

Cytotoxic Effect of Antioxidant products from *Nitraria retusa* Leaves in Combination with Temozolomide on Glioblastoma Cell Growth

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Abstract

The aim of this research was to study of inhibition glioblastoma (GBM) cell growth by proved antioxidant component identified in *Nitraria retusa* leaves extracts. The antioxidant power has been evaluated in non-enzymatic, enzymatic and cellular systems. Next, the apoptosis was evidenced by investigating inhibition glioblastoma cell growth. The methanol (Nr-Meoh) and ethyl acetate (Nr-EA) extracts showed with highest reducing capacity, the Trolox equivalent antioxidant capacity values of 1.81 and 1.62 mM respectively and a highest 50 % inhibitory concentration of 2',7'-Dichlorofluorescein radicals (0.44 and 0.51 respectively). The most potent extract in inhibiting xanthine oxidase activity is Nr-Meoh with an 50 % inhibitory concentration value of 86 µg/mL. The same extract produced a 94.46 % decrease of nitro-blue tetrazolium photoreduction at a concentration of 10 mg/mL and an 50 % inhibitory concentration value of 5 mg/mL. The cytotoxic study of Nr-extracts and molecule whether in the absence or presence of TEMODAL (TPZ) revealed a: IC₅₀ Nr-Meoh: 450µg/mL, IC₅₀ Nr-Meoh + TPZ: 125µg/mL, IC₅₀ Nr-EA: 187µg/mL, IC₅₀ Nr-EA + TPZ: 63,5µg/mL , IC₅₀ of isorhamnetin-3-O-robinobioside (Nr-I3-O-Rob) = 190 µg/mL, IC₅₀ Nr-I3-O-Rob + TPZ: 68 µg/mL and IC₅₀ Nr-Chl: 90µg/mL, IC₅₀ Nr-Chl + TPZ: 25µg/mL. Nr-Chl extract through β-sitosterol combined with temodal exhibited the most apoptotic effect on glioblastoma than temodal alone.

In conclusion, flavonols showed strong free radical scavenging activity compared to sterols which showed stronger apoptotic power than flavonols and a potentially synergistic therapeutic approach for glioma can be planned.

Introduction

Natural antioxidants and various phytochemicals are advocated as adjuvant therapies in cancer treatment due to their well-documented antitumor properties. until 2020, 102 unaltered natural product, botanical drug (defined mixture) and natural product derivative, have received approval based on their anticancer activities[1]. Consequently, the exploration of plant-derived anticancer agents and compounds has emerged as a crucial strategy for the development of new anticancer therapeutics [2].

Glioblastoma (GBM) characteristically exhibits aggressive cell proliferation and rapid invasion of normal brain tissue or the spinal cord, resulting in a poor clinical prognosis. [3], [4]. Temozolomide is an active anticancer agent marketed under the name Temodal and administered orally. It is indicated for the treatment of newly diagnosed glioblastoma multiforme in combination with radiotherapy and subsequently as monotherapy [5]. Conventional interventions involving pharmacological and radiological treatments present significant challenges related to resistance to chemotherapy and radiotherapy, particularly in the context of glioma stem cells[6] the ineffectiveness of chemotherapeutic agents may be attributed to challenges in drug delivery to the tumor [3] Consequently, there is a pressing need for alternative and adjunctive therapies that may effectively address these challenges [6]. In GBM, there are genetic alterations and deregulations of molecular and metabolic pathways, such as elevated reactive oxygen species (ROS), which cause, among other things, a decrease in the antioxidant system

[7]. Oxidative stress is an imbalance between the production of radical species and the antioxidant defense systems, which can cause damage to cellular biomolecules, including lipids, proteins, and DNA [8]. Consequently, leading to a wide variety of human diseases and cancers. Fortunately, there are antioxidant enzymes and other molecules that scavenge free radicals [9], [10]. The former includes several enzymes such as catalase and glutathione peroxidase, while the latter comprises a range of antioxidants obtained from dietary sources, including vitamin C, carotenoids, flavonoids, and polyphenols, which possess strong antioxidant activities [9], [11]. Flavonols and phytosterols exhibit strong antioxidant properties [12], [13] through various mechanisms of action, such as electron transfer and hydrogen atom transfer, which are involved in free radical quenching or [14], [15], [16]. These mechanisms can occur simultaneously during antioxidant-free radical scavenging [17]. Plant extracts may contain compounds with antioxidant properties that provide protection against a range of carcinomas [18]. Therefore, dietary natural substances are being evaluated for their potential as agents in GBM treatment [3], [6] .

Many researchers have sought chemopreventive and antioxidant agents from natural products to identify dietary treatments for humans. This is the case for *Nitraria retusa* (Forssk.) Asch, a genus in the Nitrariaceae family. Known for its fleshy red fruits, it is consumed by humans and used in beverage preparation. Its leaves serve as a tea supplement and poultice, while the ashes can absorb exudates from infected wounds. A decoction of fresh leaves is utilized for treating various diseases. Recently, it has been studied for its antimutagenic, antigenotoxic, and antitumor activities [19], [20], [21].

The richness of this plant in active compounds and its biological potential led us to study its effects on glioblastoma while studying its antioxidant potential, while knowing that the evaluation of the antiproliferative effect of β -sitosterol against glioblastoma is almost rare.

Materials and methods

2.1. Reagents

All the organic solvents were obtained from Carlo ERBA (Paris, France). Xanthine oxidase (XOD) and 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 6-hydroxy-2,5,7,8-tetramethylchroman carboxylic acid (Trolox) were obtained from Wako (Osaka, Japan). Nitrotetrazolium blue chloride (NBT) was purchased from Fluka (Buchs, Switzerland). All the other chemicals and solvents were of the highest commercial grade and obtained from Carlo ERBA (Paris, France). 2',7'-Dichlorofluorescein diacetate (DCFH-DA). 2,2'-azobis(2-amidinopropane) dihydrochloride (ABAP). Temozolomide (temodal), DMEM, RPMI-1640 Glutamax, foetal bovine serum, sodium pyruvate and non-essential amino acids and gentamicin were bought from GIBCO BRL Life technologie (Grand Island, NY, USA).

