High Performance Liquid Chromatographic Separation And Identification Of A Toxic Fraction From Aloe Barbadensis Miller Leaf Gel Using The Artemia Nauplii Bioassay

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Citation

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Abstract

The current study was undertaken to detect toxicity in purified Aloe vera gel fractions using the Artemia nauplii lethality bioassay, thereby allowing for the identification of compounds of interest for further investigation. The work presented here therefore seeks to not only detect toxicity in gel extracts, but also to assign this toxicity to individual fractions. Methanol extraction and RP-HPLC were used to purify fractions from Aloe vera gel leading to the isolation of 13 major components. Of these 13 fractions tested using the Artemia nauplii lethality bioassay, one proved to be toxic with a 24 h LC50 of 435 µg mL-1. Compared to the tested reference toxins, this Aloe vera gel fraction was approximately three times more toxic than the organophosphate insecticide Mevinphos (24 h LC50 1336 µg mL-1) and approximately six fold less toxic than potassium dichromate (LC50 73 µg mL-1). Of particular interest was the rapid onset of toxicity against the Artemia nauplii. Dilutions of the gel extract were capable of causing 100% mortality within 90 min. The isolated fraction induced 100% mortality within 120 min at a concentration of approximately 800 µg mL-1. In contrast, greater than 36 h was required for Mevinphos and 18 h for potassium dichromate to produce 100% mortality, even at high concentrations (2000 µg mL-1 or 800 µg mL-1 respectively). These results confirm the presence of toxic compounds in Aloe vera gel. As this bioassay correlates well with pesticidal activity and cytotoxic activity in some human tumours, this bioactive fraction may hold promise as a natural pesticide and/or antitumoral agent.

INTRODUCTION

The mucilaginous gel from Aloe barbadensis Miller (Aloe vera) has a long history of use as a therapeutic in various capacities. Its therapeutic properties include anti-inflammatory activity [1,2], immunostimulatory activity [3], antibacterial activity [4], antiviral activity [5,6], accelerated wound healing [7], and a reduction of radiation induced skin reactions [8].

In addition to the multitude of positive effects attributed to Aloe vera gel, there have also been reports of negative actions of gel components [3,9,10,11,12]. The gel is known to contain a large number of anthraquinones including aloe-emodin and aloin [13,14]. Some reports have shown these compounds to be mutagenic in Salmonella typhimurium [15] and to have genotoxic and tumour-promoting effects [10]. In contrast, other studies have reported antitumour activity of the anthraquinones [16]. Various low molecular weight components of Aloe vera gel including the anthrones and anthraquinones have also been reported to be cytotoxic to immortalised cells [12] and both normal human skin cells and tumour cells in vitro [17,18]. These cytotoxic agents may show promise as antitumour agents.

However, much of the work in this area has used whole gel or crude extracts, providing conflicting and difficult to interpret results. For example, Aloe vera gel is known to contain compounds that stimulate cell proliferation [19] as well as containing cytotoxic compounds [9,11,12]. Crude extracts would be expected to contain both these classes of compounds making interpretation of the bioactivities of these extracts difficult to understand and account for conflicting reports.

MATERIALS AND METHODS

PLANT MATERIAL

Fresh clean whole Aloe barbadensis Miller leaves were supplied by Aloe Wellness Pty Ltd, Australia. The leaves were cut and the outer green rind was discarded. The

mucilaginous inner pulp was minced and thoroughly homogenised using a hand held blender. Each leaf produced approximately 125 mL of gel. The homogenised gel was lyophilised in vacuo at 22° C and the resultant lyophilised material was stored frozen until further extraction.

PREPARATION OF CRUDE EXTRACT

1 g of lyophilised A. barbadensis gel was extensively extracted by adding 50 mL methanol (AR grade, Ajax) to the lyophilised gel and mixing gently for 2 h at 22 ° C. The extract was then filtered through filter paper (Whatman No. 54) under vacuum followed by drying by rotary evaporation in an Eppendorf Concentrator 5301. The resultant waxy red pellet was dissolved in 1 mL 20% methanol giving a dark red solution. The extract was passed through 0.2 µm filter (Sarstedt) and stored at 4 ° C.

