

Full Length Research Paper

Determination of total phenolic and flavonoid content, antioxidant and cytotoxic activity of the methanol extract of *Acacia nilotica* pods

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***Acacia nilotica* is a medicinal plant used in Niger republic for the treatment of gastrointestinal diseases. The aim of this study was to investigate total phenolic and flavonoid content, antioxidant and cytotoxic activity of the *A. nilotica* pods collected from Niger republic. The phytochemical contents were examined by colorimetric methods. Antioxidant activity of the methanol extract was screened using DPPH, ABTS and H₂O₂ free radical scavenging inhibition assays. Cytotoxicity potential was assessed using the *in vitro* MTT assay. The phytochemical screening of the plant extract revealed the presence of higher amounts of total phenolic, flavonoid and condensed tannins in the extract. IC₅₀ values for the scavenging of DPPH, ABTS and H₂O₂ were found lower though manifesting potential antiradical capacities. The extract showed no cytotoxicity against five different cell line models. Taken together, the present results suggested the therapeutic potential of the methanol extract of *A. nilotica* pods. Further studies to isolate and characterize the bioactive compounds and to investigate their possible cytotoxic effects *in vivo* are in view.**

Key words: *Acacia nilotica*, methanol extract, antioxidant activity, cytotoxicity potential, polyphenol contents.

INTRODUCTION

Acacia nilotica subsp. *adstringens* (Schmach. & Thonn.) Roberty, commonly known as 'Bagaroua' in Hausa community of the dry area of West Africa. It is an economically and medicinally important tree. Its different parts which include pods, bark, gum, root, flowers and leaves are widely used in traditional medicine for the treatment of various ailments (Diallo et al., 2003; Raphael

et al., 2012; Koubé et al., 2016).

The traditional healers of Niger republic use the pods for the treatment of gastrointestinal diseases (Lawaly et al., 2017a). The pods are found to be very rich in phenolic acids, tannins and proteins (Sotohy et al., 1997). The antibacterial potential of different parts of the plant has been reported by numerous investigators (Muluh et

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al., 2008; Mohammed et al., 2014; Rwarinda, 2015; Yohana et al., 2015; Rupal and Rupesh, 2016). Antiplatelet activity of the plant has been reported (Bukhtiar et al., 1997). Leaves extracts were found to possess significant antioxidant activity (Kalaivani and Methew, 2010a; Nasir et al., 2013). Plants used as food or as recipe to treat a disease could have mutagenic, cytotoxic, and geno-toxic effects (Schoonen et al., 2005). Numerous phytochemical compounds are reported to have hemolytic or anti-hemolytic effect on animal erythrocytes (De Freitas et al., 2008). Inhibition of the viability of the tested human intestinal Caco-2 cells by the crude extract of the fruits of *A. nilotica* was reported by Dikti et al. (2017).

In this context, this study was carried out to identify phytochemical constituents and to evaluate the antioxidant and cytotoxic activities of *A. nilotica* pods.

MATERIALS AND METHODS

Plant sample

A properly packed and well conserved powder of *A. nilotica* fruits was purchased from the Laboratory of Natural Substances and Organic Synthesis (LASNASO) of the Department of Chemistry, University Abdou Moumouni of Niamey (UAM). Procedures for the plant material (Pods) collection and its further laboratory authentication were highlighted in our previous published research paper (Lawaly et al., 2017b).

Chemicals

Ascorbic acid, aluminium chloride, 2,2-azino-bis-(3-zthylbenzothiazoline-6-sulphonic acid) (ABTS), ferric chloride (FeCl_3), Tween 80, gallic acid, tannic acid, quercitin, potassium persulphate, Folin-Ciocalteu reagent, ferrozine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), disodium hydrogen phosphate (Na_2HPO_4), Triton X-100, sodium dihydrogen phosphate (NaH_2PO_4), potassium chloride (KCl), aluminium trichloride (AlCl_3) and hydrogen peroxide (H_2O_2) were purchased from Solarbio Life Science (Beijing Solarbio Science & Technology Co., Ltd). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)2, 5-diphenyltetrazolium bromide assay (MTT), penicillin-streptomycin and trypsin were purchased from Sigma-Aldrich (USA). Fetal Bovine Serum (FBS) and medium for cell culture were purchased from Gibco (Scotland). All solvents used were of analytical grade purchased from Qindao Sigma Chemical Co., Ltd (China, mainland).

