

# Anthelmintic Activity of Punicalagin from *Anogeissus Leiocarpus*

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**Abstract** Leaves of *Anogeissus leiocarpus* Guill. & Perr. have been traditionally used as an African botanical treatment for parasitic worm infections in both humans and livestock. This current study documents the presence of punicalagin isomers ( $\alpha$  &  $\beta$ ) and its galloyl ester, 1-*O*-galloyl-punicalagin as major components in the leaf extract of *A. leiocarpus*. Identification was performed using HPLC and LC-ion trap-top of flight-mass spectrometry (LC-IT-TOF-MS). The activity of punicalagin was assessed with *Caenorhabditis elegans* using 24 and 48 hour motility assays. Punicalagin exhibited activity against *C. elegans* with a 24 hour LD<sub>50</sub> of 1.69 mM and a 48 hour LD<sub>50</sub> of 0.98 mM. The concentration of punicalagin in the leaf extract accounted for 47.5  $\mu\text{g mg}^{-1}$ , while the concentration of 1-*O*-galloyl-punicalagin was 24.5  $\mu\text{g mg}^{-1}$ , quantified as punicalagin equivalent. This level of punicalagin alone was not sufficient to account for the activity seen in the leaf aqueous extract of *A. leiocarpus*, indicating that other compounds present in the extract such as 1-*O*-galloyl-punicalagin and gallic acid may have also contributed to the activity. However, the level of punicalagin in the leaf aqueous extract of *A. leiocarpus* was demonstrated at concentrations comparable to those found in *A. leiocarpus* treatment regimens for ruminant parasites.

**Keywords** *Anogeissus Leiocarpus*, Anthelmintic Activity, Punicalagin, Traditional Medicine, *Caenorhabditis elegans*

## 1. Introduction

The leaves and other parts of the West African plant *Anogeissus leiocarpus* Guill. & Perr. (Combretaceae), or axelwood tree, have historically been used as a traditional treatment for worm and other infections of the digestive tract,

especially in livestock [1,2]. The high cost of modern drugs is often cited as a major factor in many countries for continued use of plants such as *A. leiocarpus* to reduce parasitic infections in livestock [1]. Subsequently, extracts and isolated compounds from this plant have been the subject of numerous studies investigating its efficacy against helminth infections [1,3-6]. Aqueous extracts from the leaves in particular have shown strong activity against nematodes both in vitro and in vivo [6-8]. In addition to crude extracts, some of the chemical constituents from *A. leiocarpus* have been evaluated for anthelmintic activity. These include gallic and gentisic acids [4] against *C. elegans* and gallic, gentisic and ellagic acids against *Onchocerca ochengi* and *C. elegans* [5]. In the present study, we investigated the chemical composition of a crude aqueous leaf extract of *A. leiocarpus* using high performance liquid chromatography (HPLC) and LC-ion trap-top of flight mass spectrometry (HPLC-IT-TOF-MS) and evaluated the contribution of the major component punicalagin to the anthelmintic activity of plant aqueous extract in the model organism *Caenorhabditis elegans*. Punicalagins are ellagitannins most well known for their presence in pomegranates (*Punica granatum*), but have also been found in members of the Combretaceae family including the *Terminalia* and *Combretum* genera [9,10]. 1-*O*-galloyl-punicalagin has previously been reported in *Combretum glutinosum* [11] and *Terminalia calamansanai* [12].

## 2. Materials and Methods

### 2.1. Plant Materials and Chemicals

The aqueous leaf extract of *A. leiocarpus* Guill. & Perr. was provided by the National Cancer Institute (National Cancer Institute, 1003 W 7<sup>th</sup> Street, Suite 206, Frederick, MD, 21701) open repository and plant voucher specimens

were deposited at the National Herbarium in Washington, DC as previously described [7]. The standard compounds gallic acid, ellagic acid and quercetin were purchased from Sigma-Aldrich (St. Louis, MO). Punicalagins were supplied from Chromadex (Irvine, CA). HPLC grade water and methanol were obtained from Sigma-Aldrich.

