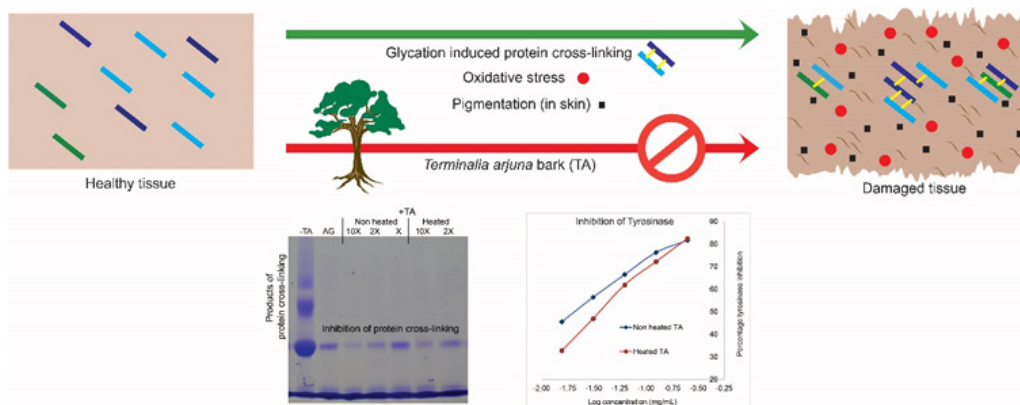


RESEARCH ARTICLE

Protective Effects of *Terminalia arjuna* Bark Extracts Against Glycation Induced Protein Damage, Oxidative Stress, and Hyperpigmentation

H.K.I. Perera *, J.A.V.R. Jayasinghe, A.P.C.P. Lankarathna, K.G. Nilmanel & R. Sivakanesan



Highlights

- *Terminalia arjuna* bark extracts were analyzed *in vitro*.
- Strong inhibitory effects on glycation induced protein cross-linking were found.
- Strong inhibitory effects on tyrosinase were observed.
- Inhibitory effects showed heat stability.
- High antioxidant capacity, total phenolic and flavonoid content were detected.

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Protective Effects of *Terminalia arjuna* Bark Extracts Against Glycation Induced Protein Damage, Oxidative Stress, and Hyperpigmentation

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Abstract: Glycation induced protein damage, and oxidative stress play a critical role in the development of chronic diabetic complications and skin aging. *Terminalia arjuna* (Kumbuk) bark has been used for medicinal purposes for thousands of years. Main objectives of the study were to assess in vitro inhibitory effects of *T. arjuna* bark extracts on glycation induced protein cross-linking and to assess heat stability of those effects. Additional objectives were to assess antityrosinase effects, total antioxidant activity, phenolic content, and flavonoid content of *T. arjuna* extracts. The effects of *T. arjuna* bark extracts were assessed using electrophoresis-based and colorimetric methods. Experiments were repeated on three separate occasions with appropriate controls. Fiji/ImageJ software was used to quantify the extent of protein cross-linking. Statistical significance of the differences of colorimetric assays was calculated using ANOVA. $P < 0.05$ was considered statistically significant. *T. arjuna* extracts showed strong inhibitory effects on glycation induced protein cross-linking and on tyrosinase activity. These effects showed heat stability to a great extent. Methanol extract demonstrated higher inhibitory effects on protein cross-linking compared to that of ethanol extracts. *T. arjuna* extracts demonstrated high total antioxidant capacity, total phenolic and flavonoid content. Furthermore, antioxidant capacity showed a significantly positive correlation with polyphenolic and flavonoid contents. The results provide evidence to suggest the beneficial effects of *T. arjuna* bark in alleviating some mechanisms that cause chronic diabetic complications and skin aging. Findings also provide in vitro evidence to validate the traditional use of *T. arjuna* bark in diabetes mellitus and skin disorders.

Keywords: *Terminalia arjuna*; Glycation; Cross-linking; Antityrosinase; Antioxidant

INTRODUCTION

Accumulation of advanced glycation end products (AGEs) thorough non enzymatic glycation of proteins and oxidative stress are major contributors in the development of chronic diabetic complications such as cardiovascular diseases and nephropathy (Mengstie et al., 2022; Wijetunge & Perera, 2014; Giacco & Brownlee, 2010). Non enzymatic glycation is commenced with a reaction between reducing sugars and amine residues of biomolecules. Accumulation of AGEs is proportional to the concentration and half-life of substrates. Hence chronic hyperglycemia is a main contributor for accelerating the generation of AGEs and long-lived abundant proteins such as collagen are primarily affected by AGE mediated damage (Khalid et al., 2022).

Chronic diabetic complications are mainly mediated through direct extracellular matrix destruction caused by AGEs by protein cross-linking and through receptor mediated signaling pathways (Indyk et al., 2021) in various tissues/ organs such as retina and lens of the eye, kidney, blood vessels and brain (Vadakedath & Kandi, 2018). Some AGEs such as crossline and glyoxal derived lysine dimer are cross-linking AGEs and promote intra and inter molecular cross-linking of proteins, impairing the function of various tissues (Twarda-clapa et al., 2022).