Plant extraction

The plant material extraction was carried out based on the method described by Gilani et al. (1999) with some modifications. 500 g of the powder of *A. nilotica* pods was weighed out into a conical flask and soaked in 1.5 L of methanol for 7 days with occasional stirring and shaking. Filtrate obtained first through a fresh cotton plug and finally with Whatman filter paper No. 1 was concentrated under reduced pressure at the temperature of 40°C using rotary evaporator (Buchi Rotavapor R-200, Germany). The solvent was

completely removed by using rotary evaporator and dried crude extract was obtained.

Determination of phenolic substances

The quantitative analysis of total polyphenols, of flavonoids and of tannins in crude methanol extracts were carried out based on the spectrophotometric methods described by Magalhães et al. (2010) and Brighente et al. (2007).

Determination of total phenolic content (TPC)

The total polyphenols content of crude methanol extract was determined with the use of Folin-Ciocalteu reagent. A 100 μL of the extract/standard conveniently diluted is placed in a tube containing 500 μL of Folin-Ciocalteu reagent (1:10). After vigorous agitation and stand of the mix for 5 min at room temperature, 400 μL of Na_2CO_3 at 75 g/L were added. Following incubation period for 5 min at 40°C, 250 μL from the mix were then introduced in triplicate into a well of a 96 well microplate. After 90 min of incubation, the absorbance is read at 735 nm. The results were expressed as equivalent gallic acid.

Determination of total flavonoid content (TFC)

The total flavonoids content of crude methanol extract was determined according to the AlCl_3 method with few modifications. A 500 μL of the extract/standard conveniently diluted is placed in a tube containing 2 mL of distilled water and 150 μL of NaNO_2 , 50%. After 6 min of incubation at room temperature, 150 μL of freshly prepared AlCl_3 (at 10% concentration) are introduced in the mix. All is brought to 2 mL with NaOH (4%) after 6 min then completed to 5 mL with distilled water. After 15 min of incubation, the absorbance is read at 510 nm. The results are expressed as equivalent Quercitin.

Determination of condensed tannin content (CTC)

A 100 μL of the crude methanol extract/standard conveniently diluted is placed in a tube containing 7.5 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent. After progressive mixing, 1 mL of NaCO_3 (35%) is added and the volume is brought to 10 mL with distilled water. After 30 min incubation at room temperature, the absorbance is read at 700 nm. The results are expressed as equivalent tannic acid.

Antioxidant assays

The investigation of the antioxidant potentials of the crude methanol extract of *A. nilotica* pods was realized by three biochemical methods shown in the following.

DPPH free radical scavenging activity

An aliquot of methanol solutions (10 μL) of extract and positive control (ascorbic acid) at various concentrations (0.1 to 0.6 mg/mL) were added to 195 μL of DPPH in methanol (40 $\mu\text{g}/\text{mL}$) in a 96-well microplate. The absorbance of the mixture was measured at 540 nm using a spectrophotometer (Hitachi U-1800, Japan) after

incubated at room temperature for 30 min away from light. Methanol was used as blank. The percentage of DPPH inhibition was calculated using the following equation:

$$\% \text{DPPH inhibition} = (1 - A_s/A_b) \times 100$$

where A_s and A_b correspond to the absorbance at 540 nm of DPPH in the absence and presence of antioxidant, respectively.

The extract concentration providing 50% inhibition (IC_{50}) was determined from the graph of percentage of inhibition against extract concentration using Graphpad prism. The antioxidant reducing power (ARP) is calculated as the inverse of IC_{50} value (Brand-Williams et al., 1995).

ABTS free radical scavenging activity

ABTS (7 mM) was dissolved in water and was allowed to react with potassium persulfate (2.54 mM) for 16 h to give a dark blue solution. The solution was further diluted with ethanol until the absorbance reached 0.7 at 734 nm. In a 96 well microplate, 190 μL of the resulting solution from the diluted ABTS and 10 μL of diluted crude methanol extract of *A. nilotica* or standard (0.1-0.6 mg/mL) were loaded. The absorbance of the mixture was measured at 734 nm using a spectrophotometer (Hitachi U-1800, Japan) after incubated at room temperature for 6 min away from light. The percentage of ABTS inhibition was calculated using the following equation:

$$\text{ABTS inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

where A_c is absorbance of control and A_s is absorbance of the sample. Ascorbic acid was used as a standard control (Re et al., 1999).

