



Original Article

Volatile compounds produced by *Calotropis procera* (Family: Asclepiadaceae) leaves that aid in the repulsion of grazers

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Abstract

Calotropis procera, a desert shrub with large succulent leaves, but without protective spines, was investigated for volatile substances that aid in repelling grazing animals. It was found that fresh *C. perociera* leaves produced volatile organic compounds that included thioacetic acid, 2,3-dihydro-3,5-dihydroxy-6-mehtyl-4H-pyran-4-one, and 5-hydroxymethyl-2-furancarboxaldehyde. The most probable repellent of grazers from leaves of *C. procera* was thioacetic acid. The ecological significance is that *C. procera* uses irritating volatiles to repel desert grazers in addition to toxins rather than the usual plant defense mechanisms, such as the production of numerous spines.

Keywords: *Calotropis procera*, grazing, repellants, thioacetic acid, irritating volatiles

INTRODUCTION

Calotropis procera [Family: *Ascepiadaceae*] is a common native desert milkweed shrub found throughout the tropical arid world from Morocco to India and South East Asia [Orwa, et al. 2009; Western, 1989]. In the arid regions it is most often found in disturbed and over-grazed areas [Millerand Morris, 1988]. It has also been introduced accidentally or as an ornamental shrub to similar habitats as far West as Hawaii and South into Australia. *Calotropis procera* is an important species in the Northern deserts of the United Arab Emirates and its presence is essential for the support of a unique community of animals [Khan, 1989] and provides a critical nursery habitat for other valuable plants [Campolucci and Paolini, 1990].

Calotropis procera is a unique desert plant because it lacks protective spines and has abundant broad leaf succulent foliage year around. Nevertheless, grazers such as sheep, goats, camels and Oryx actively avoid eating it [Bovey and Mandaville, 1978]. Grazing animals will eat the surrounding plant species, but will leave *C. procera* completely untouched. Often, it is the only plant of size remaining in an over-grazed desert area [Jongbloed, 2003; Shuaib, 1995]. It has been assumed by the several authors

mentioned above that the non-volatile edible toxins are the reason grazers avoid *C. procera*, but this does not explain why the grazers avoid tasting the fresh leaves.

The sap from *C. procera* is mildly toxic to humans and other animals, causing inflammation of the skin, vomiting, diarrhea, blindness, lowered blood pressure and even death [Boulos, 2000]. The toxins are akundarin, gigantol, calcium oxalate, crystalline alcohols, terenes, and other alcohols. Specific compounds in *C. procera* sap include lup-20[29]-en-3-one, beta-sitosterol, lupeol, cycloeucalenol, banaleric acid, giaganteol, taraxasterol, beta-calotropeol, mudarol, alpha-amyrin benzoate and others [Harbi, 2004; Khan, 1989]. The leaves contain an active chemical that is bitter [mudarine] and the four toxic glycosides calotropin, uscharin, calotoxin and calactin [Meena et al. 2010]. However, none of these investigations included an investigation into the lightest and most volatile compounds that are being emitted by the fresh leaves.

Our preliminary observations of *C. procera* shrubs located within fenced areas that contain numerous grazers, were that the grazers did not even taste the leaves. None of the fresh leaves in the enclosures showed any bite marks. The animals would come close to the leaves, smell them and

then avoid the leaves without tasting them. This suggested that neither the toxins nor the bitter flavor were causing the grazers to avoid eating the leaves, but rather, there could be a volatile odor or other compound that is acting as a repellent. Gallacher and Hill [2006] observed Gazelle using their front hooves to knock off fresh *C. procera* leaves and then, after the leaves had dried, they would come back and eat the dry leaves. This suggests that the repulsive volatile substances or toxins contained in the leaves are changed by enzymatic action or evaporate as the leaves dry. Further evidence for this hypothesis comes from a study reporting that when the leaves are chopped, dried and mixed with other feed, sheep, goats and camels can eat it without any noticeable repulsion or toxic effects [Abbas, et al. 1992]. It is the purpose of this paper to describe the most volatile substances being emitted by *C. procera* leaves that could be acting as an effective repellent to grazers. Gas Chromatograph-Mass Spectrometer [GCMS] analytical techniques were used to detect and describe these compounds.

