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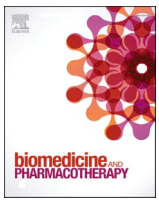

3,3'4-trimethoxy-4'-rutinosylellagic acid and its acetylated derivative: Antioxidant activity and antiproliferative effects on breast cancer cells and molecular docking study

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ABSTRACT

Cancers account for many deaths worldwide and natural compounds and their derivatives are interesting chemotherapeutic agents for cancer drug development. In this study, a natural compound 3,3'4-trimethoxy-4'-rutinosylellagic acid (**TR2**) and its acetylated derivative 3,3'4-trimethoxy-4'-hexaacetylrutinosylellagic acid (**TR22**) were evaluated for their antioxidant and anticancer effects against estrogen sensitive (MCF-7) and estrogen non-sensitive (MDA-MB 231) breast adenocarcinoma. In the β-Carotene-linoleic acid assay, $DPPH^{\bullet}$ radical scavenging and CUPRAC assay, the compound TR2 had better activity than the standard α-Tocopherol, while in the $ABTS^{\bullet+}$ assay, it was more active than both standards α- α-Tocopherol and BHA. Both compounds had good antioxidant effects with TR2 being more active than TR22. Both compounds inhibited growth of breast carcinoma cells when compared to the untreated controls after 72 h. Compound TR22 significantly ($p < 0.001$) inhibited proliferation of both MCF-7 and MDA-MB 231 breast carcinoma cell lines suggesting that acetylation reaction improves inhibition of breast cancer cells growth. On the contrary, TR2 exhibited better inhibitory effect of clone formation than TR22 suggesting that acetylation reduces the activity in this assay. Both compounds inhibited migration of the cancer cells when compared to the untreated control cells and compound TR2 exhibited greater cellular anti-migration effect than TR22 at the same concentration and after the same period of incubation. Molecular docking studies supplemented the results and revealed that TR2 and TR22 had appreciable interactions with tyrosine kinase with negative binding energies suggesting that they are potent receptor tyrosine kinase inhibitors which can impede on cancer progression.

1. Introduction

Cancers constitute the second most fatal pathology worldwide, occurring in various forms and affecting different tissues, with the most prevalent being prostate cancer and breast cancer in men and women respectively [1,2]. The overall prevalence of cancers is always increasing, and it is expected to reach 28.4 million cases by 2040. Globally, new cancer patients were approximately 19.3 million with over 10.0 million cancer deaths out of which breast cancer alone accounted for an estimated 2.3 million new cases in 2020 [3]. Population growth, aging, socioeconomic factors as well as increased cancer risk factors contribute to the rapid increase of cancer incidence across

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the globe. In sub-Saharan Africa alone, 801 392 new cases with 520 158 fatalities resulted from cancer amongst which about 129 400 cases were breast cancers [4]. Breast cancer alone accounted for over 2.3 million new cases and 685000 deaths in 2020, with most cases occurring in transitioning countries and it is predicted to rise to more than 3 million new cases and 1 million deaths by 2040 with population growth and aging being the major causes [5]. Multifaceted research on breast cancers, reduction of the resulting mortalities and establishment of guidelines and treatment/control strategies especially in low and middle income countries is a global health priority [6,7].

Changes in cell functions and gene mutations are mostly accountable for the onset of cancers. Environmental stress factors, smoking and various carcinogenic chemicals contribute to gene mutations and development of cancer cells [8]. Such chemicals can indirectly or directly affect the nucleus and cytoplasm of cells creating gene mutations and genetic disorders, which can lead to cancer development [9–11]. Radiations, heavy metals and microorganisms like some viruses are also amongst the carcinogenic factors, which account for about 7 % of the different forms of cancers [12]. Cancers mostly disrupt normal cellular activities and gene functions that interplay in the cell cycle and division, leading to abnormal proliferation [13]. Summarily, sedentary lifestyles including non-breastfeeding, alcoholism and smoking, exposure to sunlight and other radiations, chemicals, hormones, pharmaceuticals, wood dust, microorganisms including fungi, viruses and bacteria, consumption of low fiber diets, salted fish, beta carotene, herbs, obesity, red and processed meats are amongst the cancer risk factors [14–16].

Oxidative stress also interferes in the onset of cancers and describes the relative excess of reactive oxygen species (ROS) in relation to antioxidants in living systems [17–20]. It has been associated with numerous illnesses, including diabetes mellitus, cardiovascular disease, and neurodegenerative diseases [18,21–23]. Reactive oxygen species (ROS) either initiate or stimulate carcinogenesis, promote the transformation and proliferation of cancer cells, and tumor cells alter NADPH synthesis, sulfur-based metabolism, and the activity of antioxidant transcription factors to adapt to elevated ROS levels [24–26]. Greater pharmacological success can therefore be achieved by comprehending how oxidative stress and redox signaling contribute to pathology and by targeting oxidative stress through antioxidant mechanisms in diseases such as cancer, chronic obstructive pulmonary disease, atherosclerosis, and Alzheimer's disease [21,27]. As oxidative stress from high ROS accumulation, resulting from metabolic disruptions and signaling aberrations, causing activation of pro-oncogenic signaling and promoting carcinogenesis and malignant progression, antioxidant therapies offer a compelling rationale for targeting cancer treatment as well [28,29].

High tumor-inhibiting effects is exhibited by natural products, particularly phenolic antioxidants and there is a significant interest in developing novel, cheap and highly effective antioxidant and anticancer medications from natural resources [30–32]. It can be observed that antioxidants can prevent oxidative stress as well as the onset of cancers. Ellagic acid and its derivatives are natural phenolic compounds with potent antioxidant effects. This study aimed at investigating the antioxidant properties of 3,3'4-trimethoxy-4'-rutinosylellagic acid and its acetylated derivative as well as their cytotoxic effects against breast cancer cell lines.

2. Materials and methods

2.1. Extraction, isolation and characterization of TR2

Extraction, isolation and characterization of TR2 (3,3'4-trimethoxy-4'-rutinosylellagic acid) from the plant *Pterocarpus erinaceus* was performed as described previously [33].

2.2. Hemisynthesis and purification of TR22

In a test tube, 30 mg of 3,3′4-trimethoxy-4′-rutinosylellagic acid (TR2) was dissolved in 5 mL of acetic anhydride, then 5 mL pyridine was added to the reaction medium. The mixture was left in the dark for 24 hours. The solution was transferred to a separatory funnel and suspended in distilled water. A liquid-lipid extraction was carried out using methylene chloride several times. After separation of the two phases, organic phase was dried on a rotary evaporator under reduced pressure to give 56.1 mg of residue. Purification on silica gel (0.063–0.200 mm) column chromatography (10 × 200 mm) of this residue using an isocratic elution with the mixture CH_2Cl_2-MeOH (97.5:2.5, v/v) yielded 51.4 mg of the acetylated derivative, 3,3',4-Trimethoxy-4' hexaacetylrutinosylellagic acid (TR22).

