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6C potency of Terminalia chebula reveals secondary metabolites and induces apoptosis in Triple-Negative Breast Cancer Cells (TNBCs)

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Abstract

Background: Breast cancer is a prevalent malignancy in women globally, and TNBCs, being ER-/PR-/Her2-, pose challenges in treatment options. Complementary and alternative medicines, including homoeopathic remedies, have gained popularity as potential anti-cancer agents to mitigate the side e ects of conventional drugs. Previously, the anticancer activity of homoeopathic potencies of *Terminalia chebula* (MT, 3X, 6C, and 30C) against breast cancer cell lines has been reported. Objectives: This study aimed to evaluate the mechanism of anticancer activity of 6C potency of *T. chebula* against the TNBC cell line, MDAMB231 and to understand the phytochemical composition of 6C potency. Methods: Lactate release was studied by LDH assay, and mitochondrial membrane potential was evaluated by JC- 1 assay. The mRNA expression of tumour suppressor and pro-apoptotic genes was done by qRT-PCR assay. Mother Tincture (MT) and 6C potencies were phytochemically characterised by LCMS analysis. Results: The 6C potency of TC increased lactate release in TNBCs, indicating a shift towards glycolysis and potentially inducing metabolic changes that contribute to its anticancer activity. The increased lactate release led to loss of mitochondrial membrane potential and an increase in mRNA expression of pro-apoptotic markers (caspase 3, caspase 9, and cytochrome c) and tumour suppressor proteins (p53 and pRb). LCMS analysis of 6C potency revealed phytocompounds with anticancer, anti-inflammatory, and antioxidant activities. Conclusion: The 6C potency of *T. chebula* induced apoptosis in TNBCs, however, further in vivo efficacy and safety studies are warranted to understand the anti-cancer mechanism of 6C in-depth.

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ORIGINAL ARTICLE

6C potency of *Terminalia chebula* reveals secondary metabolites and induces apoptosis in Triple-Negative Breast Cancer Cells (TNBCs)

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ABSTRACT

Background: Breast cancer is a prevalent malignancy in women globally, and TNBCs, being ER-/PR-/Her2-, pose challenges in treatment options. Complementary and alternative medicines, including homoeopathic remedies, have gained popularity as potential anti-cancer agents to mitigate the side effects of conventional drugs. Previously, the anticancer activity of homoeopathic potencies of *Terminalia chebula* (MT, 3X, 6C, and 30C) against breast cancer cell lines has been reported. **Objectives:** This study aimed to evaluate the mechanism of anticancer activity of 6C potency of *T. chebula* against the TNBC cell line, MDAMB231 and to understand the phytochemical composition of 6C potency. **Methods:** Lactate release was studied by LDH assay, and mitochondrial membrane potential was evaluated by JC-1 assay. The mRNA expression of tumour suppressor and pro-apoptotic genes was done by qRT-PCR assay. Mother Tincture (MT) and 6C potencies were phytochemically characterised by LCMS analysis. **Results:** The 6C potency of TC increased lactate release in TNBCs, indicating a shift towards glycolysis and potentially inducing metabolic changes that contribute to its anticancer activity. The increased lactate release led to loss of mitochondrial membrane potential and an increase in mRNA expression of pro-apoptotic markers (caspase 3, caspase 9, and cytochrome c) and tumour suppressor proteins (p53 and pRb). LCMS analysis of 6C potency revealed phytocompounds with anticancer, anti-inflammatory, and antioxidant activities. **Conclusion:** The 6C potency of *T. chebula* induced apoptosis in TNBCs, however, further *in vivo* efficacy and safety studies are warranted to understand the anti-cancer mechanism of 6C in-depth.

Keywords: Complementary & alternative medicine (CAM), JC-1, LCMS, LDH, *Terminalia chebula*, Triple negative breast cancer (TNBC)

Introduction

In India, breast cancer is the leading cancer among females, with an estimated 2,32,832 cases by 2025.[1] Triple-negative breast cancer (TNBC) is the most aggressive and highly prevalent subtype in India compared to Western countries, characterised by quick spread, high recurrence and metastasis, and poor disease-free survival.[2,3]

Due to the negative expression of estrogen receptor (ER), progesterone receptor (PR), and the lack of or under-expression of human epidermal growth factor receptor-2 (HER2/neu) in TNBCs, targeted therapies are unavailable, resulting in poor prognosis.

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Conventional chemotherapy, associated with severe side effects, remains the mainstay treatment modality for TNBCs.[4] Thus, nowadays, patients often seek complementary and alternative medicine (CAM) after undergoing conventional therapies to help regulate the recurrence and spread of their disease. Homoeopathic drugs are used as anticancer agents, to reduce side effects associated with conventional cancer treatments and improve patient well-being.[5–10]

Terminalia chebula (*T. chebula* / TC) is a medicinal plant widely employed in Ayurveda, Siddha, Unani, and Homoeopathic systems of medicine. Homoeopathic potencies of TC are used to control bleeding piles, diarrhea, chronic dysentery, constipation, biliary colic, glossitis, headache, vertigo, dropsy, and skin disorders.[11] We have previously reported that homoeopathic potencies of *T. chebula* (MT, 3X, 6C, and 30C) reduced the viability of breast cancer cell lines.[12] In the present study, phytochemical analysis of 6C and the mechanism of its activity against the TNBC cell line, MDAMB231 have been presented. The effect of 6C was studied on the messenger ribonucleic acid (mRNA) expression of p53, Rb, caspase-3, caspase-9, and cytochrome c genes.

Materials and methods

Homoeopathic potencies

The homoeopathic potencies MT (Batch No. M455) and 6C (Batch No. D783) were procured from Bio-India Pharma, Mumbai, India.

Chemicals and reagents

The tissue culture plasticware was purchased from BD Biosciences (San Diego, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), phenol red-free DMEM, fetal bovine serum (FBS), penicillin/streptomycin combined antibiotic were procured from Invitrogen-Gibco (Grand Island, NY, USA). N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), TRIzol reagent, and isopropanol were procured from Sigma-Aldrich (St. Louis, MO, USA). L-glutamine was obtained from MP Biomedicals. Trypsin-EDTA solution and trypan blue dye were obtained from HiMedia Laboratories. Trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP) was procured from Abcam and 5,5,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazoylcarbocyanine iodide (JC-1) dye was procured from Invitrogen. The Lactate Dehydrogenase (LDH) cytotoxicity assay kit was procured from Cayman Chemical. The predesigned KiCqStart SYBR Green primers, p53, Rb, caspase-3, caspase-9, and cytochrome c, were purchased from Sigma-Aldrich.

Cell culture

MDAMB231 was procured from the American Type Culture Collection (ATCC), USA.

MDAMB231, a highly aggressive TNBC cell line, is ER-, PR-, HER2- having high expression of Ki67 and low expression of E-cadherin, and claudins (3, 4, and 7). The cells show intermediate response to chemotherapy.[13] MDAMB231 cells were cultured in DMEM supplemented with 2 mM L-glutamine, 100 units/ml of a combination of penicillin-streptomycin, and 10% FBS and maintained in a humidified 5% CO_2 incubator at 37°C.

Lactate release

The cells were seeded at 1×10^5 cells/ml density in a 96-well plate and cultured overnight at 37°C in a CO_2 incubator for 24 h. This was followed by the treatment of cells with different preparations, including DMEM medium as a negative control, and 1:12.5, 1:25, and 1:100 dilutions of 6C potency prepared in the phenol-red-free DMEM culture medium, as discussed in Table 1. After 24 h, 100 μl of cell culture supernatants were collected and used to determine LDH activity, following the manufacturer's protocol and LDH was used as the positive control. The absorbance was measured by FluoStar Omega microplate reader (BMG Labtech) at 490 nm and the experiment was conducted twice in triplicates. Vehicle control, i.e., 100% ethanol, was not used, as ethanol is safe to cells up to 1:10 dilution in DMEM culture medium.[12]

Table 1. Working stock solution preparation for 6C homoeopathic potency of *T. chebula*.