HPLC SEPARATION OF EXTRACT COMPONENTS

The extract was analysed and further fractionated by RP-HPLC. All of the equipment was Shimadzu. The system consisted of twin LC-10AT pumps, a DGU-12A degasser, a SIL-10AD automatic injector using a 20 µL injector loop and a SPD-M10A diode array detector, all under the control of a SCL-10A system controller. Detection was monitored at 210 nm. All solvents were of HPLC grade and were obtained from Lab-Scan Australia.

HPLC separations were performed on a Spherisorb C_{18} column (50 mm × 4.6 mm). 20 µL samples of Aloe gel extract were injected and chromatographed using a gradient from 20% methanol to 60% methanol as follows: 2 min isocratically at 20% methanol followed by an 8 min gradient to 40% methanol. This was followed by isocratic elution at 40% methanol for a further 5 min. The methanol was increased to 60% over a further 10 min. The column was washed with 100% methanol before re-equilibrating to 20% methanol for further chromatograms. Samples from multiple chromatograms (10 repeats) were collected and pooled. These samples were dried by rotary evaporation in an Eppendorf Concentrator 5301 and were resuspended in 1 mL distilled water and stored at 4 ° C for further analysis.

ANTHRONE ASSAY FOR CARBOHYDRATE CONTENT

The anthrone assay was performed as described by Dische [20] with the following modifications. 0.2% anthrone was prepared by dissolving pure anthrone (Chem-Supply, Australia) in concentrated H_2SO_4 (AR grade, Unilab). Anthrone reagent was prepared fresh for each assay. D-Mannose (Chem-Supply, Australia, AR grade) was diluted by serial dilution in the range 1 mg mL^{-1} - 0.032 mg mL^{-1} and used as a standard. 50 µL of standard dilutions or of samples was added to wells of a 96 well plate. 100 µl of anthrone reagent was added to each well and mixed. The assays were incubated for 15 min at 22 ° C and colour development was measured at 630 nm using a microplate reader (Biotrak). All determinations were performed in at least triplicate.

TOTAL POLYPHENOLS ASSAY

Determination of the polyphenol content of the extract and HPLC purified compounds was performed by the method of Singleton and Rossi [21]. This method has been routinely used to determine the total polyphenolic levels of plant extracts [22]. Gallic acid (Sigma) was used as a phenol standard. Aloe extract was diluted 1 in 10 for the assay. HPLC fractions were tested undiluted. 100 µL of the standards, the diluted extract and the HPLC fractions were added to 2 mL of 2% Na_2CO_3 and 200 µl of Folin-Ciocalteu reagent (Lab-Chem) was added. The tubes were incubated for 30 min at 22 ° C. The absorbance was measured at 720 nm. All determinations were performed in at least triplicate.

REFERENCE TOXINS FOR BIOLOGICAL SCREENING

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 mg mL^{-1} solution in distilled water and was serially diluted in synthetic seawater for use in the Artemia nauplii bioassay. Mevinphos (2-methoxycarbonyl-1-methylvinyl dimethyl phosphate) was obtained from Sigma-Aldrich with a mixture of cis (76.6%) and trans (23.0%) isomers and prepared as a 4 mg mL^{-1} stock in distilled water. The stock was serially diluted in synthetic seawater for use in the bioassay.

