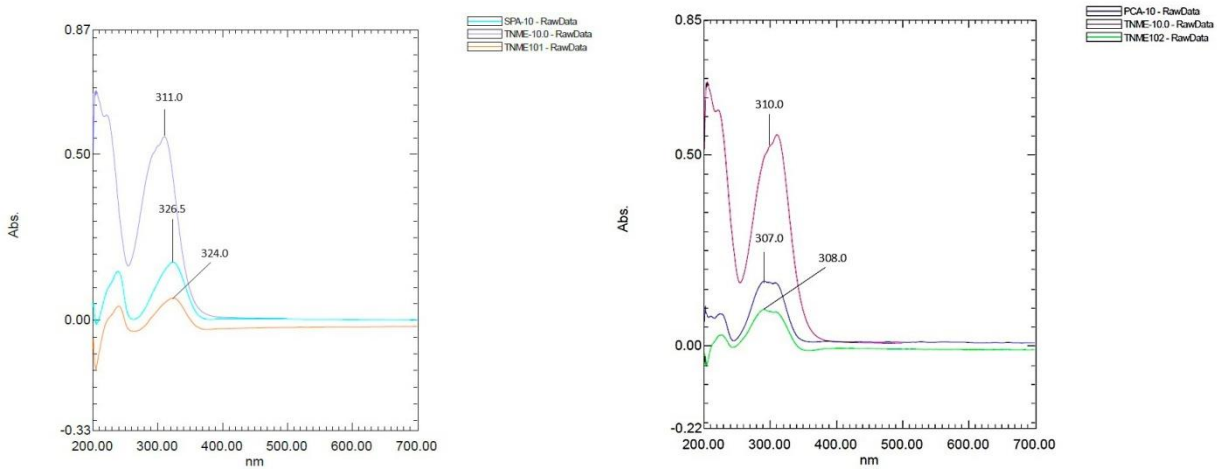


**Fig. :** UV-Vis spectral graph of (A) TNME101 and extract TNME with standard sinapic acid (B) TNME102 and extract TNME with standard p-coumaric acid

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3.7 **Structural Elucidation**

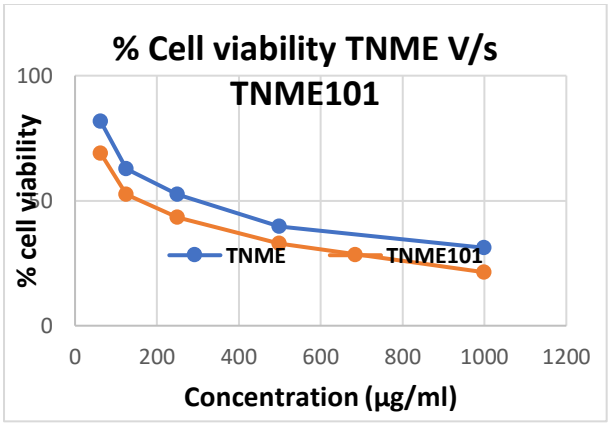
**Sinapic acid (TNME101): m.p.:** 202.4ºC; **$R_f$**( silica gel G):0.348 (Toluene: Ethyl acetate: Formic acid, 6:4:0.3); **FTIR (cm$^{-1}$)** : 3380.6 (O-H, Str); 2520.2 (C-H, Str); 1618.87 (C=O, Str.); 1514.94 (O-H, Ben); 1265.58 (C-O, Str.); **MS (ES+) m/z** 225.07 ($M^+$); **$^1$H NMR (500 Hz)**: δ 3.80 (s, 6H), 6.40 (d, 1H, J=15.9), 6.99(s, 2H), 7.48 (d, 1H, J=15.9), 8.89 (s, 1H), 12.11 (s, 1H);**$^{13}$C NMR (500Hz)**: δ 39.42, 55.99, 105.99, 115.98, 124.51, 137.97, 144.70, 147.94, 167.86

**p-coumaric acid (TNME102): m.p.:** 212.3ºC; **$R_f$** ( silica gel G):0.613 (Toluene: Ethyl acetate: Formic acid, 6:4:0.3); **FTIR (cm$^{-1}$)** : 3363.3 (O-H, Str); 2821.8 (C-H, Str.); 1667.09 (C=O, Str.); 1599.75 (O-H, Ben); **MS (ES+) m/z** 165.05 ($M^+$); **$^1$H NMR (500 Hz)**: δ 6.27 (d, 1H, J=15.9), 6.78 (t, 2H, J=8.65), 7.48 (d, 1H, J=15.95), 7.50 (t, 2H, J=8.65), 9.95 (s, 1H), 12.11 (s, 1H); **$^{13}$C NMR (500Hz)**: δ 39.42, 115.29, 115.71, 125.24, 130.02, 144.13, 159.55, 167.91

