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ORIGINAL ARTICLE

Andrographis paniculata extract as an immunity modulator against cancer via telomerase inhibition

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Abstract

In accordance with the importance of telomerase inhibition as a potential target in cancer therapy, and increasing reports on the association between short telomeres and severe COVID-19 symptoms as well as extensive application of *Andrographis paniculata* as a remedy for both cancer and SARS-CoV-2, the present study aimed at investigating the impact of the plant's extracts on telomerase activity (as an important enzyme regulating telomere length). Telomerase inhibition in MCF-7 cells treated with the Dichloromethane, ethanol, water, and methanol extracts of *A. paniculata* was assessed using Telomerase Repeated Amplification Protocol (TRAP). The above-mentioned extracts inhibited telomerase by 80.3 ± 1.4%, 78.5 ± 1.35%, 77.5 ± 1.81%, and 73.7 ± 1.81%, respectively. Furthermore, the flow cytometry analysis showed that the water and methanol extracts induced higher rates of total apoptosis by 32.8% and 25%, respectively, compared with dichloromethane (10.07%) and ethanol (10.7%) extracts. The inhibitory effect of *A. paniculata* on telomerase activity can be considered as a potential immunity modulator in cancer therapy; however, telomerase inhibition as a safe approach to SARS-CoV-2 is arguable. Two mechanisms can be considered accordingly; (a) reducing the existing population of short telomeres via telomerase inhibition in cancer cells (arresting proliferation and finally cell death) may decrease the susceptibility against SARS-CoV-2, especially in cancer patients or patients prone to cancer, and (b) increasing the population of short telomeres via telomerase inhibition in normal/somatic cells may increase the susceptibility against SARS-CoV-2. Therefore, the telomerase inhibition of *A. paniculata* as an immunity modulator in cancer and COVID-19 should be investigated, carefully.

Keywords TRAP-ELISA · Short telomere · Cancer · SARS-CoV-2

Introduction

Andrographis paniculata Nees is an annual herbaceous plant from the family Acanthaceae, native to southeast Asia (Valdiani et al. 2012). This plant had played an outstanding role to arrest the spread of the global flu pandemic (especially in India) during 1918–1919 (Hancke et al. 1995; Sharma et al. 2009). The most important bioactive compounds of *A. paniculata* are andrographolide (AG), 14-Deoxy-11,12-didehydroandrographolide (DDAG), and neoandrographolide (NAG) (Valdiani et al. 2017). The herb has shown important therapeutic properties, such as antiviral and anti-inflammatory (Gupta et al. 2017) as well as anticancer effects (Suriyo et al. 2021), mainly because of andrographolide. Different anticancer mechanisms of andrographolide, such as cell cycle arrest and apoptosis (Suriyo et al. 2014), NF-κβ inhibition (Mishra 2021), antiangiogenesis (Dai et al. 2017), cytochrome P400 and P450s regulation (Suriyo et al. 2021), and cytokine inhibition (Li et al. 2017), have been compiled and reviewed, recently (Malik et al. 2021). The modulatory effect of *A. paniculata* on cancer, has been attributed to its effects on the tumour suppressors, such as p53 (Zhau et al. 2008; Sato et al. 2018).

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Telomerase is an enzyme responsible for the regulation of telomere length in eukaryotes. In general, activation and inhibition of telomerase result in the elongation (maintenance) and shortening of telomeres, respectively (Aviv et al. 2017). These mechanisms have probably evolved against ageing and cancer development (Shay 2016). Sometimes, genomic mutations lead to telomerase reconstitution and trafficking, and finally, cancer initiation and tumour development (Aviv et al. 2017). Hypothetically, under such circumstances, telomerase reactivation causes excessive cell division (Smith et al. 2003), and telomere shortening, instead of telomere maintenance (Jafri et al. 2016). Therefore, telomere maintenance, via reactivation of telomerase and expression of human Telomerase Reverse Transcriptase (hTERT), is a common hallmark of cancer cells to support replicative immortality against replicative senescence (Guterres and Villanueva 2020). In contrast, telomerase activity is silenced in most adult somatic cells. Despite the paradox of cancer-telomere length (Aviv et al. 2017), telomere length in tumours is usually shorter than that of matched normal tissue (Barthel et al. 2017). Considering these facts, the inhibition of telomerase activity has been suggested as a potential target in cancer therapy, since mid-90 s [Kim et al. 1994; Holt and Shay 1999; Zhang et al. 1999), until recently (Guterres and Villanueva 2020; Liu et al. 2019).

As a matter of fact, the telomere/telomerase-related effects of *A. paniculata* have seldom been subject to any investigation. In this regard, the only valuable effort has been made by Budiatin et al. (2021). Their objective was to evaluate the effect of *A. paniculata* extract on the proliferation of lung cancer cells and induction of abnormal cell death. The results showed that the extract inhibited the development of cancer at the hyperplasia stage by reducing telomerase activity and increasing apoptosis, "qualitatively", which was marked by an increase of caspase-3 expressions (Budiatin et al. 2021).

Therefore, considering the lack of any "quantitative" evidence, the present research tended to assess the direct impact of different *A. paniculata* extracts on the inhibition of "telomerase", quantitatively in the genomic level, by using the Telomerase Repeated Amplification Protocol—Enzyme-Linked Immunosorbent Assay (TRAP-ELISA), as the main objective. In addition, the effect of solvent polarity on the toxicity and telomerase inhibition of *A. paniculata* extracts was assessed as a dependent variable. To this end, four different extracts of *A. paniculata*, including ethanol, methanol, dichloromethane (DCM), and water extracts were applied to Michigan Cancer Foundation-7 (MCF-7) cancer cell lines. The TRAP assay is a reliable and fast genomic approach to assess the inhibition of telomerase. The assay is considered as a Polymerase Chain Reaction (PCR)-based technique that is not involved with any complicated staining procedure and imaging methods/equipment (Mender and Shay 2015).