BIOLOGICAL SCREENING

Toxicity was tested using the Artemia nauplii lethality assay developed by Meyer et al. [23] for the screening of active plant constituents. This bioassay has previously been used to examine the toxicity of a wide variety of compounds. It is an efficient, inexpensive and relatively rapid way to detect toxic compounds, requiring only low amounts of sample (<20 mg). This test correlates well with cytotoxic activity of some human tumours and therefore has the potential to detect new antitumour agents [24]. The assay was performed as described by Meyer et al. [23] with the following

modifications. Artemia franciscana Kellogg cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). Synthetic seawater was prepared using aquarium salt (Reef Salt, AZOO Co., USA) at 34 g L^{-1} distilled water. 2 g of A. franciscana cysts were incubated in 1 L synthetic seawater under artificial light at 25 ° C, 2000 Lux with continuous aeration. Hatching commenced within 16-18 h of incubation. Newly hatched A. franciscana (nauplii) were used within 10 h of hatching. Nauplii were separated from the shells and remaining cysts and were concentrated to a suitable density by placing an artificial light at one end of their incubation vessel and the nauplii rich water closest to the light was removed for biological assays. 400 µL of seawater containing approximately 60 (mean 59, n = 114, SD 17) nauplii were added to wells of a 48 well plate and immediately used for bioassay. The extract was diluted 1:4 and diluted serially in synthetic seawater. HPLC purified fractions were similarly diluted for bioassay. 400 µL of diluted Aloe extract samples, HPLC fractions and the reference toxins were transferred to the wells and incubated at 25 ± 1 ° C under artificial light (500 Lux). A negative control (400 µL seawater) was run in at least triplicate for each plate. All concentrations of treatments also were performed in at least triplicate. The wells were checked at regular intervals and the number of dead counted. The nauplii were considered dead if no movement of the appendages was observed within 10 seconds. After 72 h all nauplii were sacrificed and counted to determine the total number per well. The LC_{50} with 95% confidence limits for each treatment was calculated using Probit analysis [25].

RESULTS

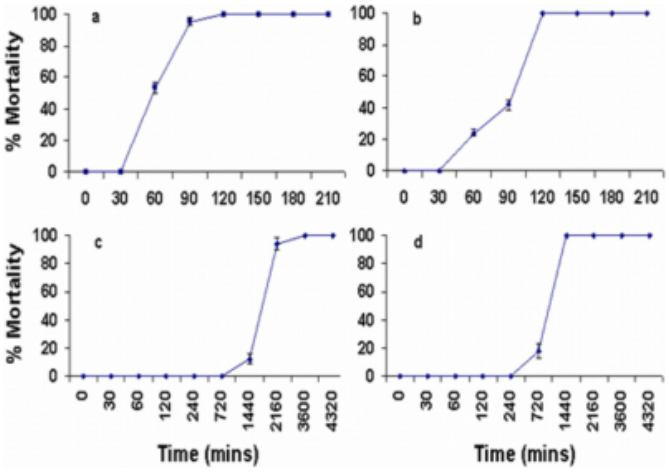
Lyophilisation of 100 mL of Aloe vera gel by rotary evaporation produced approximately 1g of dried fraction (1% of original weight). When further processed with methanol extraction and drying by rotary evaporation, the net result was 391 mg of dried extracted material. Resuspension of the dried fraction in 1 mL distilled water resulted in 1.2 mL of concentrated extract. This equated to an approximate 80 fold concentration of the extractable, non-volatile compounds of the Aloe vera gel compared to the original gel.

Quantitative analysis of the carbohydrate content of the extract performed using a modified anthrone assay, revealed that the extract had 201 mg total carbohydrate at a concentration of 167.5 mg mL^{-1} . The polyphenol content, determined by the method of Singleton and Rossi [21], of the Aloe vera extract was 46.8 mg total extractable polyphenolics.

