ORIGINAL ARTICLE

Antiproliferative activity of *Neolamarckia cadamba* (kelempayan) leaves methanol extract on human glioblastoma (U-87 MG) cells

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ABSTRACT

Introduction: Glioblastoma is a malignant brain tumor with a poor prognosis and high fatality rates. Current chemotherapy, primarily with temozolomide (TMZ), is limited by resistance and toxic side effects, underscoring the need for alternative treatments. This study investigates the antiproliferative properties of *Neolamarckia cadamba* (NC) leaves methanol extract on glioblastoma (U-87 MG) cells as a potential alternative treatment.

Materials and Methods: NC leaves were collected, dried, and extracted using methanol via maceration. Phytochemical screening identified active compounds, including phenolics, flavonoids and tannins. U-87 MG cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum and 1% Penicillin-Streptomycin. The cells were treated with serial dilutions of NC extract and TMZ as a positive control for 24, 48 and 72 hours. Cytoselective of NC extract is conducted on non-cancerous (Vero) cells. Cytotoxicity was assessed using the MTT assay, with IC50 values determined from dose-response curves. Apoptotic induction was evaluated using Acridine Orange/Propidium Iodide (AO/PI) staining under a fluorescent microscope.

Results: Phytochemical screening of NC leaves extract revealed active compounds such as phenolics, flavonoids, tannins and saponins. The MTT assay demonstrated dose- and time-dependent cytotoxicity of NC extract, with IC50 values of 198.7 µg/mL (24 hours), 321.4 µg/mL (48 hours), and 126.3 µg/mL (72 hours). NC extract also shows cytoselective on Vero cells with an IC50 value of more than 100 µg/mL for all timeframes. AO/PI staining confirmed apoptosis induction of the NC extract on U-87 MG cells.

Conclusion: NC extract exhibited low potential as an alternative therapeutic agent for glioblastoma by inducing early apoptosis on U-87 MG cells.

KEYWORDS:
Neolamarckia cadamba, kelempayan, antiproliferative, anticancer, glioblastoma, medicinal plants

INTRODUCTION

Gliomas are the most common primary malignant brain tumours, with glioblastoma being the most prevalent and aggressive subtype. The global incidence rate of glioblastoma is approximately 3.23 per 100,000 population.[1] Gliomas account for about 24.5% of all primary brain tumours and 80.9% of all malignant brain tumours in adults.[1] The incidence of gliomas varies significantly by age, sex, and geographical region. For instance, the incidence rate is higher in males compared to females.[2,3] Additionally, the higher incidence observed in American and Northern European populations compared to Asian populations suggests a possible hereditary predisposition.[1,4] In Malaysia, gliomas are less common compared to other cancers. According to the Malaysia National Cancer Registry Report 2017-2021, gliomas are not listed among the top ten most common cancers in the country.[5] However, the incidence of brain and central nervous system (CNS) cancers, which include gliomas, has been documented.5 The report indicates that the age-standardized rate (ASR) for brain and CNS cancers in Malaysia is relatively low compared to other types of cancer. In 2022, there were 870 new cases of brain and CNS cancers reported, with an ASR of 0.23 per 100,000 population.[5] The mortality rate for these cancers was 2.5%, with 799 deaths reported.[5,6]

Glioma presents a significant therapeutic challenge due to its high invasiveness, poor prognosis, and resistance to conventional treatments.[7] Current therapies, including surgery, radiation, and chemotherapy with agents like temozolomide (TMZ), are often limited by systemic toxicity and the emergence of drug resistance, emphasizing the urgent need for novel therapeutic approaches.[8] TMZ is an oral alkylating agent that has become a cornerstone in the treatment of gliomas, particularly glioblastoma (GB). TMZ is most commonly used in combination with radiotherapy, after surgery and collectively known as the Stupp protocol. This combination has been shown to improve median survival rates for patients with GBM from approximately 12.1 months with radiotherapy alone to 14.6 months with the addition of TMZ. While the landmark Stupp protocol demonstrated an increase in 2-year survival of glioblastoma patients from 10.4% to 26.5% with combined radiotherapy