Dilution	Volume of *T. chebula* (6C) (μl)	Volume of culture media (μl)
DMEM medium (negative control)	–	1000
1:12.5	80	920 (Made up to 1000)
1:25	40	960 (Made up to 1000)
1:100	10	990 (Made up to 1000)

Mitochondrial membrane potential ($\Delta\psi m$)

MDAMB231 cells at a density of 1×10^5 cells/ml were seeded in a 96-well black plate, maintained overnight at 37°C in a CO_2 incubator to allow their adherence. The next day, the cells were treated with different dilutions, including DMEM medium as negative control, and 1:12.5, 1:25, and 1:100 dilutions

of 6C potency in the culture medium for 24 h. 1 ml working stocks of these dilutions were prepared as shown in Table 1. This was followed by incubating the cells with fresh culture medium containing 0.5 μg/ml JC-1 dye for 30 minutes at 37°C in a CO_2 incubator in the dark. FCCP (20 μM) was used as a positive control. The fluorescence was measured at 520 nm for JC-1 monomers and 590 nm for JC-1 aggregates by Fluostar Omega microplate reader (BMG Labtech).[14]

Quantitative real time-polymerase chain reaction (qRT-PCR)

MDAMB231 cells were seeded at a density of 4×10^5 cells/ml in a 6-well plate and incubated overnight at 37°C in a CO_2 incubator. The next day, the cells were either untreated i.e., only treated with culture medium or with 1:100 dilutions of 6C potency and cultured for 24 h. Untreated cells served as the negative control to represent baseline gene expression levels. To run the samples in a 6-well plate in duplicate, a total of 3 mL stock solution of the mentioned dilutions was prepared as discussed in Table 1. This was followed by total RNA extraction from the control as well as treated cells using TRIzol reagent. The isolated RNA was precipitated with isopropanol and quantified using a NanoDrop spectrophotometer (NanoDrop®2000, Thermo Scientific, Waltham, MA, USA). RNA purity and integrity were evaluated based on A260/A280 absorbance ratio, and samples exhibiting a ratio of 2.0 were used in the study. 5 μg of total RNA was used to synthesize complementary DNA (cDNA) by reverse transcription using a high-capacity cDNA reverse transcription kit (PrimeScript 1st Strand cDNA Synthesis Kit, Takara) performed in a MiniAmp Thermal Cycler (Applied Biosystems). The expression of p53, Rb, caspase 3, caspase 9, and cytochrome c genes was examined by amplifying the cDNA template using gene-specific primers performed in a PCR system (7500 Real-Time PCR, Applied Biosystems). The primers used were p53-F: 5′-ACCTATGGAAACTACTTCCTG-3′, p53-R: 3′-ACCATTGTTCAATATCGTCC-5′; Rb-F: 5′-ACCAGATCATGTCAGAGAG—3′, Rb-R: 3′-TAACCTCCCAATACTCCATC—5′; Caspase-3-F: 5′-AAAGCACTGGAATGACATC-3′, Caspase-3-R: 3′-CGCATCAATTCCACAATTTC-5′; Caspase-9-F: 5′-CTCTACTTTCCCAGGTTTTG-3′, Caspase-9-R: 3′-TTTCACCGAAACAGCATTAG-5′; Cytochrome C-F: 5′-AAGAACAAAGGCATCATCTG-3′, Cytochrome C-R: 3′-GCTATTAAGTCTGCCCTTTC-5′, and β-actin-F: 5′-GACGACATGGAGAAAATCTG-3′, β-actin-R: 3′-ATGATCTGGGTCATCTTCTC-5′. The reaction was performed using TB Green Premix Ex Taq II, Takara kit. qRT-PCR cycling conditions were performed according to the manufacturer's recommendations (1 cycle at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec). The relative expression levels of test genes were normalized with the expression of β-actin, which was used as a housekeeping gene. The samples were run twice in triplicate, and average values were used for the calculation. The relative quantification of gene expression was done by $\Delta\Delta$Ct method.

Liquid chromatography mass spectrometry (LCMS)

High-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (HPLC-MS QTOF) analysis of MT and 6C potencies of TC was performed using the Agilent 1290 HPLC system, where an Agilent 6530 Quadrupole time-of-flight spectrometer was fitted with a source of electrospray ionization in positive as well as negative mode.[15] Briefly, 10 μL of sample was injected onto an Agilent 1290 HPLC system having Zorbax Eclipse Plus C18 column (2.1 mm × 100 mm, 1.8 μm particle sizes). The mobile phases consisted of (A) water and (B) acetonitrile (LCMS grade, J. T. Baker) with flow rate of 0.3 mL/min and 95:5 acetonitrile/water at a flow rate of 0.7 mL/min. Both mobile phases were modified with 0.1% (v/v) formic acid for MS analysis in positive mode and with 5 mm ammonium acetate for analysis in negative mode. The chromatographic conditions utilized for the study consisted of the first 5 min run isocratically at 5% B; a gradient of B from 95% to 5% was applied from 5 min to 30 min, followed by 3 min isocratically at 100%. MS analysis was performed on an Agilent 6530 Quadrupole time-of-flight spectrometer fitted with an electrospray ionization source in both positive and negative mode. Mass Hunter Qualitative Analysis Software Package (Agilent Technologies) and Metlin database were used for data analysis. Blanks using each of the solvent extraction systems were analyzed using "Find by Molecular Feature" algorithm in the software package to generate a compound list of molecules with significant abundances >10,000 counts. This was then used as an exclusion list to eliminate background contaminant compounds from the analysis of the extracts. The data were analyzed using "Find by Molecular Feature" function to generate a list of compounds with empirical formula in the extracts. Compound lists were then screened against online mass databases; METLIN Metabolomics Database and MassBank Database.[15,16]

Statistical analysis

The data were expressed as mean ± standard deviation (SD), and statistical analysis was conducted using GraphPad Prism 9 (San Diego, CA, USA) with one-way analysis of variance (ANOVA) followed by Dunnett's, Tukey's, Sidak's, or Repeated Measures multiple comparison tests. The values for $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ indicated statistically significant data.

Results

6C potency of T. chebula induced lactate release

The effect of 6C homoeopathic potency of *T. chebula* was studied on lactate release, an indicator of cell death, in MDAMB231 cells. Compared to the control untreated cells, 6C treated cells showed significantly high levels of lactate release of 37.74 ± 6.37% ($p < 0.001$) and 35.16 ± 5.21% ($p < 0.001$) at 1:12.5 and 1:25 dilutions, respectively and 12.06 ± 3.73% [non-significant (ns), p = 0.1967] at 1:100 dilution (Fig. 1). Lactate release was inversely proportional to the increased dilution of the potency, that is at lower dilutions, the release was high.

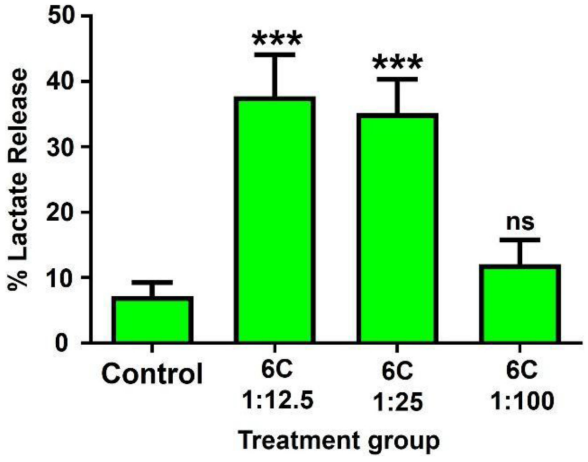


Fig. 1. 6C homoeopathic potency of *T. chebula* regulated MDAMB231 growth through apoptosis by increasing the lactate release at 1:12.5 and 1:25 dilutions. The values for $***p < 0.001$ indicated statistically significant data.

6C potency of T. chebula induced apoptosis by decreasing mitochondrial membrane potential

Homoeopathic potency of *T. chebula,* 6C significantly reduced %JC-1 aggregates, suggesting a decrease in mitochondrial membrane potential ($\Delta\psi$m) in MDAMB231 cells, indicative of apoptosis (Fig. 2). At 1:12.5, 1:25, and 1:100 dilutions, the aggregates

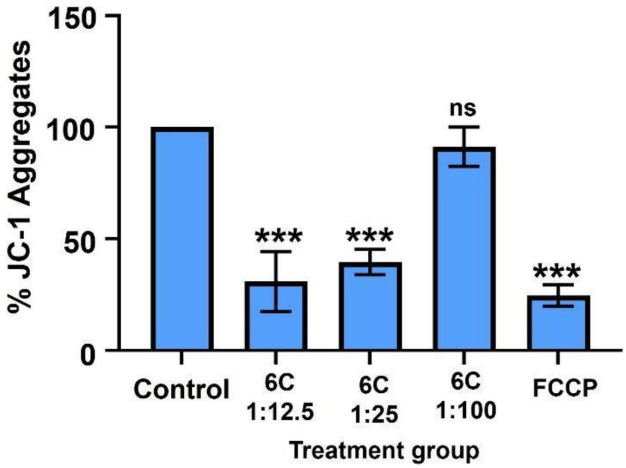


Fig. 2. 6C homoeopathic potency of *T. chebula* regulated MDAMB231 growth through apoptosis by decreasing the mitochondrial membrane potential at 1:12.5 and 1:25 dilutions. The values for $***p < 0.001$ indicated statistically significant data.

reduced to 30.93 ± 13.43% ($p < 0.001$), 39.67 ± 5.69% ($p < 0.001$), and 91.19 ± 8.81% (ns, p = 0.192), respectively, compared to the untreated control cells. The positive control, FCCP, decreased the $\Delta\psi$m to 24.61 ± 4.77% ($p < 0.001$).