Oxidative stress is increased in hyperglycaemic situations by several mechanisms such as glycolytic pathway, increased AGE generation, hexosamine pathway, and polyol pathway. Oxidative stress damages pancreatic β -cells and contributes to diabetic manifestations. Oxidative stress has a crucial role in the pathogenesis of diabetic complications as well (Darenskaya et al., 2021).

Accumulation of AGEs and oxidative stress are interconnected, and they aggravate the progression of each other. Free radicals formed during glycoxidation reactions of the middle stage reactions of glycation process enhance AGE accumulation (Reddy et al., 2022). On the other hand, AGE mediated intra cellular signaling cascades induce production of free radicals in target cells (Mengstie et al., 2022). Free radical scavenging activity of plant extracts provides protection against oxidative stress. Among the broad spectrum of medicinal effects reported, phenolic and flavonoid contents are highly correlated with antioxidant and antiglycation effects (Dearlove et al., 2008). Hence, estimation of total antioxidant activity, phenolic and flavonoid content in plant extracts is important. In addition to the contribution in pathogenesis of chronic diabetic complications, AGEs, and oxidative stress, together with pigmentation disorders adversely affect the skin structure. As the key regulatory enzyme of melanogenesis, tyrosinase has become the prime target in therapeutics and cosmetics in order to minimize skin pigmentation (Pillaiyar et al., 2017). Many reports show evidence for the effectiveness of phenolic compounds which include flavonoids in the prevention of skin disorders (Działo et al., 2016). Several antiglycation agents including the extensively studied aminoguanidine (AG) failed at clinical trials due to their side effects. Existing skin lightening agents present

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limitations in terms of their high toxicity.

Terminalia arjuna (Combretaceae) (Kumbuk) is being used for all three humors *kapa*, *pitta* and *vayu* for over 2,500 years in traditional medicine (Viswanatha et al., 2010). *T. arjuna* bark is traditionally used to treat diabetes mellitus and scientifically proven for its antihyperglycemic effects (Ansari et al., 2021; Shengule et al., 2018) and to treat patients with melasma (Angadi & Gowda, 2014; Konwar & Das, 2016; Sreelekshmi & Geethesh, 2018). *T. arjuna* bark is used as a decoction in many traditional remedies (Sadowska-bartosz & Bartosz, 2015). Therefore, it is important for the active ingredients to be heat stable.

Main objectives of the study were to assess in vitro inhibitory effects of *T. arjuna* bark extracts on glycation induced protein cross-linking and to assess heat stability of those effects. Additional objectives were to assess antityrosinase effects, total antioxidant activity, phenolic content, and flavonoid content of *T. arjuna* extracts.

MATERIALS AND METHODS

Preparation of Plant Extracts

Terminalia arjuna (Roxb. Ex DC.) Wight & Arn. bark was collected from Middeniya, Sri Lanka. Plant was authenticated (Tag No. APCP-HKIP-BIO-PDN-10) by the Deputy Director, National Herbarium and the voucher samples were deposited at the National Herbarium, Royal Botanical Gardens, Peradeniya, Sri Lanka. *T. arjuna* bark was cleaned, dried under shade for one week and powdered using an electric grinder. Dry powder (10 g) of *T. arjuna* bark was extracted with 100 mL of solvent (ethanol and methanol separately) three times using a sonicator. Filtrates were dried by evaporating solvents below 50°C using a rotary evaporator. Ethanol and methanol extracts were stored at room temperature until further investigation. Aqueous extracts were prepared by adding 100 mL deionized water to 10 g bark powder and sonicating for 1 h (3X 20 min). Supernatant was collected by centrifuging and filtering. Aqueous extracts were stored at -20°C. Extracts were diluted in phosphate buffer prior to the assay to make required concentrations [DMSO (<0.5%) was used prior to use of buffer for ethanol and methanol extracts]. Ethanol and methanol extracts and dry powder of *T. arjuna* were prepared in deionized water (5 mg/mL) for estimation of total antioxidant activity, total phenolic content, and total flavonoid content.

Glycation induced protein cross-linking inhibitory effect of *T. arjuna*

Assessment of glycation induced protein cross-linking inhibitory effect of *T. arjuna* extracts

Glycation induced protein cross-linking inhibitory effect of *T. arjuna* extracts was assessed using an electrophoresis based method (Perera & Ranasinghe, 2015). Briefly, chicken egg lysozyme (Sigma) (10 mg/mL) [used as an ideal protein to detect glycation induced cross-linking, because lysozyme is a small protein in which high molecular weight products of cross-linking can be easily differentiated in SDS-PAGE and it is a protein rich in

arginine and lysine which are the target sites for glycation (Perera & Ranasinghe, 2015)] was incubated with 0.5 M fructose (Sigma) [fructose is used instead of glucose, because fructose is much more reactive than glucose and enhances the speed of glycation rapidly, so that the detection and differentiation of the effects of glycation is much quicker and clearer compared to that of glucose (Perera & Ranasinghe, 2015)] in the presence and absence of *T. arjuna* extracts (12.5, 25, 50 and 100 µg/mL) for 21 days at 37 °C and pH 7.4. Aminoguanidine (Sigma) (AG) was used as the positive control. Aliquots collected after 7 and 21 days of incubation were analyzed (with the sample buffer containing reducing agent β-mercaptoethanol) for the appearance of high molecular weight products using sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). The appearance of high molecular weight products of lysozyme in the aliquots was compared after staining the gels with Coomassie brilliant blue (Biorad). Broad range molecular weight markers (Promega) were included in some gels. Experiments were repeated three times.