2.3. Bioassays

2.3.1. Antioxidant activity

The antioxidant potential of the compounds (TR2 and TR22) was measured using four different methods: β-carotene-linoleic acid assay, DPPH (2,2-diphenyl-1-picrylhydrazylhydrazyl) radical scavenging assay, ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid) radical cation, and CUPRAC (cupric reducing antioxidant capacity). A prior work [34,35] described the β-carotene-linoleic acid assay, which was used to assess inhibition of lipid peroxidation. As previously reported [36,37], spectrophotometric methods were utilized to assess the radical scavenging potentials on $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. CUPRAC was measured using a technique that has been previously described [38]. To compare the activity of the compounds, α-tocopherol and BHA (Butylated hydroxyanisole) were used as antioxidant standards in the aforementioned experiments.

2.3.2. Cell lines and cell cultures

Human estrogen non-sensitive (MDA-MB 231) and sensitive (MCF-7) cell lines were supplied by American Type Culture Collection (ATC/LGC) Promochem (Wesel, Germany). MCF-7 and MDA-MB 231 cells were grown and subcultured in RPMI-1640 media supplemented with 10 % fetal bovine serum (FBS), and 1 % penicillin (100 U/mL)/streptomycin (100 g/mL). They were incubated in a humid 5 % CO_2 incubator maintained at 37°C and pH of 7.4. For cell passage, 90 % of the supernatant was replaced with fresh medium every two days. Before each experiment, the number of viable cells was estimated through the enzyme-linked immunosorbent assay (ELISA) Multiskan TECAN reader counter system.

2.3.3. Evaluation of the inhibition of cell growth and proliferation by compounds

2.3.3.1. Assessment of cell growth. Cell growth was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (Roche Diagnostics, Penzberg, Germany). Treated versus non- treated MCF-7 and MDA-MB 231 cells (100 µL, 1×10^5 cells/mL) were seeded onto 96-well plates. TR2 & TR22 were dissolved in DMSO, and tested in final concentrations of 2.5–100 µg/mL. Controls was exposed to vehicle (DMSO 0.01 %), only. At 0, 24, 48 and 72 h, 10 µL of MTT (5 mg/mL) was added and incubated at 37°C, 5 % CO_2 for 2 h; afterward the cells were lysed in a buffer containing 10 % SDS in 0.01 M HCl for 2 additional hours. Absorbance at 550 nm was determined for each well using a microplate ELISA TECAN© SPARK reader (Crailsheim, Germany).

2.3.3.2. Assessment of cell proliferation. The ability of TR2 & TR22 to inhibit the cell proliferation was determined by the stable and non-toxic GLPBio Cell Counting Kit-8 (CCK-8) (Hamburg, Germany). Treated versus non-treated MCF-7 and MDA-MB 231 cells (100 µL, 1×10^4 cells/

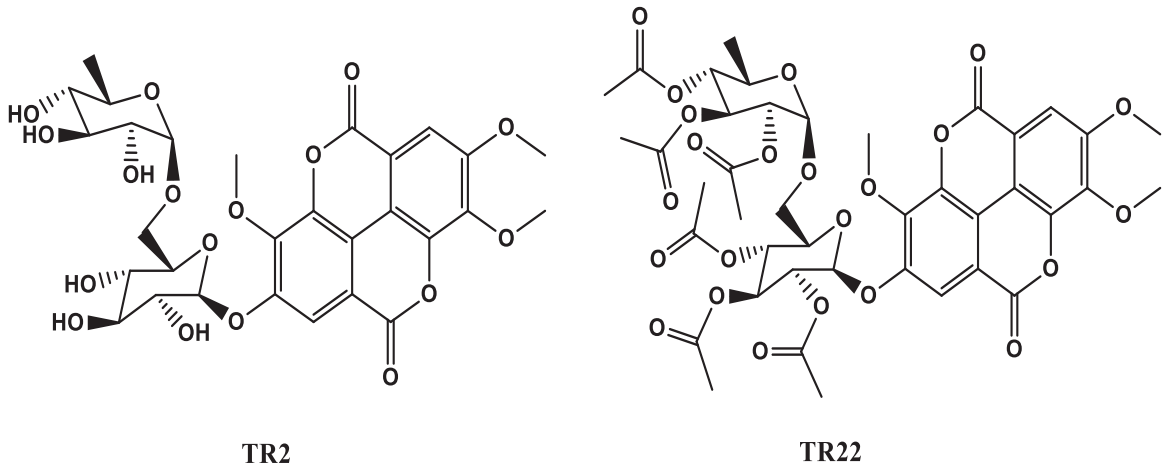

Fig. 1. structures of compounds TR2 and TR22.

mL) were seeded onto 96-well plates and incubated (5 % CO_2, 37°C) for 24 hours. Thereafter 10 µL of TR2 & TR22 were tested at concentrations of 10–20 µg/mL, while control was exposed to vehicle (DMSO 0.01 %). After 48 h of incubation, 10 µL of CCK8 solution was added to each well of the plate and incubated for 4 hours (5 % CO_2, 37°C). Before plates were gently homogenate on a shaker and absorbance read at 450 nm using a microplate TECAN reader.

2.3.3.3. Assessment of the inhibition of clone formation. Treated versus non-treated MDA-MB 231 cells were transferred onto 6-well plates at 500 cells per well. TR2 & TR22 were then added at optimal concentration. Following 7 days incubation, colonies ≥ 50 cells were counted. The number of clones in control was set to 100 % and compared to the number of clones of treated tumor cells.

2.3.3.4. Assessment of anti-metastatic potential using Wound-healing assay. This assay assesses the inhibition of MDA-MB 231 cell migration in presence of TR2 & TR22. The cells were seeded on 6-well plates at 5×10^5 cells/well and leave to grow until the confluence. Furthermore, a scratch wound was created using a 100 µL pipette tip and then washed twice with PBS to remove cells mechanically detached. Four hours before the creation of wound, the medium was replaced by a serum-free RPMI 1640 medium. TR2, TR22 or control solvent (DMSO) were added in serum-free RPMI 1640 medium and cells were allowed for 72 h. The variation in recovery of the wounded area by migrating cells was recorded under a fluorescent microscope (10×) Olympus CK2/ULWCCD 0.030 (Olympus, Japan). Microphotographs were made every 24 h and area of wound healing was evaluated by ImageJ ® software.