H_2O_2 free radical scavenging activity

A solution of hydrogen peroxide (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4). In a 96 well plate, 18.2 μL of the crude methanol extract of *A. nilotica* and/or standard, 72.7 μL of 50 mM phosphate buffer and then 109 μL of H_2O_2 (2 mM) were respectively introduced. After mixing, the absorbance is read at 230 nm after incubation timing scale. 50 mM phosphate buffer without hydrogen peroxide was used as blank (Ruch et al., 1989). Hydrogen peroxide scavenging ability (in triplicate) was calculated by the formula:

$$\% \text{ scavenging} = (1 - A_s/A_0) \times 100$$

where A_0 is the absorbance without sample and A_s is absorbance with sample.

Cytotoxicity towards mammalian cells

The cytotoxicity of the methanol extract of *A. nilotica* pods towards human liver cancer cell line (HepG2), human glioblastoma cell line (U87), human breast adenocarcinoma (MCF7), normal fibroblast cell line (L929) and Chinese hamster ovary (CHO) cell line was determined by routine MTT assay as described in our previous studies (Liu et al., 2015). The cell lines were kindly provided by a collaborative key laboratory for drug screening of the China Pharmaceutical University (Research Main Building) and were

maintained in log phase growth in T-75 culture flasks (Corning, NY, USA) by subculturing at either 24- or 48-h intervals until the beginning of the cytotoxicity assay. Well growing cells were exposed to serially diluted plant extract (1.563-200 $\mu\text{g/mL}$) for 48 h at 37°C. The amount of reduced MTT was measured as absorbance at 570 nm using micro-titre plate reader. Cell cytotoxicity was calculated as a percentage of corresponding control value (non-treated cells) obtained in a minimum of three independent experiments. The half-maximal inhibitory concentration values (IC_{50}), defined as the concentration that inhibits 50% of cell growth, were calculated from concentration-response curves. Cytotoxicity was measured using following formula:

$$\text{Cell survival (\%)} = \frac{A_t - A_b}{A_c - A_b} \times 100$$

where A_t = absorbance value of test compound, A_b = absorbance value of blank, A_c = absorbance value of control,

$$\text{Cell death (\%)} = 100 - \% \text{ cell survival}$$

IC_{50} values were calculated using Graphpad prism 5.

RESULTS

Phenolic composition of the crude methanol extract

A. nilotica crude methanol extract yield was 18% of the dry powder. The extract was found significantly rich in polyphenols (Figure 1). The total phenolic content of the extract was found highest (257.61 \pm 4.22 mg GAE/g) compared to that of total flavonoids and that of total tannins, respectively.

Antioxidant activity assessment

The results output concerning the antioxidant potentials of the crude methanol extract of *A. nilotica* pods with the application of more than one technique are shown in the following.

DPPH radical scavenging potential

Absorbance obtained after spectrophotometry were used to determine the percentage scavenging inhibition. The values that were obtained have permitted to draw the curves which represent the percentage variation of inhibition viz sample concentrations. It was found that the percentage of DPPH radical scavenging activity of all the extracts increases with the increase in the concentration of the tested extract or standard antioxidants as illustrated in Figure 2. The antiradical activity was further analyzed by determining the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50% (IC_{50}). The antiradical power (ARP) which defines antioxidant action of an antioxidant is also determined for