Assessment to detect whether the extracts can inhibit cross-linking when added after commencement of protein glycation

T. arjuna extracts (100 µg/mL) were added on day 0, 1 or 2 of the incubation separately, to the lysozyme, fructose mixture (Perera & Premadasa, 2016). Aliquots from the uninhibited control were collected on day 0, 1 and 2. Aliquots were stored at -20 °C until further analysis. Appearance of high molecular weight products in the aliquots collected on day 7 and control samples collected were compared using SDS-PAGE.

Quantification of extent of protein cross-linking

Color intensity of the bands representing the high molecular bands (products of protein cross-linking) were quantified using the Fiji/ImageJ software after subtracting the background color. Percentage of the extent of cross-linking under different lanes were calculated taking the cross-linking of negative control as 100%. Percentage inhibition of protein cross-linking was calculated (100-% cross-linking).

Measurement of tyrosinase inhibitory effect

Tyrosinase inhibitory effect of *T. arjuna* extracts (15.6 to 250.0 µg/mL to reach the IC₅₀ value) were measured by the method of Di Petrillo et al. (Di Petrillo et al., 2016) with modifications (Perera et al., 2018). Kojic acid (Sigma) was used as the positive control. Each measurement was taken in duplicate and on three separate occasions. Inhibitory effects on tyrosinase activity were calculated using the formula stated below.

$$\text{Percentage inhibition} = \frac{(\text{Control} - \text{Control blank}) - (\text{Test} - \text{Test blank})}{(\text{Control} - \text{Control blank})} \times 100$$

Measurement of IC₅₀ for tyrosinase inhibitory effect

IC₅₀ values of the extracts were measured using a series of *T. arjuna* extract concentrations. IC₅₀ values were determined by plotting percent inhibition (Y axis) versus log10 extract concentration (X axis) and calculated by logarithmic regression analysis from the mean inhibitory

values (Perera et al., 2018).

Assessment of heat stability of inhibitory compounds

A fraction of diluted samples of *T. arjuna* extracts were heated in a boiling water bath for 30 minutes. Heated and non-heated extracts were analyzed to determine the heat stability of inhibitory compounds responsible for protein cross-linking inhibition and tyrosinase inhibition (Perera & Premadasa, 2016).

Estimation of total antioxidant capacity

Total antioxidant capacity (TAC) of *T. arjuna* extracts was assessed using ferric reducing antioxidant power (FRAP) assay as described previously (Mathiventhan & Sivakanesan, 2013). Ascorbic acid (1 mM) was used as the control.

Estimation of total phenolic content

Total phenolic content (TPC) of *T. arjuna* extracts was determined using the method described previously (Mathiventhan & Sivakanesan, 2013). Tannic acid (0.1 g/L) was used as the control.

Estimation of total flavonoid content

Total flavonoid content (TFC) of *T. arjuna* extracts was determined using a method described previously (Enujiugha, 2010). Tannic acid (1 g/L) was used as the control.

Statistical analysis

Means and standard deviations of data collected were calculated using measurements taken on three separate occasions. Statistical significance of the differences was calculated using ANOVA. $P < 0.05$ was considered statistically significant (Mishra et al., 2019).

RESULTS

Glycation induced protein cross-linking inhibitory effects

Assessment of glycation induced protein cross-linking inhibitory effects of *T. arjuna* extracts and effect of heating on this effect

Glycation induced protein cross-linking inhibitory effects of the non-heated and heated *T. arjuna* extracts were determined using lysozyme and fructose. SDS-PAGE showed presence of high molecular weight products in the samples incubated with lysozyme and fructose (Figures 1, 2 and 3). The sizes of high molecular weight bands were previously reported by comparing molecular weight of lysozyme monomer (~12 kD) with molecular weight markers. Those products corresponded to dimer (~24 kD), trimer (~36 kD) and tetramer (~48 kD) of lysozyme (Perera & Ranasinghe, 2015; Perera & Handuwalage, 2015; Perera & Premadasa, 2016; Perera et al., 2017). These high molecular weight bands which represent the stable products of lysozyme cross-linking, were not observed in the samples incubated in the absence of fructose (Figures 1 and 2). Previously it was demonstrated that the degree of the appearance of high molecular weight bands depends on the extent of glycation (Perera & Ranasinghe, 2015).

Effect of the presence of non-heated and heated ethanol and methanol extracts of *T. arjuna* for 1 week is shown in figures 1 A and 1 B. Both non heated and heated samples of ethanol and methanol extracts at the concentrations of 100, 50 and 25 µg/mL showed strong inhibitory effects [$>88\%$ (densitometer values of negative control and with 25 µg/mL ethanol extract without deducting the values of their respective blanks)] of protein cross-linking after 1 week of incubation in the presence of fructose. These effects were greater than that of the standard inhibitor AG which was used at a much higher concentration (1 mg/mL). Methanol extract showed a better inhibition on protein cross-linking at lower concentrations (12.5 µg/mL) when compared with that of ethanol extract (Figures 1 A and 1 B). Furthermore, it shows that the inhibitory effects are mostly heat stable even though some reduction of effects seem to be there, as the effect was much lower in the heated sample of ethanol extract at 12.5 µg/mL (Figure 1 A).

