IN VITRO THYMIDINE PHOSPHORYLASE INHIBITORY AND ANTIGLYCATION POTENTIALS OF *ANABASIS ARTICULATA* HPLC PURIFIED FRACTIONS

JAN, M.[1] – ZAHOOR, M.[1*] – KHAN, M.[2] – HAYAT, M. F.[3] – ESA, M.[4] – ULLAH, R.[5] – ALOTAIBI, A.[6]

[1]Department of Biochemistry, University of Malakand, Chakdara, Dir Lower, KPK 18800, Pakistan
(e-mail: jmarwa084@gmail.com)

[2]College of Chemistry, Fuzhou University, 350116 Fuzhou, China
(e-mail: majidk166@yahoo.com)

[3]North West Institute of Health Sciences, Hayatabad Peshawar, KPK, Pakistan
(e-mail: fhayat004@gmail.com)

[4]Drug Delivery System Excellence Center, Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand
(e-mail: esakhan5595@gmail.com)

[5]Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia
(e-mail: rullah@ksa.edu.sa)

[6]Department of Basic Science, College of Medicine, Princess Nourah bint Abdulrahman University, Riyadh 11671, Saudi Arabia
(e-mail: amaalotaibi@pnu.edu.sa)

**Corresponding author*
e-mail: mohammadzahoorus@yahoo.com

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Abstract. *Anabasis articulata*, a medicinal plant renowned for its abundance of bioactive constituents, has been studied for its antioxidant, anticancer, and antidiabetic potentials. Our previous research highlighted its significant antioxidant, anti-inflammatory, and antidiabetic activities. Herein, we extend on thymidine phosphorylase (TP) inhibitory and antiglycation potential of HPLC-purified fractions of *A. articulata* stem and leaves methanolic extract. TP is a key enzyme in angiogenesis and cancer progression, whereas protein glycation is associated with metabolic illnesses, including diabetes and neurological disorders. The methanolic extract of *A. articulata* was subjected to silica gel chromatography, resulting in four pure fractions (A-1, D-1, B-1, C-1). Results showed that A-1 and B-1 exhibited highest TP inhibition (87.1 and 90.4%) with an IC_{50} of 1.9 ± 0.9 and 3.1 ± 0.2 mg/mL, respectively. Furthermore, the antiglycation test showed significant inhibitory effects, with fraction C-1 exhibiting the strongest antiglycation activity (89.7%, IC_{50} = 0.11 ± 0.002 mg/mL), followed by A-1 (87.3%, IC_{50} = 2.68 ± 0.098 mg/mL). The findings indicated that *A. articulata* exhibited considerable therapeutic effects in combating cancer-related angiogenesis and glycation-associated metabolic diseases such as diabetes.
Keywords: *angiogenesis, bioactive compounds, protein glycation, in vitro activities, natural inhibitors, medicinal plants*

Introduction

Plants have long been utilized for the treatment of cancer, neurodegenerative, cardiovascular, inflammatory, and metabolic disorders, and other diseases, due to their

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rich reservoir of bioactive compounds (Teodoro, 2019). Specifically, they produce diverse secondary metabolites that exhibit significant anticancer, neuroprotective, and metabolic disorder-modulating properties (Seca and Pinto, 2018). Some of these metabolites exert anticancer effects by inhibiting cancer cell proliferation, preventing metastasis, and suppressing angiogenesis, etc. Angiogenesis, a key factor in tumor growth, has been a major therapeutic target for the past five decades (Majnooni et al., 2023). Thymidine phosphorylase (TP) is one such factor that promotes blood vessel growth and is recognized as a validated target for antiangiogenic drug development (Alam et al., 2024). In metabolic disorders like diabetes, it acts as an inhibitor of α-amylase, α-glucosidase, and advanced glycation end-product (AGE) formation, which is also implicated in Alzheimer's disease, among other mechanisms (Lan et al., 2024).