2.4. Molecular docking study

Further to the experimental investigation of the cytotoxic effects against breast cancer cell lines of 3,3'4-trimethoxy-4'-rutinosylellagic acid (**TR2**) and its acetylated derivative (**TR22**), their binding affinities into the binding site of tyrosine kinase, the enzyme involved in the replication of cancer cells have been determined using molecular docking. The tyrosine kinase and its original ligand were downloaded from the RCSB database (PDB 1T46) [39]. The validity of the molecular docking is investigated by re-docking the original ligand into the binding site of tyrosine kinase, which yields an RMSD of 0.71Å and -14.41 kcal-mol^{-1} binding energy. Further details on molecular docking steps may be found in our previously reported study [40]. Molecular docking calculations have been carried out using the Autodock package [41].

2.5. Statistical analysis

GraphPad Prism software version 5.00 was used for data analysis. Each experiment was repeated at least thrice. Data from 3 or more groups with one variable were analyzed by ANOVA followed by Dunnett's post-hoc test for multiple comparison. Statistical significance was set at p value < 0.05.

3. Results

3.1. Characterization of compounds TR2 and TR22

3.1.1. 3,3'4-trimethoxy-4'-rutinosylellagic acid (TR2)

White solid. ESI-TOF-MS: [M+H] at m/z = 653. IR: 3375, 2825, 1715, 1610, 1500, 1375, 1060 cm^{-1}. ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H **ppm** 7.62 (1 H, s, H-5), 7.83 (1 H, s, H-5′), 4.05 (3 H, s, 3-OCH_3), 4.00 (3 H, s,4-OCH_3), 4.11 (3 H, s,3′-OCH_3), 5.16 (1 H, d, J = 8,7 Hz, H-1''), 3.64 (1 H, m, H-2''), 3.55 (1 H, m, H-3''), 3.84 (1 H, m, H-4''), 3.49 (1 H, m, H-5''), 3.45 (2 H, m, H-6''), 4.50 (1 H,d, J = 0,99 Hz; H-1'''), 3.47 (1 H, m, H-2'''), 3.31 (1 H, m, H-3'''), 3.29 (1 H, m, H-4'''), 3.42 (1 H, m, H-5'''). ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 112.7 (C-1), 140.9 (C-2), 140.1 (C-3), 154.2 (C-4), 107.4 (C-5), 112.4 (C-6), 157.9 (COO-), 113.6 (C-1′), 141.1 (C-2′), 141.8 (C-3′), 151.6 (C-4′), 112.4 (C-5′), 112.2 (C-6′), 158.2 (COO-), 61.3 (3-OCH_3), 56.7 (4-OCH_3), 61.6 (3′-OCH_3), 101.4 (C-1''), 78.2 (C-2''), 75.7 (C-3''), 73.9 (C-4''), 77.3 (C-5''), 68.6 (C-6''), 100.4 (C-1'''), 71.4 (C-2'''), 72.1 (C-3'''), 72.2 (C-4'''), 69.7 (C-5'''), 17.9 (C-6''').

3.1.2. 3,3'4-trimethoxy-4'-hexaacetylrutinosylellagic acid (TR22)

White solid. ESI-TOF-MS: [M+Na] at m/z = 927.7. IR: 2825, 1750, 1620, 1243, 1033 cm^{-1}. ^{1}H NMR (DMSO-d_6, 500 MHz): δ_H **ppm** 7.61 (1 H, s, H-5), 7.81 (1 H, s, H-5′), 4.05 (3 H, s, 3-OCH_3), 4.00 (3 H, s,4-OCH_3), 4.11 (3 H, s, 3′-OCH_3), 5.16 (1 H, d, J = 8.7 Hz, H-1''), 3.64 (1 H, m, H-2''), 3.55 (1 H, m, H-3''), 3.84 (1 H, m, H-4''), 3.49 (1 H, m, H-5''), 3.45 (2 H, m, H-6''), 4.50 (1 H,d, J = 0.99 Hz; H-1'''), 3.47 (1 H, m, H-2'''), 3.31 (1 H, m, H-3'''), 3.29 (1 H, m, H-4'''), 3.42 (1 H, m, H-5'''), 2.06 (2'''-COO-CH_3), 2.05 (3''-COO-CH_3), 2.01 (4''-COO-CH_3), 1.98 (2'''-COO-CH_3), 1.98 (3'''-COO-CH_3), 1.87 (4''-COO-CH_3). ^{13}C NMR (DMSO-d_6, 125 MHz): δ_C 112.9 (C-1), 140.9 (C-2), 141.9 (C-3), 154.3 (C-4), 107.5 (C-5), 112.4 (C-6), 158.1 (COO-), 113.8 (C-1'), 141.2 (C-2'), 141.9 (C-3'), 151.8 (C-4'), 112.6 (C-5'), 112.5 (C-6'), 158.4 (COO-), 61.4 (3-OCH_3), 56.8 (4-OCH_3), 61.7 (3'-OCH_3), 102.3 (C-1''), 74.0 (C-2''), 71.5 (C-3''), 68.4 (C-4''), 73.4 (C-5''), 65.6 (C-6''), 100.5 (C-1′''), 70.9 (C-2′''), 71.1 (C-3′''), 72.3 (C-4′''), 68.7 (C-5′''), 18.3 (C-6′''), 169.6 (2''-COO-), 169.5 (3''-COO-), 169.3 (4''-COO-), 169.1 (2'''-COO-), 169.2 (3'''-COO-), 169.1 (4'''-COO-), 20.4 (2''-COO-CH_3), 20.4 (3''-

Table 1
Antioxidant activity of the compounds.

Sample	β-Carotene-linoleic acid	DPPH•	ABTS•+	CUPRAC
	IC_{50} (μg/mL)	**IC_{50} (μg/mL)**	**IC_{50} (μg/mL)**	**$A_{0.50}$ (μg/mL)**
TR2	3.87 ± 0.92	25.31 ± 0.85	11.58 ± 0.39	40.10 ± 0.67
TR22	5.81 ± 0.75	32.70 ± 0.36	24.65 ± 0.21	51.10 ± 0.44
α-Tocopherol	4.50 ± 0.22	41.35 ± 0.71	34.80 ± 0.21	63.17 ± 0.52
BHA	2.10 ± 0.31	22.41 ± 0.50	15.25 ± 0.11	28.33 ± 0.42

Values are means ± SEM of triple measurements ($p < 0.05$).

COO-CH_3), 20.3 (4''-COO-CH_3), 20.3 (2'''-COO-CH_3), 20.2 (3'''-COO-CH_3), 20.2 (4'''-COO-CH_3).

The structures of compounds TR2 and TR22 are presented on Fig. 1. The IR and NMR spectra of the compounds are provided in the supplementary material (Figures S1-S8) and complete NMR data on Table S1.