Effect of the presence of *T. arjuna* extracts for a longer period of incubation (3 weeks) is shown in figures 1 C and 1 D. Strong inhibitory [$>76\%$ (densitometer values of negative control and with 50 µg/mL methanol extract without deducting the values of their respective blanks)] and effects of *T. arjuna* ethanol and methanol extracts on protein cross-linking persisted even after 3 weeks at the concentrations of 100 and 50 µg/mL. However, the effect at 12.5 µg/mL was not retained at three weeks as high molecular weight bands appeared to a level like that of negative control (Figures 1 C and 1 D). There was a dose-dependent decline in the inhibitory effect when the concentration of extract was reduced (from 100 to 12.5 µg/mL). Inhibitory effect of methanol extract (Figure 1 D) was greater at 25 µg/mL when compared with the ethanol extract (Figure 1 C) at three weeks.

Figure 2 shows the effect of heated and non-heated water extracts on glycation induced protein cross-linking. Observed inhibitory effects were mostly heat stable. Results indicate $>83\%$ inhibition of protein cross-linking after 1 week of incubation in the presence of both heated and non-heated *T. arjuna* water extracts at concentrations as low as 0.0025 % w/v (Figures 2 A1 and 2 A2). Both heated and non-heated extracts showed a dose-dependent inhibition (Figure 2A and 2B). Inhibitory effects were observed even after 3 weeks of incubation with water extracts.

Assessment to detect whether the extracts can inhibit protein cross-linking when added after commencement of glycation

T. arjuna extracts were added to the lysozyme and fructose mixtures at day 0, 1 and 2 separately. Figure 3 A corresponds to the inhibitory effects obtained with ethanol and methanol extracts and figure 3 B demonstrates the effects of water extract. Considerable degree of inhibition was seen when the extracts were added on day 1 even though the degree of inhibition is lesser when compared with that of day 0. Addition at day 2 seem to be less effective. Standard inhibitor AG showed a better inhibitory effect on protein cross-linking even when added after 2 days of commencement of glycation (Figures 3 A and 3 B).

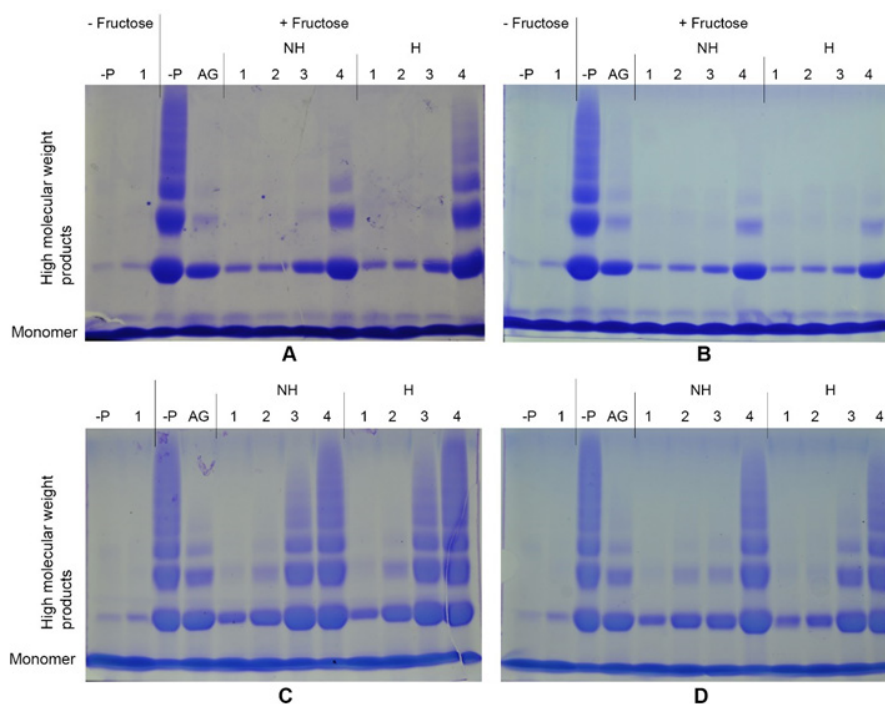


Figure 1: Glycation induced protein cross-linking inhibitory effects of ethanol and methanol extracts of *Terminalia arjuna* and influence of heating on this effect; A: Effect of *T. arjuna* bark ethanol extract after 1 week of incubation, B: Effect of *T. arjuna* bark methanol extract after 1 week of incubation, C: Effect of *T. arjuna* bark ethanol extract after 3 weeks of incubation, D: Effect of *T. arjuna* bark methanol extract after 3 weeks of incubation. -P: lysozyme without extract, AG: Aminoguanidine 1 mg/mL, NH: Non-heated *T. arjuna* extracts, H: Heated *T. arjuna* extracts, - Fructose: Absence of fructose, + Fructose: Presence of fructose. Concentrations of extracts used were (1): 100, (2): 50, (3): 25 and (4): 12.5 $\mu\text{g/mL}$.