## 2.2. HPLC Analysis of *A. Leiocarpus* Aqueous Extract

Standard stock solutions of gallic acid, ellagic acid, quercetin and punicalagin were prepared in methanol or water at concentrations of 0.5 to 1 mg ml<sup>-1</sup>. The crude aqueous extract was prepared in methanol at a concentration of 4 mg ml<sup>-1</sup>. HPLC analysis was performed on an Agilent 1100 Series HPLC system. A Zorbax 300 SB-C8 4.6 mm x 15 cm column was used for separation. The UV absorption was monitored at 280 and 365 nm. The injection volumes were 50 µl for both the plant extract and the standard compounds. The mobile phase consisted of (A) water containing 0.1% acetic acid and (B) 100% methanol. The gradient was linear from 1 to 10% B, 0-55 min, linear from 10 to 15% B, 55-75 min, linear from 15 to 20%, 75-95 min, linear from 20 to 50%, 95-115 min, linear from 50 to 90%, 115-160 min, and linear from 90 to 1%, 160-165 min. The flow rate was set at 0.2 ml/min.

Quantification of punicalagins and 1-*O*-galloyl-punicalagin was performed from the peak areas recorded at 280 nm to the calibration curve obtained with punicalagin reference standard (50, 100, 300 and 500 µg ml<sup>-1</sup>).

## 2.3. LC-IT-TOF-MS Analysis of *A. Leiocarpus* Aqueous Extract

LC-MS analysis was performed using a Shimadzu LC-MS-IT-TOF instrument (Shimadzu, Tokyo, Japan) equipped with an ESI interface, and attached to a Prominence HPLC system (SIL-20A HT autosampler, LC-20AD pump system, SDP-M20A photo diode array detector). The LC separation was performed using a C<sub>18</sub> reverse-phase column (Shim-pack XR-ODS column, 50 mm x 3.0 mm id x 2.2 µm, Shimadzu Scientific Inst., Columbia, MD, USA) with a binary solvent system comprising of (A) 0.1% formic acid in H<sub>2</sub>O and (B) methanol. Compounds were eluted into the ion source at the flow rate of 0.35 ml/min with a step gradient of 5-95% B over 30 min, isocratic at 95% B over 2 min, and return to 5% B over 1 min. Prior to the next injection, the column was re-equilibrated for 5 min at initial conditions (5% B). The heat block and curved desolvation line (CDL) were maintained at 200°C. Nitrogen gas was used as nebulizer and drying gas with the flow rate set at 1.5 L/min. The ESI source voltage was set at 4.5 kV and the detector was set at 1.5 V. The instrument was calibrated to < 5 ppm error in mass accuracy with an external standard of sodium TFA solution. Ionization was performed in negative ionization mode. Data was acquired at *m/z* 150-1500 range. Shimadzu's LCMS Solution software (Shimadzu Scientific Inst., Columbia, MD, USA) was used for system control and data analysis.

## 2.4. Anthelmintic Assay

Age-synchronized adult populations of a levamisole-resistant *C. elegans* strain were grown on Nematode Growth Medium (NGM) seeded with *Escherichia coli* strain OP50, at 25°C as previously described [4]. The plates were incubated at 25°C for 2 days, after which worms were harvested for testing. Worms were suspended in purified water to obtain a final density of 50 to 100 worms per replicate.

Standard punicalagin was dissolved in purified water at a concentration equal to twice the final concentration being tested. To this was added an equal volume of nematodes suspended in purified water, bringing the concentration of drug to the final concentration being tested (0.12, 0.23, 0.46, 0.92, and 1.84 mM). The plant extract was suspended in purified water at a final concentration of 2 mg ml<sup>-1</sup> for comparison. All tests were conducted in sterile 15 ml centrifuge tubes. Negative control replicates consisted of worms suspended in purified water. All tubes were incubated at 25°C for 24 and 48 hrs. Viability was evaluated by comparing the number of motile worms to the total number of worms (motile and non-motile). Non-motile worms were considered dead if they did not resume motion immediately after prodding. Each assay was performed in triplicate.

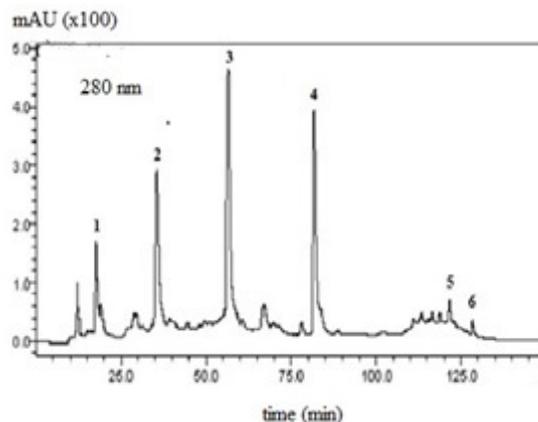
## 2.5. Statistics

The 50% lethal dose (LD<sub>50</sub>) at 24 and 48 hours for punicalagin was calculated fitting a three-parameter logistic function using R language for statistical computing [13] and R packages drc [14]. The results are reported as estimated LD<sub>50</sub> (95% confidence interval).