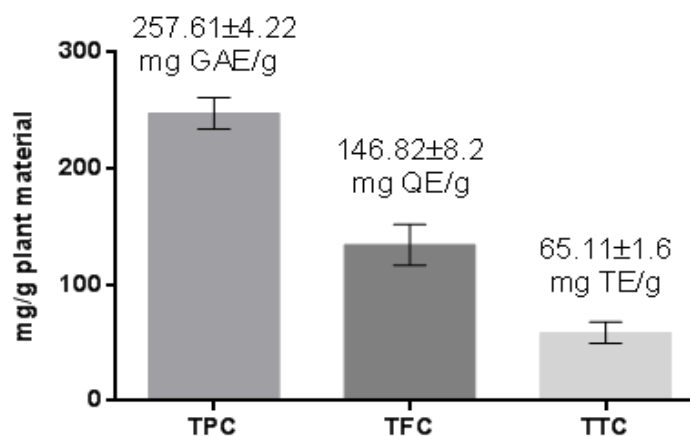


Figure 1. Phenolic composition of the crude methanol extract. TPC: Total phenolic content; TFC: total flavonoids content; TTC: total tannins content; GAE: gallic acid equivalent; QE: quercetin equivalent; TE: tannic acid equivalent.

Source Author's 2022

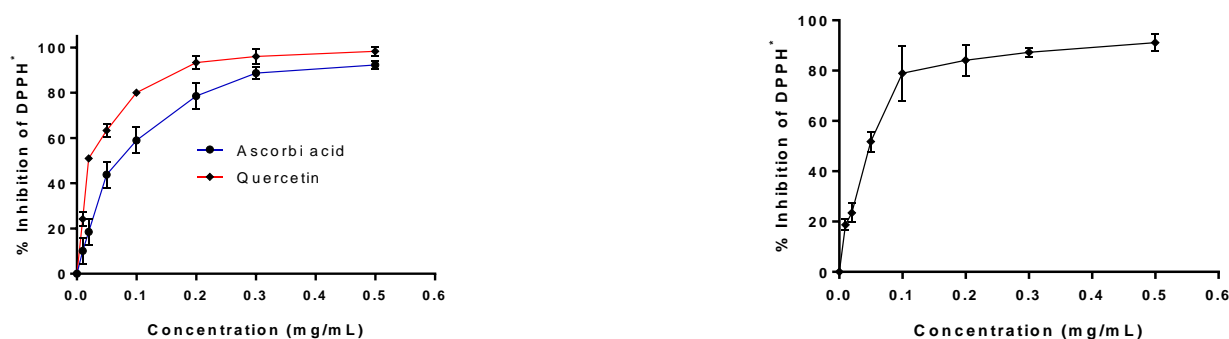


Figure 2. Percentage inhibition of DPPH. Each value represents a mean \pm SD (n = 3).

Source Author's 2022

each IC_{50} values. These values are presented in Table 1. IC_{50} values for ascorbic acid and quercetin were calculated as 0.201 and 0.211 whereas the corresponding ARP values were 4.975 and 4.739, respectively. The lower the IC_{50} value is the higher its antioxidant potential. In this case, the IC_{50} concentration of the crude methanol extract (0.218 mg/mL) presented the highest DPPH free radical scavenging when compared with IC_{50} of the reference compounds (ascorbic acid).

ABTS radical scavenging capacity of the extract

A concentration-response relationship was found in the ABTS radical scavenging assay (Figure 3); the activity increased as the concentration increased for extract. The plant extract at the highest concentration (0.5 mg/mL), showed $64.12 \pm 0.34\%$ while ascorbic acid used as

standard antioxidant showed $83.56 \pm 1.17\%$ scavenging activity. From the estimated IC_{50} values, the order potency is ascorbic acid (0.21 mg/mL) > crude methanol extract (0.23 mg/mL).

H_2O_2 radical scavenging capacity of the extract

The curves which represent the percentage variation of inhibition viz sample concentrations were presented in Figure 4. From these curves, IC_{50} was deduced. The crude methanol extract in line with the standard compound had H_2O_2 scavenging activity of 5.12 ± 1.61 and $59.61 \pm 0.55\%$, respectively.