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and temozolomide, it also underscored the considerable systemic toxicity and economic burden associated with this treatment, highlighting the urgent need for safer, more cost-effective therapeutic alternatives.[6]

Neolamarckia cadamba (NC), commonly known as Kelempayan or Bur flower, is a fast-growing tropical deciduous tree native to South and Southeast Asia and widely distributed across India, Nepal, Bangladesh, Sri Lanka, Myanmar, Thailand, Malaysia, Indonesia, and the Philippines,[9] with introductions to other tropical regions for ornamental, timber, and medicinal purposes.[10,11] The species thrives in moist, evergreen, and mixed deciduous forests, particularly in fertile, well-drained alluvial soils. Traditionally, various parts of NC, including leaves, bark, and fruits,[12] have been employed in ethnomedicine, and modern studies have reported that its phytochemicals, particularly phenolics, flavonoids, and alkaloids[12] exhibit diverse bioactivities, including antioxidant,[13] antidiabetic,[14] anti-inflammatory,[15] antitumor,[16] and anticancer effects.[17,18] However, despite these promising findings, systematic investigations into the mechanisms underlying its anticancer activity and its potential for development as a therapeutic agent remain limited, highlighting the need for further research.

Therefore, the current study was designed to evaluate the anticancer effect of NC leaves methanol extract on cell cytotoxicity and apoptosis induction in human glioblastoma cell line.

MATERIALS AND METHODS

Plant material authentication

The leaves of NC were collected and verified by local residents of Telemong, Terengganu, Malaysia. The plant materials were further authenticated at the International Islamic University Malaysia (IIUM) Herbarium Centre in Pahang, Malaysia, and the voucher specimen (PIIUM 0266-1) was deposited in the IIUM Herbarium Centre (Voucher No.: PIIUM 0266-1)

Plant material extraction

The leaves were properly washed and dried in a drying cabinet. The dried leaves were then finely powdered and 50 g of the powdered leaves were extracted using the maceration technique with 450 mL of methanol solvent. The leaves were soaked for six days and filtered every two days. The crude plant extract was evaporated to dryness in a rotary vacuum evaporator. The crude plant extract was dried and then collected into vials, with the amount being recorded.[19] The percentage of yield was calculated using the formula given below:

$$\text{Percentage yield (\%)} = \frac{\text{weight of crude extract (g)}}{\text{weight of plant material (g)}} \times 100$$

Phytochemical analysis of NC extract

NC leaves methanol extract was subjected to various qualitative phytochemical tests to screen for phytochemical constituents[20]

i. Detection of phenolic compound
250 mg (0.25g) of extract was dissolved in 5 ml distilled water. Then, 5 drops of 5% ferric chloride (FeCl3) were added. The appearance of intense green, purple or blue-black coloration indicates the presence of phenolic compound.

ii. Detection of flavonoid compound
100 mg (0.1 g) of extract was dissolved in 2 ml of distilled water. Then, the solution was mixed with 4 drops of 10% sodium hydroxide (NaOH) solution. Appearance of yellow coloration indicates presence of flavonoid compound.

iii. Detection of tannin compound
500 mg (0.5 g) of extract was dissolved in 10 ml of distilled water. In 2 ml of the solution, 4 drops of 1% ferric chloride (FeCl3) were added. Appearance of blue-black coloration indicates the presence of tannin compound.

iv. Detection of coumarin compound
100 mg (0.1 g) of extract was dissolved in 1 ml of distilled water. Then, 1 ml of 10% sodium hydroxide (NaOH) was added followed by 1 ml of chloroform. Appearance of yellowish brown shows the presence of coumarin.