Previous reports [26,27] express Artemia nauplii bioassay LC_{50} values for toxins at 24 h of exposure. However, throughout the course of these studies a notable feature of the toxic Aloe vera extract was the rapid onset of toxicity at which mortality occurred. The 1 in 5 diluted extract produced 100% brine shrimp mortality within 2 h of treatment. Neither Mevinphos (2000 µg mL^{-1}) nor potassium dichromate (800 µg mL^{-1}) was seen to produce notable mortality compared to the negative controls within 2 h. For this reason, a time course study was run to determine the rate of toxicity of the extract. As is seen in Figure 1a, the onset of the extract's toxicity, as defined by mortality, was evident at approximately 60 min, and approximately 100% mortality was seen by 90 min. In contrast, both Mevinphos (Figure 1c) and potassium dichromate (Figure 1d) took much longer to exert their effect. The onset of Mevinphos toxicity was approximately 24 h and more than 36 h was required for 100% mortality. Similarly, potassium dichromate toxicity was not evident until 12 h and approximately 24 h was required for 100 % mortality (spontaneous mortality in all seawater controls was < 1% 24 h). Due to the rapid toxicity of the extract, LC_{50} values have been reported for 2 h (Table 1). To enable comparison to the LC_{50} values of the reference toxins the LC_{50} at 24, 48 and 72 h are also reported.

Figure 1

Figure 1: Brine shrimp lethality of (a) Aloe vera extract (1 in 5 dilution in synthetic seawater), (b) Fraction 1 (800 Âµg/ml) from RP-HPLC fractionation of the Aloe vera extract, (c) Mevinphos (2000 Âµg/ml), (d) potassium dichromate (800 Âµg/ml). All bioassays were performed in at least triplicate.



The LC_{50} of the extract (1443 µg mL^{-1}) was similar to the Mevinphos LC_{50} (1336 µg mL^{-1}) at 24 h demonstrating its toxicity. However, potassium dichromate had a LC_{50} of 73 µg mL^{-1} at 24 h, nearly twenty fold more toxic than the extract. The sensitivity of the Artemia nauplii in this study compared well with a previous report [28] of LC_{50} 34 µg mL^{-1} for potassium dichromate tested with an Artemia species also sourced from the USA. No LC_{50} values were found in the literature for Mevinphos using the Artemia nauplii lethality assay. Interestingly, whilst the toxicity of the extract was observed very rapidly, it reached its maximum lethality within 24 h. Only small decreases in LC_{50} were seen over the next 2 days. In contrast, while both Mevinphos and potassium dichromate took longer to exert their effects, the mortality due to these toxins continued to increase over time. Whether these contrasting effects are due to a difference in the mechanism of toxicity of these compounds, or whether the active compounds in the extract are labile and lose effect over time, is not evident from these studies. Future studies need to focus on the mechanism by which the extract compounds exert toxicity.

Figure 2

Table 1: LC (95% confidence interval) for brine shrimp nauplii exposed to Aloe vera extract, Fraction 1 from RP-HPLC separation of extract and the reference toxins Mevinphos and potassium dichromate.