6C potency of T. chebula increased mRNA expression of tumor suppressor and pro-apoptotic genes

The effect of 6C homoeopathic potency was evaluated further on the expression of tumor suppressor and proapoptotic genes at mRNA level, and a higher dilution (1:100) of 6C potency was used to treat the cells. 6C increased the mRNA expression of p53, Rb, caspase-3, caspase-9, and cytochrome-c by 4.54 ± 0.58 ($p = 0.001$), 2.97 ± 0.25 ($p < 0.001$), 11.81 ± 1.27 ($p < 0.001$), 4.24 ± 1.73 ($p = 0.0198$), and 15.15 ± 3.84 ($p = 0.0010$)-folds (Fig. 3), respectively, compared to the untreated control cells. Thus, 6C exhibited anticancer activity by regulating mRNA expression of tumor suppressor genes, p53, and Rb, as well as pro-apoptotic genes caspase-3, caspase-9, and cytochrome-c.

6C potency of T. chebula revealed the presence of phytocompounds

MT and 6C potencies were subjected to LCMS analysis and revealed the presence of phytochemicals such as tannins, phenolic acids, flavonoids, triterpenes, ketone, and fatty acids. The compounds were identified by matching the m/z values or observed masses with their monoisotopic masses. A total of 17 compounds were identified in MT (Table 2, Fig. 4)

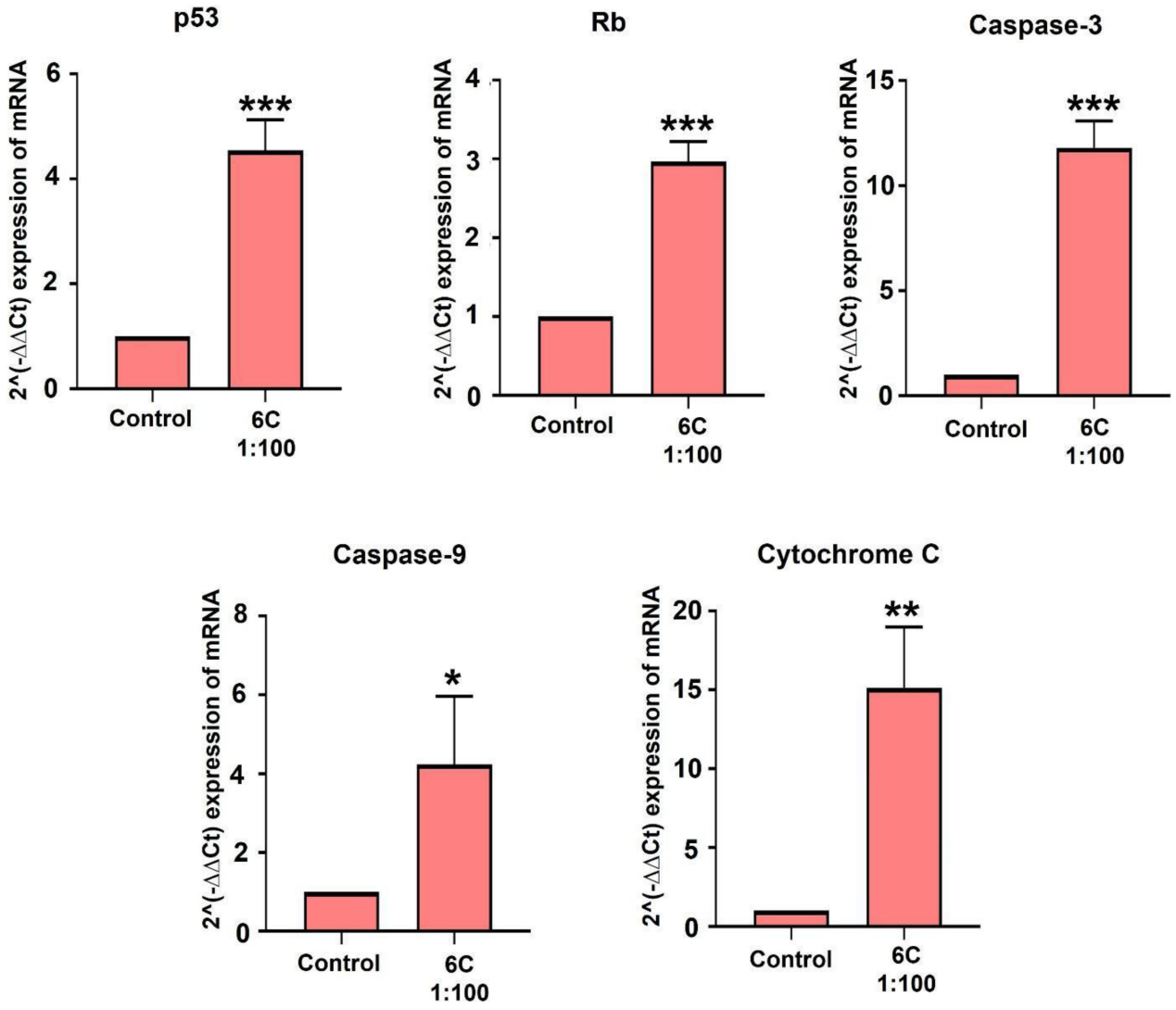


Fig. 3. 6C homoeopathic potency of *T. chebula* regulated MDAMB231 growth through apoptosis and increased mRNA expression of p53, Rb, caspase-3, caspase-9, and cytochrome-c genes at 1:100 dilution.

and 7 compounds were found in 6C potency (Table 3, Fig. 5).

Discussion

Homoeopathic remedies are being used at the clinical level for breast cancer management and are also helpful in other health-related issues.[7,17–21] They signify a substantial part of the global drug market in both developed and developing countries as therapeutic adjuncts to allopathic treatments. Homoeopathy remedies are mostly sourced from plants, herbs, minerals, or animal products.[22]

In the present study, 6C-induced LDH release in the TNBC cell line, MDA-MB231, a measure of cell death.[23] TNBCs are heterogeneous and aggressive in nature, and the absence of ERs, PRs, and HER2 makes their treatment more difficult;[24] hence, a search for anti-cancer agents against TNBCs is the need of the hour. Hydroalcoholic extract of TC was reported to induce lactate release in the HCT-116 human colorectal carcinoma cell line.[25] CO_2 and methanolic extracts of TC have been reported for their anticancer activity against human breast cancer and mouse breast cancer cells; 7,12-dimethylbenz[a]anthracene (DMBA)-induced murine mammary carcinoma model; and Ehrlich ascites carcinoma (EAC)-induced tumour model.[26–30] Various other studies have reported the anticancer activity of TC against other cancer cell types.[28,31–36]

LDH upregulation has been reported to induce apoptosis.[25] 6C induced cell death in MDAMB231 through a decrease in mitochondrial membrane

Table 2. LCMS pattern of compounds identified in MT potency of *T. chebula*.