Inhibiting the process of angiogenesis is considered a promising strategy to combat cancer progression, while the inhibition of glycation is crucial in managing in metabolic disorders such as diabetes, and age-related diseases like Alzheimer's disease. TP facilitates tumor angiogenesis and is an important contributor to cancer progression, thus serving as an optimal target for the development of anti-angiogenic drugs and preclinical experiments (Bronckaers et al., 2009). The overexpression of TP has been linked to cancer aggressiveness and survival. Various solid tumors, such as breast, colorectal, bladder, and esophageal cancers, are associated with elevated levels of TP (Javaid et al., 2019). Moreover, other conditions, like rheumatoid arthritis, psoriasis, and inflammation, also have elevated TP activity levels (Hammiche and Maiza, 2006). Multiple TP inhibitors have demonstrated significant in vitro efficacy; however, only one, tipiracil, used in combination with the cytotoxic drug trifluridine, has received US FDA approval for the treatment of gastrointestinal malignancies (Peeters et al., 2018). The administration of this combination medicine (Lonsruf®) is hindered by numerous adverse effects, including neutropenia, anemia, and myelosuppression, among others (Lee and Chu, 2017).

Given the importance of TP in cancer and of antiglycation in diabetes mellitus, Alzheimer's disease, and the progression of atherosclerosis, plant extracts and phytochemical fractions (pure compounds) isolated from plants have been reported to exhibit antiglycation activity. Saffron (*Crocus sativus L.*) extract, with crocin as the key bioactive carotenoid, demonstrated antiglycation activity, with Greek and Sicilian saffron extracts inhibiting AGE formation by ~40%, while Iranian saffron showed 30% inhibition (Ronsisvalle et al., 2023). The ethyl acetate fraction of *Ephedra fragilis* (joint-pine) extract exhibited the strongest antiglycation activity (IC_{50} = 0.375 mg/mL), attributed to its high phenolic and flavonoid contents (Guenaou et al., 2021). Sesquiterpene lactones obtained from *Inula helenium* (commonly knwon as elecampane, or horse-heal), particularly alantolactone and isoalantolactone, showed the highest antiglycation activity (97.16% inhibition) (Özcan et al., 2024). Polyphenol-rich agro-residues of pistachio (*Pistachia vera*) green hull and pomegranate (*Punica granatum* L.) peel demonstrated high antiglycation activity, with pomegranate peel extract showing greater inhibition (IC_{50} = 94 mg/mL) than pistachio green hull (IC_{50} = 142 mg/mL) (Roudbari et al., 2024). *Argania spinosa* (argan) and *Opuntia dillenii* (erect prickly) have also been reported to possess antiglycation activity with active compounds being catechin, kaempferol-O-acetylhexoside, luteolin, and quercetin (Rhizlan et al., 2024). Quercetin, catechin, epicatechin, vanillic acid, and caffeic acid, obtained from *Cocos nucifera Linn.* (coconut) husk fiber extract showed good antiglycation activity (IC_{50} = 4.50 µg/mL) (Oliveira et al., 2021). Similarly, polyphenol extracts of *Arctium lappa* (burdock) and *Symphytum*

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officinale (comfrey) showed the strongest antiglycation activity (89.4%) (Chociej et al., 2024). The methanolic extract of *Pfaffia glomerata* (Brazilian ginseng or canela-velh), with 20-hydroxyecdysone being the major compound, showed below 50% antiglycation activity at a concentration of 10 mg/mL, compared to quercetin control (99%) (Franco et al., 2024).

Anabasis articulata (Forssk) Moq. (Amaranthaceae), also known as 'Ajrem' is a shrub spread over halic and xeric regions of Khyber Pakhtunkhwa, Pakistan (Al-Joufi et al., 2022). The phytochemical constituents showed the presence of alkaloids (Boutrif et al., 2024), triterpenoids (Gamal et al., 2022), 30-noroleanane triterpene saponins (El Dine et al., 2019), phenolic compounds (Ben Menni et al., 2024; Kambouche et al., 2019), flavonoids, and carotenoids (Benhammou et al., 2013), sterols (Ben Menni et al., 2022), etc. To date, multiple studies reported that *A. articulata* exhibit antioxidant (Al-Joufi et al., 2022; Benhammou et al., 2013), anti-inflammatory (Ben Menni et al., 2024, 2022; Boutrif et al., 2024), antibacterial (Al-Joufi et al., 2022), antimicrobial (Belyagoubi-Benhammou et al., 2019), larvicidal (Alamri et al., 2024), antidiabetic (Al-Joufi et al., 2022; El Dine et al., 2019), gastroprotective effect (Gamal et al., 2022), anti-arthritic (Abdulhusain et al., 2022), anti-tyrosinase (Jan et al., 2025), anti-acetylcholinesterase (Ben Menni et al., 2022), antiangiogenic (Abdulsahib et al., 2016), and anticancer potentials (Alamri et al., 2024; Monteleone et al., 2024), among others.