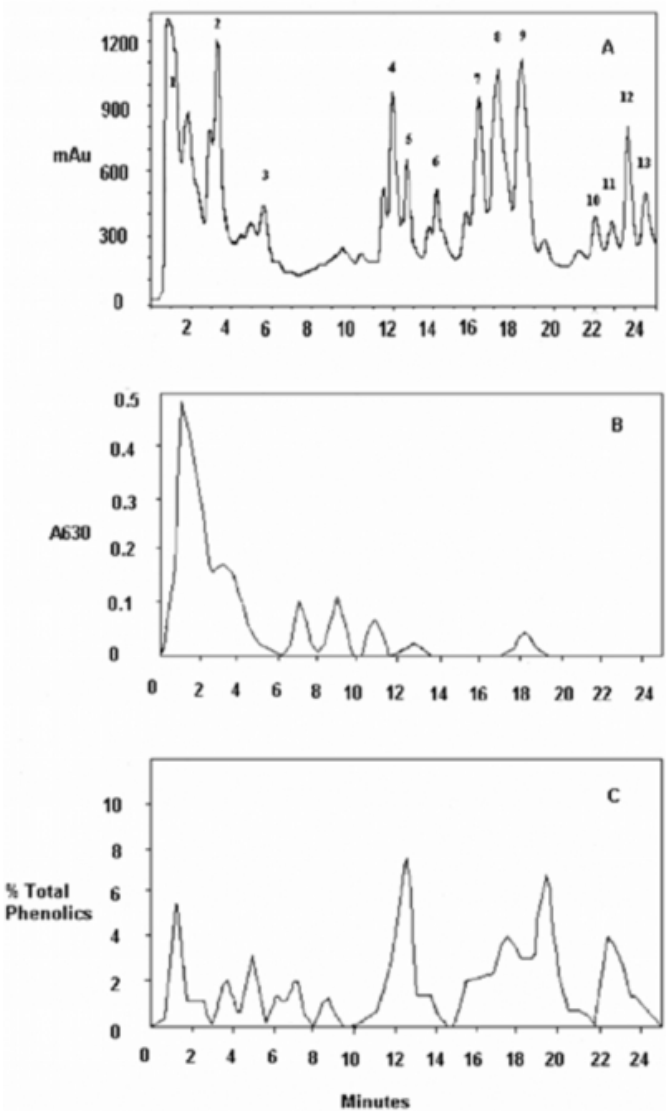
Treatment	LC_{50} value (µg ml^{-1}) at time (h)			
	2	24	48	72
Aloe Extract	3426 (3294-3557)	1443 (1368-1522)	1404 (1329-1481)	1404 (1329-1481)
Fraction 1	947 (905-995)	435 (413-459)	266 (252-281)	260 (246-275)
Mevinphos	*	1336 (1266-1414)	501 (468-537)	109 (97-122)
Potassium Dichromate	*	73 (69-78)	12 (11-13)	3.7 (3.5-4.0)

*2 h LC_{50} values for Mevinphos and potassium dichromate were not obtained as no increase in mortality above that of the negative control was evident.

The HPLC gradient elution system used in this study facilitated the simultaneous separation of Aloe vera components of varying hydrophobicities. Figure 2a shows the typical HPLC profile of the fractionated methanol extract.

Figure 3

Figure 2: (a) A typical RP-HPLC chromatogram of an Aloe vera gel extract fraction with detection at 210 nm. Chromatography conditions were as described in Materials and Methods. Fractions collected for further analysis are indicated with numbers on the chromatogram. (b) Detection of relative carbohydrate levels in the RP-HPLC fractions using the anthrone assay. Carbohydrates were detected at 630 nm. (c) Detection of relative levels of phenolic compounds in the RP-HPLC fractions using the method of Singleton and Rossi [ ] as described in the Materials and Methods. All carbohydrate and polyphenolic determinations were performed in at least triplicate.



The HPLC fractions were evaluated by chemical methods to study their chemical nature. As shown in Figure 2b, the majority of the carbohydrate components of the extract eluted early in the chromatogram. Figure 2c shows the distribution of polyphenolic compounds across the HPLC profile. Polyphenolic molecules were seen to be widely

distributed with most present in the latter half of the chromatogram.

The 13 HPLC separated fractions were tested for toxicity using the using the Artemia nauplii lethality bioassay. Only Fraction 1 was found to show toxicity above the levels observed in the negative controls. Like the extract, this fraction was seen to have a rapid onset of toxicity, killing within 2 h (LC_{50} 947 µg mL^{-1}). As shown in Table 1, Fraction 1 had a 24 h LC_{50} of 435 µg mL^{-1}. This is significantly lower than the LC_{50} of Mevinphos (1336 µg mL^{-1}) attesting to the toxicity of this fraction. However, the 24 h LC_{50} of the fraction is approximately six fold less toxic than potassium dichromate with a LC_{50} 73 µg mL^{-1}. Overall, Fraction 1 showed good brine shrimp larvicidal toxicity according to Meyer et al. [23] who classified purified plant fractions as toxic when 24 h LC_{50} value <1000 µg mL^{-1}.