3.8 **Antiproliferative action of isolated compounds**

The antiproliferative activity of the isolated compounds wereevaluated against MDA-MB-231 cell lines over a test concentration range of 62.5 – 1000 µg/ml. 5-fluorouracil was taken as the standard. The percentage of cell viability and $IC_{50}$ values of the compounds have been depicted graphically in figure…. .Among the isolated compounds, TNME101 reported an $IC_{50}$ value of 244.98 ± 1.23µg/ml while the compound TNME102 showed an $IC_{50}$ value of 287.09 ± 2.03µg/ml.Analysis reported statistical significance among the groups ($p < 0.001$). With and $IC_{50}$ value of 496.27 ± 2.63 µg/ml the crude extract TNME showed a significantly lower antiproliferative potential. Statistical analysis showed a significant difference in the values of the standard 5-FU and the samples ($p < 0.001$). TNME101 had a substantially lower $IC_{50}$ value than TNME102 ($P < 0.01$), suggesting that it had a relatively higher anti-proliferative potential.

However, the $IC_{50}$values of both isolated compounds were much higher than those of 5-fluorouracil (5-FU), a common chemotherapeutic drug. This highlights the necessity of structural modification or synergistic formulation to increase efficacy.



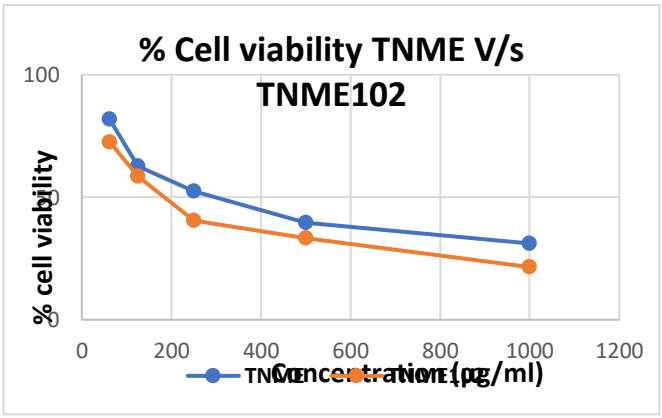




**Figure:** Graphical representation of antiproliferative action crude extract TNME Vs isolated compound a) TNME101 and b)TNME102 against MDA-MB-231 cell line

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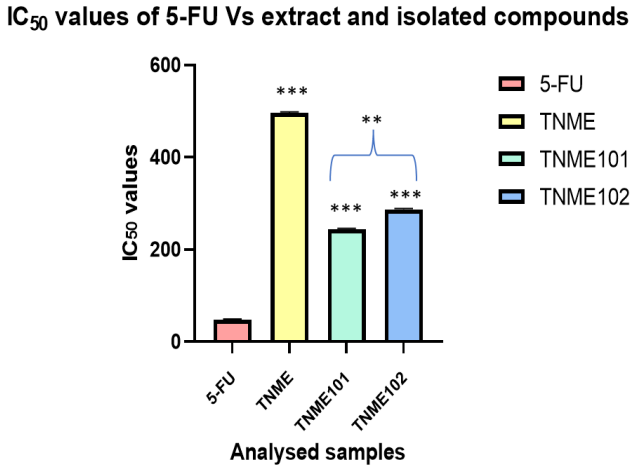


**Figure:** Graphical representation of comparativeIC50 values of standard (5-FU), crude extract (TNME) and isolated compounds (TNME101 & TNME102).