v. Detection of alkaloid compound
250 mg (0.25 g) of extract was dissolved with 5 ml of 1% hydrochloric acid (HCl). Then, 3 drops of Dragendorff's reagent were added. Appearance of orange red precipitate indicate the presence of alkaloid compound.

vi. Detection of glycosides compound
2 ml of sulphuric acid was added into 250 mg (0.25 g) of extract. A reddish-brown colour formation indicates the presence of glycosides compound.

vii. Detection of saponin compound
300 mg (0.3 g) of extract was dissolved in 3 ml of distilled water and shaken vigorously for 2 minutes. Persistent frothing indicates the presence of saponin compound.

viii. Detection of steroid compound
250 mg (0.25 g) of extract was dissolved in 2.5 ml distilled water. Then, 1 ml of chloroform was added. Then, 1 ml of sulphuric acid was carefully added to form a layer. In the lower of chloroform layer, the appearance of red colour indicates the presence of steroid compound.

ix. Detection of terpenoid compound
250 mg (0.25 g) of extract was dissolved in 2.5 ml of distilled water. Then, 1 ml of chloroform and 1 ml of concentrated sulphuric acid (H2SO4) were added carefully to form a layer. After that, solution was mixed. A reddish-brown coloration indicates terpenoids compound.

x. Detection of quinone compound
100 mg of extract was dissolved in 1 ml of distilled water. Then, 1 ml of dilute sodium hydroxide (NaOH) was added. The appearance of blue-green or red coloration indicates the presence of quinones.

Cell culture

U-87 MG (human glioblastoma cells) and Vero (African Green Monkey normal kidney cells) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Both cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC, Manassas, VA) supplemented with 10% foetal bovine serum (FBS) and penicillin–streptomycin 1% (v/v) (Gibco, Grand Island, NY). The cells were incubated at 37°C and 5% CO2 in a humidified atmosphere.

Antiproliferative assay

U-87 MG and Vero cells were seeded into a 96-well plate at a density 5.0×10^4 cells per well and incubated for 24 hours, respectively. After 24 h, complete media with gradient concentrations (0, 3.9,7.8, 15.6, 31.3, 62.5, 125 and 250 µg/mL) of NC extracts and TMZ (used as positive control drug) were added into respective wells. Whereby, Vero cells (negative control cells) were treated with six different concentrations of NC extract ranging from 150µg/ml to 12.5µg/ml.

The plates were incubated at 37°C for 24, 48 and 72 hours. Then, MTT solution (5 mg/mL) was added into each of the wells. The plates were incubated in humidified incubator with at 37°C for 4 hours. Absolute DMSO was added into the wells and absorbance (OD) was measured at wavelength 570 nm. The half maximal inhibitory concentration (IC50) of each solvent was determined using graph percentage of viable cells against concentration (µg/mL). The percentage of viable cells was calculated using formula as follows:

$$\text{Percentage of viable cells (\%)} = \frac{\text{(OD value of treated cells)}}{\text{(OD value of untreated cells)}} \times 100$$

Cell morphology analysis

Morphological detection of apoptosis was examined using acridine orange and propidium iodide (AO/PI) staining. U-87 MG cells at a concentration of 3.0×10^4 cells/mL were treated with NC extract for 24, 48 and 72 hours at their IC50 concentrations in triplicates. The treated cells were trypsinised and harvested with 1 mL cold phosphate buffered saline (PBS), followed by centrifuged at 1500 rpm for 10 minutes at 4°C. This process was repeated twice. The cell suspension was mixed with 20 µL of AO/PI solution (1:1) and the mixture (10 mL) was transferred onto a slide and covered with a cover slip. Viable, apoptotic and necrotic cells were quantified in a population of 200 cells using a fluorescence microscope equipped with a B-2A filter (Nikon TE2000-U, Japan). Untreated U-87 MG cells served as a negative control and TMZ-treated cells were used as a positive control.