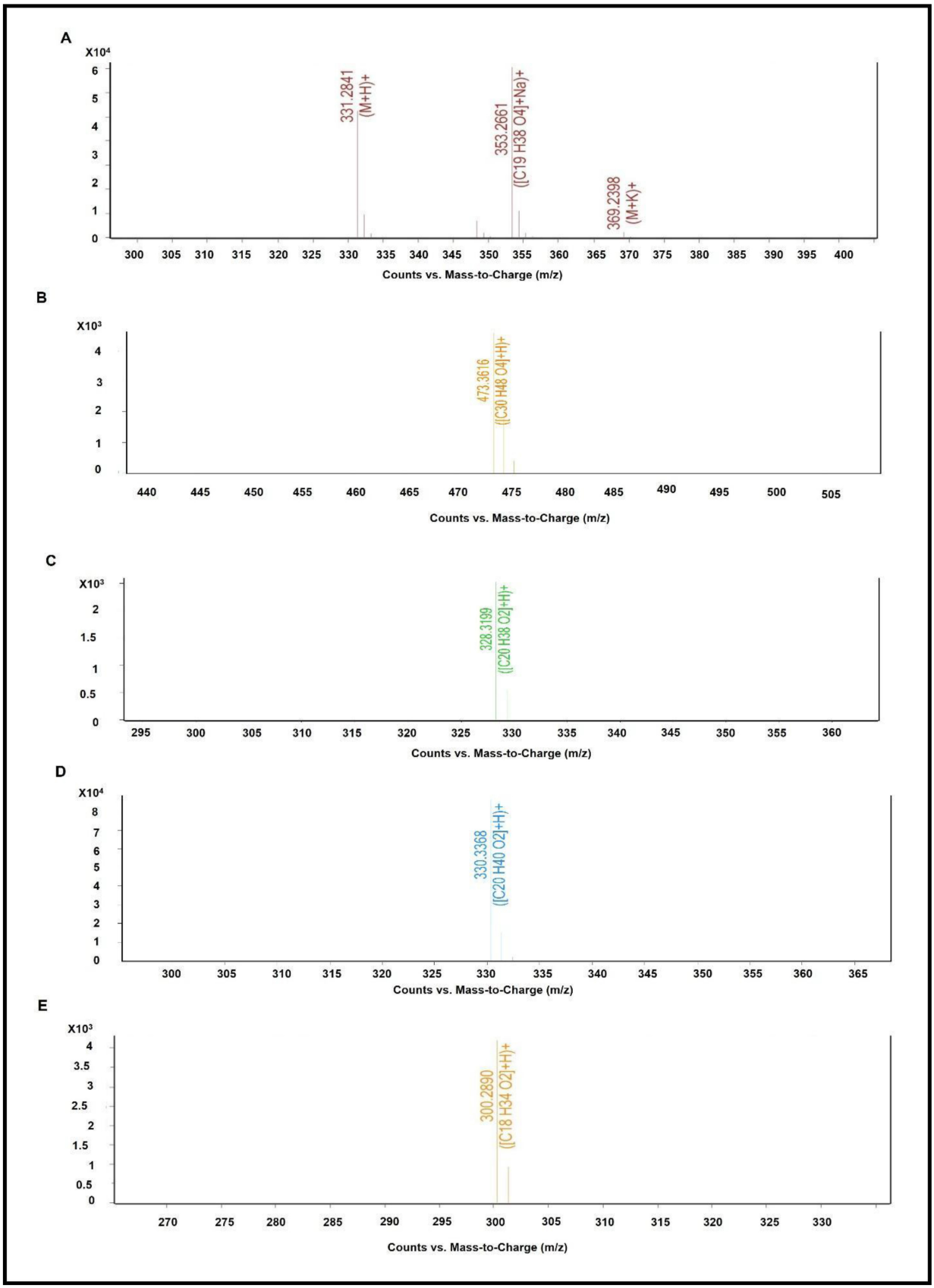
S. No.	Compound	Empirical Formula	Observed RT (min)	m/z	Observed Mass (Da)	DB Mass Error	Abundance
1	1,3-Dihydroxypropan-2-yl palmitate	$C_{19}H_{38}O_4$	18.16	353.2661	330.2768	0.74	70664.46
2	2-alpha-Hydroxyursolic acid	$C_{30}H_{48}O_4$	14.61	473.3616	472.3545	1.73	4590.3
3	9-Octadecenoic acid ethyl ester	$C_{20}H_{38}O_2$	14.02	328.3199	310.2858	4.37	2532.02
4	Arachidic acid	$C_{20}H_{40}O_2$	13.71	330.3368	312.3029	–0.19	86478.79
5	Oleic acid [(9Z)-Octadec-9-enoic acid]	$C_{18}H_{34}O_2$	12.65	300.289	282.2552	2.53	4203.86
6	Chebuloside I	$C_{30}H_{48}O_5$	12.44	506.3824	488.3484	3.77	5376.22
7	Ricinoleic acid	$C_{18}H_{34}O_3$	11.63	316.2846	298.2508	0.06	6933.66
8	Arjunglucoside II	$C_{36}H_{58}O_{10}$	9.88	668.4352	650.4008	3.34	7911.75
9	Chebulanin	$C_{27}H_{24}O_{19}$	9.21	651.085	652.0918	–0.98	4740.4
10	Luteolin	$C_{15}H_{10}O_6$	8.86	287.055	286.0477	–0.04	3281.12
11	Bellericoside	$C_{36}H_{58}O_{11}$	8.61	689.385	666.3959	3.03	4195.99
12	Arjungenin	$C_{30}H_{48}O_6$	8.53	505.3513	504.3439	2.28	9942.68
13	Ethyl gallate	$C_9H_{10}O_5$	5.97	199.0598	198.0526	1.2	7996.91
14	Corilagin	$C_{27}H_{22}O_{18}$	5.12	652.1127	634.0794	1.86	6842.27
15	Punicalin	$C_{34}H_{22}O_{22}$	0.82	800.0913	782.0582	2.71	3849.79
16	2,4,6-Trihydroxybenzoic acid	$C_7H_6O_5$	0.77	169.0148	170.022	–3.02	2592418.25
17	Chebulic acid	$C_{14}H_{12}O_{11}$	0.65	357.0456	356.0383	–0.93	140346.2

Da: Daltons, RT: Retention Time, min: minutes, m/z: mass-to-charge ratio.

potential and an increase in the mRNA expression of caspase 3, 9, and cytochrome c, the key factors involved in the execution of apoptosis.[37] An alcoholic extract of TC was previously shown to induce apoptosis in A549 human lung cancer cells via mitochondria-mediated pathways.[34] 6C increased the mRNA expression of tumour suppressors, p53 and Rb, in the TNBC cell line, which are known to act as checkpoint proteins, regulating cell growth and inducing apoptosis.[38] p53, a prognostic TNBC marker, is a well-studied tumour suppressor protein that is known to regulate cell cycle progression and apoptosis, whereas retinoblastoma (Rb) protein plays a chief role in the regulation of cell cycle and is frequently lost in TNBCs.[39,40] p53 activates several genes involved in apoptosis, leading to mitochondrial membrane depolarization, which further leads to the release of cytochrome c and further activation of caspases. These are a group of cysteine-aspartic proteases that generally exist in the state of inactive zymogens or procaspases, which, upon proteolytic cleavage, get transformed into caspases.[41] Cytochrome c is known to activate the initiator caspase-9, which subsequently activates executioner caspase-3 that in turn inhibits the activity of poly (ADP-ribose) polymerase (PARP), cleaving it and leading to the process of cell death or apoptosis.[26] *T. chebula* has been previously reported to activate p53 in MCF-7 breast cancer cells, leading to the activation of caspases and reducing their viability.[26,42]

Further, *T. chebula* fruit extract has been reported to downregulate Bcl-2 expression and upregulate Bax expression with an increased Bax/Bcl-2 ratio, resulting in elevated levels of cytochrome c, leading to activation of caspase-3 and 9 in MCF-7 cell line.[26] Although Bcl-2 and Bax proteins were not evaluated in the current study, the homoeopathic potency of 6C increased the mRNA expression of tumour suppressor genes, p53, and Rb, as well as pro-apoptotic caspase-3, caspase-9, and cytochrome-c in the TNBC cell line, MDAMB231. The present data has verified the previously advocated studies suggesting the potential of homoeopathic remedies to induce epigenetic modifications.[43–47] Various homoeopathic potencies has been shown to induce cell cycle arrest or apoptosis in cancer cells. Homoeopathic remedies e.g., *Arnica montana* has been reported to induce apoptosis in TNBCs.[37] *Ginnalin A* induced apoptosis and regulated metastasis-associated genes in liver and prostate cancer cells;[22] *Hydrastis canadensis* potencies caused cell cycle arrest at G0/G1 phase and caspase-3 upregulation in breast cancer cells;[48] and *Hepatitis C* 30C induced apoptosis in liver cancer cells.[49]

The phytochemical composition of homoeopathic potencies, MT, and 6C of *T. chebula* has been reported here to understand the underlying mechanism of anticancer activity in breast cancer cells. Recently, *T. chebula* potency has been reported against candidiasis, wherein 33 major phytocomponents such as phenolic acids, flavonoids, tannins, terpenoids were identified.[50] Likewise, in the present study, MT potency showed the presence of chebuloside I and bellericoside (triterpenes); 1,3-dihydroxypropan-2-yl-palmitate (monoacylglyceride) with no reported pharmacological activities. However, it revealed other phytocompounds with reported anticancer activities that include tannins, namely punicalin,[51] corilagin,[52] chebulanin,[53] ethyl gallate;[54] triterpenes