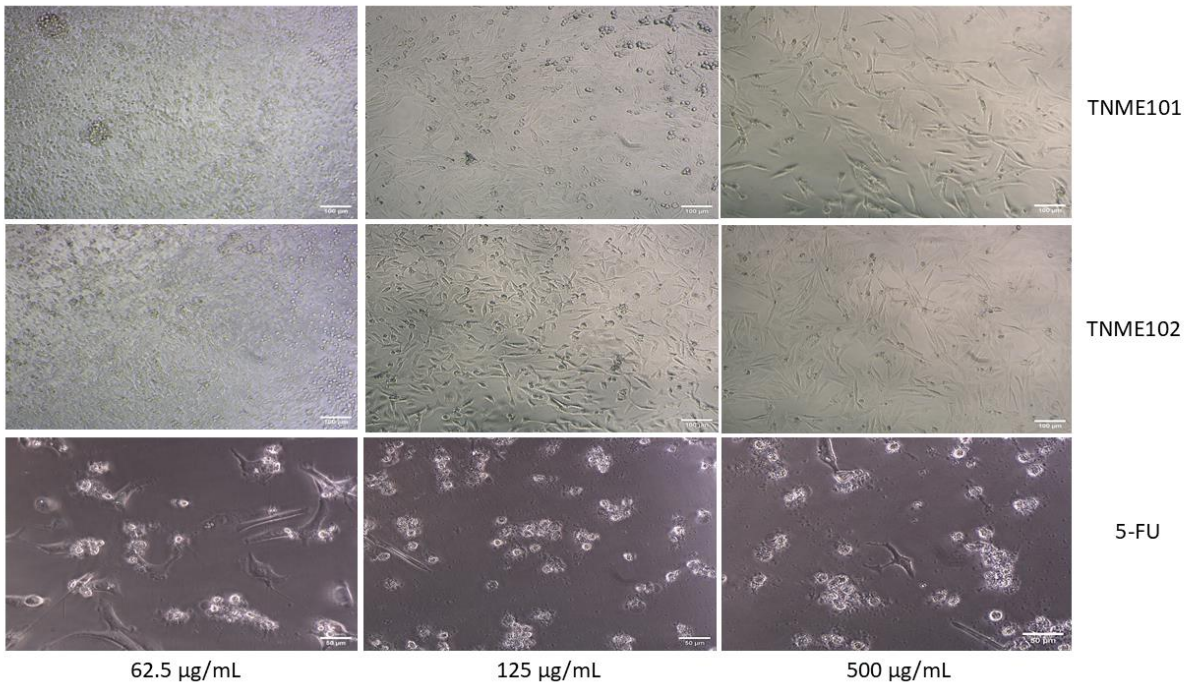


**Figure:** Images of MTT Assay of isolated compounds and standard drug at concentrations 62.5, 125 and 500 µg/ml

## 4. Conclusion

The study underscored the potential antiproliferativeaction against MDA-MB-231 cells and antioxidant actions of the isolated compounds from the methanolic fruit extract of *Trapa natans* after successive extraction. The isolated compounds TNME101 and TNME102 were identified as a) sinapic acid and b) p-coumaric acid respectively after structural elucidation through spectroscopical analysis.

Structurally defined by the presence of a phenolic ring with conjugated double bonds and different substituents on the phenyl ring, both the compounds belong to the class of hydroxycinnamic acid. With two methoxy groups and one hydroxyl group on the aromatic ring, sinapic acid has a greater ability to donate electrons,

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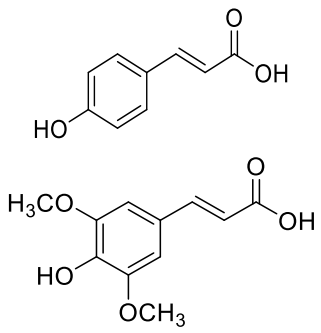
which helps cancer cells undergo ROS-mediated oxidative stress, which results in death and the prevention of cell growth [33]. This is also in accordance with the reported antiproliferative action of sinapic acid against HT-29 human colon cancer cell line and PC-3 and LNCaP human prostate cancer cell lines [34, 35]. The second isolated compound, p-coumaric acid with a single hydroxyl group at the para position of the phenyl ring contributes to its moderate anticancer activity in comparison to sinapic acid. The difference in the structural characteristics emphasizes the differences in the therapeutic potentials ofthe two compounds and affirm the hypothesis that alterations in the substituents of phenyl ringprovide a significant impact on the bioactivity of hydroxycinnamic acids.Studies have already proved the active role of phenolic phytoconstituents in combating cancer cell proliferation by inducing oxidative stress and promoting apoptosis [36].

However, to validate these theories, further research involving mechanistic analyses, apoptotic markers, and in vivo studies is necessary.

This study contributes to the underexplored area of anticancer potential of *T. natans* specifically against triple negative breast cancer. Previously, studies emphasized on the hepatoprotective and antidiabetic actions of the plant with few preliminary data indicating the cytotoxic potential against breast cancer. Our research not only validate the therapeutic relevance of *Trapa natans* fruits but also identify TNME101 as a promising candidate for future anticancer drug development




**Fig**

**5. Acknowledgement**

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**7. Conflict of Interest**

None