3.2. Antioxidant activity of TR2 and TR22

The antioxidant effects of the compounds was evaluated through four (β-Carotene-linoleic acid, DPPH•, ABTS•+, CUPRAC assays) complementary assays and the results are presented on Table 1. In the β-Carotene-linoleic acid assay, DPPH• radical scavenging and CUPRAC assay, the compound TR2 had better activity than the standard α-Tocopherol while in the ABTS•+ assay, it was more active (IC_{50} = 11.58 ± 0.39 μg/mL) than both standards α-Tocopherol (IC_{50} = 34.80 ± 0.21 μg/mL) and BHA (IC_{50} = 15.25 ± 0.11 μg/mL). The compound TR22 had greater antioxidant capacity than α-Tocopherol in the DPPH•, ABTS•+ and CUPRAC assays. The overall results indicated good antioxidant effects for both compounds with TR2 being showing greater antioxidant potential than TR22.

3.3. Inhibitory effects of breast cancer cell growth by the compounds

The inhibitory effects of the compounds TR2 and TR22 were evaluated against the two breast cancer cell lines, that is, estrogen-sensitive MCF-7 and non-sensitive MDA-MB 231 at 3.75, 7.5, 15 and 30 μg/mL overtime at 0 h, 24 h, 48 h and 72 h and the results presented on Fig. 2. It is generally observed that, the inhibitory effects followed a concentration-dependent trend. In all experiments, the compounds inhibited cell growth when compared to the untreated controls after 72 h. TR2 exhibited the most potent inhibitory effect in both MCF-7 and MDA-MB 231 cells with high magnitude at 15 μg/mL ($p < 0.05$) and 30 μg/mL ($p < 0.01$). TR22 significantly reduced the MCF-7 cell growth after 72 h and failed to do so in MDA-MB 231 cells.

3.4. Inhibitory effects of cell proliferation by the compounds

The inhibitory effects of the compounds on the cell proliferation of both breast carcinoma, estrogen-sensitive MCF-7 and non-sensitive MDA-MB 231, were evaluated at 10 and 20 μg/mL and presented on Fig. 3. Compound TR2 significantly ($p < 0.05$) inhibited the proliferation of estrogen-sensitive MCF-7 when compared to the untreated control but did not exhibited any visible inhibition of the non-sensitive MDA-MB 231 cell lines. Compound TR22 significantly ($p < 0.001$) inhibited proliferation of both estrogen-sensitive MCF-7 and non-

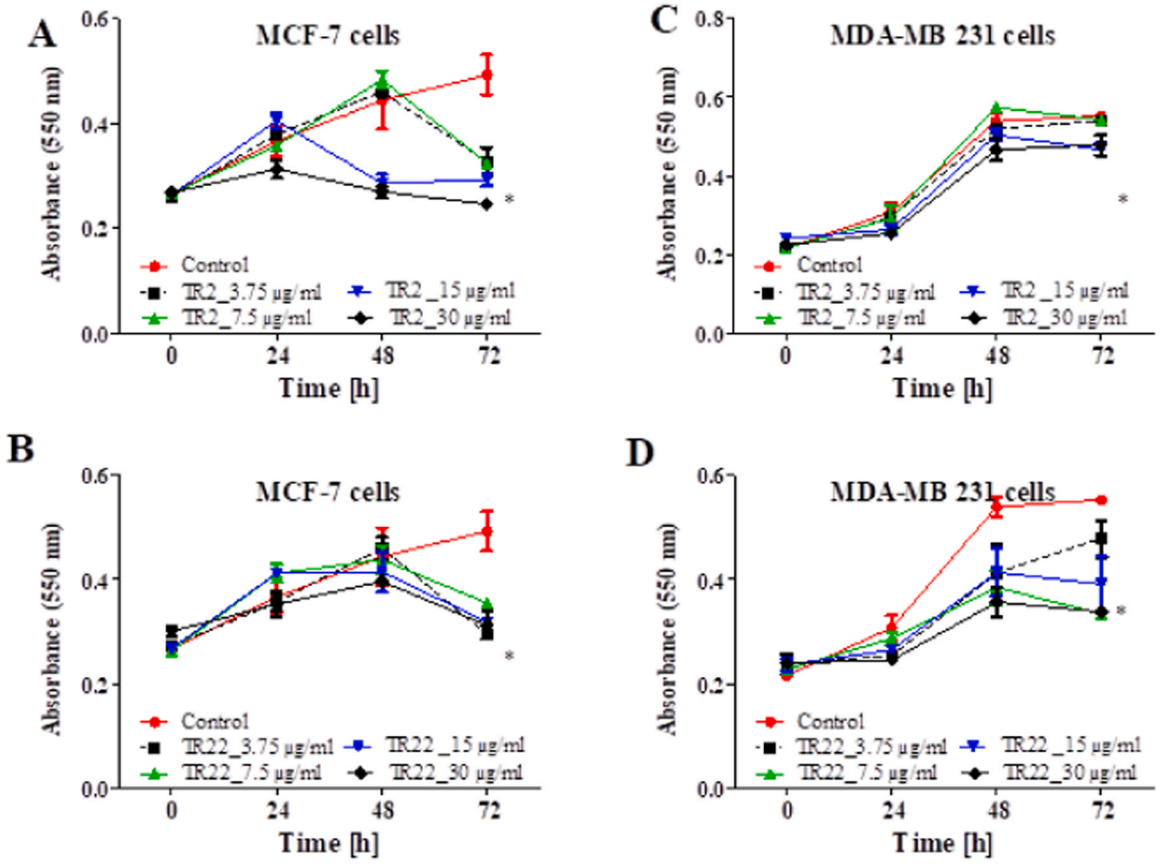

Fig. 2. Growth and proliferation of estrogen-sensitive MCF-7 (A & B) and non-sensitive MDA-MB 231 (C & D) breast carcinoma cells treated as well as with different concentrations of compound TR2 & TR22 after 24, 48 and 72 hours. Controls remained untreated. (n = 3). Treated cancer cell cultures were compared to non-treated control cultures of the same passage and cell numbers per well. *$p < 0.05$ and **$p < 0.01$ compared to control.