Data analysis

The statistical analysis was performed using the GraphPad PRISM software version 10 (GraphPad Software, Inc., San Diego, CA, USA). The dose-dependent curve analysis for the determination of IC_{50} was analysed and plotted using GraphPad Prism. The differences between the means were assessed with the two-way analysis of variance (ANOVA) and Tukey's post hoc test. The difference with p values less than 0.05 was considered statistically significant ($p<0.05$).

RESULTS

Phytochemical analysis of NC extract

The phytochemical analysis of NC leaves methanol extract revealed a diverse profile of bioactive compounds, which likely contribute to its observed anticancer effects. This qualitative screening identified the presence of key phytoconstituents, including phenolics, flavonoids, tannins, coumarins, alkaloids, glycosides, saponins, and steroids. However, terpenoids and quinones were not detected in the extract as represented in Table I.

Antiproliferative activity of NC extract

The MTT assay results demonstrated the antiproliferative effects of NC extract and TMZ on U-87 MG cells in a time- and dose-dependent manner, as shown in Figure 1. Table II shows the IC50 values of NC extract and TMZ on U-87 MG cells obtained from the dose-response curve of percentage of cell viability against log10 concentration. Based on the results, IC50 values of NC extract on U-87 MG cells were 198.7, 321.4, and 126.3 µg/mL at 24, 48, and 72 hours, respectively. TMZ achieved much lower IC50 value than NC extract; 18.70, 83.90 and 67.12 µg/mL at 24,48 and 72 hours, respectively.

Two-way ANOVA revealed that both NC extract and TMZ exerted concentration-dependent effects on U-87 MG cell viability, although with different patterns. For NC extract, concentration was the dominant factor ($F(7,48)=193.72$, $p<0.0001$), explaining 79.5% of the total variance, with a significant contribution from treatment duration ($F(2,48)=27.97$, $p<0.0001$; 3.3% variance) and a strong interaction effect ($F(14,48)=17.53$, $p<0.0001$; 14.4% variance). In contrast, TMZ activity was almost exclusively driven by concentration ($F(7,36)=247.53$, $p<0.0001$), which accounted for 87.9% of the variance, with no significant effect of treatment duration ($F(2,36)=0.57$, $p=0.571$), although the interaction between concentration and time remained significant ($F(14,36)=12.55$, $p<0.0001$; 8.9% variance). Collectively, these results indicate that while both agents are strongly concentration-dependent, NC extract's antiproliferative activity is more influenced by treatment duration compared to TMZ, suggesting differences in the kinetics or mechanisms of action between the crude extract and the chemotherapeutic drug.

Figure 2 shows the IC50 values of NC extract against non-cancerous (Vero) cells were 321.6, 479.9, and 169.6 µg/mL at 24-, 48-, and 72-hour treatment, respectively. Two-way ANOVA showed a significant main effect of concentration ($p<0.0001$) and a significant interaction between concentration and treatment duration ($p<0.0001$), whereas treatment duration alone was not significant ($p=0.0870$). Concentration accounted for 74.42% of the total variation, while the interaction contributed 18.71%, indicating that the effect of concentration on the response was dependent on treatment duration.

Apoptosis analysis from AO/PI staining

The Acridine Orange/Propidium Iodide (AO/PI) staining results provided valuable insights into the induction of apoptosis by NC extract in U-87 MG cells. Following 72 hours of treatment with the IC50 concentration of NC extract, a substantial increase in apoptotic cells was observed compared to untreated controls. As shown in Figure 3, NC-