2.2. Plant Material and analysis extracts:

Leaves of *N. retusa* were collected from halophilic soils in Sahline, a coastal area in Tunisia. Their identification was carried out by Professor M. Cheieb from the Department of Botany at the Faculty of Sciences, University of Sfax, Tunisia, following the Flora of Tunisia [19]. A total of three hundred and fifty grams of powdered dried leaves were extracted sequentially using a Soxhlet apparatus for 6 hours (AM Glassware, Aberdeen, Scotland, United Kingdom) successively with different solvents: chloroform, ethyl acetate, and methanol. The chloroform (Nr-Chl) extract, ethyl acetate (Nr-EA) extract and methanol (Nr-Meoh) extract was obtained.

HPLC/ESI-MS² is an efficient method for the separation, identification, and characterization of chemical compounds. High-Performance Liquid Chromatography (HPLC) separates the components of a mixture by passing them through a column filled with an adsorbent material, utilizing a liquid solvent. Electrospray Ionization (ESI) is an ionization technique used in mass spectrometry that converts molecules into ions by spraying them into an electric field. Mass Spectrometry (MS²) involves a second stage of analysis (indicated by the "2"), where the ions generated by ESI are analyzed in a mass spectrometer to determine the structure of the molecules. The results of this technique are presented in the form of a diagram displaying various peaks, with each peak corresponding to a specific retention time (elution time) for each chemical molecule as it passes through the HPLC column. The area of each peak reflects the quantity or proportion of each molecule.

The analysis of HPLC/ESI-MS² was conducted using a Thermo Finnigan LCQ Advantage ion trap mass spectrometer equipped with an electrospray ionization (ESI) source, which was coupled to a Thermo Scientific Accela HPLC system comprising an MS pump, an autosampler, and a PDA detector. The separation was achieved on a Nucleodur 100-3 C18ec column (Macherey-Nagel). Gradient elution was performed using water and acetonitrile (ACN) without formic acid for the ESI negative mode, transitioning from 5% to 30% ACN over 60 minutes, followed by an increase from 30% to 90% ACN over an additional 35 minutes, all at a temperature of 30 °C. The flow rate was maintained at 0.3 mL/min, with an injection volume of approximately 25 µL. All samples were analyzed in negative ion mode. The mass spectrometer operated with a capillary voltage set at 10 V, a source temperature of 240 °C, and high-purity nitrogen was used as sheath and auxiliary gas at flow rates of 70 and 10 arbitrary units, respectively. Ions were detected within a mass range of 50-2000 m/z, with a collision energy of 35% applied for fragmentation during MS/MS analysis. Data acquisition was performed using Xcalibur™ 2.0.7 software (Thermo Scientific).

2.3. Cell culture

Human lymphoblastoid cell line TK6 (Kindly provided by Pr. Pierre Bischoff, Centre Paul Strauss, Strasbourg, France). Cells were cultured in RPMI-1640 glutamax supplemented with 10% (v / v) foetal bovine serum, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 50 µg / mL gentamicin at 37 °C in humid atmosphere of 5% CO₂.

U373 (kindly provided by Laurent Pelletier (Univ. Grenoble Alpes, Grenoble Institut of Neurosciences, GIN, F-38000 Grenoble, France) were initially purchased from the American Type Culture Collection (ATCC,

Rockville, MD), stored in DMEM (Cambrex Biosciences, New Jersey, USA) supplemented with 10% fetal bovine serum (v/v; AbCys, Paris, France). Cells were maintained in 5% CO₂ at 37°C in a humidified incubator.

2.4. Cellular Antioxidant Activity (CAA) Assay

Human lymphoblastoid cell line TK6 cells were seeded at a density of 6×10^4 /well on a 96-well microplate in 100 μ L of growth medium/well. Twenty-four hours after seeding, the growth medium was removed and the wells were washed with PBS. Triplicate wells were treated for 1 h with 25 μ M DCFH-DA dissolved in treatment medium. Then 600 μ M ABAP was applied to the cells in 100 μ L of PBS, and the 96-well microplate was placed into a Fluoroskan Ascent FL plate-reader (ThermoLabsystems, Franklin, MA) at 37 °C. The fluorescence was measured with an excitation wave length of 485nm and emission wave length of 538 every 5min for 1h. Each plate included triplicate control and blank wells: control wells contained cells treated with DCFH-DA and oxidant; blank wells contained cells treated with dye and HBSS without oxidant [22], [23] .

2.5. Quantification of CAA

After blank subtraction from the fluorescence readings, the area under the curve of fluorescence versus time was integrated to calculate the CAA value at each concentration of extracts as follows:

$$\text{CAA unit} = 1 - (f_{\text{SA}} / f_{\text{CA}}) \times 100$$

where f_{SA} is the integrated area under the sample fluorescence versus time curve and f_{CA} is the integrated area from the control curve. The median effective dose (IC_{50}) was determined for the pure phytochemical compounds and fruit extracts from the median effect plot of $\log (f_a / f_u)$ versus $\log (\text{dose})$, where f_a is the fraction affected and f_u is the fraction unaffected by the treatment. To quantify intraexperimental variation, the IC_{50} values were stated as mean (SD for triplicate sets of data obtained from the same experiment) [22], [23] .

2.6. ABTS radical-scavenging activity

ABTS was dissolved in water to a 7 mM concentration. $\text{ABTS}^{+\bullet}$ was produced by reacting an ABTS stock solution with 2.45 mM of potassium persulfate (final concentration) The $\text{ABTS}^{+\bullet}$ solution was diluted with ethanol to an absorbance of 0.7 (± 0.02) at 734 nm. In order to measure the antioxidant activity of the extracts, 10 μ L of each sample at various concentrations (0.05; 0.5; 2.5; 4.5 mg/mL) were added to 990 μ L of diluted $\text{ABTS}^{+\bullet}$ and the absorbance was recorded every 1 minute until it became stable. The percentage decrease of absorbance at 734 nm was calculated for each point, and the antioxidant capacity of the test compounds was expressed as percent inhibition (%). The 50% inhibitory concentration (IC_{50}) value was calculated from regression analysis [24] .