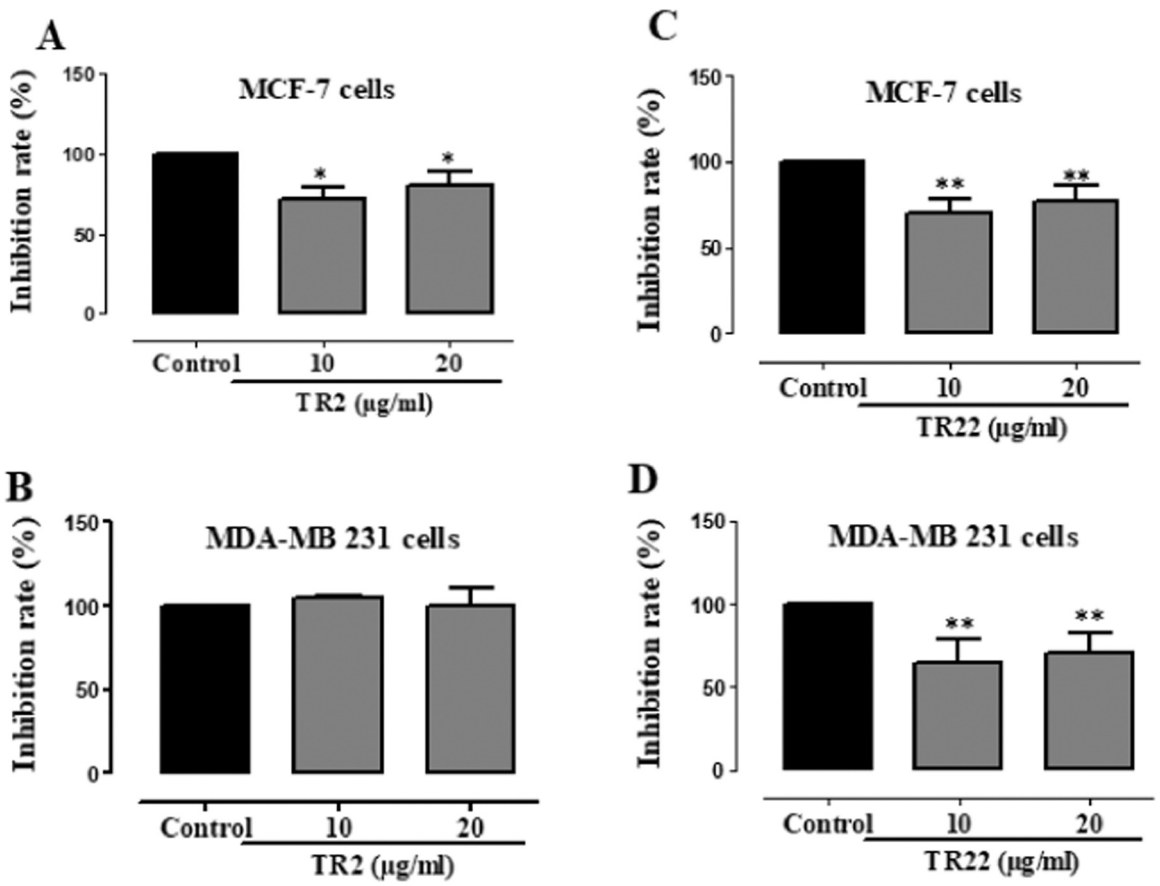

Fig. 3. Cell proliferation of estrogen-sensitive MCF-7 (A) and non-sensitive MDA-MB 231 (B) breast carcinoma cells. Cells were treated with compound TR2 and TR22 at 10 and 20 µg/mL after 48 hours. Control remained untreated. *$p < 0.05$ and **$p < 0.01$ compared to control.

sensitive MDA-MB 231 breast carcinoma cell lines when compared to the control. This indicates that acetylation reaction could improve inhibition of cell proliferation of breast cancer cells.

3.5. Inhibitory effects of clone formation by the compounds

The potential effect of the compounds to inhibit the formation of clones by the non-sensitive MDA-MB 231 cell lines was evaluated at 10 and 20 µg/mL. The results presented on Fig. 4 indicates that both compounds significantly inhibited the formation of clones compared to the untreated controls. This is evident in the photomicrographs showing the cell densities as indicated on Fig. 4 and inhibition of clone formation was dose-dependent. TR2 exhibited better inhibitory effect of clone formation than TR22 suggesting that acetylation reduced the activity in this assay.

3.6. Inhibitory effects on cellular migration by the compounds

The ability of the compounds to inhibit the non-sensitive MDA-MB 231 cells migration was evaluated at different times (0 h, 24 h, 48 h, 72 h) and at two concentrations of 10 and 20 µg/mL and the results presented on Fig. 5. It was observed that the cell migration fronts reduced over time and that the compounds were able to inhibit the migration of the cells when compared to the untreated controls. Compound TR2 exhibited greater cellular migration inhibition than compound TR22 at the same concentration and after the same period of time. Three independent wound-healing assays were performed after 24 h, 48 h and 72 h and the results plotted on Fig. 5. The compound TR2 significantly ($p < 0.05$) reduced the percentage of cells compared to untreated controls, thereby indicating good potential wound healing effects.

3.7. Molecular docking results

As mentioned above, 3,3'4-trimethoxy-4'-rutinosylellagic acid (**TR2**) and its acetylated derivative (**TR22**) showed the potency to inhibit the proliferation of breast cancer cells. In an attempt to shed light on this activity, their binding affinities into the binding site of tyrosine kinase were determined. The binding energies, the number of intermolecular hydrogen bonds, and the number of active residues in stable TR2-tyrosine kinase and TR22-tyrosine kinase complexes are represented in Table 2.

TR2 and **TR22** form stable complexes into the binding site of tyrosine kinase with binding energy –10.37 and –6.74 kcal/mol, respectively. The negative binding energy may indicate that these compounds have potency to inhibit tyrosine kinase and the process is thermodynamically favorable and spontaneous. 2D and 3D binding affinities of **TR2** and **TR22** into the binding site of tyrosinase kinase and the plausible interactions they form with the amino acids of tyrosine kinase are displayed in Fig. 6. Molecular docking results reveals that TR2 may have higher potency to inhibit tyrosine kinase compared to TR22, which may return to the stability of TR2-tyrosine kinase complex, and to the number of hydrogen bonding formed between TR2 and the amino acids of tyrosine kinase. TR22 is derived from TR2 by acetylation of sugars groups. In TR2, the sugar moiety forms five hydrogen bonds with ARG A518, ASP A820, ASP A677, ASN A680, and LEU A595 amino acids of tyrosine kinase, while in TR22 the acetylation of the sugar hydroxyl groups leads to the disappearance of these hydrogen bonds, and hence the decrease of the binding affinity of TR22 (Fig. 6).