Keeping in view the literature mentioning plant extracts and pure compounds that have shown potent thymidine phosphorylase inhibition and antiglycation activities, herein, we attempt to assess the *in vitro* thymidine phosphorylase inhibitory and antiglycation potentials of *A. articulata* HPLC-purified fractions (A-1, D-1, B-1, C-1). In our previous study, we already reported the antibacterial, antioxidant, and antidiabetic potential of *A. articulata* obtained fractions (Al-Joufi et al., 2022). In this study, we further explore the thymidine phosphorylase inhibitory and antiglycation activities of these fractions to evaluate their potential as therapeutic agents for metabolic and age-related disorders.

Material and methods

Plant sample collection

A. articulata (Ajrem) plant stems and leaves were collected from their natural habitat in Mohmand Agency, Pakistan. The collected plant sample (1 kg) was brought to the Herbarium at the Department of Botany, University of Malakand, Khyber Pakhtunkhwa Pakistan, and authenticated by the incharge, who has prior experience in plant taxonomy and identification. The plant material was cleaned, shade-dried, and pulverized into a fine powder.

Extraction

The powdered sample (200 g) was immersed in methanol with continuous vigorous agitation for six days. The mixture was filtered using a Whattman filter paper, and the filtrate was subsequently soaked in methanol for an additional six days. The filtrate was concentrated using a rotary evaporator (Rota vapour R-200 Buchi, Switzerland) under reduced pressure at 40°C. The residual solvent in the semi-solid extract was evaporated by exposing it to open air.

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Fractionation

A previously documented procedure utilizing a silica gel column was followed to obtain purified fractions (Haq et al., 2013). The methanolic extract was applied to a silica gel column and eluted with a suitable solvent system. Based on TLC profiling, similar fractions were pooled and designated as pure fractions. The four purified fractions were designated as A1 (5% ethyl acetate solution), B1 (10% ethyl acetate), C1 (30% ethyl acetate), D1 (40% ethyl acetate solution), along with an additional n-hexane fraction. All fractions were stored at 4°C in a refrigerator. The obtained fractions, crude extract, and oil were subsequently subjected to subsequent experimentation.

Thymidine phosphorylase inhibition assay

Thymidine phosphorylase inhibition assay was conducted in triplicate using 96-well plates. Each 200 µL reaction mixture contained 10 µL of the test fraction (0.5 mM; dissolved in DMSO), 150 µL of potassium phosphate buffer (pH 7.0, 50 mM), and 20 µL of TP enzyme (0.058 unit/well). The 96-well plate containing all reagents was subsequent incubated at 30°C for 10 min. Following, 20 µL of 1.5 mM thymidine substrate was added, and the change in optical density was measured at 290 nm for 10 min using a microtiter plate reader (SpectraMax 384, Molecular Devices, San Jose, CA, USA) (Bera et al., 2013). The percentage of inhibition was calculated using *Equation 1*:

$$\% \text{ Inhibition } = \left(1 - \frac{\text{Abs.of sample}}{\text{Abs.of control}}\right) \times 100 \qquad \text{(Eq.1)}$$

Antiglycation assay

The antiglycation test was carried out as described in the literature, with few modifications (Dos Santos et al., 2022). The assay was performed in 96-well plates, with each well containing 50 µL of bovine serum albumin (BSA) at a concentration of 10 mg/mL, prepared in 100 mM phosphate buffer (pH 7.4), 50 µL of a 14 mM methylglyoxal solution, 20 µL of each fraction (dissolved in DMSO), and 80 µL of phosphate buffer. Sodium azide (3 mM) was incorporated into each well as an antibacterial agent. The plates were incubated at 37 °C for nine days. After incubation, absorbance was measured at 330 nm (excitation) and 420 nm (emission) using a microplate reader (Spectramax M5, Molecular Devices, CA, USA). Rutin served as the reference compound for comparing the activity of test compounds. Percentage inhibition was determined using *Equation 2*:

$$\% \text{ Inhibition } = \left(1 - \frac{\text{Abs.of test sample}}{\text{Abs.of glycated sample}}\right) \times 100 \qquad \text{(Eq.2)}$$

