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In vitro acetylcholinesterase inhibitory activity and chemical composition of the dichloromethane extracts of the leaves and inflorescences of the tumbleweed, *Trachyandra laxa* (N.E.Br.) Oberm. var. *rigida* (Suesseng.) Roessl

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ABSTRACT

Trachyandra laxa, which belongs to the family Asphodelaceae, is a poisonous geophyte, often consumed by livestock, particularly when pastures are poor. Ingestion of *T. laxa* by livestock causes paresis and paralysis of the animals. However, the chemical composition of this plant has never been studied before and its toxic components are still unknown. In this study, an acetylcholinesterase (AChE) inhibition assay of different extracts of the parts of *Trachyandra laxa* (N.E.Br.) Oberm. var. *rigida* (Suesseng.) Roessl. revealed that the DCM extracts possess the highest AChE inhibitory activity. In addition, using qualitative phytochemical tests, it was determined that all the plant parts contain polyphenols and tannins, while only the leaves contain flavonoids. Saponins were detected in all plant parts, except in the flowers and anthraquinones were detected in all plant parts expect the roots. Using GC–MS analysis, 13 volatile constituents were identified in the DCM extracts of the leaves and inflorescences, including α,β-unsaturated lactones, carboxylic acids and esters, a diterpenoid, phytol, and a phytosterol, β-sitosterol. Of particular interest are the α,β-unsaturated lactones, which were identified as dihydroactinidiolide (DHA) and its hydroxylated analogue, hydroxy-DHA, as well as phytol, a diterpenoid. DHA and phytol have been reported to possess potent AChE inhibition activity. It is therefore plausible that these compounds and potentially also hydroxy-DHA could contribute to the AChE inhibitory activity of the DCM extracts, and thus the neurotoxicity of *T. laxa* var. *rigida*.

1. Introduction

Poisonous plants are a major concern for livestock farming in southern Africa (Mannheimer et al., 2012; Schubert, 2002). Usually, plant poisoning in animals occurs accidentally during unfavourable conditions when pastures are poor due to drought, veld fires, and overstocking, when animals are forced to eat poisonous plants which would normally be avoided. Poisonous plants can affect the entire spectrum of organ systems, with some plants having several toxic principles that affect different systems (Schubert, 2002). Two varieties of *Trachyandra laxa* occur in Namibia namely, *Trachyandra laxa* (N.E.Br.) Oberm. var. *laxa* and *Trachyandra laxa* (N.E.Br.) Oberm. var. *rigida* (Suesseng.) Roessl. *Trachyandra laxa* (N.E.Br.) Oberm. var. *rigida* (Suesseng.) Roessl are robust and coarse plants that have trichotomous lower inflorescence branches, clavate peduncles at the apex and swollen (but not tuberous) long and spreading roots. They are perennial geophytes, with tough, fleshy, bilaterally flattened, terete leaves in a basal rosette. The tepals are white with a narrow purple-brownish central stripe. *Trachyandra laxa* (N.E.Br.) Oberm. var. *laxa* are slender plants that have alternate inflorescence branches from the base upwards, peduncles not clavate at the apex and tuberous roots (Grant et al., 1985; Kativu, 1996).

Consumption of *Trachyandra laxa* (N.E.Br.) Oberm. var. *laxa* causes progressive paresis (partial paralysis) and paralysis in cattle, goats, sheep, horses and pigs and is characterized by the accumulation of a brownish pigment, consistent with lipofuscin, mainly in the larger neurons of the brain and spinal cord (Grant et al., 1985). Poisoned animals develop a number of symptoms consistent with central nervous system toxicity, including tremors or muscle twitching, paresis and paralysis which results in sternal recumbence (Botha and

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Penrith, 2008; Grant et al., 1985; Mannheimer et al., 2012; Schubert, 2002). In another study it was reported that a similar lipofuscin storage disease caused paresis in sheep with the ingestion of another *Trachyandra* species, *T. divaricata* (Jacq.) Kunth (Newsholme et al., 1985).

Plant toxicity may be due to the presence of a diversity of primary and secondary metabolites that include alkaloids, triterpenes, steroids, saponins, anthraquinones, glycosides, proteins and amino acids (Kellerman et al., 2005). Numerous phytoconstituents, including alkaloids, coumarins, terpenoids, and polyphenols have been identified as a natural source of acetylcholinesterase (AChE) inhibitors (Dos Santos et al., 2018). When compounds inhibit AChE enzymes and cause a disruption of neurotransmission, they are essentially acting as neurotoxins (Čolović et al., 2013). Therefore, in vitro AChE inhibition assays are commonly used for the determination of the presence of neurotoxins (Hendricks and Pool, 2012; Lionetto et al., 2013). AChE hydrolyses the substrate acetylcholine (ACh) at the membrane of mammalian neurons and, while predominantly associated with the neuromuscular junction and cholinergic synapses in the central nervous system (CNS), it is also found in the blood where it is associated with erythrocytes, lymphocytes, megakaryocytes, and platelets (Husain, 1994; Paulus et al., 1981; Zajicek, 1957).