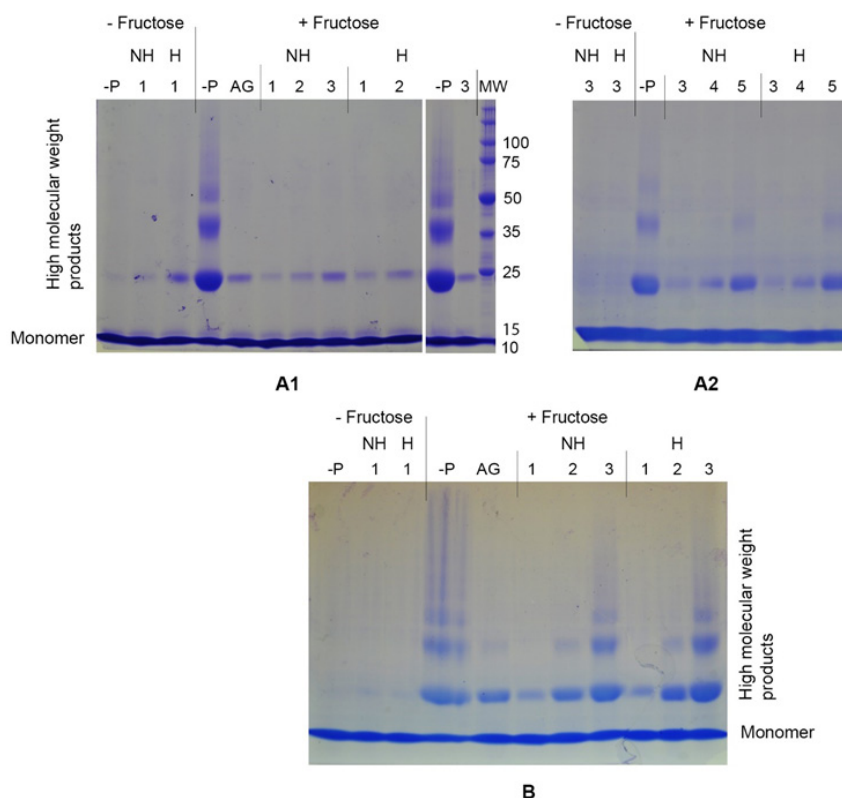


Figure 2: Glycation induced protein cross-linking inhibitory effects of *Terminalia arjuna* water extracts and influence of heating on this effect; A1 and A2: Effect of *T. arjuna* bark water extracts after 1 week of incubation, B: Effect of *T. arjuna* bark water extracts after 3 weeks of incubation. -P: lysozyme without extract, AG: Aminoguanidine 1.5 mg/mL, NH: Non-heated *T. arjuna* extracts, H: Heated *T. arjuna* extracts, - Fructose: Absence of fructose, + Fructose: Presence of fructose. Concentrations of extracts used were (1): 0.05, (2): 0.01, (3): 0.005, (4): 0.0025 and (5): 0.001 % (w/v).

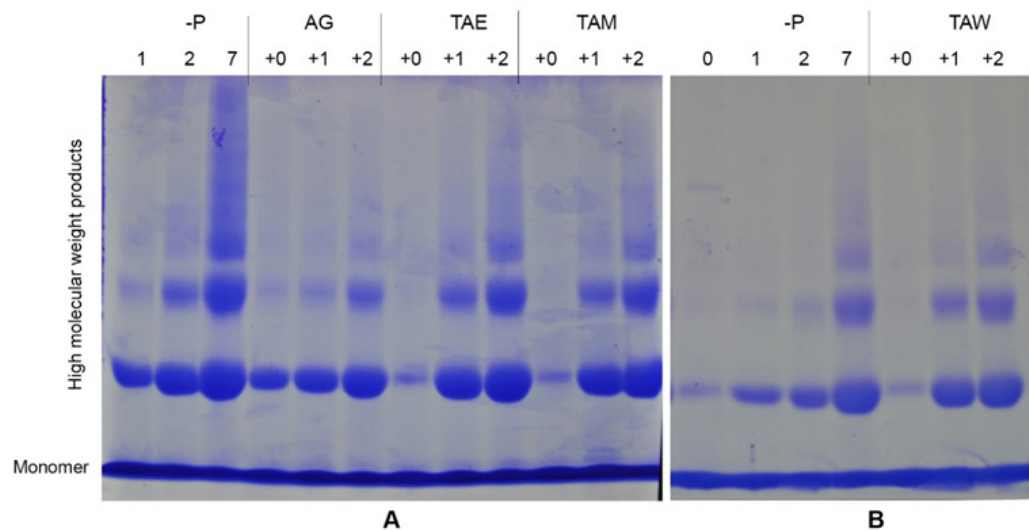


Figure 3: Protein cross-linking inhibitory effects of *Terminalia arjuna* extracts when added after commencement of protein glycation; A: Effect of *T. arjuna* bark ethanol and methanol extracts (100 µg/mL), B: Effect of *T. arjuna* bark water extract [0.05%(w/v)]. Incubation period was 1 week. -P: lysozyme without extract (aliquots were collected at day 0, 1, 2 and 7), AG: Aminoguanidine 1 mg/mL, TAE: *T. arjuna* ethanol extract, TAM: *T. arjuna* methanol extract. TAW: *T. arjuna* water extract. +0, +1, +2: Plant extracts and AG were added to the incubation mixtures containing lysozyme and fructose at day 0, 1 and 2 and tested after 1 week of incubation.