Statistical analysis

All assays were conducted in triplicate, and the results are presented as mean ± standard error of the mean (SEM). IC_{50} values for antiglycation activity were calculated by nonlinear regression analysis using GraphPad Prism (version 8.0.2). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was applied to determine statistically significant differences among fractions and the standard. A *p*-value less than 0.05 was considered statistically significant.

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Results and discussion

Thymidine phosphorylase (TP) inhibition

TP plays a key role in nucleoside metabolism by catalyzing the breaking down of thymidine, a process essential for DNA repair and replication. In various cancers, TP is overexpressed, leading to increased thymidine degradation and the subsequent production of 2-deoxyribose (Warfield and Reigan, 2022). This byproduct contributes to cancer progression by facilitating protein glycation, altering metabolic pathways, modifying the extracellular matrix, and promoting angiogenesis. As a result, targeting TP inhibition has emerged as a potential anticancer strategy (Murmu et al., 2024).

In the present study, inhibition of TP was evaluated for the test compounds, crude extract, oil and standard, as shown in *Table 1*. Purified fractions A-1, D-1, B-1, and C-1 exhibited strong TP inhibition, with % inhibition values ranging from 87.1% to 90.4%. Notably, B-1 and C-1 showed the highest inhibition at 90.4%. A-1 demonstrated the lowest IC_{50} value (1.9 ± 0.9 mg/mL, $p < 0.01$), followed by B-1 (3.1 ± 0.2 mg/mL, $p < 0.001$) suggesting that both are the most potent inhibitors among the tested compounds. In contrast, C-1 had a much higher IC_{50} value (80.9 ± 0.2 mg/mL, $p < 0.001$), indicating lower potency despite its high TP inhibition. Fraction D-1 also showed strong TP inhibition with an IC_{50} value of 6.8 ± 0.7 mg/mL ($p < 0.05$). The crude and oil samples showed moderate inhibition (47.6% and 39.5%, respectively) but were not active enough to determine an IC_{50} value. The standard inhibitor, 7-deazaxanthine, showed 80.6% inhibition with an IC_{50} of 15.1 ± 0.1 mg/mL, which was less potent than A-1 but more potent than C-1.

Table 1. *Thymidine phosphorylase inhibition activity of tested fractions*

Code	% Inhibition	IC_{50} ± SEM (mg/mL)
A-1	87.1	1.9 ± 0.9**
D-1	89.2	6.8 ± 0.7*
B-1	90.4	3.1 ± 0.2***
C-1	90.4	80.9 ± 0.2***
Crude	47.6	Not active
Oil	39.5	Not active
Standard (7-deazaxanthine)	80.6	15.1 ± 0.1

IC_{50} values are expressed as mean ± SEM (n = 3). Statistical comparisons were made between each sample and the standard (7-deazaxanthine) using unpaired t-tests. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

In the literature, the majority of studies used plant extracts, and pure compounds for the determination of % TP inhibition. Among the three bioactive compounds of *Pistacia integerrima* (zebrawood) namely spinacetin, patuletin, and triterpene pistagremic acid, spinacetin showed the most potent TP inhibition, with an IC_{50} of 1.9 ± 0.9 μM, followed by patuletin (IC_{50} of 3.1 ± 0.2 μM), and pistagremic acid (IC_{50} of 6.8 ± 0.7 μM) (Rauf et al., 2024). Zerumbone, a sesquiterpene found in *Zingiber zerumbet* (bitter ginger) possesses potent TP inhibitory activity (IC_{50} value of 50.3 ± 0.31 μg/mL) (Albaayit and Mohammed, 2023). Some studies also reported the highest TP inhibition for synthetic compounds, such as benzothiazole-based thiadiazole derivatives (1–15). Notably, compounds 2, 5, 6, and 12 demonstrated excellent inhibition, with IC_{50} values of