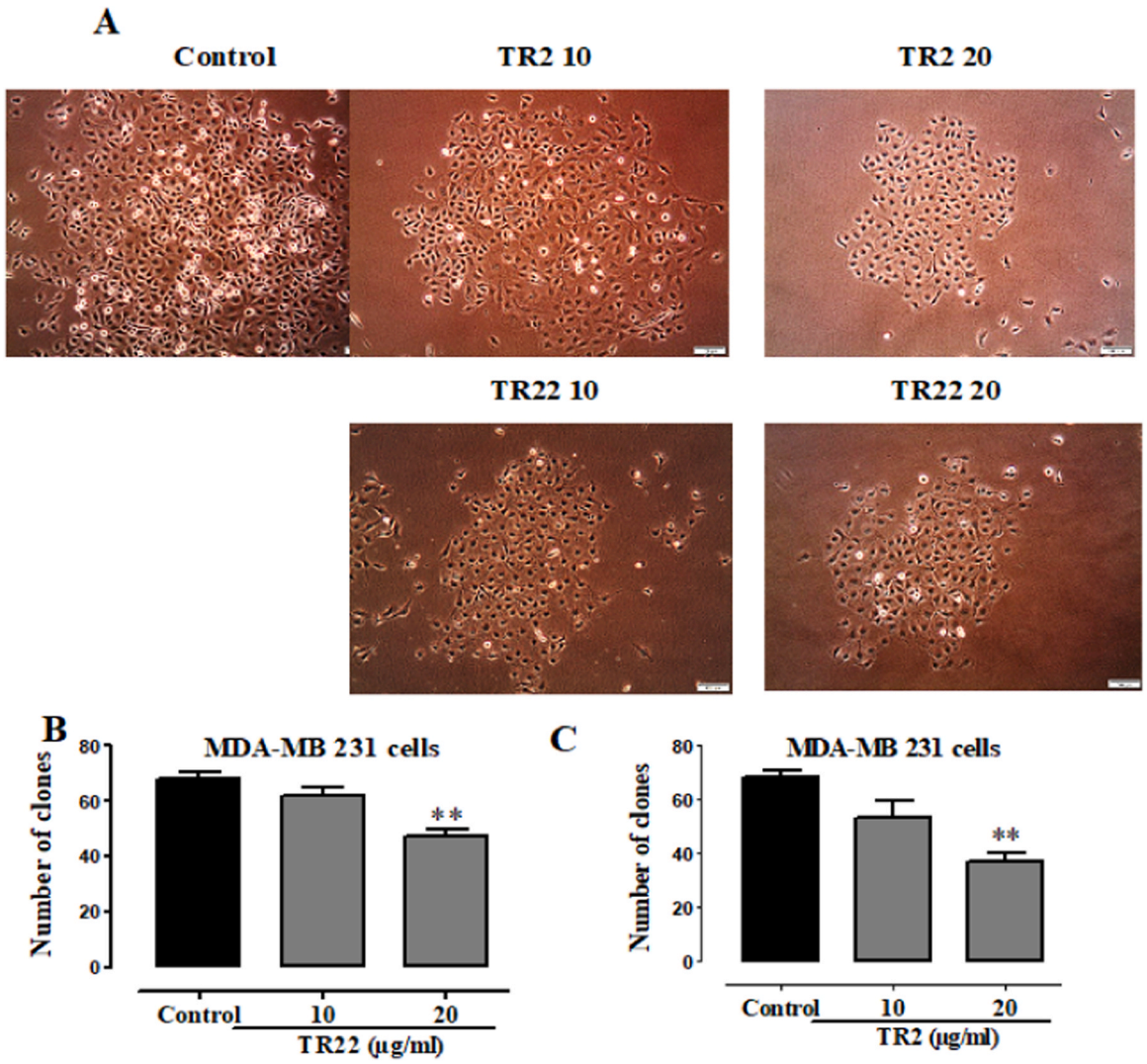

Fig. 4. Clonogenic growth of MDA-MB 231 cells exposed to compound TR2 and TR22 at 10 and 20 µg/mL after 48 hours: graphic representation (A) and photomicrographs (B). Control remained untreated. $^{**}p < 0.01$ compared to control.

4. Discussion

Ellagic acid is a polyphenolic chromene dione compound which occurs in many edible and medicinal plants in free form, as ellagitannins or as glycosylated derivatives [42]. 3,3'4-trimethoxy-4'-rutinosylellagic acid (TR2) is an important glycosylated derivative of ellagic acid which was isolated, characterized and used for this study alongside its semi-synthetic derivative 3,3'4-trimethoxy-4'-hexaacetylrutinosylellagic acid (TR22). These compounds are very relevant in diets since they have highly potent antioxidant compounds capable of preventing the risks of oxidative stress and its associated diseases. Antioxidant derivatives of ellagic acid have been described from various sources as dietary polyphenols which act through various mechanisms to potentially prevent various disease onsets and progression or reduce the symptoms of numerous chronic diseases [43]. Both derivatives of ellagic acid (TR2 and TR22) used in this study exhibited good antioxidant capacities in four complementary assays (β-Carotene-linoleic acid assay, DPPH$^{\bullet}$ assay, ABTS$^{\bullet+}$ assay, CUPRAC assay). Ellagic acid and its derivatives are known to inhibit lipid peroxidation, improve antioxidant enzymes activities and reduce the production of various reactive oxygen species (ROS) thereby contributing to the prevention of various diseases [44,45]. The presence of methoxy, hydroxyl groups, sugar moieties together with aromatic rings and lactone functions improves the ability of the ellagic acid derivatives to receive free electrons in an antioxidant mechanism which is capable to quench free radicals, chelate metal ions and intervene in various signaling pathways [45,46]. In addition, ellagic acid and derivatives can also regulate the potential of antioxidant enzymes and parameters including superoxide dismutase, glutathione peroxidase and catalase towards the reduction of ROS generation [47].

The potential anticancer activity of 3,3'4-trimethoxy-4'-rutinosylellagic acid (TR2) and 3,3'4-trimethoxy-4'-hexaacetylrutinosylellagic acid (TR22) described in this study is not surprising since as the compounds are derivatives of ellagic acid which has been shown to possess antioxidant and anticancer properties. Common features of cancer cells include resistance to antigrowth signals and uncontrolled production of growth signals, inhibition of the apoptosis, continuous cellular replication, angiogenesis and metastasis [48]. Cancer involves a complex process that modifies the normal physiological behaviour and growth of cells as well as their homeostasis, leading to abnormal tissue growth and malignant tumors with adverse effects. Many natural and synthetic compounds have promising chemopreventive actions towards the reduction of cancer incidence and in the inhibition of growth of cancerous cells or suppression of malignant lesions [49,50]. Various plant phenolic compounds including ellagic acid have demonstrated encouraging anticancer effects in *in vitro*, *in vivo* and *in silico* studies. They act as modulators of various genes expressions associated with cancers, inflammation, oxidative stress and are also involved in