2.7. Evaluation of the antioxidant activity by an enzymatic assay

The assay mixture consisted of 100 μL of the tested compound solution, 200 μL (final concentration 0.1 mM) of xanthine as the substrate, hydroxylamine (final concentration, 0.2 mM), 200 μL EDTA (0.1 mM), and 300 μL distilled water. The reaction was initiated by adding 200 μL XOD ($11\text{mU}\cdot\text{mL}^{-1}$) dissolved in phosphate buffer (KH_2PO_4 20.8 mM, pH 7.5). The assay mixture was incubated at 37°C for 30 minutes and stopped by adding 0.1 mL of HCl 0.5 mol/L. The absorbance was measured spectrophotometrically against a blank solution, prepared as described above, but replacing XOD with buffer solution at 290 nm. Another control solution without the tested compound was prepared in the same manner as the assay mixture to measure the total uric acid production (100%). The uric acid production was calculated from the differential absorbance.

2.8. Superoxide radical-scavenging activity

The reaction mixture contains EDTA (6.5 mM), riboflavin (4 μM), NBT (96 μM) and phosphate buffer (51.5 mM, pH 7.4). The volume of the tested sample was of 100 μL /assay. The occurrence of superoxide was indirectly evaluated by the increase in the absorbance of formazan at 560 nm, after 5 min incubation at 30°C from the beginning of illumination. The assay run without any test compound was used as control. The results were calculated as the percentage inhibition according to the following formula:

$$(\%) = 1 - (A_{560} \text{ sample} / A_{560} \text{ control}) \times 100.$$

2.9. Assay for cytotoxic activity

Cytotoxicity of *Nitraria retusa* extracts against U373 MG cells was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, based on the reduction of the MTT by mitochondrial dehydrogenases in viable cells. The resulting blue formazan product is measured spectrophotometrically (Carmichael, et al., 1987). Cells were seeded in a 96-well plate at a concentration of 5×10^4 cells/well and incubated at 37°C for 24h in a 5% CO_2 enriched atmosphere. The extracts were firstly dissolved in 0.2% DMSO, then in the cell growth medium. Cells were incubated again at 37°C for 96 h with each of the tested extract at successive concentrations of 0.03125/0.0625/0.125/0.25/0.5/1/2/4 mg/mL. In parallel, each concentration of each extract was tested in the presence of TEMODAL at 3% under the same conditions. Next, the medium was removed and cells in each well were incubated with 50 μL of MTT solution (5 mg/mL) at 37°C for 4 h. MTT solution was then discarded and 50 μL of 100% DMSO were added to dissolve the insoluble formazan crystal. The optical density was measured at 540 nm. Each drug concentration was tested in triplicate.

The cytotoxic effects of the extracts were estimated in terms of cell population growth and expressed as IC_{50} which is the concentration of extract that reduces the absorbance of the treated cells by 50% with reference to the control (cells treated with DMSO 0.2%). The IC_{50} values were graphically obtained from the dose–response curves. We determined IC_{50} values when cytotoxicity resulted more than 50% at screening concentrations.

2.10. Statistical analysis

Data were collected and expressed as the mean $\pm$ standard deviation of three independent experiments and analyzed for statistical significance from control. The data were tested for statistical differences by student test. The criterion for significance was set at $p < 0.05$. The correlations coefficients between studied parameters were demonstrated by linear regression analysis. The data cytotoxic study was tested for statistical differences by i two way anova test. The criterion for significance : $p < 0.0001$ (tuk test)

Results

3.1. Determination of the various molecules present in each extract derived from the leaves of *Nitraria retusa*:

Screening analysis with HPLC/ESI-MS² of Nr-Chl extract reveals two major peaks with retention times of 8.6 and 25.5, corresponding respectively to palmitic acid, which accounts for 16.9 % of the chloroform extract, and β -sitosterol, which represents 19.6% of the chloroform extract. Several other peaks are also observed, indicating the presence of multiple minor molecules that are infinitely present.

Screening analysis with HPLC/ESI-MS² of EA-Chl extract reveals a major product at a retention time of 19.3 corresponding to isorhamnetin-3-O-robinobioside. Several other peaks are revealed, indicating the presence of multiple molecules, all of which are minor and present in infinitesimal amounts. Reveals

Screening analysis with HPLC/ESI-MS² of Meoh extract revealed five major peaks with retention times of (15.07); (15.53); (16.95); (19.33) and (22.36) corresponding respectively to quercetin-O-hexoside which accounts for 9.54 %, isorhamnetin-3-O-glucoside which accounts for 19.27 %, isorhamnetin-3-O-rutinoside which accounts for 19.71 %, isorhamnetin glucuronide which accounts for 17.21 % and isorhamnetin which accounts for 13.75 % in Nr-Meoh extract. Several other peaks are revealed, indicating the presence of multiple molecules, all of which are minor and present in infinitesimal amounts.

Electrospray Ionization (ESI) coupled to Mass Spectrometry (MS²) has enabled the determination of the chemical structure of each compound represented below the diagram of each extract (Figure1).

3.2. Extracts capacity to scavenge ABTS $\cdot$ Radical:

The results obtained are summarized in Table 1. Nr-Meoh and Nr-EA extracts exhibited a high antioxidant potential with TEAC values of 1.81 and 1.62 mM, respectively. IC₅₀ values of Nr-Meoh and Nr-EA extracts were 0.50 mg/mL and 0.75 mg/mL, respectively. Nr- Chl extracts antioxidant capacity were also significant, but less potent with TEAC values of 1.11 mM, respectively. IC₅₀ values of Nr-Chl extract is 1.25 mg/mL.