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5.70 ± 0.20 μM, 4.50 ± 0.50 μM, 4.60 ± 0.20 μM, and 6.30 ± 0.20 μM, respectively (Khan et al., 2023). Three new acrylic acid derivatives (1–3) isolated from the ethyl acetate fraction of *Achillea millefolium* (common yarrow) demonstrated that compound 3 exhibited the most potent TP inhibitory activity with an IC_{50} value of 57.81 ± 3.41 μM, followed by compound 2 (IC_{50} = 89.92 ± 0.37 μM) and compound 1 (IC_{50} = 158.9 ± 0.97 μM) (Naz et al., 2021). Novel synthesized dihydropyrimidone derivatives (n = 40) act as non-competitive TP inhibitors, and show weak % TP inhibition activity with low IC_{50} values ranging from 314 to 322.6 μM, respectively (Cui et al., 2023). Bis-thiadiazole bis-Schiff bases were shown to be more potent than the standard drug in inhibiting the thymidine phosphorylase enzyme, with IC_{50} values ranging from 1.16 to 24.87 μM (Hussain et al., 2023).

Antiglycation assay

Results of the antiglycation activity of the compounds are shown in *Table 2*. All tested compounds demonstrated significant antiglycation activity, with % inhibition values ranging from 81.6% to 90.3%. The crude extract and oil showed particularly high inhibition with values of 90.3% and 90.1%, respectively. Fraction C-1 exhibited the lowest IC_{50} value (0.11 ± 0.002 mg/mL, $p < 0.01$), and an 87.3% inhibition serving as the most potent, followed by oil (0.17 ± 0.004 mg/mL, $p < 0.01$) and crude extract (1.02 ± 0.013 mg/mL, $p < 0.001$). Rutin, the standard antiglycation agent, showed the highest % inhibition (96.2%) and the lowest IC_{50} value (0.02 ± 0.01 mg/mL). However, C-1, oil, and crude extract demonstrated comparable potency, suggesting their potential as natural antiglycation agents. A-1 and B-1 also showed strong activity but with high IC_{50} values (2.68 ± 0.098 mg/mL and 3.69 ± 0.016 mg/mL, both $p < 0.001$), respectively.

Table 2. *Antiglycation activity of tested fractions*

Code	% Inhibition	IC_{50} ± SEM (mg/mL)
A-1	87.3	2.68 ± 0.098***
D-1	81.6	3.54 ± 0.028***
B-1	88.0	3.69 ± 0.016***
C-1	89.7	0.11 ± 0.002**
Crude	90.3	1.02 ± 0.013***
Oil	90.1	0.17 ± 0.004**
Standard (Rutin)	96.2	0.02 ± 0.01

IC_{50} values are expressed as mean ± SEM (n = 3). Statistical comparisons were made between each sample and the standard (Rutin) using unpaired t-tests. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

In the literature, the hydroalcoholic extract of *Coffea arabica* (Arabian coffee) and *Coffea canephora Pierre* (robusta coffee) demonstrated the highest antiglycation activity (65.2%) compared to the low-temperature vacuum mediated extract, which showed lower inhibition (47.6%) (Dias et al., 2024). *Solanum aculeatissimum* (Dutch eggplant) aqueous extract showed the highest antiglycation activity (72.8%) in comparison to silver nanoparticles of the extract (67.9%) (Silva et al., 2024). *Punica granatum* L. (pomegranate) peel extract exhibited greater antiglycation activity (IC_{50} = 94 mg/mL) than *Pistachia vera* (pistachio) green hull extract (IC_{50} = 142 mg/mL) (Roudbari et al., 2024).

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Another study reported that high phenolic and flavonoid contents correlated with the highest antiglycation activity. The ethyl acetate fraction of *Ephedra fragilis* (joint-pine) exhibited the strongest inhibitory activity, with an IC_{50} value of 0.375 mg/mL, followed by water-saturated n-butanol fraction (IC_{50} = 0.595 mg/mL), dichloromethane fraction (IC_{50} = 0.857 mg/mL), crude ethanol extract (IC_{50} = 0.951 mg/mL), and diluted water fraction (IC_{50} = 1.044 mg/mL), while the least activity was shown by hexane fraction with an IC_{50} of 1.212 mg/mL (Guenaou et al., 2021). The sesquiterpene lactones, particularly alantolactone and isoalantolactone, from the roots of *Inula helenium* (horse-heal) were extracted exhibited high antiglycation activity (97.1%) compared to the extract obtained using maceration (Özcan et al., 2024). *Crocus sativus L.* (saffron) contains crocin, a glycoside carotenoid that possesses antioxidant and antiglycation activity. Sicilian and Greek saffron extracts exhibited comparable antiglycation activities, each providing approximately 40% inhibition of AGEs formation. In contrast, Iranian saffron was significantly less effective, showing only 30% inhibition ($p < 0.05$) (Ronsisvalle et al., 2023).