A secondary objective of the current article is to discuss telomerase inhibition as a potential therapeutic target in Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), since reports of short telomeres in patients with severe symptoms of coronavirus disease 2019 (COVID-19) (Sanchez-Vazquez et al. 2021; Kamal et al. 2020)and interest in using *A. paniculata* as a treatment for SARS-CoV-2 both have been increasing (Sa-ngiamsuntorn et al. 2021; Enmozhi et al. 2021; Verma et al. 2021; Rehan et al. 2021). Figure 1

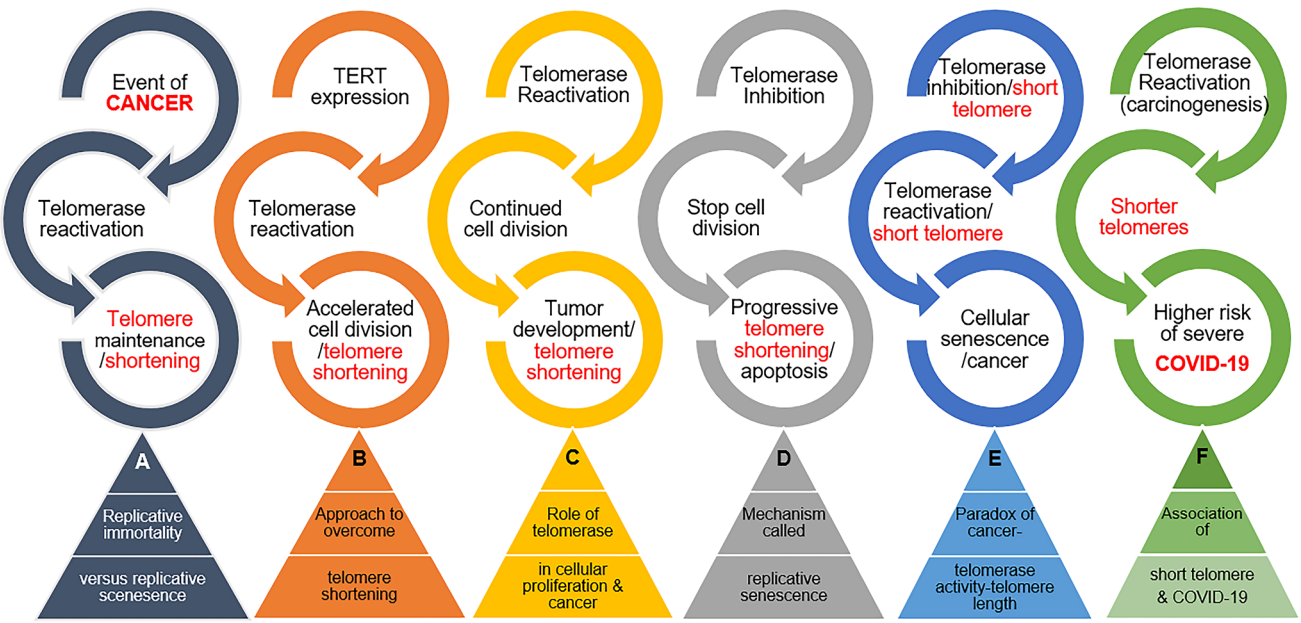

Fig. 1 Schematic depiction of the telomerase activity paradox, cancer, telomere shortening, and COVID-19

represents the cancer-telomere length paradox as well as the association of short telomeres, cancer, and COVID-19. In another word, we tried to make an indirect link between the results of the present research and SARS-CoV-2, since short telomeres increase the risk of SARS-CoV-2 (Froidure et al. 2020). This article is not an attempt to promote or reject *A. paniculata* as a treatment for SARS-CoV-2 via its telomerase inhibition property, but it suggests that the association between telomerase inhibition and COVID-19 as well as the application of any agent with telomerase inhibition/activation property against SARS-CoV-2 must be examined, carefully.

Materials and methods

Plant material

The seeds of *A paniculata* (accession 261) were obtained from the Medicinal Plants Research Center, Shahed University—Tehran. The seeds were then grown in an open field located in Rasht, Gilan—Iran (coordinates: 37° 20′ 89.7″ N and 49° 58′ 68.0″ E).

Plant sample preparation and crude extract isolation

The aerial parts of *A. paniculata* plants, including the entire leaves and stems were harvested at the pre-flowering stage (18 weeks after sowing), due to the highest content of AG, NAG, and DDAG at this stage (Tajidin et al. 2019). The harvested parts were dried in a universal ventilated electric oven (Memmert, Germany) at 55 °C for 48 h. The dried material was then chopped into small pieces and ground into fine powder, consequently. The extraction was done using four different solvents according to their polarity index (PI), including 100% ethanol (PI = 5.2), 100% methanol (PI = 6.6), 100% dichloromethane (DCM) (PI = 3.4), and 100% double-distilled water (PI = 9.0) (Luque de Castro and García-Ayuso 1998). The reason of using the four solvents was an initial assessment of the impact of extracts on telomerase inhibition and cytotoxicity in MCF-7 cells, based on the each solvent's polarity. However, the polarity of solvent was a dependent variable in this study, and the effect of polarity was not independently studied on telomerase inhibition and cytotoxicity. To this end, 50 g of *A. paniculata* powder was soaked in 350 mL of each solvent, separately. The soaked samples were shaken on an orbital shaker for 72 h at room temperature. The solvent extracts were filtered through Whatman No. 1 filter papers. The residues were rinsed several times with the same solvent to achieve the maximum extract recovery. The solvent extracts were then concentrated under reduced pressure using a rotary evaporator and transferred into conical flasks. The final drying process of the concentrated extracts was performed in the ventilated electric oven (Memmert, Germany) at 25 ± 1 °C for removal of the residual solvents. The dried extracts were placed into small size capped glass containers and after labelling and sealing were kept in a − 20 °C freezer until conducting the cytotoxicity tests.

Cell lines and culture condition

The MCF-7 cell line was received from Pasteur Institute of Iran. The cells were cultured in RPMI-1640 medium composed of 10% Fetal Bovine Serum (FBS) (Gibco, USA), and 1% penicillin–streptomycin (Gibco, USA). The cultures were incubated at 37 °C in a CO_2 incubator (Memmert, Germany), under anaerobic incubation of 5% CO_2, as explained by Akbarizare et al. (2019).

Cytotoxicity test

The half-maximal inhibitory concentration (IC_{50}) of each extract was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The MCF-7 cells were treated with trypsin 25% (Gibco, USA) and centrifuged at 1500 rpm and 4 °C for 5 min. The cells were then counted using hemocytometer lam and dispensed in 96-well microplate at the rate of 1×10^4 cells in each well. The dispensed cells were then treated with different concentrations (ranged within 3.1–100 µg/mL) of each extract in three replicates and incubated for 24 h at 37 °C. Consequently, 100 µL of the MTT solution (0.5 mg/mL) was added to each well, and the plates were incubated again for 4 h at 37 °C followed by the treatment with 100 µL of dimethyl sulfoxide (DMSO) (Merck, Germany). The adsorption was measured at 570 nm using a microplate ELISA Reader (Bio Tek Instruments, USA), as explained by Akbarizare et al. (2021). The morphological changes of MCF-7 cells were evaluated at the magnification of 40 × using a CETi inverted microscope (Ceti Inverso TC-100). The untreated MCF-7 cells were used as the control treatment.

Flow cytometry

To further investigate the effect of four *A. paniculata* extracts on MCF-7 cell cycle, flow cytometry assay was performed by employing the IC_{50} dose of each extract. The rates of apoptotic cells were determined by flow cytometry based on the fact that phosphatidylserines (PS) becomes exposed on the cell surface by flipping from the inner to outer leaflet of the cytoplasmic membrane, during early apoptosis process.