Measurement of tyrosinase activity

Percentage inhibitions of extracts at different concentrations ranging from 15.6 to 250.0 µg/mL are shown in figure 4. IC₅₀ values of non-heated ethanol and methanol extracts of *T. arjuna* against tyrosinase were 26.8±5.0 and 19.8±4.0 µg/mL, respectively. IC₅₀ values of heated ethanol and methanol

extracts were 58.1±6.5 and 37.5±3.6 µg/mL, respectively. IC₅₀ of Kojic acid (3.4±0.4 µg/mL) was significantly lower (p<0.02) than that of *T. arjuna* extracts. Extracts retained >70% of initial antityrosinase activity even after boiling for 30 min. However, there was a significant increase (p<0.02) in the IC₅₀ values after heating.

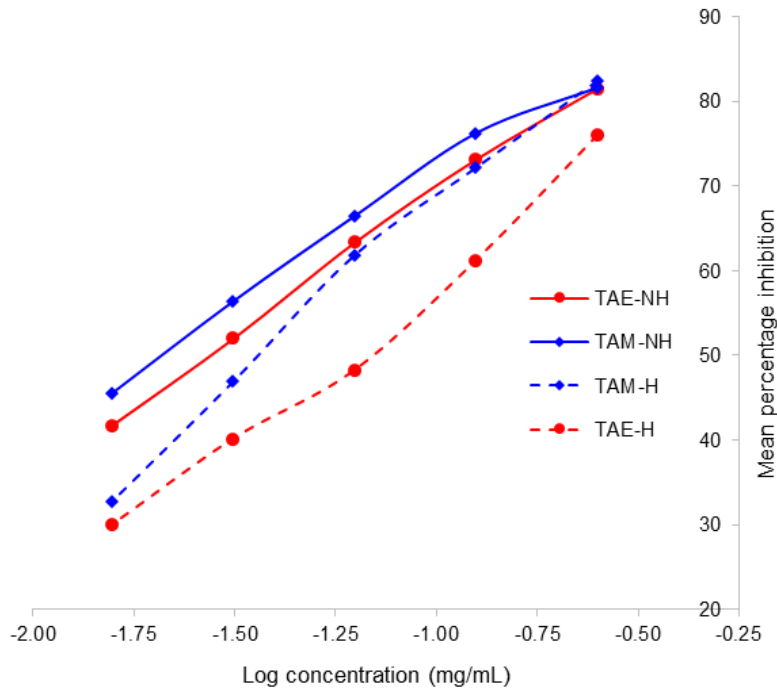


Figure 4: Percentage tyrosinase inhibitory effects of heated and non-heated *Terminalia arjuna* extracts; TAE-NH: *T. arjuna* non-heated ethanol extract, TAE-H: *T. arjuna* heated ethanol extract TAM-NH: *T. arjuna* non-heated methanol extract, TAM-H: *T. arjuna* heated methanol extract.

Estimation of total antioxidant capacity

T. arjuna ethanol and methanol extracts have shown very high TAC 519.0 ± 8.5 and 482.1 ± 7.1 mM $\text{Fe}^{2+}\text{E}/100$ g, respectively. The TAC (112.1 ± 7.8 mM $\text{Fe}^{2+}\text{E}/100$ g) of water extract obtained from dry bark powder was significantly lower ($p < 0.001$) compared to that of ethanol and methanol extracts. TAC value of ascorbic acid (control) was 2 mM.

Estimation of total phenolic content

TPC of ethanol and methanol extracts were 52.9 ± 3.1 and 53.1 ± 3.0 g gallic acid equivalents/100 g, respectively. The TPC of 14.3 ± 1.4 g GAE/100 g in water extract of the dry bark powder was significantly lower ($p < 0.001$) compared to that of ethanol and methanol extracts. Tannic acid used as the control gave a value of 12.3 g GAE/100 g.

Estimation of total flavonoid content

TFC of ethanol and methanol extracts were 5.96 ± 0.16 and 6.25 ± 0.25 g catechin equivalents/100 g, respectively. The TFC (1.09 ± 0.15 g CE/100 g) of water extract obtained from dry bark powder was significantly lower ($p < 0.001$) compared to that of ethanol and methanol extracts. Tannic acid used as the control gave a value of 8.2 g CE/100 g.

A correlation analysis between TAC and TPC ($r = 0.997$, $p < 0.001$) and between TAC and TFC ($r = 0.991$, $p < 0.001$) of the extracts indicated a significant positive correlation (Figures 5 and 6).