3.3. Extract capacity to inhibit xanthine oxidase activity:

In Table 1, the results showed that the Nr-Meoh extract seems to be the most efficient inhibitor of uric acid production than other extracts tested (IC_{50} = 86 μ g/mL). the Nr-Chl and Nr-EA extracts inhibit uric acid production at IC_{50} = 350 μ g/mL and 334 μ g/mL respectively.

3.4. Extracts capacity to scavenge $O_2^{\cdot}$ radical:

The Nr-Meoh extract decreased NBT photoreduction by 94.46 % at a concentration of 10 mg/mL which exceeds that of positive control, quercetin (72.90 %). Nevertheless, the IC_{50} value of Nr-Meoh extract is 5 mg/mL, less significant than that obtained with Nr-EA extract (IC_{50} = 1mg/mL). However, the highest inhibition percentage obtained with the Nr-Chl extract was 41.51 % at a concentration of 10 mg/mL (Table 1).

3.5. Extracts capacity to prevent DCFH formation:

The extracts from the leaves of *N. retusa* showed in general a significant cellular antioxidant activity at the intracellular environment. The chloroform, ethyl acetate and methanol extracts have, respectively, an IC_{50} > 0.8 mg / mL, 0.510 mg / mL and 0.44 mg / mL on TK6 cells (Figure 2,3) (Table 2).

3.6. Extracts and molecule inhibitory effect in combination with temozolomide on glioblastoma proliferation:

Using the MTT assay, We have examined, on the U 373 MG cell population growth *in-vitro*, the effect of *Nitraria retusa* extract and the isorhamnetin-3-O-robinobioside(Nr-I3-O-Rob), a pure molecule previously extracted from the ethyl acetate extract [25]. The results of this assay were reported in Figure 4.

The results show that the effect of Nr-extracts whether in the absence or presence of TEMODAL is dose dependent in proportion to the dose used and revealed a: IC_{50} Nr-Meoh: 450 μ g/mL, IC_{50} Nr-Meoh + TPZ: 125 μ g/mL IC_{50} Nr-EA: 187 μ g/mL, IC_{50} Nr-EA + TPZ: 63,5 μ g/mL, IC_{50} of Nr-I3-O-Rob = 190 μ g/mL, IC_{50} Nr-I3-O-Rob + TPZ: 68 μ g/mL and IC_{50} Nr-Chl: 90 μ g/mL, IC_{50} Nr-Chl + TPZ: 25 μ g/mL. the combination of temodal improves the effectiveness of Nr-EA and Nr-Meoh against glioblastoma; this combination does not affect the effectiveness of Nr-Chl against glioblastoma. Knowing that the temodal median IC_{50} at cell lines was 65,3 μ g/mL.

Discussion

The CAA of the Nr-EA and Nr-MeOH extracts to scavenge free radicals is attributed to isorhamnetin and its glycosylated analogues. Isorhamnetin, a 3'-O-methylated metabolite of quercetin, protects cells against oxidative stress by activating the nuclear factor erythroid 2-related factor 2 (Nrf2), which binds to the antioxidant response element (ARE) and regulates the induction of genes encoding antioxidant proteins and phase II detoxifying enzymes, [26] such as heme oxygenase-1 (HO-1) [27]. Additionally, glycosylated isorhamnetin enhances the production of superoxide dismutase (SOD), catalase, and

glutathione reductase [28]. Isorhamnetin decreased LPS-induced ROS production (HO-1), inhibit ROS-mediated accumulation of hypoxia-inducible factor-1 α (HIF-1 α), and repress pro-oxidant factors [27],

It has been shown that, isorhamnetin to provide a protective effect against oxidative stress in human cells, with its mechanism of action linked to the activation of the PI3K/Akt signaling pathway. [29]. Additionally, Isorhamnetin demonstrates antioxidant effects against H₂O₂ [30].

Some flavonoids act as competitive inhibitors of xanthine oxidase (XO), but the Nr-EA and Nr-MeOH extracts demonstrate superior radical scavenging capabilities compared to their inhibitory effects on XO. The inhibition of XO by flavanols in these extracts correlates with their chemical structure, with correlation coefficients of $r = 0.757$ for Nr-MeOH and $r = 0.995$ for Nr-EA. The structure-activity relationship of isorhamnetin and its glycosylated derivatives indicates that hydroxyl groups at C-5 and C-7, along with a double bond between C-2 and C-3, are crucial for significant XO inhibition [31], [32]. For effective superoxide scavenging, hydroxyl groups at C-3 and C-3' are essential. The planar configuration of the benzopyran ring and the torsion angle of specific atoms also influence XO inhibition. Isorhamnetin is recognized as a potent XO inhibitor, which can lower uric acid levels [31]. [32]. [33].

The direct ROS-scavenging activity was evaluated using ABTS^{•+} and NBT/Riboflavin, revealing strong correlations between flavonols in the extracts and their scavenging capacity for superoxide (O₂^{•-}), with coefficients of 0.988 and 0.862, respectively. Both extracts exhibited significant antiradical activity against ABTS radicals, linked to their chemical constituents. The presence of multiple phenolic hydroxyl groups enhances antioxidant capacity, while glycosylation and methylation can further improve efficacy. Isorhamnetin's ability to scavenge ABTS radicals underscores its significant antioxidant activity [34]. [35]. [36]. [37], [38], suggesting that its anticancer effects may be related to its antioxidant properties.

Our study shown that Nr- Chl extract seem to react as a braking system against some types of radicals in some systems. This behavior is correlated with the presence of palmitic acid and β -Sitosterol in the Nr-Chl extract; ($r = 0.988$ with Nr-Chl extract against the production of uric acid). Palmitic acid revealed in Nr-Chl extract showed an antioxidant activity against radical are considered because of their hydrogen contributing ability and can function as free radical inhibitors [15] and significantly decreased radicals generation by the xanthine-xanthine oxidase system levels [39].