Cell preparation and annexin V-FITC/PI staining for flow cytometry

Initially, the collected cells in microtubes were washed with 2 mL of 1 × phosphate-buffered saline (PBS). The cells were centrifuged at 1500 rpm and 4 °C for 5 min, and the supernatants were decanted carefully. The cells were gently suspended in 500 µL of the 1 × binding buffer, and the content of each tube was transferred equally into four microtubes (125 µL per tube × 16 microtubes). Considering spectral overlap of Fluorescein Isothiocyanate (FITC) and Propidium Iodide (PI), these fluorochromes were added together and separately to each microtube. The negative control contained no fluorochrome. Following the manufacturer's assay protocol for TACS™ Annexin V-FITC Apoptosis Detection Kit (USA R&D Systems, Inc.), all samples were incubated for 15 min at 4 °C in the dark. Subsequently, an additional 5 µL of annexin V-FITC dye was added only to those samples containing both annexin V-FITC and PI dyes, prior to the incubation in the dark. In the next step, 1 mL of the 1 × binding buffer was added to each sample, and centrifugation was performed at 1500 rpm and 4 °C for 5 min. The supernatants were gently decanted, and an additional 500 µL of the 1 × binding buffer was added to each microtube to achieve a target cell density of 1×10^5 cells/mL. Finally, 3 µL of PI dye was added to each microtube. The FITC and PI dyes were excited by 488 nm laser. The emission was collected between standard channels of FITC/GFP at 530 nm and PE/Cy3 at 575–610 nm. The apoptotic and necrotic cells were analysed using a flow cytometer BD FACSCalibur ™ (BD biosciences, San Jose, CA, USA). The data were analysed by FlowJo v10 software. Two-parameter dot plot is displayed in four quadrants. Plotting annexin V-FITC binding on the x-axis of a two-dimensional dot/density plot and PI on the y-axis enables the identification of necrotic (annexin V^-/PI^+, upper left quadrant "Q1"), late apoptotic/dead (annexin V^+/PI^+, upper right quadrant "Q2"), early apoptotic (annexin V^+/PI^-, lower right quadrant "Q3"), and dynamic/non-apoptotic (annexin V^-/PI^-, lower left quadrant "Q4") cells (Yao et al. 2016; Cossarizza et al. 2017).

Telomerase Repeated Amplification Protocol (TRAP assay)

Initially, the MCF-7 cells were treated at IC_{50} and 100 µg/mL concentrations of the four *A. paniculata* extracts (three replicates), for 24 h at 37 °C. The cells were then treated with 0.25% of sterile trypsin for 3–4 min at room temperature and counted under the microscope. A seeding density of 2×10^5 MCF-7 cells was transferred into each sterile microtube, per single reaction. The cells were then centrifuged for 5 min using a refrigerate centrifuge at 1500 rpm and 2–8 °C. The supernatants were discarded and 200 µL of the lysis buffer was added to each pellet. The pellets were immediately incubated in a polystyrene ice rack for 30 min. The lysates were centrifuged at 16,926 rpm and 2–8 °C for 20 min. The supernatants were transferred into sterile 1.5 mL microtubes. The content of these tubes were shock frozen in liquid nitrogen and stored at –80 °C for TRAP assay, as explained by Herbert et al. (2006). The TRAP assay was performed using TeloTAGGG™ telomerase PCR-ELISA kit, version 8 (Roche, Germany).

Each PCR was performed in a total volume of 50 µL. The PCR master mix contained Tris buffer, telomerase substrate, biotin-labelled P1-TS primer (5′-AATCCGTCG AGCAGAGTT-3′), P2 primer (5′-CCCTTACCCTTACCC TTACCCTAA-3′), PCR nucleotide mix (dNTP), and Taq DNA polymerase. The PCR was performed using a Thermal Cycler machine, model iCycler™ (Bio-Rad, USA). A pre-PCR was carried out at 25 °C (30 min) and 95 °C (5 min), for primer elongation and telomerase inactivation, respectively, as explained by Akbarizare et al. (2021). The extended products were then subjected to the main PCR with an initial denaturation at 95 °C for 3 min, followed by 30 cycles of 30 s at 94 °C (denaturation), 30 s at 50 °C (annealing), and 90 s at 72 °C (extension). A final extension was performed for 10 min at 72 °C (Akbarizare et al. 2021).

Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay method was used for quantitative analysis of telomerase activity using the confirmed PCR products. The PCR products were denatured and hybridized with digoxigenin-labelled as telomeric repeat-specific detection probe. The hybridized products were immobilized in streptavidin-coated microplate by biotin–streptavidin reaction. Finally, TRAP products were detected and visualized via peroxidase reaction method by using anti-digoxigenin antibody conjugated peroxidase and TMB (3,3′,5,5′-Tetramethylbenzidine) chromogenic substrate (Sigma-Aldrich, USA). The absorbance was separately recorded at 450 nm for each sample by using a microplate ELISA reader (Bio Tek, USA) (Akbarizare et al. 2021).

Statistical analysis

The MTT and TRAP-ELISA experiments were performed in triplicate. The results were analysed using SAS software version 9.3 (SAS Institute Inc., Cary, NC). The analysis of variance of telomerase inhibition was performed at $P \leq 0.01$. The mean comparisons were performed by using Duncan multiple range test. The results are presented as mean ± standard error of mean.

Results

Cytotoxicity analysis based on MTT test

The effects of four *A. paniculata* extracts, including ethanol, methanol, water, and DCM extracts, on the viability of MCF-7 cells showed that the ethanol extract had the lowest IC_{50} dose (37 µg/mL) and the water extract possessed the highest IC_{50} dose (105 µg/mL). Indeed, the water extract was the only treatment of this experiment with an IC_{50} value greater than the value of 100 µg/mL dose (Fig. 2A). The IC_{50} doses of DCM and methanol extracts were 61 µg/mL and 70 µg/mL, respectively (Fig. 2A). Higher concentrations of *A. paniculata* extracts decreased the cell viability. Figure 2B represents the dose–response curves of MCF-7 cells viability against each extract, which implies the dose-dependent inhibition of *A. paniculata* extracts. Additionally, the results showed that the IC_{50} values of the four extracts were significantly different at 1% level (Fig. 2A).