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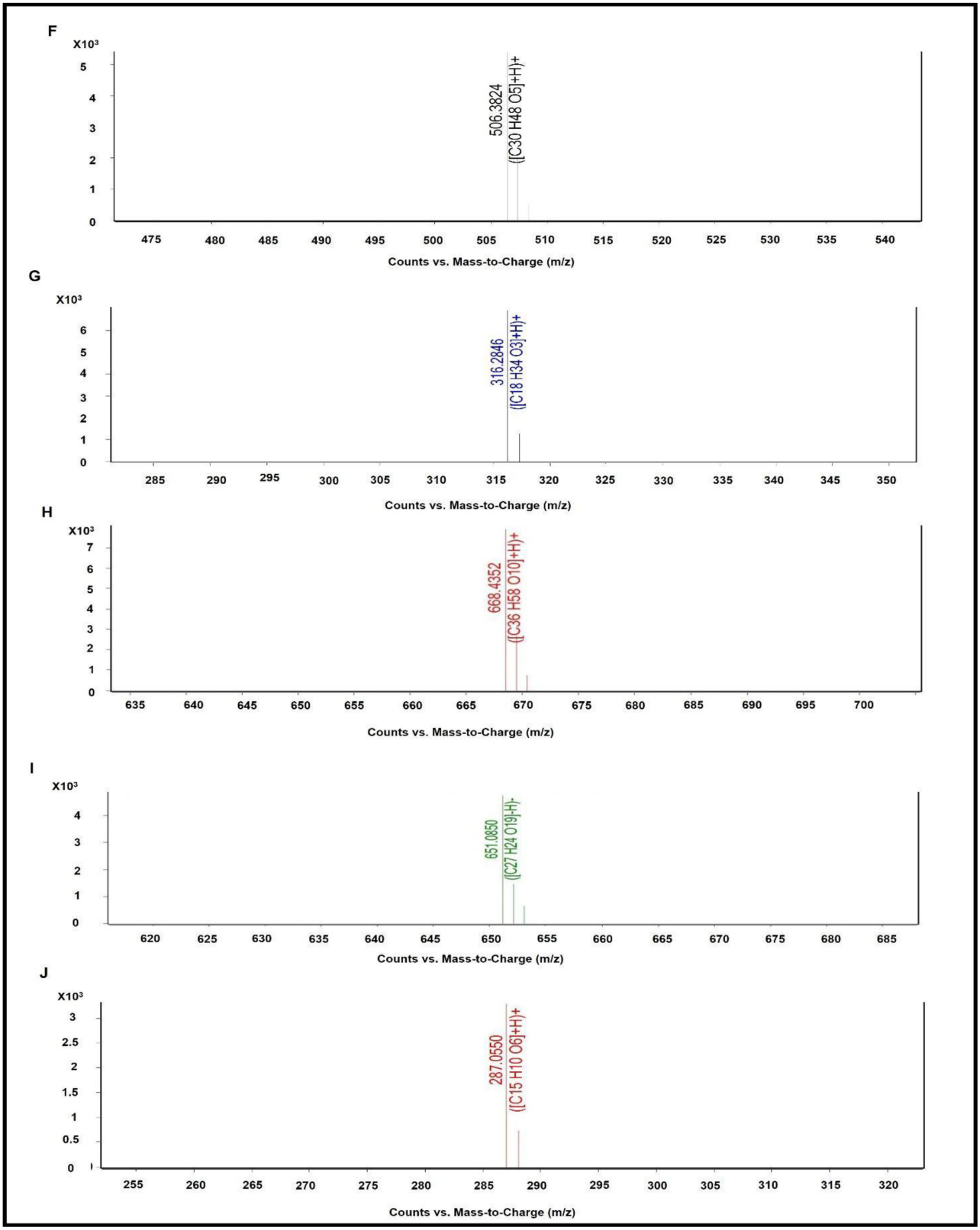

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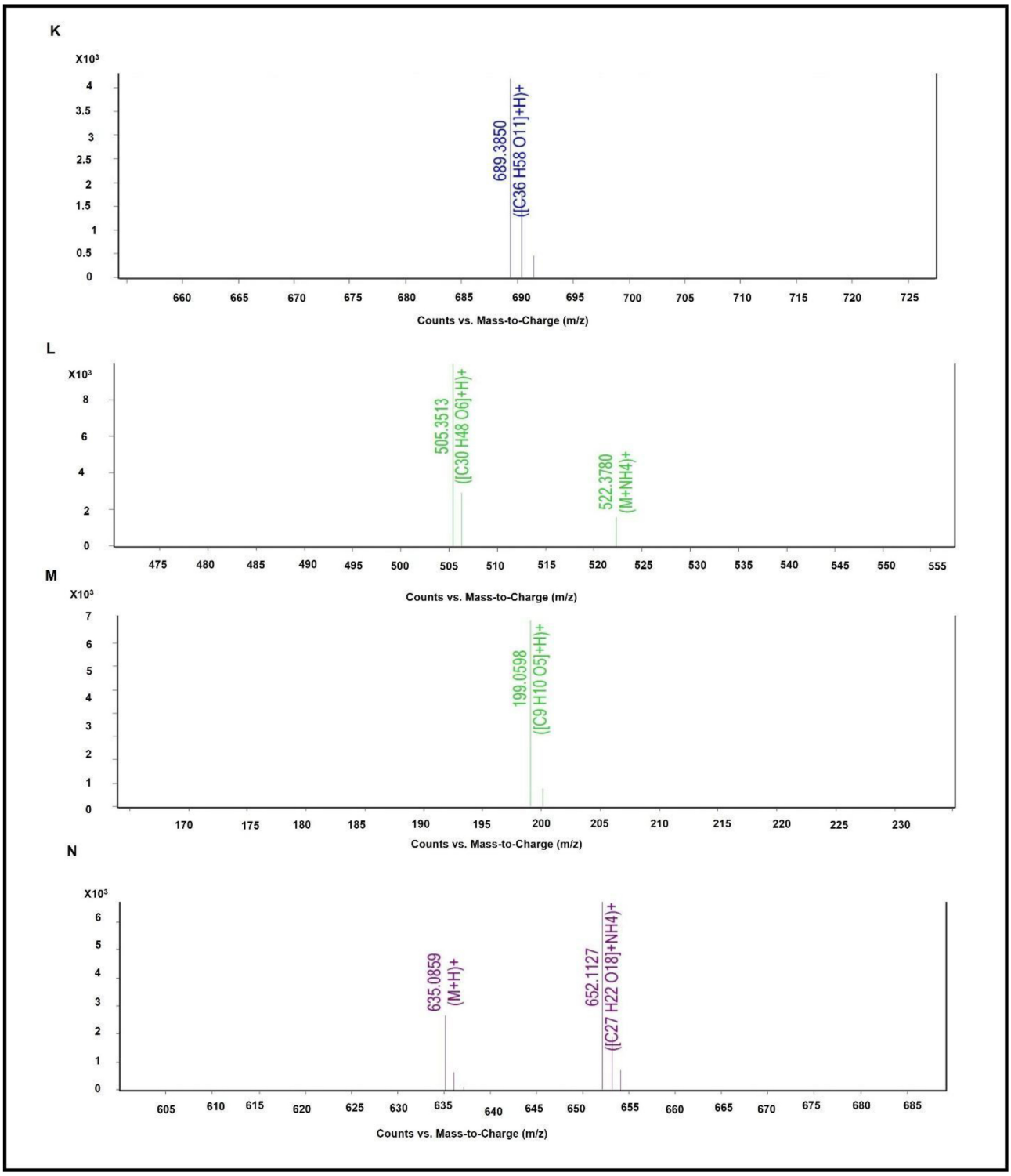

Fig. 4. Continued.