MATERIAL & METHODS

GCMS/MS Setting:

A Varian 3600 Gas Chromatograph coupled to a Varian Saturn 2000 MS system was used with the model 1078 Universal Capillary Injector equipped and fitted with the ChromatoProbe kit. This kit allows the thermal desorption of small amounts of solids or liquids contained in quartz microvials without any prior handling of the samples. Small pieces of the leaf [~10 mg] were placed in the ChromatoProbe microvial and the vial was loaded into the probe, which was then inserted into the modified injector. Thus, either fresh or dried *C. procera* leaves could be loaded directly to the machine where all volatile compounds are captured and separated by Gas Chromatography [GC]. The compounds coming off the leaves were then identified through their mass spectra as compared to the MS library on the machine [Skoog et al. 2007]. The GC/MS used helium as its mobile phase and the column was a factor four VF-5ms, 30m x 0.25, df= 0.25 equivalent to 5% phenyl 95% and dimethyl polysiloxane. To acquire the volatiles from *C. procera*, the GC/MS the injector was started at 40°C and raised slowly to 200°C at a rate of 16°C / min, after which it would remain at 200°C for two minutes before cooling back to 40°C. Meanwhile, the column oven was adjusted to start from 40°C for five minutes then go up to 290°C at a rate of °C /min for 12.5 minutes in which the total time would be 30 minutes.

Comparing Volatiles in Dry and Fresh Leaves:

Leaves that had fallen off the plant naturally and dried in the sun were used as a control to be compared with fresh leaves. Each leaf was cut into small pieces [0.009 g] and put into a microvial and into the ChromatoProbe. Comparison between fresh leaves and dried leaves indicated which compounds had evaporated from the leaves and left them palatable to grazers. The dried leaves were treated and analyzed in the same manner as the fresh leaves.

Monitoring Volatiles in Fresh Leaves over Time:

Three replicate samples of fresh leaves were taken from *C. procera*, young leaves from the upper branches, older leaves from the middle part of the plant, and the oldest leaves from the lower branches. Each leaf was cut into small pieces [0.009 g] and put into a microvial and into the ChromatoProbe. This was done every day for 8 days to follow reductions of constituents in the leaves as they slowly withered and dried.

Analysis of Solvent-Extract of Leaves:

Top, middle and lower *C. procera* fresh leaves were cut into 5 g pieces and placed into a Soxhlet distillation apparatus. A solvent [ethanol or ethyl acetate] was used to extract the leaves at its boiling point for four hours. The solution was concentrated by evaporation under vacuum. A 10 mL sample of the concentrate was collected and injected into the GCMS.

RESULTS & DISCUSSION

Before comparing the volatiles available in fresh leaves with the dry ones, it was important to compare the composition of the volatiles in the fresh leaves to rule out the effect of the leaf's age on its components. Thus, fresh leaves were taken from *Calotropis procera* from the upper, the middle, and the lower part of the branches to have samples of young, older and the oldest leaves, respectively. The GCMS chromatogram [Figure 1] of these leaves showed no significant difference in the number or intensity of the peaks indicating similar compositions. Therefore, all age groups of fresh leaves contained the same compounds.

On the other hand, the chromatogram of the fresh leaves was compared with that of the dry leaves [collected from the leaves falling on the ground around the plant], there were several peaks either missing or greatly diminished, from the chromatogram [Figure 2]. The intensity of three compounds that came out at 6.93 mins, 10.34

mins and 10.93 mins [peaks I, II and III in Figure 2] were significantly diminished in the chromatogram of the dry leaves as compared to the fresh leaves. This suggests that one or more of these compounds which are present in the fresh leaves, but not in the dry leaves could be a repellent to the grazers.

Furthermore, three replicates of fresh leaves were allowed to air-dry on the bench top and their chromatograms were monitored daily over an 8-day period. A comparison of these chromatograms [Figure 3] showed clearly that there are three peaks at retention time of 6.93 mins, 10.34 mins and 10.93 mins which were decreasing overtime. These were the same peaks missing from the chromatograms of the dry leaves. It is highly probable that one of the compounds is responsible for repelling the grazers and evaporates over time. It is also possible these compounds may be continuing to be produced by enzymatic actions as the plant leaf tissues wither and die.

To identify the compounds missing from the dry leaves, the mass spectra of the compounds I, II and III were compared to a library of MS spectra. As a result, I, II and III were identified as thioacetic acid, 2, 3-dihydro-3,5-dihydroxy-6-mehtyl-4H-pyran-4-one, and 5-hydroxymethyl-2-furancarboxaldehyde. Thioacetic acid [Figure 4] has the lowest molecular weight of the three [76.13 g/mol] and the lowest boiling point range [88°C-91.5°C]. It also gives off a stench and it is both a powerful eye and sinus irritant and a lacrymator [tear inducer] which is used in tear gas mixtures [Crouch, 1952]. Thioacetic acid is toxic when swallowed or inhaled and its odor causes headache, dizziness, and nausea [Arkema, 2004]. On the other hand, the two compounds, 2,3-dihydro-3,5-dihydroxy-6-mehtyl-4H-pyran-4-one, and 5-hydroxymethyl-2-furancarboxaldehyde are also byproducts of heated foods especially carbohydrates [Sun et al, 1993]. The first has a toasty and caramel smell [Cutzach et al, 1997] and it is found in many types of food such as potatoes, soya, peas, beans, onion, garlic [Walton et al., 1999], and honey [Bhandari et al., 1998]. The second has been identified in a variety of heat-processed foods such as milk, fruit juices, and honey [Jiang et al, 2008].