The CAA capacity of Nr-Chl extracts is due to palmitic acid that decreases ROS production during stress and reduces the need of peroxide detoxification through GPx as well as of GSH resulting in diminished cell injury and death, Palmitic acid effect may be due to the cAMP/PKA signaling [40]. In the other hand, β -sitosterol decreases O₂^{•-} by inhibition ROS generating system [41]. Also, β -Sitosterol regulates also the GSH redox cycle by preventing the accumulation of reactive oxygen species (ROS). It significantly increases the expression of Nrf2, thereby activating the GSH metabolism pathway at the genetic level. Additionally, β -sitosterol can enhance cellular antioxidant capacity by upregulating uric acid transporter expression [42], and stimulates antioxidant enzymes by an estrogen receptor/PI3-kinase-dependent

pathway [43], character which can be attributed to its ability to bind by hydrogen bonds between the hydroxyl group (OH) of the 3-carbon of β -sitosterol and several amino acids [44].

Furthermore, the capacity of our extracts to scavenge free radical was assessed. This suggests that the Nr-extracts contains potent antioxidants that are highly effective at scavenging ROS, potentially providing a protective effect against oxidative stress, which is a hallmark of cancer progression., the Nr-extracts could have a protective effect on normal cells and could help sensitize cancer cells to therapies like temozolomide, as antioxidants may enhance the efficacy of chemotherapy while minimizing side effects [45].

Our data demonstrated a dose-dependent effect of the *Nitraria retusa* extracts on the U373 MG cell growth. As the concentration of the extracts increases, the inhibition of cell growth also increases, which is a typical response for cytotoxic compounds. These findings suggest that the extracts of *Nitraria retusa* can effectively reduce glioblastoma cell proliferation at tested concentrations. Nr-Meoh had the highest IC₅₀ value in the absence of temozolomide (450 μ g/mL), suggesting that it is less potent compared to the ethyl acetate, Nr-I3-O-Rob and chloroform extracts in inhibiting cell growth. However, Nr-EA and Nr-I3-O-Rob was more potent than the methanol extract, with respectively an IC₅₀ of 187 μ g/mL and 190 μ g/mL. Nr-Chl exhibited the strongest cytotoxic effect on its own (90 μ g/mL). The combination of temozolomide (TPZ) with the Nr extracts improved the effectiveness of both the methanol, ethyl acetate extracts and Nr-I3-O-Rob against glioblastoma cells. Specifically: The IC₅₀ for Nr-Meoh dropped from 450 μ g/mL to 125 μ g/mL when combined with temozolomide, indicating that temozolomide enhances the cytotoxicity of the methanol extract. Similarly, The IC₅₀ for Nr-EA decreased from 187 μ g/mL to 63.5 μ g/mL when combined with temozolomide, and, the IC₅₀ for Nr-I3-O-Rob decreased from 190 μ g/mL to 68 μ g/mL when combined with temozolomide, showing a similar synergistic effect. The best combinatory effect with temozolomide is achieved with Nr-Chl, yielding an IC₅₀ of 25 μ g/mL, which is even better than temozolomide alone (IC₅₀ = 65,3 μ g/mL), suggesting an amplifying effect of temozolomide on chloroform extract. These results suggest that temozolomide may sensitize the glioblastoma cells to the effects of the *Nitraria retusa* extracts, possibly by increasing oxidative stress, which temozolomide induces.

Based on our results, we hypothesize that this study presents a potentially synergistic therapeutic approach for glioma. This effect is likely due to the induction of apoptosis via the activation of apoptotic signaling pathways, thereby enhancing TMZ-induced apoptosis. Various compounds present in *Nitraria* extracts may synergistically enhance their therapeutic effects against glioblastoma. Indeed, flavonoids inhibit cell proliferation and induce apoptosis by suppressing the PI3K/Akt/mTOR and JAK/STAT signaling pathways [46] [47] thereby blocking pro-oncogenic cascades mediated by MAPK, NF- κ B, and Akt[48]. Flavonoids also induce cell death in U-373MG cells via a mitochondrial pathway, increasing p53 expression and facilitating cytochrome c release into the cytosol while activating apoptotic pathways involving caspase-3 and caspase-9[49] [50]. It has been demonstrated that combinations of flavonoids exhibit greater efficacy in inhibiting cell population growth compared to individual flavonoids [51],

[52] and the different flavonols present in Nr-EA and Nr-Meoh extracts may act synergistically against glioblastoma.

Additionally, the strong antiproliferative effect of Nr-Chl is likely due to β -sitosterol, which induces apoptosis and G2/M phase cell cycle arrest in U87 cells while enhancing E-cadherin expression and suppressing β -catenin and vimentin, implicating its role in the epithelial-mesenchymal transition (EMT). EMT activation can lead to a more invasive phenotype and reduced chemotherapy sensitivity. Furthermore, β -sitosterol has been shown to induce apoptosis via the mitochondria-mediated apoptotic signaling pathway [2]. Our results suggest that β -sitosterol may be a promising therapeutic agent for the treatment of glioma. On the other hand, the role of palmitic acid in the inhibition of glioblastoma remains to be further developed; however, it is involved in several signaling pathways relevant to glioma, including EGFR/PI3K/Akt/mTOR, p53, and the retinoblastoma protein [53]. While the role of palmitic acid in glioblastoma inhibition warrants further investigation, it is implicated in key signaling pathways associated with glioma.

Our results and the findings from El-Hag et al. (2015) [54] align in demonstrating the potential of combining temozolomide with natural compounds to enhance glioblastoma treatment. The authors reported similar findings, showing that natural products can potentiate the effects of temozolomide in glioblastoma therapy. They suggested that natural compounds could enhance temozolomide's cytotoxic effects by inhibiting DNA repair mechanisms, increasing oxidative stress, or modulating cell signaling pathways involved in resistance. The combination of temozolomide with natural agents could help overcome the chemoresistance that often limits the efficacy of temozolomide alone. This encourages to study in more detail from a mechanistic point of view the effect of β -sitosterol which through the Nr-Chl extract shows a more pronounced effect than the temodal.