Cell viability and apoptosis based on the flow cytometry

The flow cytometry results revealed that the water extract of *A. paniculata* led to a massive rate of apoptosis (32.8%) in MCF-7 cells, only 24 h after inducing the extract (Fig. 3C). More specifically, the water extract appeared more toxic and achieved the highest rate of necrotic cells (annexin V^-/PI^+, Q1 = 8.04%), late apoptotic/dead cells (annexin V^+/PI^+, Q2 = 25.4%), early apoptotic cells (annexin V^+/PI^-, Q3 = 7.39%), as well as the lowest rate of dynamic/non-apoptotic cells (annexin V^-/PI^-, Q4 = 59.2%), when compared to other extracts (Fig. 3). The methanol extract of *A. paniculata* resulted in a total of 24.99% apoptosis, including Q3 = 8.99% of early apoptotic cells (annexin V^+/PI^-), and Q2 = 16% of late apoptotic/dead cells (annexin V^+/PI^+). The rate of non-apoptotic cells was determined as Q4 = 72.3% (annexin V^-/PI^-), as shown in Fig. 3D. As a remarkable result, the DCM and ethanol extracts achieved the highest toxicity (lowest IC_{50} values: 61 µg/mL and 37 µg/mL, respectively, as shown in Fig. 2A) as well as the highest mean telomerase inhibition (80.3 ± 1.4% and 78.5 ± 1.35%,

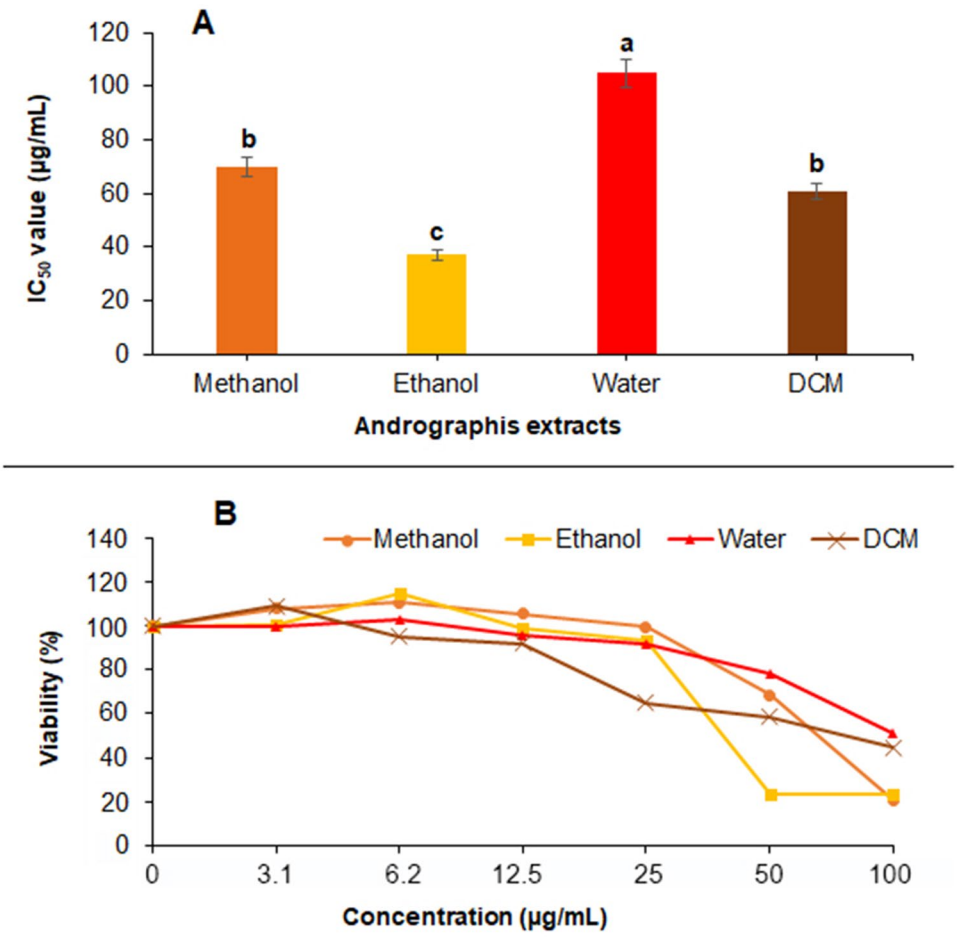

Fig. 2 Cellular viability of MCF-7 after treating with different concentrations of *A. paniculata* extracts: **A** IC_{50} values in MCF-7 cells, and **B** Dose–response curves of MCF-7 cells to *A. paniculata* extracts. Different lowercase letters on top of the error bars of the columns indicate a significant difference between the values of pairs of treatment within columns using Duncan's multiple comparison test at $P \leq 0.05$. Untreated MCF-7 cells were used as the control treatment