The above results strongly suggest that thioacetic acid is responsible for repelling grazers from the fresh leaves of *C. procera*. To the best of our knowledge, this is the first time where thioacetic acid has been reported as a product or a component of *C. procera*. However, previously published investigations only used a solvent extraction technique to isolate the products of *C.*

procera leaves and during such extraction processes, fresh leaves were extracted with boiling solvents for few hours. Thus, it would be expected that all highly volatile and reactive products such as thioacetic acid would not be detected because they would have been lost with the solvent vapors during boiling / condensation processes or because they would have reacted with other products during the heating process. To further test this hypothesis, fresh leaves that were taken from different parts of the plant and as well as dry leaves of *C. procera* were extracted using Soxhlet's apparatus using either ethanol or ethyl acetate as a solvent. Neither of the resulting chromatograms showed any traces of thioacetic acid [Figure 5] but did show the same compounds that had been reported previously in the literature, such as alpha-amyrin and lup-20[29]-en-3-ol [Harbi, 2004]. Finally, the solvent extractants from both fresh leaves and dry leaves produced very similar chromatograms, because, as expected, the highly volatile compounds were missing from both.

Finally, fresh *C. procera* leaves were placed inside a paper bag and the air and leave vapors breathed in through our nose to try and detect an odor directly. This was done to simulate what grazing animals might be detecting, but would not be able to communicate to us. No offensive odor was detected, but almost immediately there was very noticeable sinus irritation and inflammation that lasted for 30 minutes after we stopped breathing the fresh leaf vapors.

CONCLUSION

1. *Calotropis procera* produces volatile organic compounds that were not identified before this report and these include, but are not limited to thioacetic acid, 2,3-dihydro-3,5-dihydroxy-6-mehtyl-4H-pyran-4-one, and 5-hydroxymethyl-2-furancarboxaldehyde.
2. The most probable repellent of grazers from the fresh leaves of *C. procera* is most likely thioacetic acid due to its potential, when concentrated, to produce bad odor and case irritation to the sinuses and eyes along with it is corrosiveness.
3. GCMS equipped with the ChromatoProbe kit was shown to be an efficient and easy analytical technique to identify highly volatile organic compounds from natural sources.
4. The ecological significance is that *C. procera* uses irritating volatiles to repel desert grazers rather than the usual plant defense mechanisms, such as the production of numerous spines.

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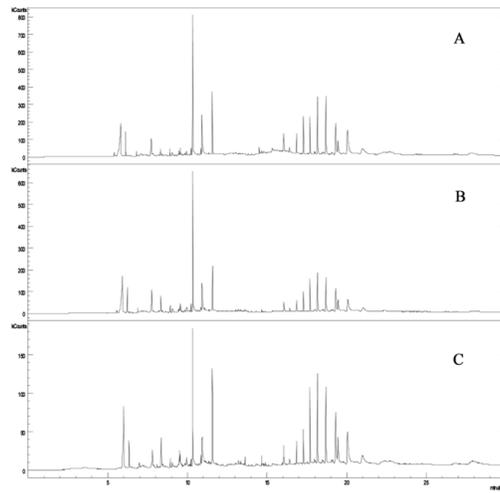


Fig1. GCMS chromatogram of samples from (A) top, (B) middle, and (C) lower parts of *C. procera* branches

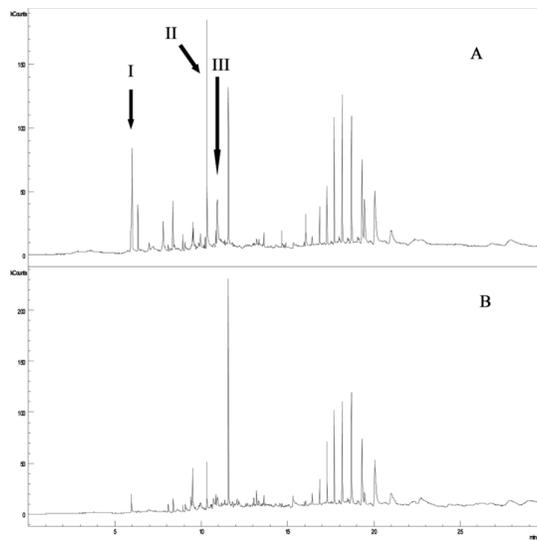


Fig2. GCMS chromatogram of samples from (A) fresh and (B) dry leaves of *C. procera*.