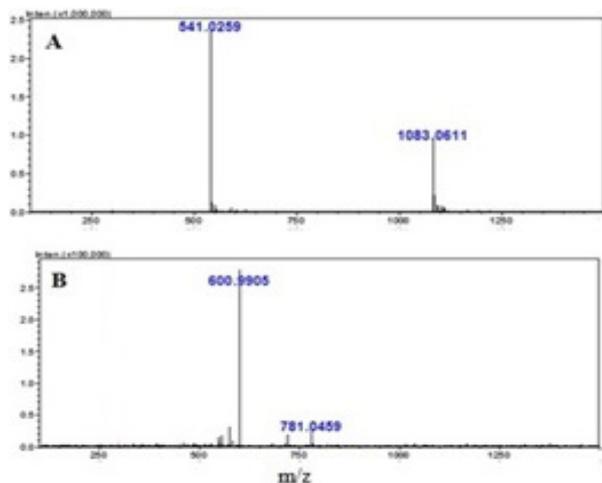
## 3. Results and Discussion

### 3.1. HPLC and LC-MS Analyses of the Crude Extract

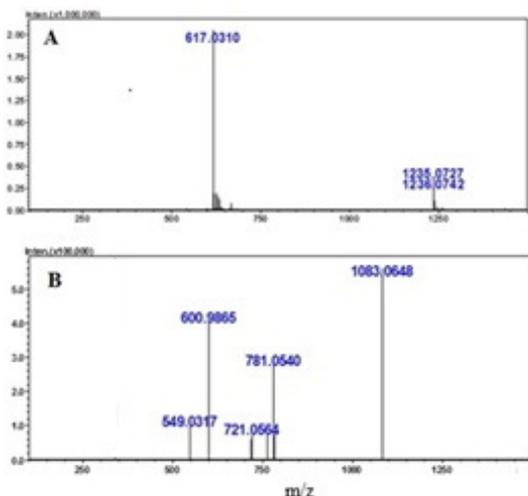
HPLC and LC-MS analyses of the crude aqueous leaf extract of *A. leiocarpus* revealed matching retention times and mass detections in comparison to five standard compounds (Figure 1).



**Figure 1.** HPLC of the crude aqueous extract of *A. leiocarpus*. Detection at 280 nm. Six peaks were identified by comparison of retention time and mass spectra against standards or the literature: gallic acid (1); punicalagin isomers (2, 3), 1-*O*-galloyl-punicalagin (4); ellagic acid (5); quercetin (6).



**Figure 2.** (A) Mass spectra showing the  $m/z$  1083  $[M-H]^-$  and  $m/z$  541  $[M-H]^{2-}$  for punicalagin; (B) MS/MS of punicalagin showing main fragment ions at  $m/z$  781 and  $m/z$  601.



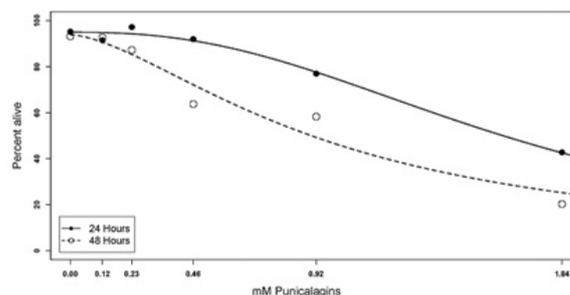
**Figure 3.** (A) Mass spectra showing the  $m/z$  1235  $[M-H]^-$  and  $m/z$  617  $[M-H]^{2-}$  for 1-*O*-galloyl-punicalagin; (B) MS/MS showing the main fragment ions at  $m/z$  1083, 781, and 601.

Peaks that gave clear  $[M-H]^-$  mass data and matching retention time with standard compounds were identified as gallic acid (**1**,  $m/z$  169), punicalagin  $\alpha$  &  $\beta$  (**2**, **3**,  $m/z$  1083), ellagic acid (**5**,  $m/z$  301), and quercetin (**6**,  $m/z$  301). Peak 4 gave a clear  $m/z$  1235  $[M-H]^-$  and was identified as 1-*O*-galloyl-punicalagin by MS/MS ion peaks and comparison to the literature [11,15]. In addition, LC-MS results for both punicalagin isomer peaks gave two main mass peaks at  $m/z$  1083  $[M-H]^-$  and 541  $m/z$   $[M-H]^{2-}$  (Figure 2), consistent with published results for punicalagin found in pomegranate fruit [16]. ESI-MS in the negative ion mode as well as MS/MS product ions further confirmed the presence of punicalagin and 1-*O*-galloyl-punicalagin. A mass of  $m/z$  1083.0611 (calculated  $m/z$  1083.0587) was determined for punicalagin. A mass of  $m/z$  1235.0727 (calculated  $m/z$  1235.0697) was obtained for 1-*O*-galloyl-punicalagin (Figure 3). In addition, the fragment