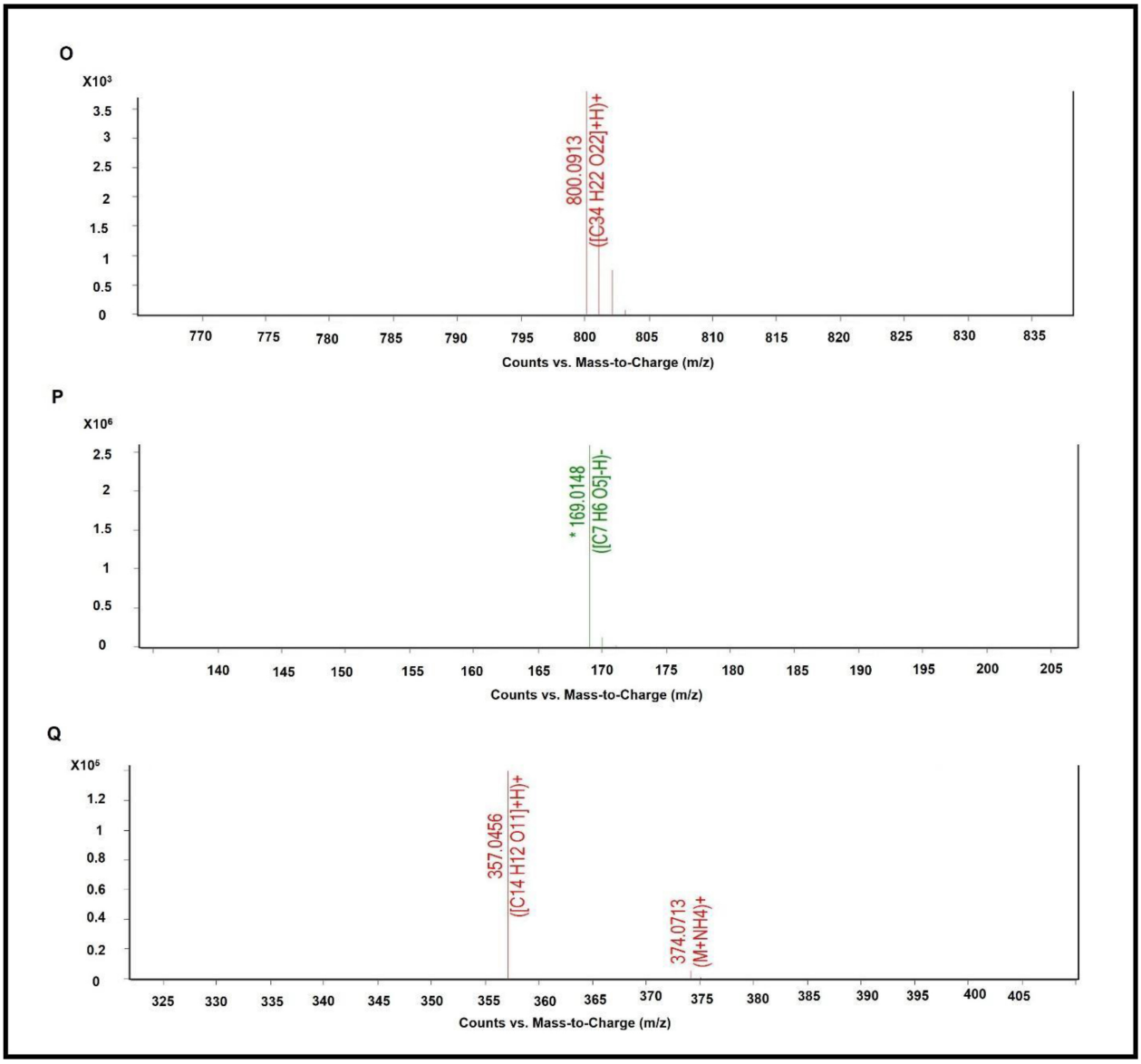

Fig. 4. LCMS pattern of compounds identified in MT potency of TC. 1,3-dihydroxypropan-2-yl palmitate (A), 2α-hydroxyursolic acid (B), 9-octadecenoic acid ethyl ester (C), arachidic acid (D), oleic acid (E), chebuloside I (F), ricinoleic acid (G), arjunglucoside II (H), chebulanin (I), luteolin (J), bellericoside (K), arjungenin (L), ethyl gallate (M), corilagin (N), punicalin (O), 2,4,6-trihydroxybenzoic acid (P), chebulic acid (Q).

Table 3. LCMS pattern of compounds identified in 6c potency of *T. chebula*.

Sr. No.	Compound	Empirical Formula	Observed RT (min)	m/z	Observed Mass (Da)	DB Mass Error	Abundance
1	1,3-Dihydroxypropan-2-yl palmitate	$C_{19}H_{38}O_4$	18.03	353.266	330.2767	1	36758.62
2	Behenic acid	$C_{22}H_{44}O_2$	15.05	358.3674	340.3336	1.59	10034.66
3	Arachidic acid	$C_{20}H_{40}O_2$	13.7	330.3368	312.3029	-0.28	57663.58
4	Stearic acid	$C_{18}H_{36}O_2$	12.34	302.3063	284.2723	–2.97	202284.53
5	Ricinoleic acid	$C_{18}H_{34}O_3$	11.79	316.2847	298.2509	–0.21	7157.36
6	8-Pentadecanone	$C_{15}H_{30}O$	11.21	244.2628	226.2291	2.78	1760.05
7	Palmitic acid	$C_{16}H_{32}O_2$	10.96	274.2753	256.2402	–4.79	1370887.5

Da: Daltons, RT: Retention Time, min: minutes, m/z: mass-to-charge ratio.

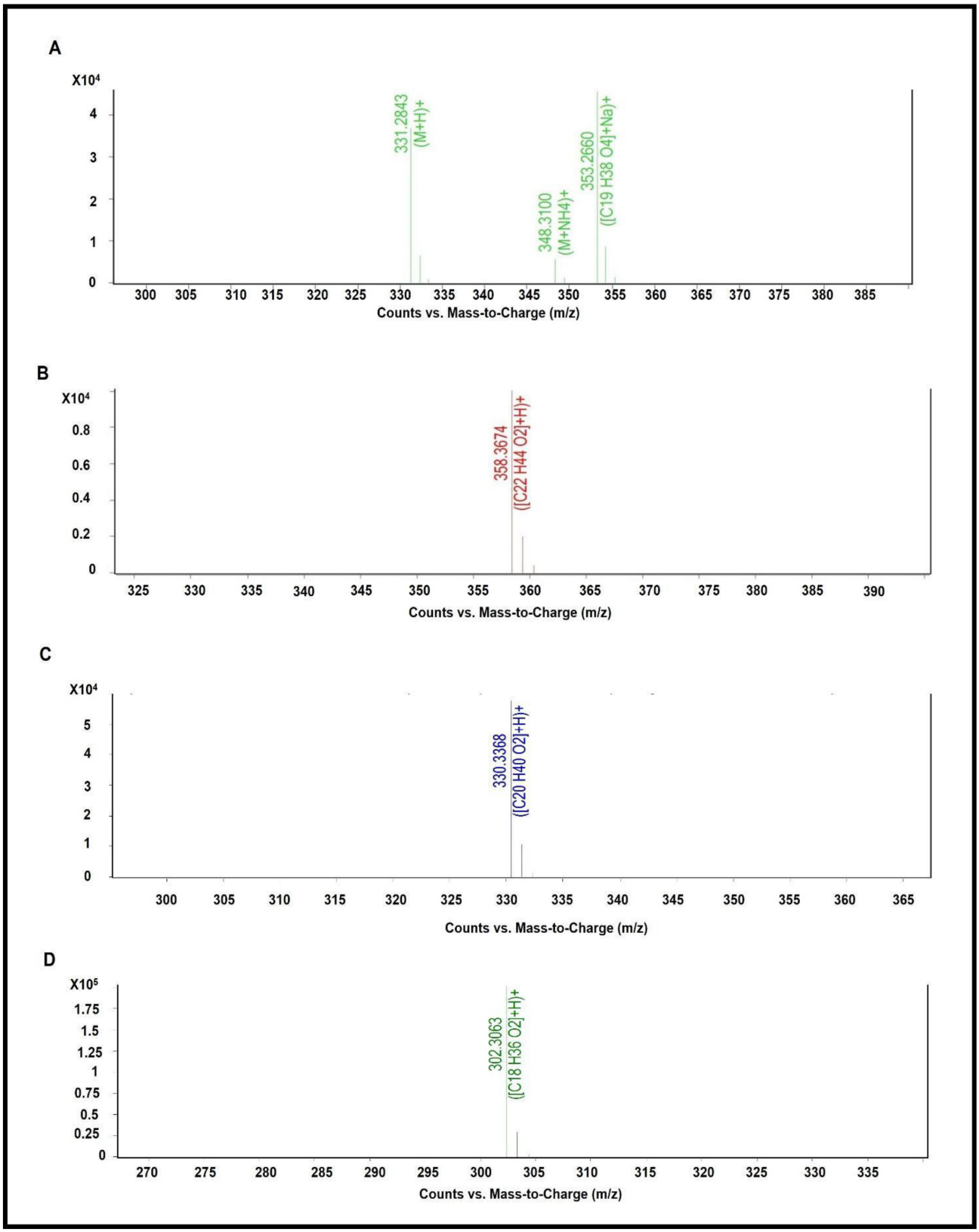

Fig. 5. Continued.