The findings of the present study are in good agreement with and greatly add to the body of knowledge on the potential of natural products as TP inhibitors and antiglycation agents. The HPLC-purified fractions from *A. articulata* exhibited significant TP inhibitory activity, with fraction A-1 presenting the lowest IC_{50} value (1.9 ± 0.9 mg/mL), followed by B-1 (3.1 ± 0.2 mg/mL), both demonstrating high inhibition percentages (87.1% and 90.4%, respectively). These values are comparable to previously documented plant-derived TP inhibitors. For instance, spinacetin and patuletin from *Pistacia integerrima* demonstrated IC_{50} values of 1.9 µM and 3.1 µM, respectively (Rauf et al., 2024), suggesting a similar order of potency in relative terms, albeit at a lower molar scale. While synthetic compounds such as benzothiazole-based thiadiazole derivatives have exhibited superior potency with IC_{50} values in the low micromolar range (4.5–6.3 µM) (Khan et al., 2023), the appeal of *A. articulata* lies in its natural origin, and dual bioactivity. Moreover, in contrast to other phytochemicals such as zerumbone (IC_{50} = 50.3 µg/mL) derived from *Zingiber zerumbet*, A-1 from *A. articulata* exhibits superior TP inhibition (Albaayit and Mohammed, 2023).

The antiglycation assay results also show strong correlation with published data. In the current study, all tested samples from *A. articulata* exhibited significant antiglycation activity, with the crude extract and oil showing inhibition above 90% and IC_{50} values of 1.02 ± 0.013 and 0.17 ± 0.004 mg/mL, respectively. Fraction C-1 was identified as the most effective sample (IC_{50} = 0.11 ± 0.002 mg/mL), exhibiting activity comparable to that of the standard, rutin (IC_{50} = 0.02 ± 0.01 mg/mL). These values surpass earlier documented extracts, including hydroalcoholic *Coffea arabica* (65.2% inhibition) (Dias et al., 2024), *Solanum aculeatissimum* aqueous extract (72.8%) (Silva et al., 2024), and even *Ephedra fragilis* fractions (ethyl acetate fraction IC_{50} = 0.375 mg/mL) (Guenaou et al., 2021). The significant antiglycation action may be ascribed to the polyphenolic compounds in *A. articulata*, although further phytochemical characterization is necessary.

The overall findings of this study suggest that the purified fractions of *A. articulata* possess promising dual activity as natural TP inhibitors and antiglycation agents. These in vitro results highlight the potential therapeutic value of *A. articulata* in managing pathological processes associated with cancer and diabetes. Despite this, we acknowledge that the study is limited to in vitro assays and lacks mechanistic insight at the molecular level, including the precise mode of TP inhibition. Furthermore, in vivo validation has not yet been performed. Therefore, further research involving detailed mechanistic

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studies, and animal model evaluations are necessary to establish the pharmacological relevance and clinical potential of these findings.

Conclusion

The present study provides preliminary evidence for the thymidine phosphorylase inhibitory and antiglycation potential of HPLC-purified fractions from *Anabasis articulata*. The strong TP inhibition reported in fractions A-1 (87.1%) and B-1 (90.4%) indicates their potential as antiangiogenic agents, which may be advantageous in cancer treatment. Furthermore, B-1 exhibited notable antiglycation activity, indicating its potential involvement in alleviating complications related to diabetes. The findings may be further validated by employing enzyme kinetics studies to determine the manner of TP inhibition, whether competitive, non-competitive, or uncompetitive. Molecular dynamics simulations may further improve the prediction of binding interactions with analogous proteins.

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