Conclusions

Despite encouraging findings, the effects of flavonoids and phytosterols on glioblastoma (GBM) remain confined to preclinical studies. Future research should investigate the physiological properties of flavonoids and β -sitosterol, including their toxicity, side effects, bioavailability, and permeability, to facilitate clinical application. When applicable, clinical trials should evaluate and validate the safety and therapeutic efficacy of combinations of flavonoids, β -sitosterol and chemotherapy.

Abbreviations

TPZ : temodal

Declarations

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Consent for publication

Not applicable.

Availability of data and materials

The dataset supporting the conclusions of this article are available from the corresponding author on reasonable request.

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The authors declare that they have no competing interests.

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Authors' contributions:

BJ: Was responsible for the conception and design, testing and data acquisition, analysis and data interpretation and drafted the manuscript.

AL: made a substantial contribution to the design and revision of the manuscript critically for important intellectual content.

SA: made contribution to the statical analysis and revised it critically.

KM: made substantial contribution to conception and revised it critically for important intellectual content.

LCG: made substantial contribution to conception and revised it critically for important intellectual content

The authors read and approved the final manuscript.

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Tables

Table 1 is available in the Supplementary Files section.

Table 2: IC₅₀ of DCFH radical formation by *Nitraria retusa* leaf extracts in TK6 cells using the cellular antioxidant activity assay

Extract ^a	CI ₅₀ (mg/ml)
Chloroform	> 0,8
Ethyl acetate	0,51* ± 0.04
Methanol	0,44* ± 0.03

^a Values were expressed as means ± standard deviation of three experiments

* P < 0.05 compared to negative control without the tested extract by student test.