Table I: Phytoconstituent from Neolamarckia cadamba leaves methanol extract

Compound	Indicator	Result
Phenolic	Appearance of intense green, purple or blue -black color upon addition of 5% FeCl3.	a
Flavonoid	Solution turns yellow upon mixing with 10% NaOH solution.	a
Tannin	Appearance of blue-black coloration upon addition of 1% FeCl3.	a
Coumarin	Appearance of a yellowish-brown solution upon addition of chloroform.	a
Alkaloid	Appearance of orange-red precipitate upon addition of Dragendorff's reagent.	a
Glycoside	A reddish-brown color formation upon addition of H2SO4.	a
Saponin	Persistent frothing upon vigorous shake.	a
Steroid	Appearance of red color in the lower of chloroform layer upon addition of sulphuric acid.	a
Terpenoid	Formation of reddish-brown coloration upon mixing.	b
Quinone	Appearance of blue-green or red coloration upon addition of dilute NaOH.	b

a: Presence of compound
b: Absence of compound

Table II: IC50 value of Neolamarckia cadamba (NC) leaves methanol extract and Temozolomide (TMZ) on U-87 MG cells

Treatment	IC50 value (µg/mL)		
	24 hours	48 hours	72 hours
NC extract	198.7	321.4	126.3
Temozolomide (TMZ)	19.46	83.90	67.12

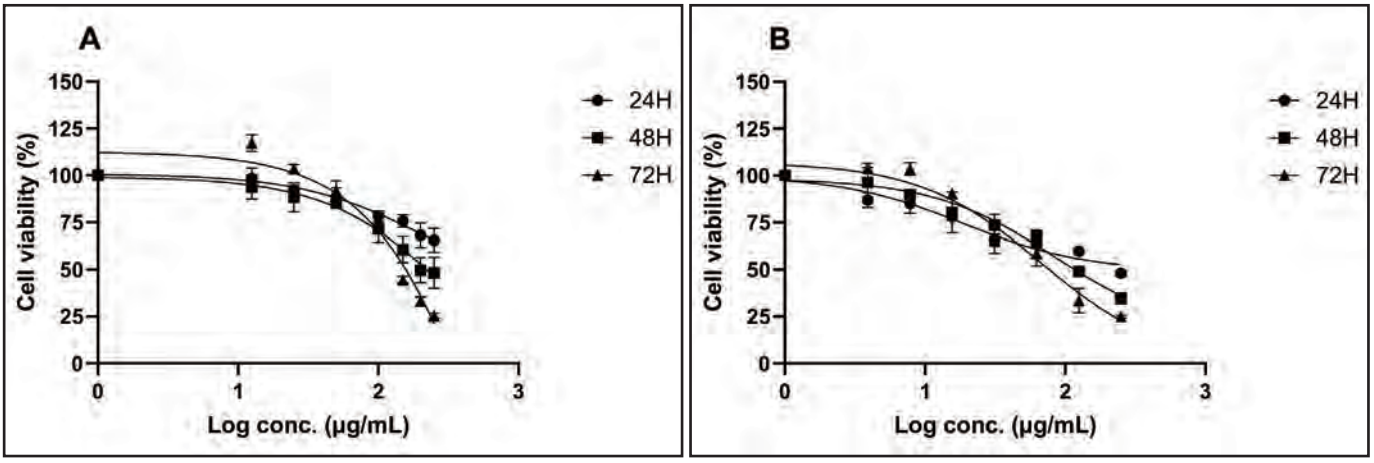

Fig. 1: Antiproliferative activity of different concentrations of (A) NC extract and (B) TMZ on U-87 MG cells at 24-, 48- and 72- hours treatment

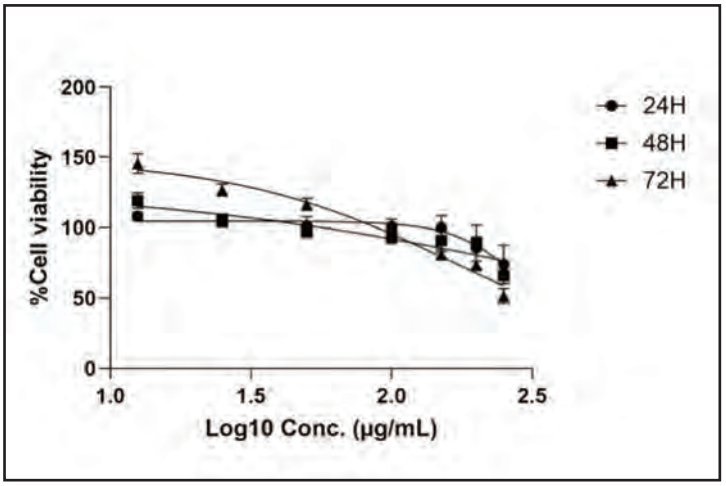

Fig. 2: Antiproliferative activity of different concentrations of NC extract on non-cancerous (Vero) cells at 24-, 48- and 72-hours treatment