DISCUSSION

Glycation and oxidative stress are strongly associated with pathogenesis of chronic diabetic complications. Natural remedies used for medicinal purposes since ancient times would be attractive targets for investigations. *Terminalia arjuna* bark is considered to be the most important part of the plant with respect to therapeutic viewpoint (Amalraj & Gopi, 2017). Many applications of *T. arjuna* bark include its use as a cardio tonic, a treatment for diabetes, cancer, inflammation and various skin disorders (Soni & Singh, 2019). *T. arjuna* stem bark is commonly used in traditional practice to reduce blood glucose concentration in diabetic patients (Chopra & Chopra, 1994). An ancient hydroalcoholic formulation “*Arjunarishta*” in which *T. arjuna* bark is the major ingredient, showed substantial modulation of genes involved in insulin signaling (Shengule et al., 2018). Another study demonstrated the effectiveness of hydroalcoholic bark extract of *T. arjuna* in lowering

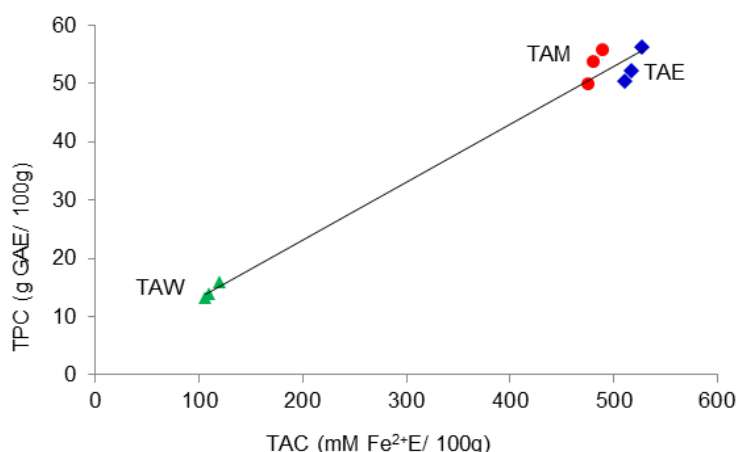


Figure 5: Correlation between total antioxidant capacity and total phenolic content of *Terminalia arjuna* extracts; TAC: Total antioxidant capacity, TPC: Total phenolic content, TAE: *T. arjuna* ethanol extract, TAM: *T. arjuna* methanol extract. TAW: *T. arjuna* water extract

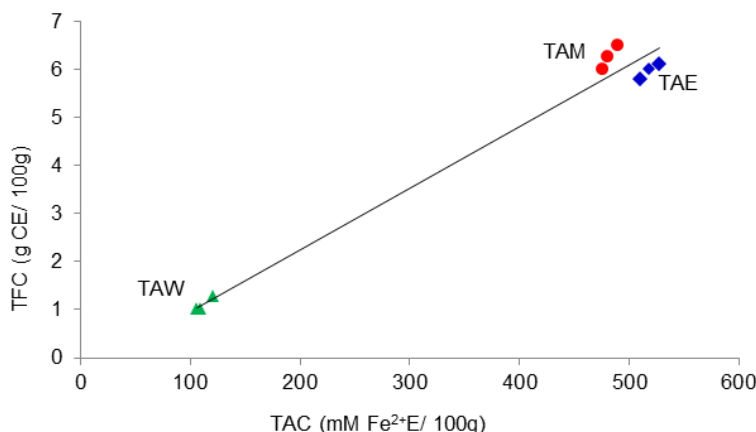


Figure 6: Correlation between total antioxidant capacity and total flavonoid content of *Terminalia arjuna* extracts; TAC: Total antioxidant capacity, TFC: Total flavonoid content, TAE: *T. arjuna* ethanol extract, TAM: *T. arjuna* methanol extract. TAW: *T. arjuna* water extract

blood glucose levels and protecting pancreatic beta cells against tissue damage (Ragavan & Krishnakumari, 2006). Amylase inhibitory effects of *T. arjuna* bark are also reported, demonstrating a mechanism responsible for lowering postprandial hyperglycemia (Saha & Verma, 2012).

Even though scientific validation data are available on antihyperglycemic effects of *T. arjuna*, only limited reports are available on their antiglycation effects. Ethanol and water extracts of *T. arjuna* bark were chosen for investigation as they are frequently used in traditional medicine, while methanol extract was included due to its ability to extract many secondary metabolites. In the current study, extracts of *T. arjuna* bark demonstrated strong inhibition of glycation induced protein cross-linking at very low concentrations when compared with that of the standard inhibitor aminoguanidine (positive control). This inhibition remained (at the upper range of the concentrations used), even after 3 weeks of incubation (as shown in figures 1C, 1D and 2B) in a medium that has promoted extensive glycation (500 mM fructose instead of approximately 10 mM glucose which is likely to be found in a diabetic patient).

With regard to limited evidence on antiglycation effects of *T. arjuna*, aqueous bark extracts (1 to 10 mg/mL) inhibited in vitro glycation of insulin with 42% to 69% inhibition when assessed using reversed phase high performance liquid chromatography (Thomson et al., 2014). In another study, *T. arjuna* bark aqueous extract (0.2% w/v) showed a significant reduction in BSA glycation when AGE fluorescence was measured. Same study showed inhibitory effects of *T. arjuna* on amyloid aggregation (Tupe et al., 2017). Another study showed antiglycation and antioxidant effects of arjunolic acid isolated from *T. arjuna* bark (Odjakova & Popova, 2012). Antiglycation effects observed in the present study were much greater than the limited data reported previously on antiglycation effects of *T. arjuna*.