Figures

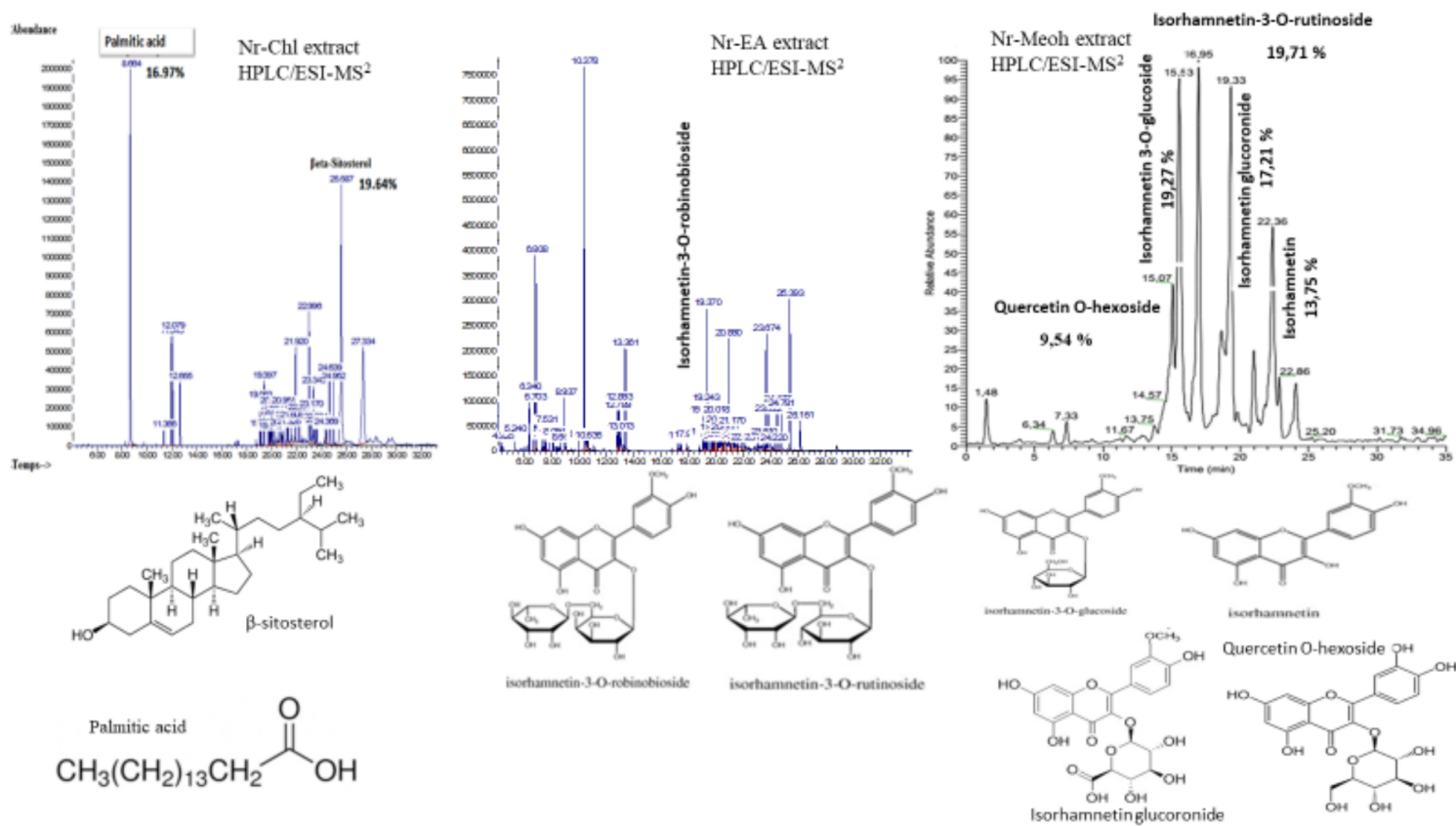


Figure 1

HPLC/ESI-MS2 profiling of compounds in *Nitraria retusa* extracts

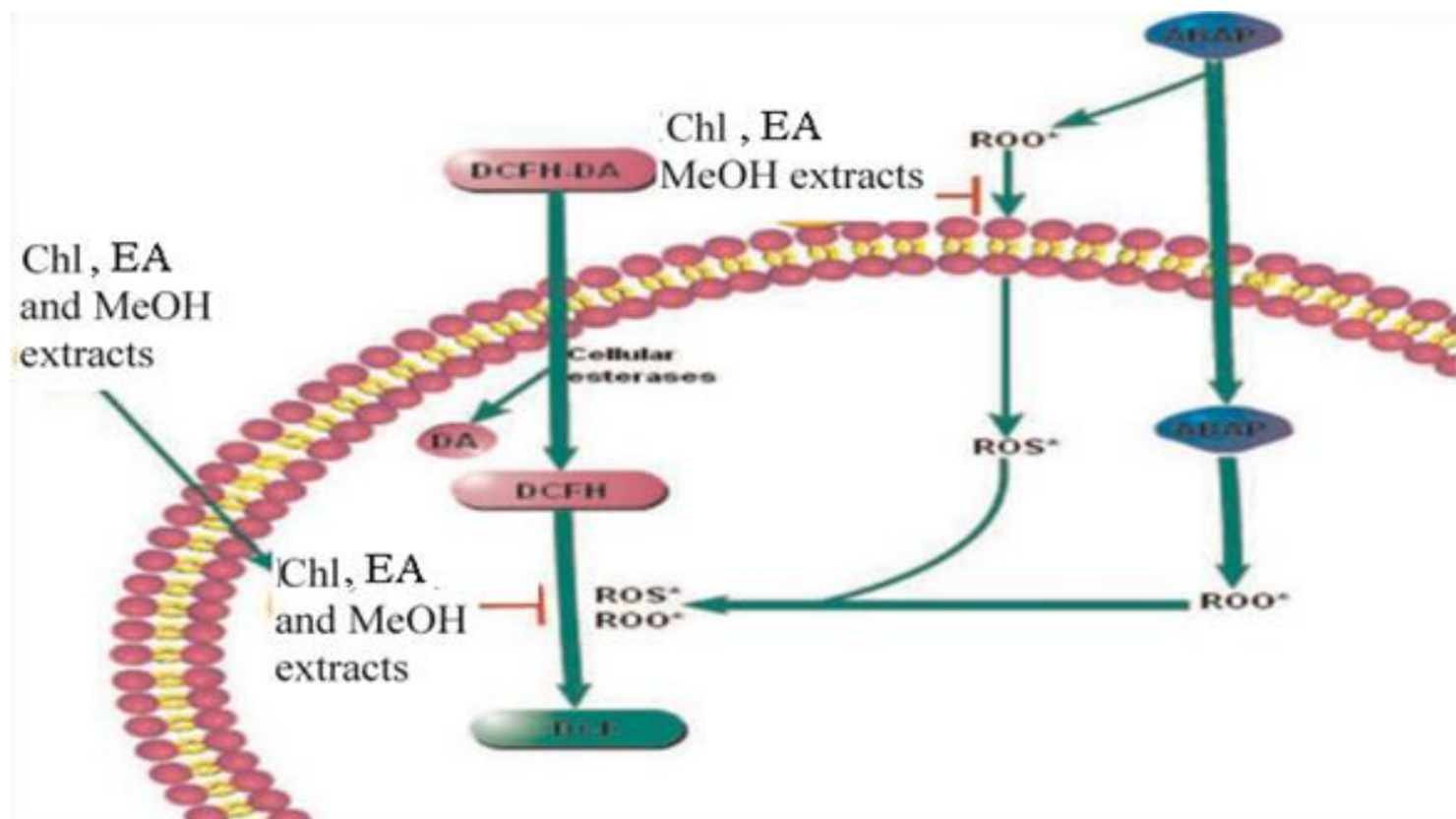


Figure 2

Cellular antioxidant activity of *Nitraria retusa* leaf extracts in human chronic myelogenous leukaemia cell line K562 (Wolfe and Liu, 2007)