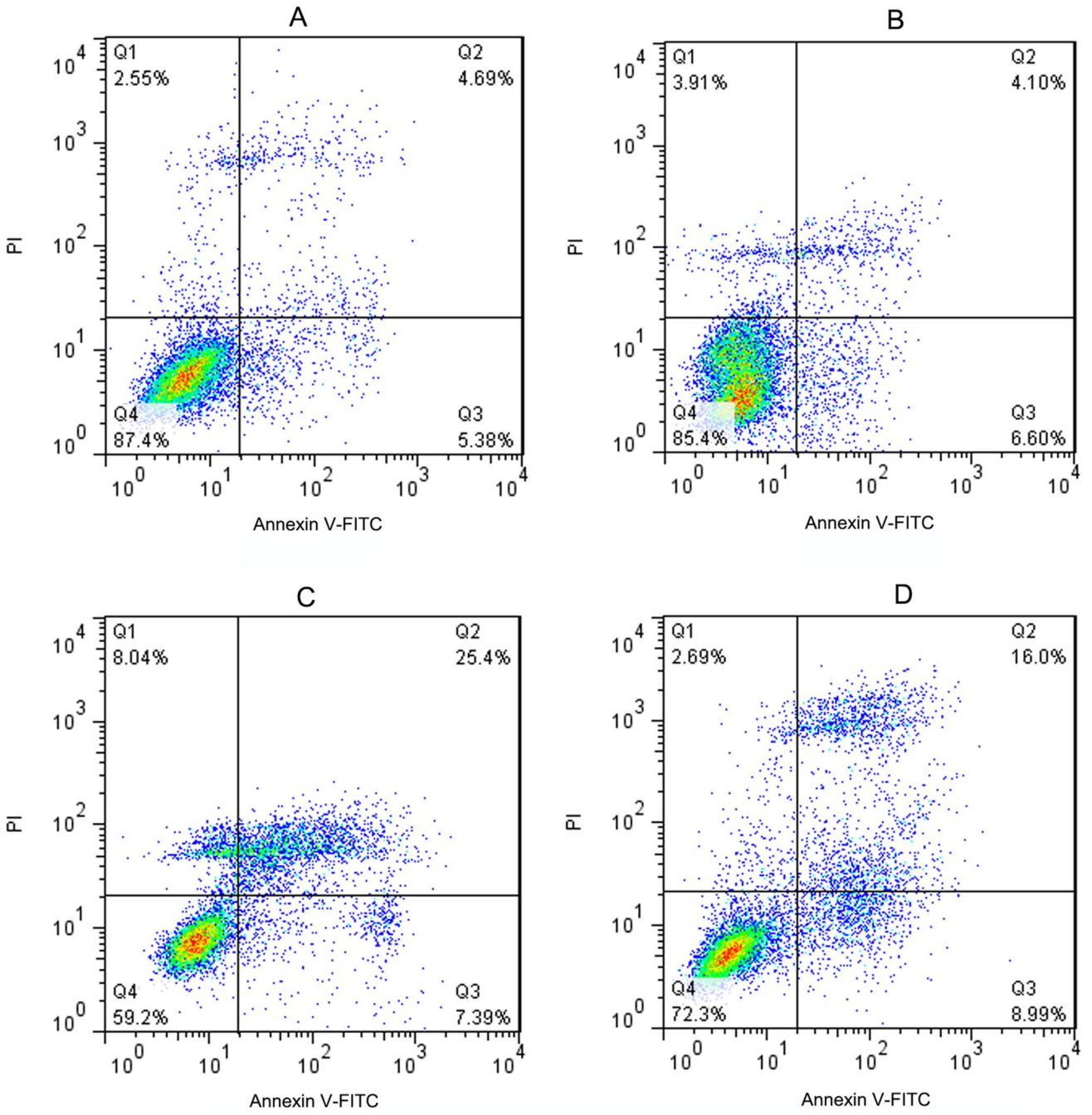

Fig. 3 Flow cytometry results of MCF-7 cells after being treated with **A** DCM, **B** ethanol, **C** water, and **D** methanol extract of *A. paniculata.* The upper left quadrant (Q1) represents necrotic cells (annexin V^-/PI^+), the upper right quadrant (Q2) represents late apoptotic/dead cells, (annexin V^+/PI^+), the lower right quadrant (Q3) represents early apoptotic cells (annexin V^+/PI^-), and the lower left quadrant (Q4) represents dynamic/non-apoptotic cells (annexin V^-/PI^-)

respectively, as shown in Fig. 4B). Surprisingly, the DCM and ethanol extracts achieved the lowest rates of apoptosis based on the flow cytometry results (Fig. 3). More specifically, the DCM extract achieved the lowest total rate of apoptotic cells, including early and late apoptotic cells (Q2 + Q3 = 10.07%) and necrotic calls (Q1 = 2.55%) as well as highest rate of non-apoptotic cells (Q4 = 87.4%), among the four extracts, as shown in Fig. 3A. Similarly, the ethanol extract showed a total rate of apoptotic cells by 10.7%, necrotic calls by 3.91%, and non-apoptotic cells by 85.4% (Fig. 3B).

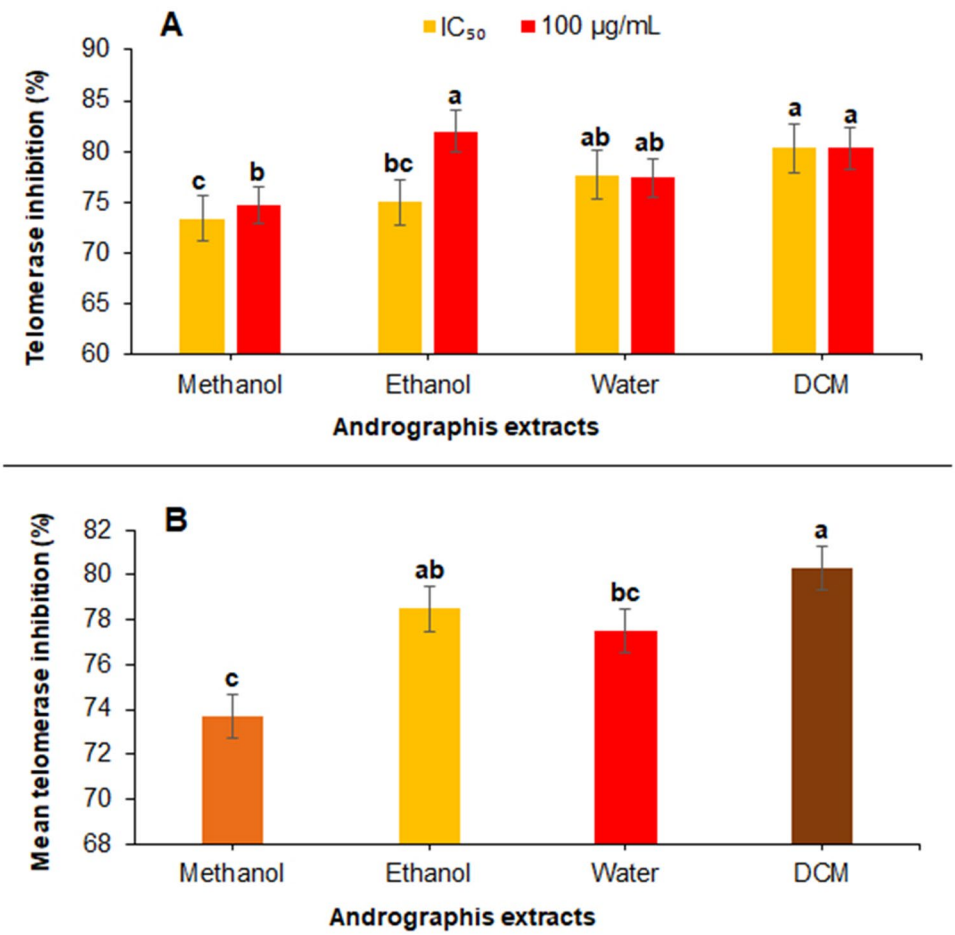

Fig. 4 TRAP-based telomerase inhibition of MCF-7 cells after treating with *A. paniculata* extracts at **A** IC_{50} dose and 100 µg/mL, and **B** mean of IC_{50} dose and 100 µg/mL. Different lowercase letters on top of the error bars of the columns indicate a significant difference between the values of pairs of treatment within columns using Duncan's multiple comparison test at $P \leq 0.05$. Untreated MCF-7 cells were used as the control treatment

Detection of telomerase inhibition by ELISA

Telomerase inhibition of MCF-7 cells treated by the *A. paniculata* extracts was double confirmed using TRAP-ELISA. Interestingly, all four *A. paniculata* extracts led to high rates of telomerase inhibition in both IC_{50} and 100 µg/mL doses (Fig. 4). The one-way analysis of variance (ANOVA) revealed that the telomerase inhibition of the extracts at IC_{50} and 100 µg/mL doses were significantly different at 1% and 5% levels, respectively. The difference of mean telomerase inhibition of the extracts was significant at 5% level (Table 1). The ethanol extract achieved the highest rate of telomerase inhibition among all four extracts by 82 ± 1.35% at 100 µg/mL. The telomerase inhibition of ethanol extract at IC_{50} dose (37 µg/mL) was determined as 75 ± 1.17% (Fig. 4). The DCM extract showed the most stable results by leading to a telomerase inhibition of 80.3 ± 0.66% at IC_{50} dose (61 µg/mL) and 80.3 ± 1.4% at 100 µg/mL (Fig. 4A). The methanol extract obtained the lowest rates of telomerase inhibition among the extracts by 73.4 ± 1.24% at IC_{50} dose (70 µg/mL) and 74.7 ± 1.81% at 100 µg/mL (Fig. 4A). The water extract achieved 77.7 ± 1.42% and 77.4 ± 1.81% of telomerase inhibition at IC_{50} dose (105 µg/mL) and 100 µg/mL, respectively (Fig. 4A). The results also showed that the DCM and methanol extracts possessed the highest and lowest rates of mean telomerase inhibition at 80.3 ± 1.4% and 73.7 ± 1.81%, respectively (Fig. 4B). The mean telomerase inhibition of the DCM and methanol extracts was statistically significant at 5% level (Fig. 4B). Furthermore, the comparison test results revealed that the mean telomerase inhibition of ethanol and water extracts as well as methanol and water extracts were not significantly different at 5% level; however, the difference between the ethanol and methanol extracts was significant at 5% level (Fig. 4B).