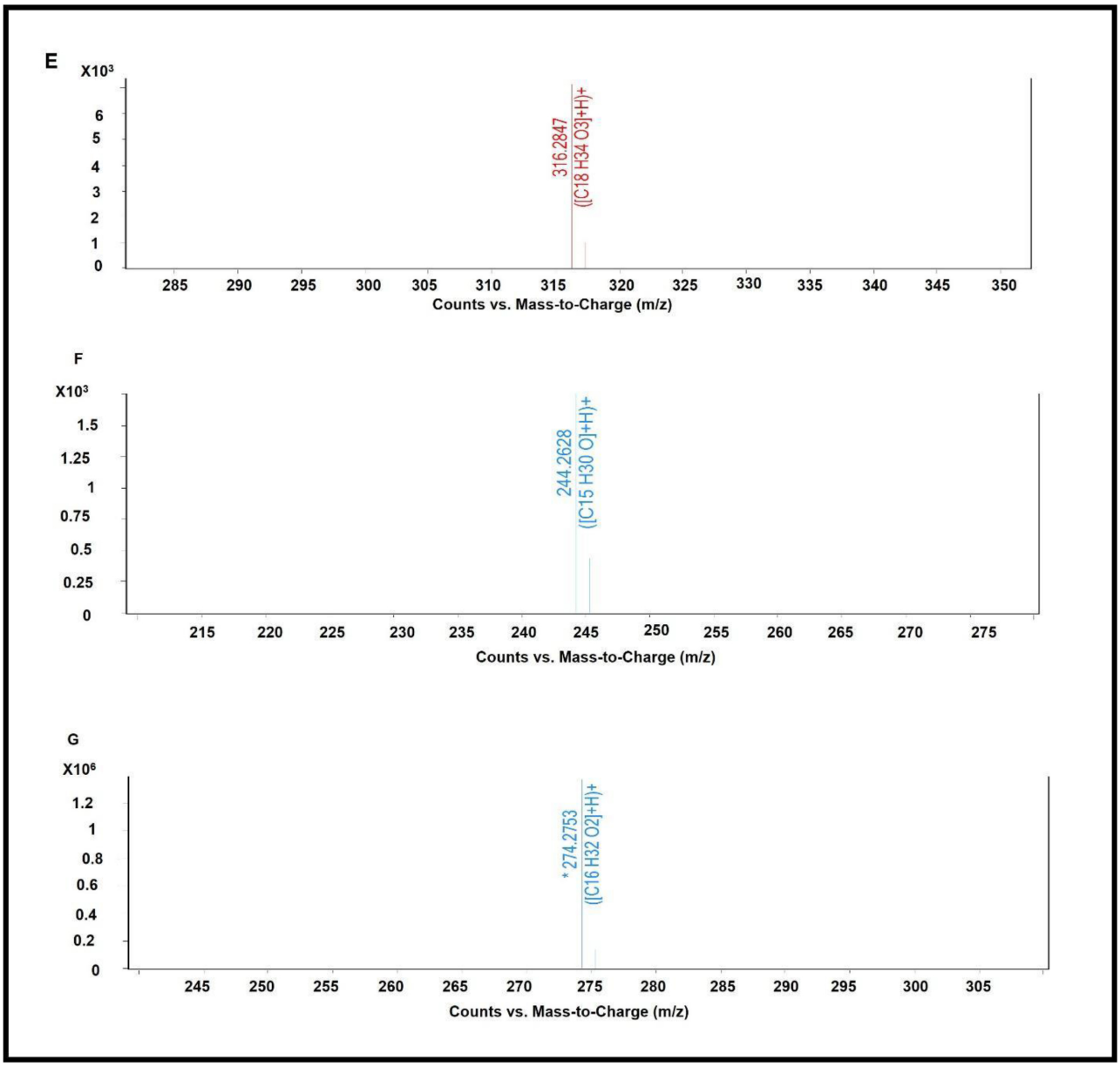

Fig. 5. LCMS pattern of compounds identified in 6C potency of TC. 1,3-Dihydroxypropan-2-yl palmitate (A), behenic acid (B), arachidic acid (C), stearic acid (D), ricinoleic acid (E), 8-pentadecanone (F), palmitic acid (G).

such as 2α-hydroxyursolic acid,[55] arjungenin and arjunglucoside II;[56] 2,4,6-trihydroxy benzoic acid (phenolic acid);[57] luteolin (flavonoid);[58] 9-octadecenoic acid ethyl ester (fatty acid ester);[59] and fatty acids such as ricinoleic acid and oleic acid.[60,61] Other phytoconstituents included arachidic acid (fatty acid) and chebulic acid (phenolic acid) with reported anti-inflammatory and/or anti-oxidant activities.[62,63] On the other hand, 6C contained 1,3-dihydroxypropan-2-yl-palmitate (monoacylglyceride), behenic acid (fatty acid), and 8-pentadecanone (ketone) with no reported pharmacological properties. It also revealed fatty acids, namely ricinoleic acid, palmitic acid, stearic acid with reported anti-cancer activity, and arachidic acid with reported anti-inflammatory property.[60,62,64,65] Interestingly, 6C, the homoeopathic potency of *T. chebula* showed the presence of 1,3-dihydroxypropan-2-yl palmitate, arachidic acid, and ricinoleic acid, which were present in MT as well, thereby showing that the diluted 6C retained few signature compounds of the parent material. Among all the phytocompounds detected from either MT or 6C, 2α-hydroxyursolic acid, chebuloside I, 8-pentadecanone, 9-octadecenoic acid ethyl ester, ricinoleic acid, arjungenin, arjunglucoside II, chebulanin, punicalin, luteolin, ethyl gallate, chebulic acid,

palmitic acid, stearic acid, oleic acid, arachidic acid, behenic acid, and corilagin have been reported earlier in TC extracts.[66] The aqueous extract of *T. chebula* fruit has been reported to contain 32-56% tannins that include gallic acid, ellagic acid, chebulic acid, chebulinic acid, punicalagin, and tannic acid.[67] It also has flavonoids such as quercetin, catechin, and kaempferol.[67]

In the current study, the homoeopathic potency, Mother tincture of *T. chebula*, being the parent material, showed more phytocompounds such as phenolic acids, flavonoids, triterpenes, ketone, and fatty acids. On the other hand, 6C potency, a diluted preparation of MT, revealed less phytocompounds compared to MT. Various studies have reported the presence of phytochemicals in mother tinctures and lower potencies of homoeopathic remedies.[68–72] For example, the MT potency of *Justicia adhatoda* showed a number of phytochemicals, whereas 6C and 30C potencies showed the signature of a few active compounds by UV-vis spectrophotometer, infrared spectroscopy, and LCMS techniques.[69] *Allium cepa* ethanolic extract and its MT potency displayed the presence of steroids, alkaloids, tannins, flavonoids, carbohydrates, saponins, amino acids, cardiac glycosides, anthraquinone glycosides, and proteins.[71] On the other hand, 30C and 200C of *Allium cepa* showed phytocompounds with absence of steroids, flavonoids, amino acids, and anthraquinone glycosides.[71] In the present study, 6C potency of TC revealed phytocompounds with reported anticancer and anti-inflammatory activities. This could be the reason behind the anticancer activity exhibited by 6C via induction of apoptosis in the TNBC cell line.

The study provides explicit evidence for the possible epigenetic modulation of TNBCs by 6C potency via the upregulation of tumour suppressors and pro-apoptotic genes, thereby signifying the future therapeutic potential of 6C in breast cancer patients.

Conclusion

The present study provides strong evidence for the potential role of 6C potency of TC against triple-negative breast cancer, a highly aggressive cancer with almost no therapeutic options. However, further *in vivo* efficacy and safety studies are warranted to understand the in-depth anti-cancer mechanism of 6C in the future.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contribution

Apoorva Parimoo: Literature search, Experimental studies, Data acquisition, Statistical analysis, Manuscript preparation / Writing original draft.
Shama Aphale: Investigation, Formal analysis.
Prajakta D. Patil: Methodology, Data acquisition.
Ruchika Kaul Ghanekar: Concept, Design, Resources, Manuscript review and editing, Supervision, Data analysis & Interpretation.

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La dilution 6C de *Terminalia chebula* révèle des métabolites secondaires et induit l'apoptose dans les cellules de cancer du sein triple négatif (CSTN)

Contexte: Le cancer du sein est une affection maligne fréquente chez les femmes dans le monde. Les CSTN, étant ER-/PR-/Her2-, représentent un défi en matière de choix thérapeutique. Les médecines complémentaires et alternatives, notamment l'homéopathie, sont de plus en plus utilisées comme agents anticancéreux potentiels pour atténuer les effets secondaires des traitements conventionnels. L'activité anticancéreuse des dilutions homéopathiques de *Terminalia chebula* (MT, 3X, 6C et 30C) contre des lignées cellulaires de cancer du sein a déjà été rapportée. **Objectifs:** Cette étude visait à évaluer le mécanisme d'action anticancéreuse de la dilution 6C de *T. chebula* contre la lignée cellulaire de CSTN MDAMB231 et à déterminer la composition phytochimique de cette dilution. **Méthodes:** La libération de lactate a été étudiée par dosage de la LDH et le potentiel de membrane mitochondrial a été évalué par le test JC-1. L'expression des ARNm des gènes suppresseurs de tumeurs et pro-apoptotiques a été analysée par qRT-PCR. Les dilutions de la teinture mère (TM) et de la fraction 6C ont été caractérisées phytochimiquement par analyse LC-MS. **Résultats:** La dilution 6C de la teinture mère a augmenté la libération de lactate dans les cancers du sein triple négatifs (TNBC), indiquant une orientation vers la glycolyse et induisant potentiellement des modifications métaboliques contribuant à son activité anticancéreuse. L'augmentation de la libération de lactate a entraîné une perte du potentiel de membrane mitochondrial et une augmentation de l'expression des ARNm des marqueurs pro-apoptotiques (caspase 3, caspase 9 et cytochrome c) et des protéines suppresseurs de tumeurs (p53 et pRb). L'analyse LC-MS de la dilution 6C a révélé la présence de composés phytochimiques aux activités anticancéreuses, anti-inflammatoires et antioxydantes. **Conclusion:** La puissance 6C de *T. chebula* a induit l'apoptose dans les cancers du sein triple négatifs (TNBC). Cependant, des études d'efficacité et d'innocuité in vivo supplémentaires sont nécessaires pour mieux comprendre le mécanisme anticancéreux de la 6C.