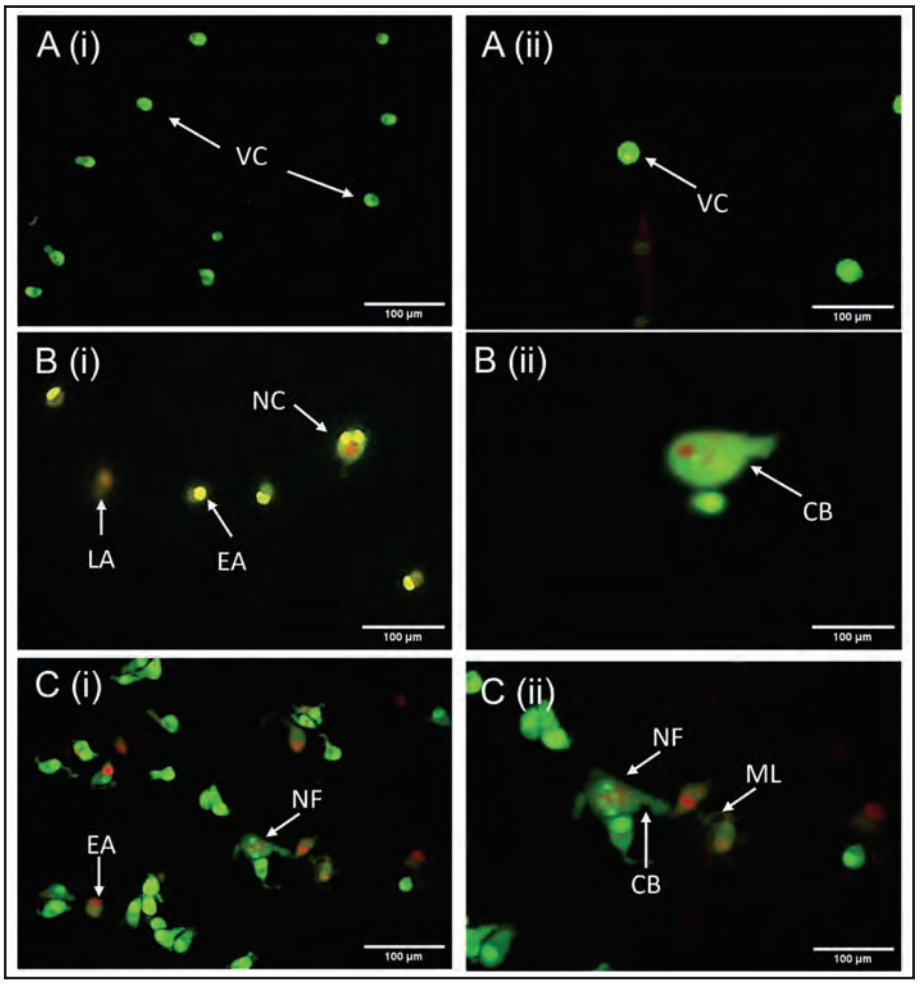

Fig. 3: Morphological changes of (A) untreated, (B) NC-treated (126.3 µg/mL) and (C) TMZ-treated (67.12 µg/mL) of U-87 MG cells stained with AO/PI staining after 72 hours. The cells were observed and captured under (i) 200x and (ii) 400x magnification, respectively, by a fluorescence microscope. EA; early apoptosis, LA; late apoptosis cell, NC; nuclear condensation; CB; cell blebbing; NF; nuclear fragmentation, ML; membrane loose, VC; viable cell

treated cells showed classic apoptotic morphological features, including chromatin condensation, membrane blebbing, and the formation of apoptotic bodies. These features were distinctly absent in untreated cells, which displayed uniformly green fluorescence indicative of healthy nuclei.