ions for punicalagin ( $m/z$  781, 601) and 1-*O*-galloyl-punicalagin ( $m/z$  1083, 781, 601) are consistent with the literature [15]. The presence of gallic and ellagic acids as well as quercetin in extracts from *A. leiocarpus* confirmed what had previously been reported [2,17]. Although castalagin [18] and other phenols, such as gentisic acid, have also been reported to be present in *A. leiocarpus*, they were not detected in our aqueous extract, most likely due to differences in extraction procedures. The concentration of punicalagin was determined from concentration curves using HPLC to be  $47.5 \mu\text{g mg}^{-1}$  of dried leaf extract. Using the same method, 1-*O*-galloyl-punicalagin was determined to be present at  $24.5 \mu\text{g mg}^{-1}$ , punicalagin equivalent, in the leaf extract.

### 3.2. Anthelmintic Activity of Punicalagins

The crude aqueous leaf extract of *A. leiocarpus* at a concentration of  $2 \text{ mg ml}^{-1}$  had an 81% average survival rate following 24 hours of incubation and a 65% average survival rate following 48 hours of incubation against *C. elegans*. Average survival rate of *C. elegans* in purified water after 24 and 48 hours was greater than 95%. In comparison, the estimated  $\text{LD}_{50}$  at 24 and 48 hours for punicalagin was found to be 1.69 mM (1.51-1.86, 95% confidence interval) and 0.98 mM (0.76-1.20, 95% confidence interval), respectively (Figure 4).



**Figure 4.** Activity of punicalagin against *Caenorhabditis elegans*. Percent alive and corresponding three-parameter logistic function after 24 hours (solid line) and 48 hours (dotted line) of incubation at  $25^\circ\text{C}$ .

The concentration of the punicalagin isomers was found to be  $47.5 \mu\text{g mg}^{-1}$  of dried leaf extract, which equates to  $95 \mu\text{g ml}^{-1}$ . At this concentration the punicalagin is likely not the only active constituent contributing to the observed activity against *C. elegans*. It may be that 1-*O*-galloyl-punicalagin or several other compounds in the mixture contribute to the activity seen in the leaf extract but not demonstrated by individual compounds. These ideas need further investigation.

Still, studies in livestock have shown that aqueous leaf extracts of *A. leiocarpus* can be consumed safely in high enough concentrations effective for anthelmintic activity. For example, Kabore et al. [19] indicated that lyophilized leaf extracts of *A. leiocarpus* traditionally are used for ruminant gastrointestinal parasites at a rate of  $160 \text{ mg kg}^{-1}$

body weight, which is well below the LD<sub>50</sub> dose of 2403.6 mg kg<sup>-1</sup> body weight. Our study has shown that punicalagin has anthelmintic activity with a 24 hour LD<sub>50</sub> at 1.69 mM or 1.6 mg ml<sup>-1</sup> against *C. elegans*. The concentration of punicalagin was 47.5 µg mg<sup>-1</sup> in our dried leaf extract. This suggests that a dose of about 7.6 mg kg<sup>-1</sup> body weight of punicalagin could be provided by traditional practices, although this comparison needs verification since the chemistry of various tannins can change seasonally and during the extraction process [20]. Still, this provides support for the traditional use of *A. leiocarpus* leaf extracts in the treatment of helminth infections and that punicalagins can contribute to the activity. This has application for human helminth infections since punicalagins are well tolerated in humans as well [21].

#### 4. Conclusions

We report for the first time the presence of punicalagin as well as 1- *O*-galloyl-punicalagin (syn. combreglutinin or teroblongin) as major constituents in the aqueous leaf extract of *A. leiocarpus*. The identification of punicalagins in an *A. leiocarpus* extract and their observed anthelmintic properties has provided an improved understanding of the biologically active phytochemicals present in this plant traditionally used to treat helminth infections. The anthelmintic activity of punicalagin was demonstrated against the free living nematode *C. elegans* at concentrations comparable to those found in *A. leiocarpus* treatment regimens for ruminant parasites. However, additional studies are needed to investigate the efficacy of these compounds against ruminant gastrointestinal parasites *in vivo*.

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