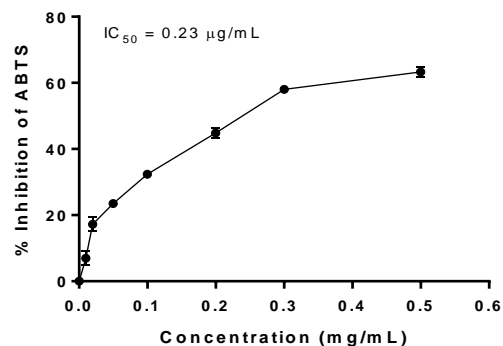
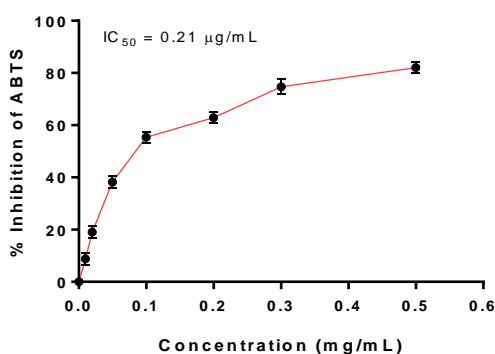
Assessment of cytotoxicity activity by MTT assay

Based on the antioxidant ability of the methanol extract of

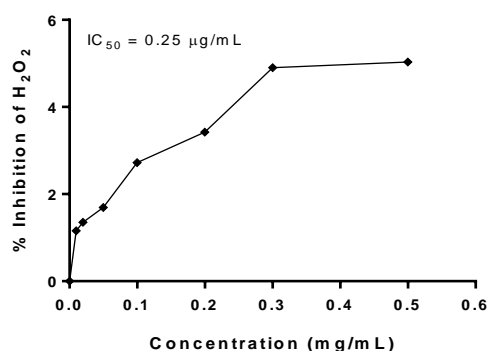
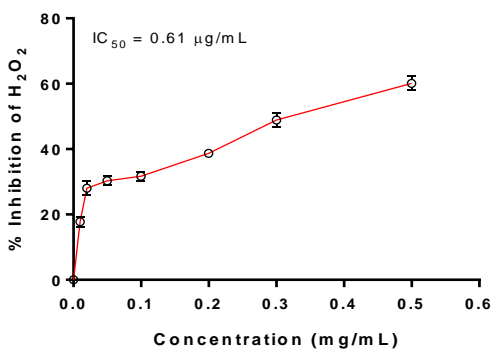
Table 1. Antiradical efficiencies of the tested standards and crude methanol extract.

Extract	IC ₅₀ (mg/mL)	ARP (1/EC ₅₀)	R ²
Ascorbic acid	0.201	4.975	0.9991
Quercetin	0.211	4.739	0.9912
MeOH	0.218	4.587	0.9807

Source Author's 2022

**Figure 3.** ABTS radical scavenging potential as percentage inhibition and as IC₅₀ (mg/mL). Each value represents a mean \pm SD (n = 3)

Source Author's 2022

**Figure 4.** H₂O₂ radical scavenging potential as percentage inhibition. Each value represents a mean \pm SD (n = 3).

Source Author's 2022

A. nilotica pods, cytotoxic testing against selected mammalian cell lines was performed. The extract was found to inhibit the proliferation of the tested cell lines in a dose dependent manner with IC₅₀ values relatively very high (IC₅₀ > 100 µg/mL) in all models (Table 2).

DISCUSSION

For many years, medicinal plant remedies have been considered to treat or alleviate various diseases which

could be infectious or chronic ones (Gurib-Fakim, 2006). *A. nilotica* is one of the plants that has been extensively used in traditional pharmacopeia of Niger republic and its neighboring countries (El-Tahir et al., 1999). Various parts of this plant including pod, bark, root, leaves and flours have been used for the treatment of different ailments.

The aim of the present study was to investigate the phenolic content and then to evaluate the antioxidant properties and the cytotoxic activities of the *A. nilotica* pods collected from Niger republic. The plant was

Table 2. The IC₅₀ (µg/mL) of the cytotoxicity assay of ANP extracts.

Extract	HepG2	U87	MCF7	L929	CHO
MeOH	299.11±0.34	105.42±0.22	141.41±0.41	658.41±0.22	940.21±0.17

*Each value represents a mean ± SD (n = 3).

Source Author's 2022

selected among others because of its vigorous use by most West African traditional healers, particularly in Niger republic to fight most gastrointestinal diseases. Also, this research assignment is part of an ongoing strategy of valorization of this valuable plant through the development of improved traditional medicines which would be very useful for the community to treat several pathologies. Three fundamental aspects were aboded in this study: the aspect relating to the estimation of phenolic content, the one related to the antioxidant property and the other one related to the cytotoxicity activity.