In the current study, antiglycation potential of *T. arjuna* extracts were demonstrated using an electrophoresis-based method to demonstrate inhibition of glycation induced protein cross-linking. Findings demonstrated that all three extracts of *T. arjuna* bark used were effective at much lower concentrations of 0.05 mg/mL and 0.05% (w/v) than those reported for *T. arjuna* (≥ 1 mg/mL and 0.2% w/v) in inhibiting glycation almost completely. At lower concentrations, methanol extracts seem to be more effective in inhibiting protein cross-linking when compared with that of ethanol extracts. Direct comparison of the water extract with the other two was not appropriate as the extract was not concentrated by eliminating the solvent. It would have been more consistent if aqueous extract was freeze dried to match with the procedure used for the alcoholic extracts, as then the masses of the dry aqueous extract could have been determined. However, results indicated that the inhibitory effect may be even higher in water extracts as almost complete inhibition was detected at very low concentrations (0.05% w/v) studied. Therefore, the compounds responsible for the glycation induced protein cross-linking inhibitory effect seem to be relatively polar.

Extracts showed the best inhibitory effect on protein cross-linking when added on day 0 (at the start of the incubation period). The effect declined when the extract was added 1 day after commencement of incubation of protein with sugar. Effect was clearly declined when the *T. arjuna* extracts were added after 2 days. AG was comparatively more effective than the *T. arjuna* extracts when added after 1 day and 2 days. AG is known to trap reactive intermediates generated during intermediate stage reactions of glycation (Sadowska-bartos & Bartosz, 2015). Phytochemicals are known to inhibit various stages of glycation (Chinchansure et al., 2015). The mode of action of the *T. arjuna* phytochemicals responsible in inhibiting glycation induced protein cross-linking may target early-stage events. None of these extracts seem to act as cross-link breakers as there was no evidence of reduction of cross-links that are already formed by the time of addition of extract.

Even though *T. arjuna* bark is used in various formulations to improve skin health, studies reported on antityrosinase effects of *T. arjuna* are lacking. However, several clinical studies provided evidence for effectiveness of *T. arjuna* preparations in patients with melasma, indicating the potential of *T. arjuna* in inhibiting hyperpigmentation. Skin whitening effect of a herbal cream containing three plant extracts was found to be highest when the cream contained *T. arjuna* bark extract (Sahu et al., 2014). External application of *Arjuna twak lepa* prepared using *T. arjuna* bark was effective in cutaneous depigmentation of patients with *vyanga* (localized facial hyperpigmentation) (Sreelekshmi & Geethesh, 2018). Likewise, when melasma was treated with a drug made using *T. arjuna* and *Rubia cordifolia*, complete relief was observed in 88% of the patients (Konwar & Das, 2016). A molecular docking approach revealed antityrosinase activity of a formulation containing *T. arjuna* extract with identification of interactions made with the active site (Gaikwad & Jadhav, 2019). Findings of the present study provide evidence for strong antityrosinase effects of *T. arjuna* bark extracts and support the evidence derived from clinical studies.

Initially extracts were prepared at $< 50^{\circ}\text{C}$ to detect any existing antiglycation and antityrosinase effects as we might have lost such effects prior to initial screening if the responsible compounds were heat labile. The observed inhibitory effects on glycation induced protein cross-linking and tyrosinase activity remained to a greater degree after boiling the extracts indicating that the effective compounds are heat stable to a great extent. Further studies are necessary to use natural remedies prepared with heat treatment to check the heat stability of the assessed effects.

Oxidative stress is enhanced with the acceleration of glycation. Effectiveness of phenolic compounds in inhibiting glycation and melanogenesis is reported (Działo et al., 2016). It is reported that formation of AGEs is reduced in the presence of higher phenol and flavonoid content (Safari et al., 2018). A good correlation was found between the antioxidant activities and glycation inhibitory activities of plant extracts (Dearlove et al., 2008).

Findings of the present study matches with previous findings which revealed high antioxidant activity of *T.*

arjuna with concomitantly high phenolic and flavonoid content (Chatha et al., 2014). TAC, TPC and TFC values in water extract of current study were comparatively lower than those of the ethanol and methanol extracts. However, this may have been due to the extraction method used (as stated in section 2.1) which may have resulted in a diluted water extract in contrast to ethanol and methanol extracts.

CONCLUSION

Strong inhibitory effects of *T. arjuna* bark extracts were demonstrated on the glycation induced protein cross-linking. Antiglycation effects observed in the present study were much greater than the limited data reported previously on antiglycation effects of *T. arjuna*. Effective antiglycation compounds seem to be heat stable and polar in nature. Antityrosinase effects were also very high as indicated by low IC₅₀ values and the effective compounds seem to be heat stable to a great extent. Antityrosinase effects observed agree with the previously identified efficacy of *T. arjuna* bark preparations on pigmentation disorders. Furthermore, very high total antioxidant capacity, total phenolic and flavonoid contents were also revealed which agree with results of previous studies. Hence, the results of the current study provide evidence to suggest the potential multiple benefits of *T. arjuna* bark extracts in alleviating some mechanisms that are implicated in chronic diabetic complications and skin aging. Findings also provide in vitro evidence to validate the traditional use of *T. arjuna* bark in diabetes mellitus and in skin disorders. Further studies are necessary to assess the natural remedies and to identify the effective compounds.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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