Telomerase inhibition, cytotoxicity, and solvent polarity

According to ANOVA results, differences among the solvents based on their polarity were significant at 1% level (Table 1). Additionally, the correlation between the polarity of solvent and the studied traits was calculated, separately. The correlations between telomerase inhibition and cytotoxicity (MTT at IC_{50} and 100 µg/mL) were significant at

Table 1 Variance analysis of *A. paniculata* extracts based on cytotoxicity, telomerase inhibition, and solvent polarity index

S. O. V.	*df*	MTT (IC_{50})			MTT (100 µg/mL)			Telomerase inhibition			Solvent polarity		
		MS	*F*	Sig	MS	*F*	Sig	MS	*F*	Sig	MS	*F*	Sig
Extracts	3	27.73	6.84	0.01	30.84	3.97	0.05	20.56	5.327	0.03	16.75	633.49	0.00
Error	8	4.05			7.78			3.86			0.03		
CV (%)		2.63			3.55			2.53			2.69		

S. O. V. source of variation, *df* degree of freedom, *MS* mean square

1% level, as shown in Table 2. Interestingly, there was no significant correlation between the polarity of solvent and telomerase inhibition as well as cytotoxicity (Table 2).

Discussion

Telomerase inhibition, short telomeres, cancer, and COVID-19

The role of herbal plants, including *A. paniculata,* as immunity modulators against COVID-19 has attracted a great deal of attention, recently (Das 2022; Sarkar and Mukhopadhyay 2022). High telomerase activity and short telomeres have been reported in cancer tumours (Piotrowska et al. 2005), while discoveries on short telomeres and telomerase inhibition have become a target for cancer diagnosis and the development of novel therapeutic agents, in recent years (Nakajima et al. 2003). The initial hypothesis on the association between short telomeres and severe COVID-19 symptoms (Aviv 2020) turned to solid reports, shortly after the outbreak of the disease (Sanchez-Vazquez et al. 2021; Kamal et al. 2020; Froidure et al. 2020; Simões et al. 2020; Benetos et al. 2021). Several molecular mechanisms have been suggested to address the connection between short telomeres and COVID-19. Aviv (2021) suggests that T-cell telomere length affects the adaptive immune response and the innate immune response to SARS-CoV-2 infection. According to this model, the individual with long T-cell telomeres shows strong T-cell response and robust suppression of the innate immune response that is accompanied by "calibrated" (moderate) activity of the innate immune response. In contrast, the individual with short T-cell telomeres shows weak T-cell response and inadequate suppression of the innate immune response that is accompanied by "calibrated" (strong) activity of the innate immune response, expressed in cytokine storm, lung injury, and severe COVID-19 (Fig. 5). Based on a very recent report, the expression of the cell receptor for the SARS-CoV-2, namely, Angiotensin Converting Enzyme-2 (ACE2), which is essential for mediating cell entry of the coronavirus, increases in the lungs of ageing mice and humans. Furthermore, ACE2 expression increases upon telomere shortening or dysfunction—common hallmarks of ageing—in cultured human cells and in mice. Such an increase depends on a DNA damage response elicited by dysfunctional telomeres (Sepe et al. 2022). Telomerase can function both as tumour suppressors by limiting the number of cell divisions and also as tumour promoters by inducing genome instability (Gobbini et al. 2014). On the other hand, the role of telomerase and virus interaction in cancer development highlights the role of telomerase-dependent therapeutic approaches (Salimi-Jeda et al. 2021). Accordingly, *A. paniculata* should be further noticed as a potent telomerase

Table 2 Correlation coefficients of the studied traits *under A. paniculata* extracts

Traits	MTT (IC_{50})	MTT (100 µg/mL)	Telomerase inhibition	Polarity of solvent
MTT (IC_{50})	1			
MTT (100 µg/mL)	0.373 ^{ns}	1		
Telomerase inhibition	0.800**	0.855**	1	
Polarity of solvent	−0.282^{ns}	−0.461^{ns}	−0.456^{ns}	1

**Significant at $P \leq 0.01$, *ns* non-significant

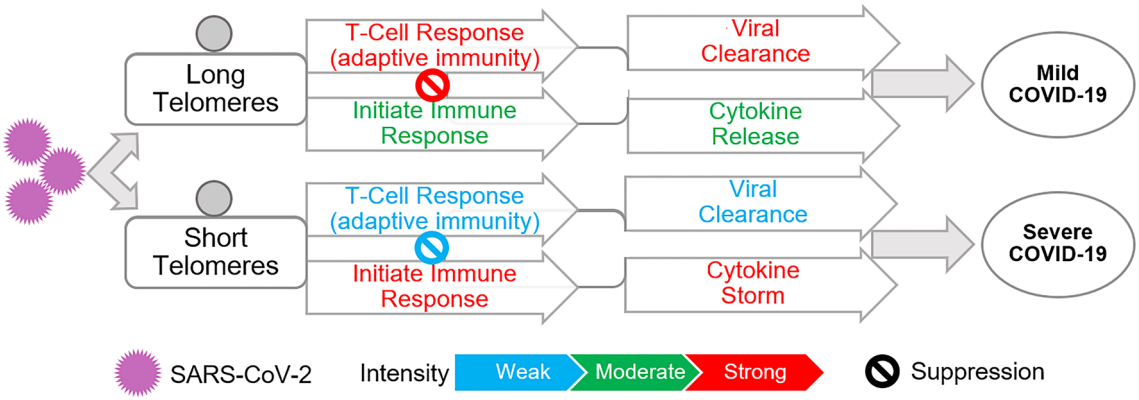

Fig. 5 T-cell telomere length effect on the adaptive immune response and the innate immune response to SARS-COV-2 infection. For the individual with long T-cell telomeres, red colour represents strong T-cell reaction and robust suppression of the innate immune response. Green colour represents "calibrated" (moderate) activity of the innate immune response. For the individual with short T-cell telomeres, blue colour represents poor T-cell response and insufficient suppression of the innate immune response. Red colour represents poorly "calibrated" (strong) activity of the innate immune response, expressed in cytokine storm, lung injury, and severe COVID-19. (This image and its caption have been adapted from Aviv 2021.)

inhibitor, since excessive activity of telomerase (telomerase reactivation + oxidative stress) in cancer cells is a key factor in telomere shortening. Hence, the inhibitory effect of *A. paniculata* on telomerase activity can be considered as a potential target in cancer therapy; however, telomerase inhibition as a safe therapy for SARS-CoV-2 is arguable.