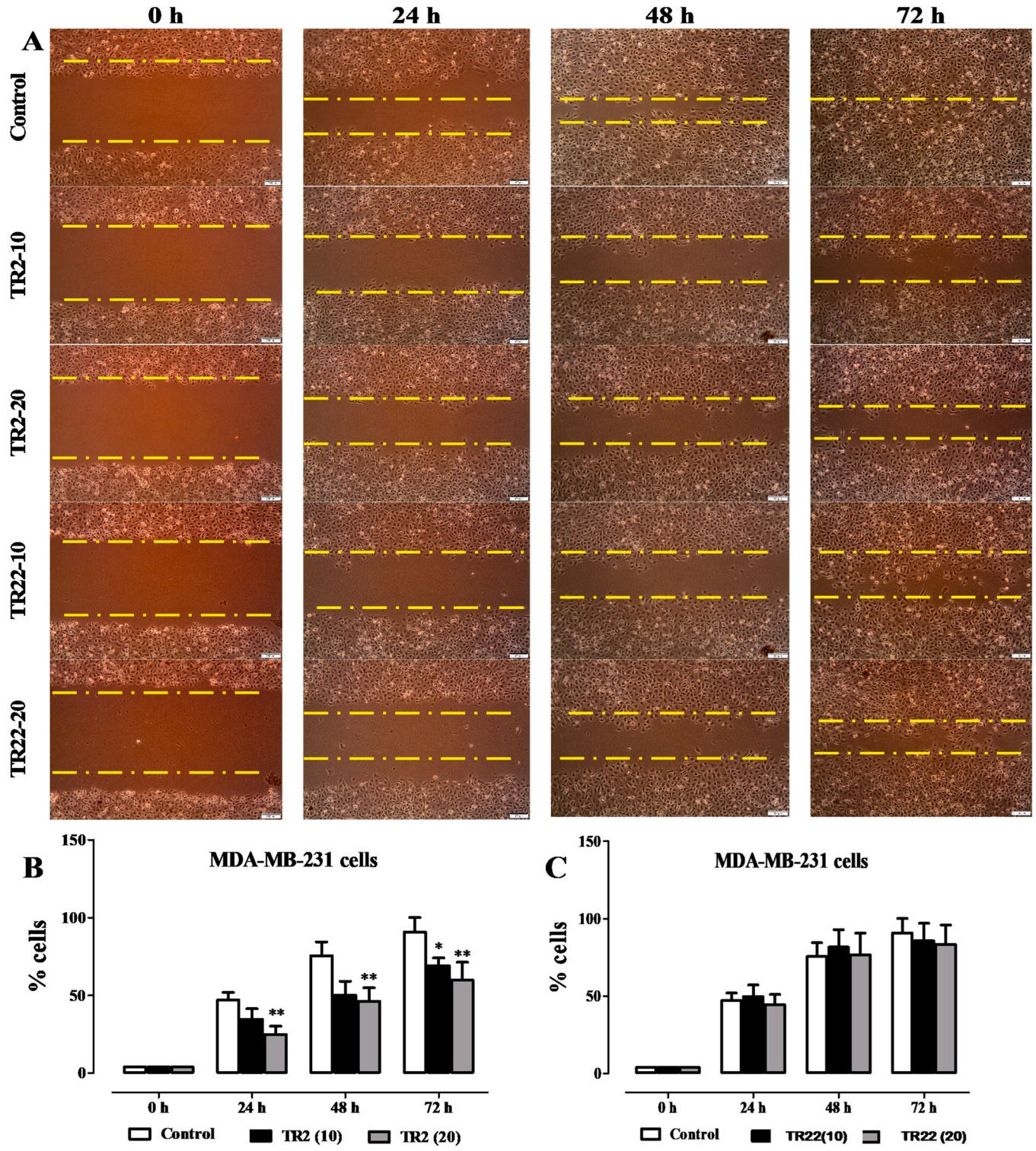

Fig. 5. Effects of TR2 and TR22 on MDA-MB 231 cells migration. Microphotographs of one assay (A) and graphic representation of three independent wound-healing assays (B & C) in MDA-MB 231 cells migration after 72 h of treatment in serum free RPMI 1640 medium. *$p < 0.05$ and **$p < 0.01$ as compared with control.

processes such as differentiation, cell cycle arrest, metastasis, proliferation, autophagy, apoptosis, inhibition of telomerase and angiogenesis [51–53]. The derivatives of ellagic acid used in this study are 3,3'4-trimethoxy-4'-rutinosylellagic acid (TR2) and 3,3'4-trimethoxy-4'-hexaacetylrutinosylellagic acid (TR22). Both compounds have demonstrated inhibitory effects of cell growth, proliferation, cloning and cellular migration on two breast carcinoma. It is possible that these compounds inhibit angiogenesis, tumor proliferation and metastasis as well as induce apoptosis, disrupt DNA binding to carcinogens, disturb inflammation and stop tumor growth, which are properties of similar structural compounds [54,55]. This results is feasible for both ellagic acid derivatives. This is so because ellagic acid has shown the capacity to prevent angiogenesis, migration, and cellular proliferation of various cancer cell lines including breast carcinomas [56–60]. 3,3'4-trimethoxy-4'-rutinosylellagic acid (TR2) and 3,3'4-trimethoxy-4'-hexaacetylrutinosylellagic acid (TR22) exhibited potential anticancer effects against estrogen-sensitive MCF-7

Table 2
Free binding energies, number of hydrogen bonds, and the number of closest residues to the docked TR2 and TR22 into the binding site of tyrosine kinase.

	Free binding energy (kcal/mol)	H-Bonds (HBs)	Number of interactions of the docked ligands into the binding site
TR2	−10.37	8	ASP A820, ASP A677, ARG A815, ALA A621, ASN A680, CYS A673, GLY A676, LEU A595, LEU A799, TYR A672, VAL A603, VAL A654
TR22	−6.74	4	ARG A815, ARG A791, ALA A636, ASN A797, ASP A810, GLY A812, LEU A813, PHE A811, SER A639, PRO A573

and non-sensitive MDA-MB 231, indicating that they can be used in the development of therapies for breast cancer independently to the estrogen pathway. This corroborates with other findings, which show that ellagic acid and its derivatives inhibit breast cancer growth through several mechanisms including cell cycle arrest, estrogen receptor gene regulation, apoptosis increase, suppression of tumor angiogenesis and antioxidant pathways [61–63]. Additionally, administration of ellagic acid inhibits cell proliferation and induces apoptosis leading to a decrease in the number of colonies (cloning) and causing a further reduction in the expression of cyclin-dependent kinase 6 (CDK6) in the breast cancer cells [64]. Ellagic acid and its derivatives express anti-tumor effects malignancies of various organs such as breast, gastric, colorectal, pancreatic, liver and lung cancers mainly through induction of apoptosis and autophagy as well as inhibition of cell proliferation and metastasis, involving several molecular mechanisms [65].