6C-Potenz von *Terminalia chebula* deckt Sekundärmetaboliten auf und induziert Apoptose in dreifach negativen Brustkrebszellen (TNBCs) zusammenfassung

Hintergrund: Brustkrebs ist weltweit eine häufige Krebserkrankung bei Frauen. TNBCs, die ER-/PR-/Her2-negativ sind, stellen eine Herausforderung für die Behandlung dar. Komplementäre und alternative Medizin, einschließlich homöopathischer Mittel, gewinnt zunehmend an Bedeutung als potenzielle Antikrebsmittel zur Linderung der Nebenwirkungen konventioneller Medikamente. Die krebshemmende Wirkung homöopathischer Potenzen von *Terminalia chebula* (MT, 3X, 6C und 30C) gegen Brustkrebszelllinien wurde bereits beschrieben. **Zielsetzung:** Diese Studie untersuchte den Wirkmechanismus der krebshemmenden Wirkung der Potenz 6C von *T. chebula* gegen die TNBC-Zelllinie MDAMB231 und analysierte die phytochemische Zusammensetzung dieser Potenz. **Methoden**: Die Laktatfreisetzung wurde mittels LDH-Assay untersucht, das mitochondriale Membranpotenzial mittels JC-1-Assay bestimmt. Die mRNA-Expression von Tumorsuppressor- und proapoptotischen Genen wurde mittels qRT-PCR analysiert. Die Urtinktur (MT) und die Potenz 6C wurden phytochemisch mittels LC-MS-Analyse charakterisiert. **Ergebnisse:** Die 6C-Potenz von *T. chebula* erhöhte die Laktatfreisetzung in TNBCs, was auf eine Verschiebung hin zur Glykolyse und potenziell auf metabolische Veränderungen hindeutet,

die zu seiner Antitumorwirkung beitragen. Die erhöhte Laktatfreisetzung führte zu einem Verlust des mitochondrialen Membranpotenzials und einer Steigerung der mRNA-Expression proapoptotischer Marker (Caspase 3, Caspase 9 und Cytochrom c) sowie von Tumorsuppressorproteinen (p53 und pRb). Die LC-MS-Analyse der 6C-Potenz identifizierte Phytokomponenten mit antikanzerogenen, entzündungshemmenden und antioxidativen Eigenschaften. **Schlussfolgerung**: Die 6C-Potenz von *T. chebula* induzierte Apoptose in TNBCs. Um den Antitumor-Mechanismus von 6C jedoch eingehend zu verstehen, sind weitere In-vivo-Studien zur Wirksamkeit und Sicherheit erforderlich.

टर्मिनलिया चेबुला की 6C क्षमता ट्रिपल-नेगेटिव ब्रेस्ट कैंसर कोशिकाओं (TNBCs) में द्वितीयक मेटाबोलाइट्स को दर्शाती है और एपोप्टोसिस को प्रेरित करती है।

पृष्ठभूमि: स्तन कैंसर विश्व स्तर पर महिलाओं में प्रचलित एक घातक रोग है, और TNBCs, जो ER-/PR-/Her2- प्रकार की होती हैं, उपचार विकल्पों में चुनौतियां पेश करती हैं। होम्योपैथिक उपचारों सहित पूरक और वैकल्पिक चिकित्सा पद्धतियां, पारंपरिक दवाओं के दुष्प्रभावों को कम करने के लिए संभावित कैंसर-रोधी एजेंटों के रूप में लोकप्रियता प्राप्त कर रही हैं। इससे पहले, स्तन कैंसर कोशिकाओं के विरुद्ध *टर्मिनलिया चेबुला* (MT, 3X, 6C और 30C) की होम्योपैथिक शक्तियों की कैंसर-रोधी गतिविधि की रिपोर्ट की गई है। **उद्देश्य**: इस अध्ययन का उद्देश्य *टर्मिनलिया चेबुला* की 6C शक्ति की TNBC कोशिका रेखा, MDAMB231 के विरुद्ध कैंसर-रोधी गतिविधि की क्रियाविधि का मूल्यांकन करना और 6C शक्ति की पादप रासायनिक संरचना को समझना था। **विधि**: लैक्टेट उत्सर्जन का अध्ययन LDH परख द्वारा किया गया, और माइटोकॉन्ड्रियल झिल्ली विभव का मूल्यांकन JC-1 परख द्वारा किया गया। ट्यूमर सप्रेसर और प्रो-एपोप्टोटिक जीनों की mRNA अभिव्यक्ति का परीक्षण qRT-PCR परख द्वारा किया गया। मदर टिंक्चर (MT) और 6C शक्तियों का पादप रासायनिक लक्षण वर्णन LCMS विश्लेषण द्वारा किया गया। **परिणाम**: *टर्मिनलिया चेबुला* की 6C क्षमता ने टीएनबीसी में लैक्टेट स्राव को बढ़ाया, जो ग्लाइकोलिसिस की ओर बदलाव का संकेत देता है और संभावित रूप से चयापचय संबंधी परिवर्तनों को प्रेरित करता है जो इसकी कैंसर-रोधी गतिविधि में योगदान करते हैं। लैक्टेट स्राव में वृद्धि से माइटोकॉन्ड्रियल झिल्ली क्षमता में कमी आई और प्रो-एपोप्टोटिक मार्करों (कैस्पेस 3, कैस्पेस 9 और साइटोक्रोम सी) और ट्यूमर सप्रेसर प्रोटीन (p53 और pRb) की mRNA अभिव्यक्ति में वृद्धि हुई। 6C क्षमता के LCMS विश्लेषण से कैंसर-रोधी, सूजन-रोधी और एंटीऑक्सीडेंट गतिविधियों वाले फाइटोयौगिकों का पता चला। **निष्कर्ष:** *टर्मिनलिया चेबुला* की 6C क्षमता ने टीएनबीसी में एपोप्टोसिस को प्रेरित किया, हालांकि, 6C के कैंसर-रोधी तंत्र को गहराई से समझने के लिए आगे के इन विवो प्रभावकारिता और सुरक्षा अध्ययनों की आवश्यकता है।

La potencia 6C de *Terminalia chebula* revela metabolitos secundarios e induce apoptosis en células de cáncer de mama triple negativo (CMTN)

Antecedentes: El cáncer de mama es una neoplasia maligna prevalente en mujeres a nivel mundial, y las CMTN, al ser ER-/PR-/Her2-, plantean desafíos en las opciones de tratamiento. Las medicinas complementarias y alternativas, incluyendo los remedios homeopáticos, han ganado popularidad como posibles agentes anticancerígenos para mitigar los efectos secundarios de los fármacos convencionales. Previamente, se ha reportado la actividad anticancerígena de las potencias homeopáticas de *Terminalia chebula* (MT, 3X, 6C y 30C) contra líneas celulares de cáncer de mama. **Objetivos**: Este estudio tuvo como objetivo evaluar el mecanismo de la actividad anticancerígena de la potencia 6C de *T. chebula* contra la

línea celular de CMTN, MDAMB231, y comprender la composición fitoquímica de la potencia 6C. **Métodos:** Se estudió la liberación de lactato mediante el ensayo de LDH y se evaluó el potencial de membrana mitocondrial mediante el ensayo JC-1. La expresión de ARNm de genes supresores de tumores y proapoptóticos se realizó mediante qRT-PCR. Las potencias de la tintura madre (MT) y 6C se caracterizaron fitoquímicamente mediante análisis de LCMS. **Resultados**: La potencia 6C del TC aumentó la liberación de lactato en los TNBC, lo que indica una tendencia hacia la glucólisis y podría inducir cambios metabólicos que contribuyen a su actividad anticancerígena. El aumento de la liberación de lactato provocó la pérdida del potencial de membrana mitocondrial y un aumento de la expresión de ARNm de marcadores proapoptóticos (caspasa 3, caspasa 9 y citocromo c) y proteínas supresoras de tumores (p53 y pRb). El análisis de LCMS de la potencia de 6C reveló fitocompuestos con actividades anticancerígenas, antiinflamatorias y antioxidantes. **Conclusión**: La potencia del 6C de *T. chebula* indujo apoptosis en TNBC; sin embargo, se justifican estudios adicionales de eficacia y seguridad in vivo para comprender a fondo el mecanismo anticancerígeno del 6C.

诃子6C效价揭示次生代谢产物并诱导三阴性乳腺癌细胞（TNBC）凋亡 摘要背景：乳腺癌是全球女性最常见的恶性肿瘤之一，而雌激素受体（ER）阴性、孕激素受体（PR）阴性、Her2阴性的三阴性乳腺癌（TNBC）给治疗带来了挑战。包括顺势疗法在内的补充和替代医学作为潜在的抗癌药物，因其能够减轻传统药物的副作用而日益受到关注。此前已有报道称，诃子顺势疗法效价（MT、3X、6C和30C）对乳腺癌细胞系具有抗癌活性。**目的**：本研究旨在评估诃子6C效价对三阴性乳腺癌细胞系MDAMB231的抗癌机制，并了解其植物化学成分。

方法：采用乳酸脱氢酶（LDH）测定法检测乳酸释放，采用JC-1测定法评估线粒体膜电位。采用实时定量PCR（qRT-PCR）法检测抑癌基因和促凋亡基因的mRNA表达。采用液相色谱-质谱联用（LCMS）分析对母酊（MT）和6C效价的植物化学成分进行表征。**结果**：TC的6C效价可增加三阴性乳腺癌（TNBC）细胞的乳酸释放，表明其代谢途径向糖酵解转变，并可能诱导有助于其抗癌活性的代谢变化。乳酸释放的增加导致线粒体膜电位下降，并增加促凋亡标志物（caspase 3、caspase 9和细胞色素c）和抑癌蛋白（p53和pRb）的mRNA表达。LCMS分析显示，6C效价中含有具有抗癌、抗炎和抗氧化活性的植物化合物。**结论**：诃子6C的效力可诱导三阴性乳腺癌细胞凋亡，但仍需进一步开展体内疗效和安全性研究，以深入了解6C的抗癌机制。