Phytochemistry of *A. paniculata* and telomerase inhibition

Telomerase inhibitors can lead to serious crisis in most of cultured cancer cells within a range of 50–80% of reduction in telomerase activity (Shay and Wright 2005). Budiatin et al. (2021) discovered that the ethanol extract of *A. paniculata* has the potential to inhibit the development of cancer at the hyperplasia stage by decreasing telomerase activity and increasing apoptosis, marked by an increase of caspase-3 expressions in female Sprague Dawley (SD) rats. Nevertheless, it is unknown whether telomerase inhibition is mediated by andrographolide or other bioactive constituents of the plant. As a comparison, ethanol extract of *A. paniculata* achieved the highest rate of telomerase inhibition (82 ± 1.35% at 100 µg/mL), and its mean telomerase inhibition (78.5 ± 1.35%) scored the second place mathematically in the present research (Fig. 4). Despite the similar telomerase inhibition rates of the four extracts, it is unclear that which bioactive compound of the plant (e.g. AG, DDAG, NAG, etc.) was responsible for the higher telomerase inhibition, since the phytochemical contents of the extracts were not analysed in the present study. According to Rafi et al. (2020), the 50% ethanol and water extracts of *A. paniculata* possessed the highest (114.56 mg/g dry weight) and lowest (25.18 mg/g dry weight) andrographolide content, respectively. On the contrary, Suriyo et al. (2014) have reported a higher content of andrographolide (8.26 mg/g dry weight) in water extract of mature leaves (MLWE) compared with ethanolic extract of the first true leaves (FTLEE), which was found richer in 14-Deoxy-11,12-didehydroandrographolide (5.74 mg/g dry weight), neoandrographolide (8.82 mg/g dry weight), and 14-deoxyandrographolide (123.73 mg/g dry weight). Since the cytotoxicity of FTLEE (rich in 14-deoxyandrographolide) was four times higher than MLWE (rich in andrographolide), it was hypothesized that the interaction between active diterpenoids, especially andrographolide

and 14-deoxyandrographolide, may play an important role in the growth inhibiting effect of FTLEE (Suriyo et al. 2014). Further interactions might occur among other analogues of andrographolide leading to cell cycle arrest in various types of cancer (Geethangili et al. 2008). Indeed, the concentration and composition of chemical constituents in plants are greatly affected by variable factors like harvesting condition, growth condition, genetics, type, and concentration of extraction solvents (Rafi et al. 2020). A separate study should be conducted to better understand the impact of each constituent (e.g. AG, DDAG, NAG, etc.) on telomerase inhibition. The application of pure powders of these diterpenoids can clarify these ambiguities in the future endeavours. Available literatures introduces andrographolide responsible for telomerase inhibition due to its role in phosphoinositide 3-kinase (PI3K)/serine/threonine kinase (AKT) (Duan et al. 2019), and/or NF-κB pathways (Li et al. 2017; Mishra 2021). The application of *A. paniculata* extracts as a treatment for COVID-19, due to its telomerase inhibition should be investigated carefully, since a recent study shows that SARS-CoV-2 infection lowers the expression of Telomeric Repeat-Binding Factor 2 (TRF2) shelterin protein complex and results in telomere shortening in Vero E6 cells (Victor et al. 2021). Moreover, the exact length of telomeres with a consistent telomerase inhibition after treating with *A. paniculata* extracts should be confirmed by the methods such as Southern blot or telomere-q PCR kit as explained by Cawthon et al. (2003). Ideally, a recently developed method, called, Telomere Length Combing Assay (TCA) can be employed to measure telomere length (Kahl et al. 2020).

Andrographis paniculata and COVID-19

Several reviews (Lim et al. 2021; Adiguna et al. 2021) and research papers (Sa-ngiamsuntorn et al. 2021; Enmozhi et al. 2021; Verma et al. 2021; Rehan et al. 2021) have regarded *A. paniculata* extract and andrographolide as a potential remedy for SARS-CoV-2. Briefly, post-infection treatment of *A. paniculata* ethanol extract and andrographolide in SARS-CoV-2-infected Calu-3 cells significantly repressed the production of infectious virions with an IC_{50} of 0.036 µg/mL and 0.034 µM, respectively, as determined by the plaque assay (Sa-ngiamsuntorn et al. 2021). In another research, andrographolide inhibited SARS-CoV-2 main protease in silico, by being docked successfully in the binding site of SARS-CoV-2 M^{pro}. Andrographolide follows Lipinski's rule, which makes it a promising candidate for future investigations on COVID-19 (Enmozhi et al. 2021). Additionally, Verma et al. (2021) have shown the major phytochemicals of *A. paniculata* as potential inhibitors of SARS-C0V-2 proteases, including papain-like protease (PL^{pro}) and main protease (M^{pro})/3-chymotrypsin-like protease ($3CL^{pro}$). Andrographolide interacted with the active sites through conventional hydrogen bonds (VAL^{159} and GLN^{269}) and with van der Waals interactions with the rest of the residues. DDAG stabilized the active site through conventional hydrogen bond (GLN^{269}), carbon–hydrogen bond (GLY^{160}), and van der Waals interactions with the rest of the residues. Neoandrographolide bound with the active sites through conventional hydrogen bonds (ASN^{109}, GLN^{269}, VA^{159}) and carbon–hydrogen bond (GLY^{160}, THR^{158}). More recently, the molecular docking method showed the potential of andrographolide in binding with Tumour Necrosis Factor (TNF) and covalent binding with NFkB1 proteins of the TNF signalling pathway, which is responsible for cytokine storm in COVID-19 patients (Rehan et al. 2021). As mentioned, telomerase inhibition as a safe therapy for SARS-CoV-2 is arguable. On one hand, eliminating (or reducing) the existing population of short telomeres via specific activity of human telomerase on immortal cells and cancer inhibition (arresting proliferation and finally cell death) as described by Kim et al. (1994) and Zhang et al. (1999) may also decrease the susceptibility against SARS-CoV-2, especially in sensitive group of individuals (e.g. elderly and cancer patients as well as patients prone to cancer). On the other hand, increasing the population of short telomeres via telomerase