From the first aspect regarding the determination of the quantity of polyphenols, results obtained in this study indicate that *A. nilotica* pods are very rich in phenolic substances. Many researchers across the world have investigated the phenolic composition of different parts of *A. nilotica* (Kalaivani and Mathew, 2010b; Enayat et al., 2012; Sulaiman and Gopalakrishnan, 2013; Sadiq et al., 2017; Foyzun et al., 2022) and most have highlighted its richness in phytochemical compounds with probable therapeutic potentials. Sulaiman and Gopalakrishnan (2013) reported that the phenolic compounds were best extracted via methanol from *A. nilotica* pods. Enayat et al. (2012) reported the presence of tannins and polyphenols in *A. nilotica* pods extract. In a recent study, Foyzun et al. (2022) have reported 256.752±10.086 mg GAE/g of total phenolic content in crude methanol extract of *A. nilotica* pods which appeared very close to those obtained in our study (257.61±4.22 mg GAE/g). However, the content of flavonoids was found with much higher content when compared to the earlier cited research works. Polyphenols are known for their biological activities (Dai and Mumper, 2010). High content of polyphenolics suggests that *A. nilotica* pods might have potential antioxidant activity (Fuchs, 1998).

Regarding the antioxidant activities, results obtained from three different *in vitro* methods (DPPH, ABTS and H₂O₂) best used to evaluate the antioxidant potential both indicated the potential radical scavenging activity of *A. nilotica* pods. With the DPPH model scavenging assay, the methanol extract of the plant was found to strongly scavenge the DPPH free radicals in a dose dependent manner. At 0.5 mg/mL concentration, methanol extract and the standards antioxidants (ascorbic acid and quercetin) all showed 90% DPPH scavenging. Similar

result was reported by Alsiddig et al. (2015), where the ethanol extract of *A. nilotica* pods was able to scavenge the DPPH with 93%. Also, from the results published by Foyzun et al. (2022), it was highlighted that at 12.5 µg/mL concentration, methanol extract of *A. nilotica* pods was able to strongly scavenge the DPPH. With the ABTS and H₂O₂ radical scavenging methods, the methanol extract of *A. nilotica* pods was found to significantly scavenge free radical in a dose dependent manner. These results reveal that the *A. nilotica* pods have pertinent antioxidant activity which might be effective in the protection against oxidative stress.

Regarding the cytotoxicity activity, different concentrations of the extract were tested against several mammalian cell lines by MTT assay. The results showed that *A. nilotica* crude methanol extract has very poor cytotoxicity effects against the tested cell models (IC₅₀ > 100 µg/mL). The Natural Cancer Institute (NCI) of the United States of America indicated that the cytotoxicity of a plant extract is considered effective when the IC₅₀ is below 20 µg/ml (Boyed and Teicher 1997). Though, the extract showed no cytotoxicity against the exposed cell lines as shown by higher IC₅₀ values. However, *in vivo* acute toxicity studies may be necessary to establish the safety level of the extract. Few studies conducted elsewhere have also studied the cytotoxicity effects of *A. nilotica* extracts against different mammalian cell lines (Kalaivani et al., 2011; Rasool et al., 2013; Manzo et al. 2019). Van den Bout-van den Beukelet al. (2008) reported highest cytotoxicity effects of the stem extracts of *A. nilotica* against Hep-G2 and HeLa human cell lines used as model. Vildina et al. (2017) reported the inhibition of human intestinal Caco-2 cells viability by the crude extract of *A. nilotica* stem.

Conclusion

In this study, the amount of phenolic composition, related antioxidant and cytotoxicity activities of the crude methanol extract of *A. nilotica* pods were evaluated. Results showed that the pods are rich in phenolics, flavonoids and tannins. This explains the use of these plants by the ancient people to treat several diseases. The extract was found to have various forms of antioxidant activities that could possibly be attributed to

the total phenolic contents. This implies that biochemical active compounds in the plant pods might be potential sources of natural antioxidants. The extract showed no cytotoxicity against the exposed cell lines models. This may imply partial safety of the extract. However, supplementary studies such as the *in vivo* acute toxicity studies may be necessary to establish the conclusive safety level of the extract of *A. nilotica* pods.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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