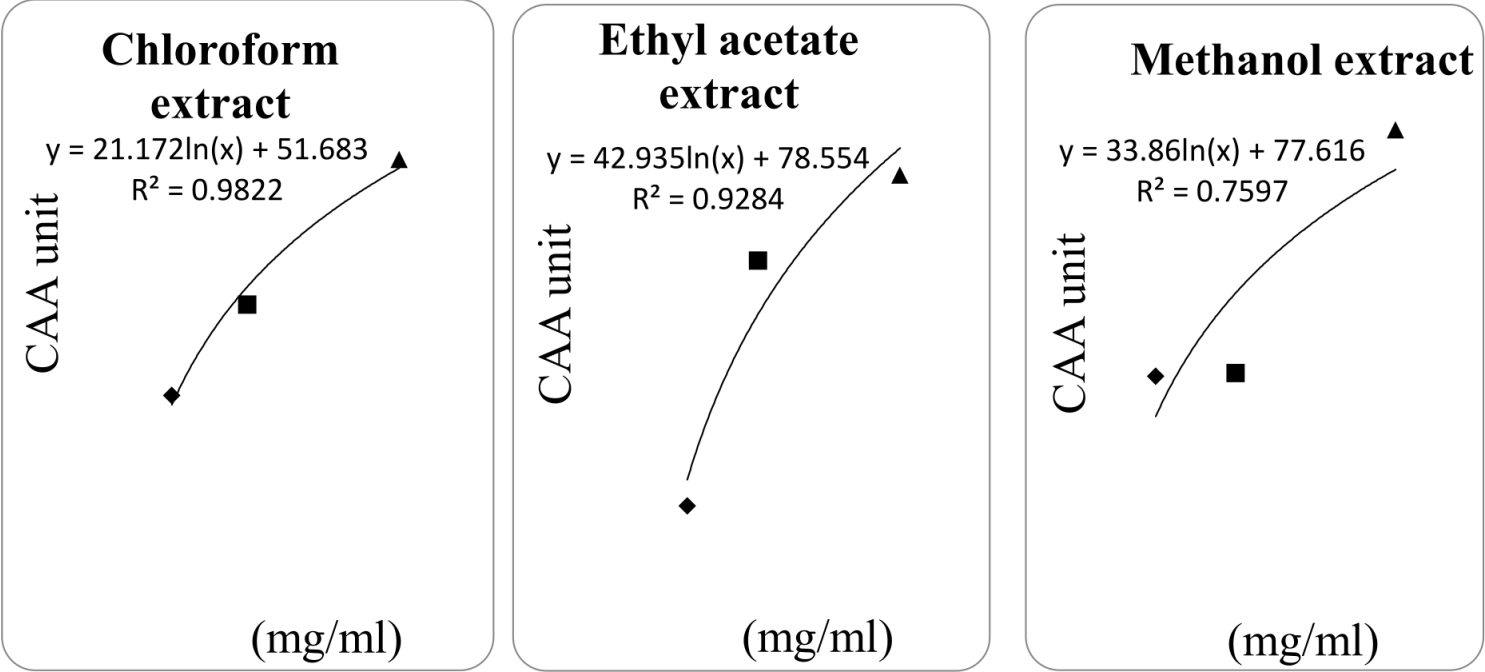


Figure 3

Dose-response curve for the inhibition of oxidation of the free-radical 2', 7'-dichlorofluorescein (DCFH) to DCF in TK6 cells using the cellular antioxidant activity assay in the presence of different extracts of *Nitraria retusa* (mean $\pm$ SD, n= 3)

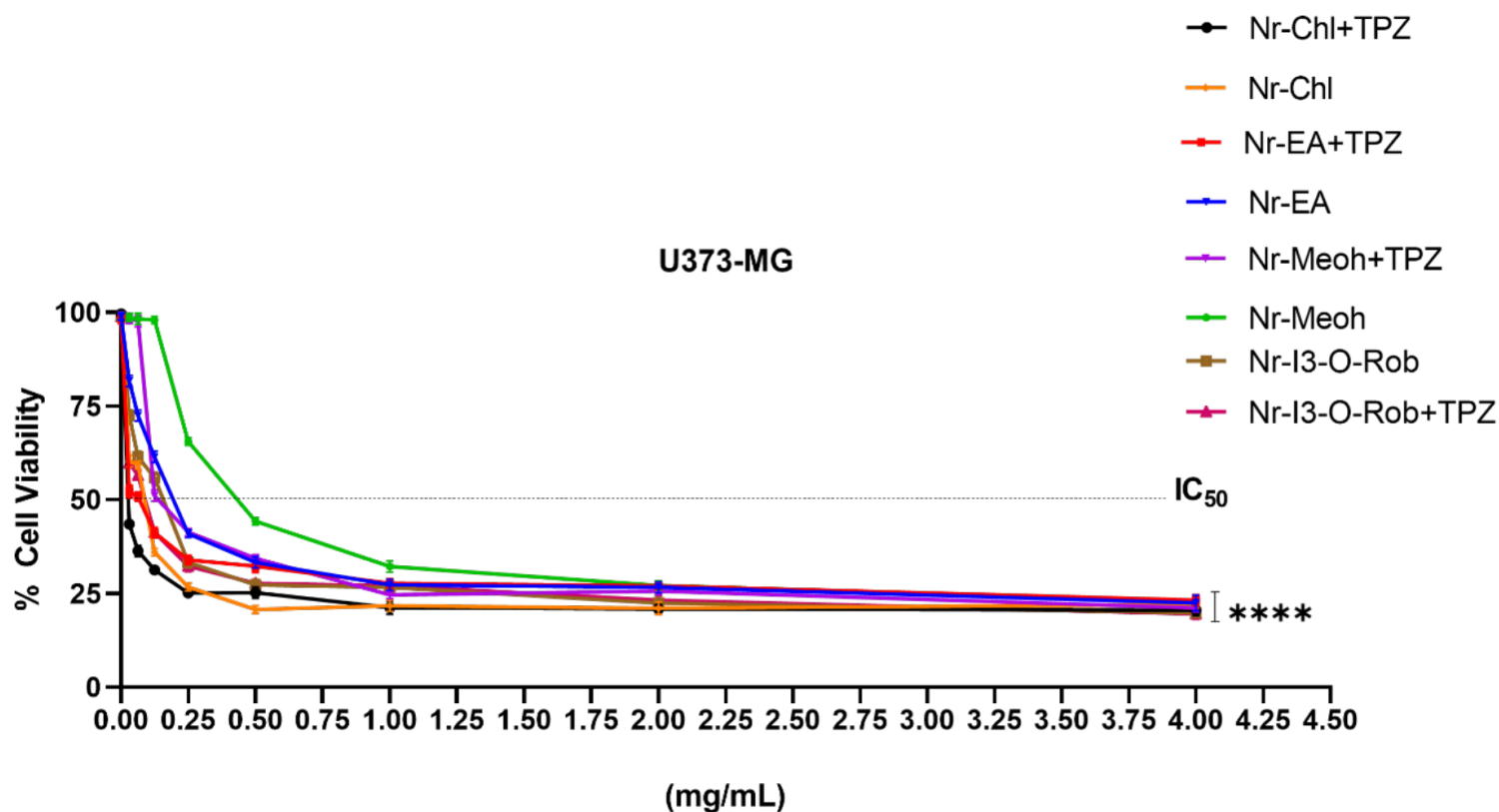


Figure 4

Inhibitory effect of *Nitraria retusa* leaf extracts and molecule on the viability of U373 cells. Results are represented by the means $\pm$ SD of $n = 3$. (*) $p < 0.0001$ means a significant difference between the treated cells (with and without temodal).

Supplementary Files

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