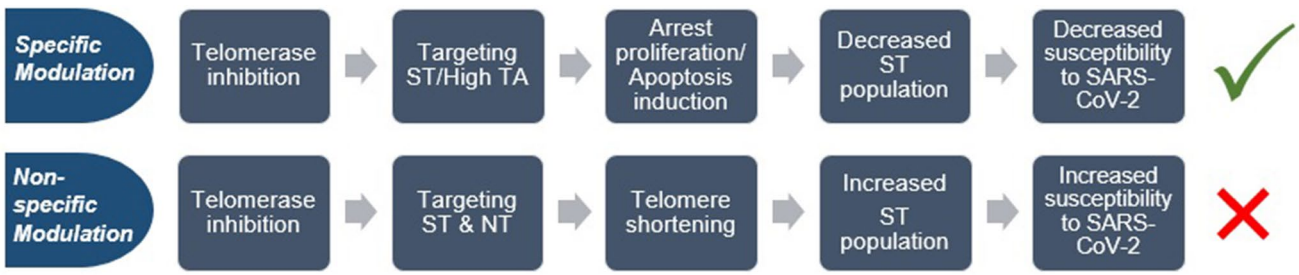

Fig. 6 Schematic representation of *A. paniculata* extract selectivity/specificity. *ST* short telomeres, *NT* normal (long) telomeres, *TA* telomerase activity

inhibition (caused by a telomerase inhibitor) may increase the susceptibility against SARS-CoV-2 (Fig. 6). Therefore, further investigation is required to establish whether telomerase inhibition (and even activation) is efficient on decreasing the rate of SARS-CoV-2 infection and severe symptoms of COVID-19. More importantly, the selectivity/specificity of *A. paniculata* extract should be indicated in future studies, meaning that it should be confirmed that the inhibitory effect of the extract is confined to the cells containing both short telomere and excessive telomerase activity (e.g. in cancer tumour). Furthermore, the impact of *A. paniculata* extract (as a telomerase inhibitor) on normal cells and long telomeres should be specified (Fig. 6).

Andrographis paniculata, growth inhibition, and apoptosis

The apoptotic and cytotoxic effects of *A. paniculata* extract and/or its major constituent "andrographolide" on different cancer cell lines, including MCF-7 have previously been studied by employing MTT and/or flow cytometry assays (Banerjee et al. 2016). Therefore, the cytotoxicity of *A. paniculata* extracts is a secondary objective of the present research. Despite this, the difference between the results of MTT assay and flow cytometric analysis should be discussed. Technically, MTT is a colorimetric method that relies on the "metabolic activity" determined mainly by the function of mitochondria, and cannot differentiate between growth inhibition and cell death. Therefore, when a treatment leads to the reduction of cellular metabolic activity, it is indirectly considered as a symptom of apoptosis in the MTT assay, while this might happen without any apoptotic processes. Therefore, an increase of apoptosis is not necessarily observed in the analysis of the same sample/treatment by flow cytometry. The lack of such capability in MTT assay often leads to misinterpreting results (Ghasemi et al. 2021). On the other hand, flow cytometry is capable of differentiating among necrotic, healthy, and early and late apoptotic cells using fluorescence labelling of annexin V-FITC/PI (Henslee et al. 2016). Differences between the MTT and flow cytometry results are reported, accordingly. The difference in sensitivity among different cancer cell lines to *A. paniculata* extracts and/or andrographolide has also been recorded in other researches (Suriyo et al. 2014; Banerjee et al. 2016). A part of these different feedback corresponds to the phytochemical content of various *A. paniculata* extracts that are greatly affected by the phenological stage, plant material and sampled organ (Tajidin et al. 2019), type and concentration of solvent, and time of exposure (as mentioned in Sect. 4.2).

Andrographis paniculata, telomerase inhibition, and apoptosis

Reportedly, apoptosis is an inseparable consequence of telomerase inhibition in cancer cells (Mondello and Scovassi 2004). The majority of evidences related to the effect of *A. paniculata* extract on various cancer cells comply with the induction of apoptosis (Suriyo et al. 2014; Budiatin et al. 2021; Sarkar et al. 2018). The results of the present research, however, was not completely in line with the above-mentioned trend, since the high rates of telomerase inhibition caused by the DCM and ethanol extracts were not accompanied by the high rates of apoptosis. The phytochemical composition and duration of induction could be the key factors in such observation, as mentioned in Sects. 4.2 and 4.4. Previous studies have assigned different roles to each diterpenoid. For example, didehydroandrographolide has been introduced as a potential P-PLCγ inhibitor that can promote apoptosis of tumour cells (Che et al. 2019). Based on recent evidences, mitochondrial enrichment with hTERT may be accompanied by reactive oxygen species (ROS) level modulation or an enhanced number of mtDNA copies that can lead to apoptosis repression and may play a protective role during cancer therapy (Lipinska et al. 2017). Interestingly, a similar behaviour (decreased ratio of apoptosis) has been recorded by Duan et al. (2019), when high glucose was the source of apoptosis via oxidation and ROS levels in human umbilical vein endothelial cells (HUVECs). Probably, the lower rates of apoptosis in MCF-7 cells under the treatments of DCM and ethanol extracts have been due to their unknown phytochemical profile and the interaction among them. This observation suggests that the inhibition of telomerase in these two treatment (DCM and ethanol) may not be a result of cell death (apoptosis) but may be correlated with cell growth inhibition. A similar condition was observed by the application of ceramide for inhibiting telomerase activity in A549 human lung adenocarcinoma cells (Ogretmen et al. 2001; Guenther et al. 2008). However, the latter mechanism does not govern the water and methanol extracts. Regardless of the rate of apoptosis, all four extracts of *A. paniculata* can be considered as a potential adjuvant, to overcome the resistance of cancer cells to drugs during cancer therapy, via inhibiting telomerase activity. The use of MCF-7 cells in this research was based on the TRAP kit manufacturer's recommendation. The MCF-7 cells are caspase-3 deficient with a partial deletion in the *CASP-3* gene. These cells display cell death that lack typical apoptotic properties (Wang et al. 2016). Thus, apoptosis induction and telomerase inhibition might have been affected by this deficiency in this study. Hence, more independent cancer cells, such as A431 cell lines, can be used in the future studies. Additional proliferation

markers, such as Ki67 and PCNA or an antibody against activated caspase-3 (not in MCF-7 cells), can be used in future studies to confirm a reduction in proliferation and apoptosis.

Conclusion

Telomerase can function as a tumour suppressor by limiting the number of cell divisions and also as a tumour promoter by inducing genome instability. The role of telomerase and virus interaction in cancer development highlights the role of telomerase-dependent therapeutic approaches. It should be noticed that short telomere is an "effect" but not a "cause". Telomerase seems to be a link between the short telomeres and the severe COVID-19 symptoms. The extract of *A. paniculata* as a telomerase inhibitor can be considered in cancer therapy; however, its safety as a telomerase inhibitor in SARS-CoV-2 therapy is currently unknown. Therefore, the application of *A. paniculata* as a treatment for COVID-19 should be investigated, carefully. The exact length of telomeres after treating with *A. paniculata* extracts should be quantified by a suitable method. Further investigation is required to establish whether telomerase inhibition (and even activation) is efficient on decreasing the rate of SARS-CoV-2 infection and severe symptoms of COVID-19. More importantly, the selectivity/specificity of *A. paniculata* extract should be indicated in the future.

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Declarations

Conflict of interest On behalf of all the authors, the corresponding author states that there is no conflict of interest.

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