Molecular docking studies supplemented the experimental potential anticancer effects against breast carcinoma of the compounds (TR2 and TR22). The evaluation of the forms of interactions between the protein kinase enzymes and the compounds indicated great potency. The signal transduction process that drives cell division, motility, metabolism, proliferation, and programmed cell death is mediated in part by tyrosine kinases and their signaling pathways can enhance the sensitivity to apoptotic stimuli and uncontrolled growth [66]. Dysregulation of signaling pathways of receptor tyrosine kinases (RTK) can cause the assortment of cancers with modifications in the genes such as HER2/ErbB2, EGFR and MET amongst others which are genes encoding RTKs [67,68]. This has led to the development of multiple small molecule-based tyrosine kinase inhibitor treatments which are clinically approved for several types of cancers as a key class of therapeutics [68,69]. Molecular docking studies revealed that both 3,3'4-trimethoxy-4'-rutinosylellagic acid (TR2) and 3,3'4-trimethoxy-4'-hexaacetylrutinosylellagic acid (TR22) had negative binding energies indicating that they are potent receptor tyrosine kinase inhibitors. Since the compounds showed appreciable interactions in molecular docking simulations, they could constitute new cancer target therapies that can target cancer cell proliferation and growth promoting enzymes and proteins such as receptor tyrosine kinases. The inhibition of tyrosine kinases can impede the progression of cancer, induce apoptosis and suppress cell cycle [70,71]. Though the results are good and the molecular docking studies also present negative binding energies of the compounds, this study can involve few limitations just like other anticancer assays. They compounds may exhibit indiscriminate toxicity as a

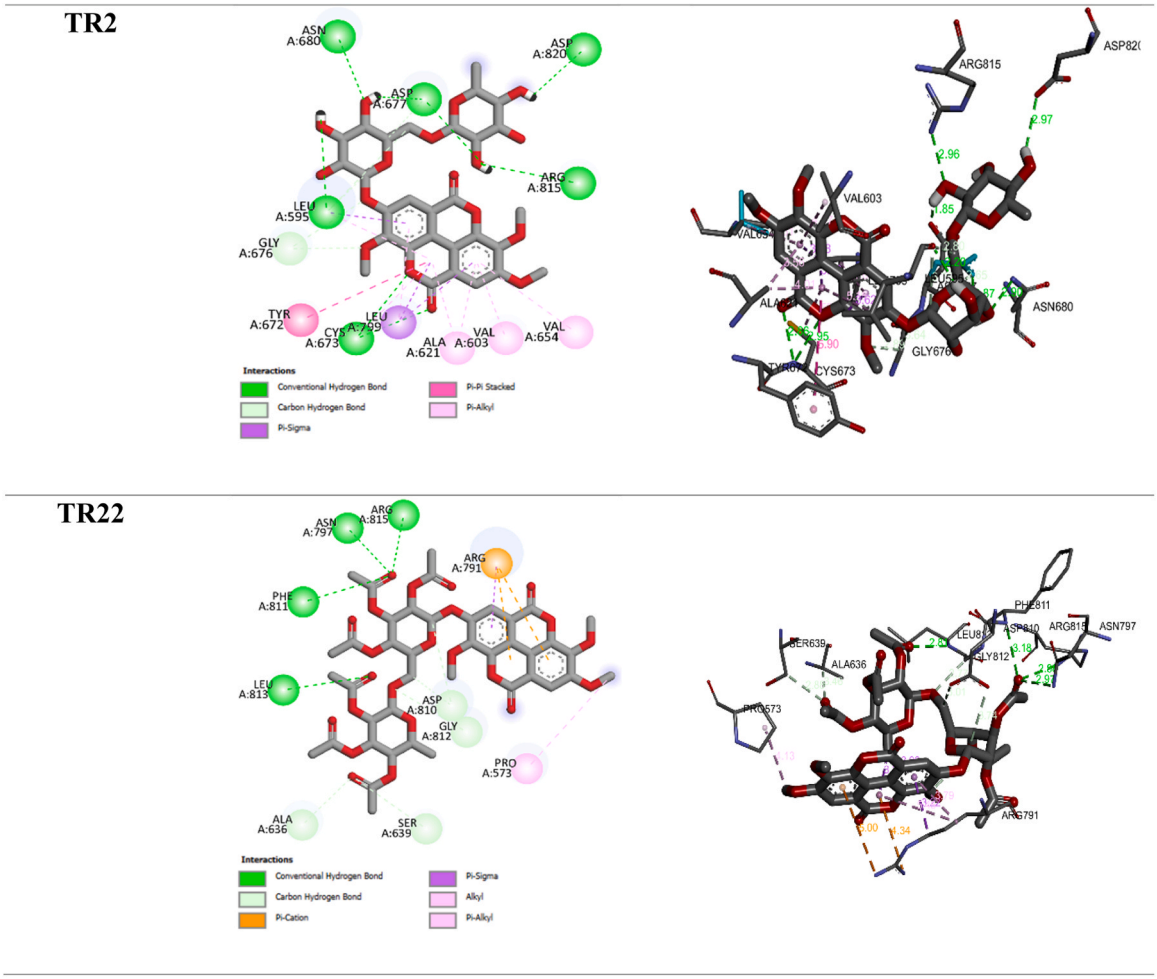

Fig. 6. 2D and 3D interaction modes of **TR2** and **TR22** into the binding site of tyrosine kinase.

result of their lack of tumor selectivity. Also, pharmacokinetic problems such as low water solubility and stability can impair the absorption, distribution and excretion of the compounds.

5. Conclusion

In this study, 3,3'4-trimethoxy-4'-rutinosylellagic acid (TR2) and 3,3'4-trimethoxy-4'-hexaacetylrutinosylellagic acid (TR22) were evaluated for their antioxidant and anticancer effects against two breast carcinoma (estrogen-sensitive MCF-7 and non-sensitive MDA-MB 231 cell lines). The compounds exhibited good antioxidant activity in four different complementary assays. Furthermore, these compounds inhibited cell growth, cell proliferation, clone formation and cellular migration against breast cancer cell lines. Formation and progression of various cancers is multifactorial and tyrosine kinases play key role in the process. It has been observed that the addition of the acetoxy group on the parent compound doesn't significantly increase its antioxidant and antiproliferative potential. These compounds showed appreciable interactions with tyrosine kinase through molecular docking simulations, indicating that they could be used in developing anticancer therapy that targets tyrosine kinases inhibition involved in cancer progression. The cytotoxic effect of these compounds could therefore be exploited for cheaper anticancer drugs development.

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CRediT authorship contribution statement

Sophie Laurent: Resources, Formal analysis, Data curation. **Stephane Zingue:** Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rodica Mihaela Dinica:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization. **Emmanuel Talla:** Supervision, Methodology, Investigation, Formal analysis, Data curation. **El Hassane Anouar:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Abel Joel Yaya Gbaweng:** Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Cyrille Leonel Tchuente Djoko:** Methodology, Investigation, Formal analysis, Data curation. **Romeo Toko Feunaing:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alfred Ngenge Tamfu:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Fidele Ntchapda:** Supervision, Resources, Formal analysis, Data curation. **Celine Henoumont:** Resources, Formal analysis, Data curation.

Declaration of Competing Interest

The authors have no competing interests to declare.

Data availability

The data and materials used in this study are available upon request from the corresponding authors upon reasonable request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117370.

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