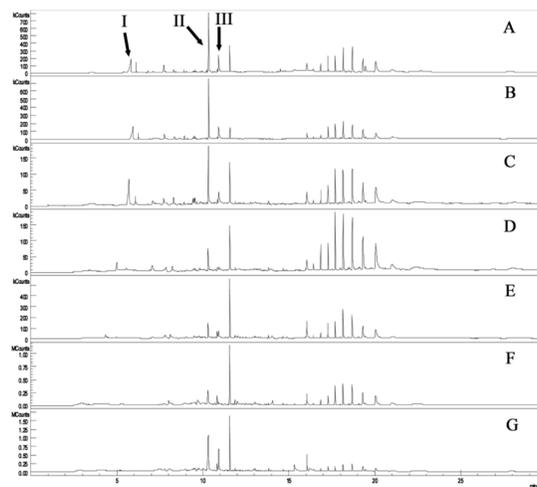


Fig 3. GCMS chromatograms of samples from a fresh leaf of *C. procera* taking every 24 hours from (A) day-1 to (G) day-8.

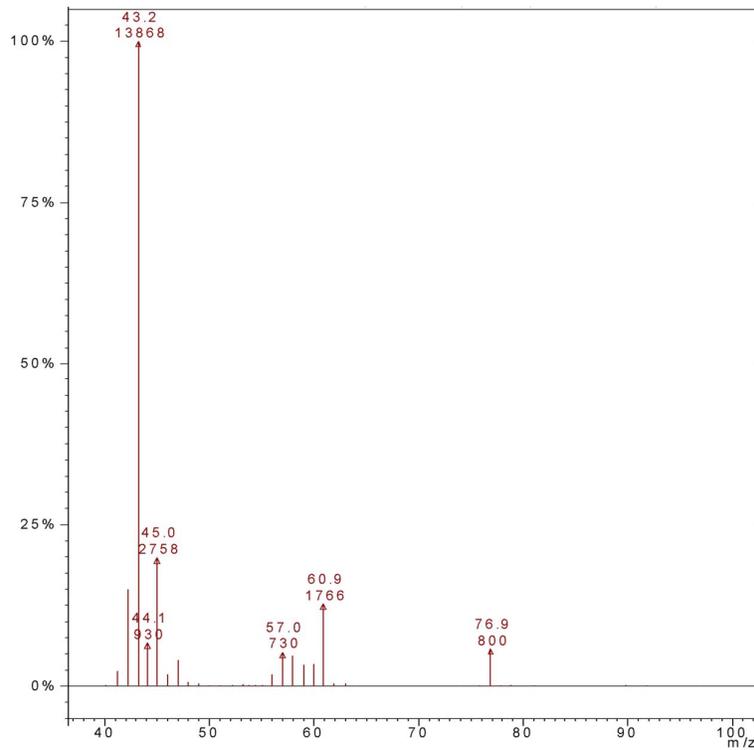


Fig4. Mass spectrum of the compound coming off the GC column at 6.93 mins and identified as thioacetic acid.

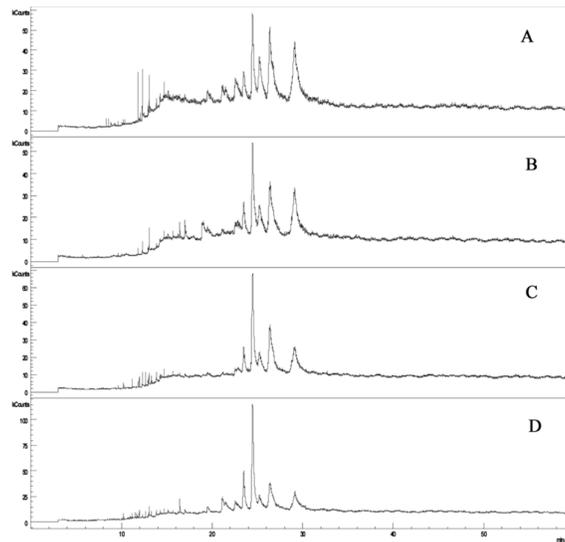


Fig5. GCMS chromatograms of ethyl acetate extractant of fresh leaves of *Calotropis procera* taken from (A) top, (B) middle and (C) bottom parts of a branch and of a (D) dry leaf.