DISCUSSION

The current study demonstrates the ability of Aloe vera gel extract and a RP-HPLC separated fraction to induce mortality in A. franciscana. The acute toxicity of Aloe vera gel exposure to A. franciscana resulted in a 24 h LC_{50} of 1443 µg mL^{-1}. The RP-HPLC purified Fraction 1 was more toxic, with a 24 h LC_{50} of 435 µg mL^{-1}. A notable feature of both the extract and Fraction 1 was the rapid onset of toxicity against the brine shrimp nauplii. Both the extract and Fraction 1 were lethal to A. franciscana at less than 2 h. Mevinphos and potassium dichromate were much slower in inducing lethality, with little induction of A. franciscana lethality seen within the first 24 h of exposure. The mechanism by which Aloe vera leaf gel components induce toxicity is unknown. However, studies within this laboratory indicate the toxicity may be due to the induction of oxidative stress (unpublished results).

Aloe vera leaf gel extract was found to have high levels of extractable carbohydrates. 201 mg of total carbohydrate were extracted from 1 g dried leaf gel (approximately 20% of the total dried weight). This equates to over 50% of the total extractable solids (391 mg) extracted from the original 1 g of Aloe vera leaf gel. The sugar composition was not analysed in this study but would be assumed to be high in β-(1,4)- linked polymannose (acemannan). Previous studies have shown that acemannan is the major fraction from Aloe vera leaf gel [29]. Acemannan is claimed to have several important therapeutic properties including acceleration of wound healing [7], immune stimulation [3,30] and antiviral effects [5].

Aloe vera leaf gel also had high levels of extractable polyphenolic compounds. 46.8mg of total phenolics were extracted from 1 g of dried Aloe vera leaf gel (approximately 4.7% of the total dried weight of the leaf gel). This equates to approximately 12% of the total solids extracted from the dried leaf gel (391 mg). This is comparable to other known sources of polyphenolics. For example, Aspalathus linearis, a plant used as a herbal tea (Rooibos) is generally thought to be a good source of polyphenolic compounds, especially flavanoids. A recent study [31] reported the polyphenolic compounds in unfermented A. linearis to account for approximately 15% of the mass of the extracted solids.

The toxic fraction obtained from RP-HPLC contains both carbohydrate and polyphenolics. This could indicate a mixture of compounds in this fraction or the presence of a glycosylated polyphenolic compound. When dried, Fraction 1 was a crystalline solid with an orange red colour with a slightly tacky consistency. The methanolic solution of the fraction absorbed UV light between 250 and 290 nm with a maxima at 265 nm in the UV region. Anthraquinones are characterised by their orange red colour and their absorbances in the UVB range. Hirata and Suga [32] have listed the UV absorbance peaks for aloe emodin as 221, 253, 266 and 289 nm and the absorbance peaks of aloin as 250-290 nm with a peak at 260 nm. A more recent paper [13] has reported UV absorbance peaks for various anthraquinones as 217-220 nm and 265-270 nm. Anthraquinones are known to be cytotoxic and have been shown to induce apoptosis in human lung squamous cell carcinoma [14]. Fraction 1 may therefore contain an anthraquinone moiety. Aloe emodin (a glycosylated anthraquinone) has been reported [29] to be one of the major constituents of the gel. Thus this compound may be present in Fraction 1. Definitive structural characterisation of this fraction was not possible. Electron ionisation mass spectroscopy (unpublished results) show complicated patterns that indicate the presence of multiple molecular species. More work is necessary to further characterise the molecular composition of this fraction.

The current report demonstrates the toxicity in the Artemia nauplii bioassay of a fraction containing a polyphenolic compound extracted from Aloe vera. This test has a good correlation with antitumour and pesticidal activity [24]. However, these authors point out that this bioassay has its limitations. There is no universally accepted standard for

determining whether the level of toxicity exhibited by the extract or compound is useful. These authors suggest that comparing the lethal concentration to that of known compounds of very high toxicity (e.g. we chose potassium dichromate and Mevinphos) would be a valid way of assessing the relative toxicity. On this basis, the Aloe vera extract and Fraction 1 described in this report show good relative toxicity and should be considered for further testing to evaluate their usefulness as pesticide and antitumour therapeutic agents.

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