The apoptotic effect of NC extract was comparable to that of TMZ, the standard chemotherapeutic agent. NC-treated cells also exhibited a less pronounced necrotic response, suggesting that the extract primarily induces programmed cell death rather than uncontrolled necrosis. This finding underscores the potential of NC extract as a targeted, less toxic alternative therapy for glioblastoma.

DISCUSSION

The interpretation of IC_{50} values is essential to evaluate the cytotoxic potential of plant extracts and to benchmark their activity against established pharmacological standards. According to American National Cancer Institute (NCI) - based criteria, crude extracts with IC_{50} values below 20–30 µg/mL are considered cytotoxic, while IC_{50} values between ~21–200 µg/mL are viewed as moderately active and values greater than 200 µg/mL are deemed weakly cytotoxic.[21] This framework is consistently applied in recent studies.[22-24] Based on these criteria, the IC_{50} values obtained for NC extract against U-87 MG cells fall within the moderate activity range, suggesting limited potency compared to standard chemotherapeutics, TMZ. However, the higher IC_{50} values of NC extract in normal Vero cells relative to U-87 MG cancer cells indicate a modest safety margin, consistent with the principle that plant extracts demonstrating reduced cytotoxicity toward normal cells are considered safer and potentially more selective.[25,26]

The antiproliferative activity of NC extract against glioblastoma cells may be attributed to its phytoconstituents, particularly flavonoids. Quercetin, identified in NC extract, has been shown to suppress phospholipase D (PLD), a key regulator of proliferation and tumorigenesis in U-87 MG cells.[27] In addition, flavonoids inhibit matrix metalloproteinase-2 (MMP-2), thereby reducing glioma cell invasion and metastatic potential. Differences in IC_{50} values across studies highlight the influence of both the plant part

used and the bioactive compounds present. Dolai et al. (2016) reported that methanolic NC extract reduced tumor volume and induced apoptosis in Dalton's lymphoma ascites cells, supporting its broad antitumor activity. Razali et al. (2021) demonstrated that ethanol extract of NC leaves inhibited proliferation of MCF-7 breast cancer cells (IC_{50} ≈ 200 µg/mL at 72 H), with apoptosis mediated through mitochondrial pathways, identifying D-pinitol and myo-inositol as key contributors. Similarly, Fatima et al. (2016) found that NC bark extract exhibited time- and dose-dependent cytotoxicity in HeLa cervical cancer cells, with IC_{50} values decreasing from 319 µg/mL (24 h) to 254 µg/mL (72 H), which was attributed to high phenolic content and strong antioxidant activity.

Extraction method and solvent type also influence cytotoxic potency. Singh et al. (2013) reported variable IC50 values of NC leaf extracts prepared with different solvents, with chloroform extract showing the most potent activity (IC_{50} < 100 µg/mL) across multiple cancer cell lines (A-549, IGR-OV-1, PC-3, SF-295), and identified indole-based compounds as likely contributors. Taken together, these findings suggest that the antiproliferative potential of NC varies depending on (i) the plant part used, which determines the profile of bioactive compounds, (ii) the type of cancer cell line tested, as different molecular pathways may be engaged, and (iii) the extraction method, which influences the recovery of active metabolites (Le et al., 2018). Such variability underscores the importance of linking IC_{50} values to phytochemical composition to better understand the mechanisms underlying NC's anticancer activity.

Although the IC_{50} values of NC extract were higher compared to TMZ, its mechanism of action, mediated through apoptosis induction, aligns closely with the goals of conventional chemotherapeutics but with potentially fewer systemic toxicities. Importantly, NC extract induced morphological changes characteristic of apoptosis, including chromatin condensation and membrane blebbing, as observed through AO/PI staining. These findings align with a study by Razali et al. (2021), which reported the cell death mechanism by NC leaves extract via apoptosis induction and cell cycle arrest in breast cancer (MCF-7) cells. The extract also exerted anticancer effects in a dose- and time-dependent manner by triggering the mitochondrial cell death pathway.

The advantages of NC extract are rooted in its phytochemical composition. The presence of phenolics, flavonoids, tannins, and saponins, among others, is consistent with its antiproliferative and pro-apoptotic effects. These compounds have been extensively studied for their ability to modulate critical cancer pathways, including cell cycle arrest, reactive oxygen species (ROS) regulation, and the activation of apoptotic cascades.[13,20] For instance, flavonoids are known to inhibit tumor growth by modulating oxidative stress and inducing apoptosis through caspase activation, while phenolics contribute to DNA damage in cancer cells. These properties underscore the multifaceted mechanisms through which NC extract exerts its anticancer effects.[18]

The absence of terpenoids and quinones does not diminish the NC extract's therapeutic potential, as the detected compounds already offer a broad spectrum of anticancer mechanisms. The combination of all bioactive constituents of NC extract likely works synergistically, providing the extract with its potent antiproliferative and pro-apoptotic effects observed in glioblastoma cells.[31,32] In addition, studies suggest that the likelihood of developing resistance is lower when cancer is targeted using a combination of bioactive compounds compared to single active molecules.[33,17] This phytochemical diversity underscores the multifaceted therapeutic potential of NC extract, not only as an anticancer agent but also as a candidate for addressing other diseases linked to oxidative stress and inflammation.[18] Future studies should focus on isolating and quantifying these active compounds to better understand their individual contributions and explore their specific mechanisms of action. Moreover, advanced analytical techniques such as high-performance liquid chromatography and mass spectrometry could be utilized to confirm and characterize these phytochemical constituents in greater detail.

When compared to TMZ, which functions as a DNA-alkylating agent, NC extract offers a natural, less toxic alternative. TMZ indiscriminately targets both healthy and cancerous cells, leading to significant side effects such as gastrointestinal distress and myelosuppression.[8] In contrast, plant-derived compounds like those in NC extract often display selective toxicity toward cancer cells, sparing normal cells and reducing the risk of systemic toxicity. This selectivity is particularly advantageous in preserving the quality of life for cancer patients undergoing treatment.[18,19]

Furthermore, the economic and practical benefits of plant-based therapies make Neolamarckia cadamba (NC) extract an attractive candidate, especially in resource-limited settings. Unlike synthetic drugs, the cultivation, extraction, and preparation of plant-based therapies are generally more cost-effective, improving accessibility to a broader population.[34] Additionally, the traditional use of NC for treating various ailments supports both its cultural acceptance and perceived safety in communities familiar with its medicinal applications.[35,36]

While this study establishes a foundation for the antiproliferative potential of NC leaves extract, further research is necessary to optimize its application. Isolation and characterization of its active compounds will help identify the specific bioactive molecules responsible for its effects. Additionally, combining NC leaves extract with TMZ or other chemotherapeutics could offer synergistic benefits, enhance efficacy while mitigating the adverse effects of conventional treatments.

CONCLUSION

The present study concluded the potential of Neolamarckia cadamba (NC) leaves methanol extract as a natural, accessible, and effective alternative for glioblastoma therapy. Its demonstrated ability to induce apoptosis, combined with its traditional medicinal use and bioactive composition, highlights its potential to complement or even partially replace conventional chemotherapeutics like TMZ. Therefore, a safety and toxicology profile of NC needs to be done. Future

investigations, including in vivo studies and clinical trials, will be critical to fully realize its potential in cancer treatment.

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