AChE is produced from a single gene and can be expressed to produce different isoforms in different tissues through alternative splicing at the 3′ terminus of the AChE pre-mRNA (Getman et al., 1992). This can produce three isoforms: the primary isoform found at synapses (AChE-S) in the CNS; a stress-induced, soluble isoform (AChE-R), and the isoform found in the membrane of erythrocytes (AChE-E) (Grisaru et al., 1999; Massoulié, 2002; Meshorer and Soreq, 2006). While AChE-S and AChE-R are predominantly found in neurons, they have also been found in peripheral blood cells (Pick et al., 2004). Despite being located in different tissues and expressed as different isoforms, the variants all share a similar catalytic domain but differ at the C-terminus, which is responsible for the molecular form, localisation, and specific features and the variants (Park et al., 2004). Furthermore, AChE-E can take part in certain reactions in the same way as the other AChE isoforms. For instance, the serine in the active site of AChE-E produces a covalent bond with an organophosphate pesticide in the same way as the AChE isoforms found in the nervous system (Dafferner et al., 2017). Consequently, one version of the AChE assay is performed by using AChE extracted from blood (Ellman et al., 1961; Hendricks and Pool, 2012). Although the AChE used in this case is mostly the erythrocyte isoform it will still provide an indication of neurotoxicity (Dafferner et al., 2017; Gupta et al., 2015).

Trachyandra species have been associated with progressive, ascending paresis and paralysis in cattle, sheep, horses, and pigs in Namibia and South Africa (Kellerman et al., 2005). However, the toxic components of these plants are still unknown (Botha and Penrith, 2008; Van Wyk et al., 2002). Moreover, no studies on the chemical composition of *T. laxa* could be found and no studies have been performed to determine the AChE inhibition properties of this plant either. In this study, the AChE inhibition activities of different extracts of the plant parts of *T. laxa* (N.E.Br.) Oberm. var. *rigida* (Suesseng.) Roessl. were determined. The chemical composition of the active extracts was subsequently investigated using gas chromatography – mass spectrometry (GC–MS). Furthermore, the different classes of phytochemicals present in the plant parts were determined using qualitative phytochemical tests.

2. Materials and methods

2.1. Chemicals and reagents

Merck high-performance liquid chromatography (HPLC) grade dichloromethane (DCM; Biodynamics, Windhoek, Namibia) was used to prepare extracts and standard solutions. The alkane mixture (C_{10}–C_{40} *n*-alkanes, all with an even number of carbons) used for retention index determinations was of analytical grade and purchased from Sigma-Aldrich (Taufkirchen, Germany). All other chemicals and reagents (methanol, hexane etc.) were of analytical reagent grade (AR). Purified water was obtained using a Milli-Q Integral 3 water purification system. Greiner 96-well microtiter plates were used for the AChE assay and HPLC vials used for storage of sample solutions.

2.2. Plant material

Plant material was collected near farm Dakota 35 (23°04′119″S; 18° 28′813″E) in the Omaheke region (research/collection permit number: 1869/2014). Samples of the plant were harvested after the rainy season in February 2014, as they were abundantly available at the time. They were randomly selected, and uprooted. The plants were identified at the National Herbarium of Namibia (National Herbarium of Namibia, identification report number: 2014/348) as *T. laxa* var. *ridiga* (Suess) Roessler, from the family Asphodelaceae (Group et al., 2016; Klopper et al., 2013). This plant is commonly known as the tumbleweed (Fig. 1A). A voucher specimen of the plant was deposited at the National Herbarium of Namibia (WIND herbarium).

2.3. Processing of plant material and preparation of extracts

The collected plants were carefully separated into their different parts, roots, rhizomes, leaves, inflorescences, and flowers (Fig. 1B).

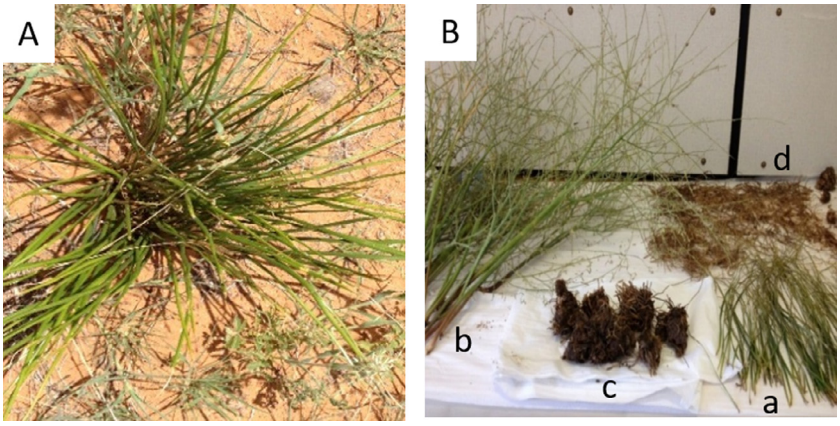

Fig. 1. (A) Young *Trachyandra laxa* var. *rigida* plant in nature. (Hedwig van Wyk: Photo taken in natural habitat); (B) The different plant parts of *T. laxa* var. *rigida*, (a) leaves, (b) inflorescences, (c) rhizomes and (d) roots.

Table 1
Yield of crude extracts produced from different plant parts of *Trachyandra laxa (N.E.Br.) Oberm.* var. *rigida* by successive maceration with hexane, DCM and MeOH.

Plant part	Dry extract yield (%)		
	Hexane	DCM	Methanol
Leaves	1.0	2.7	8.7
Flowers	1.8	2.4	9.1
Inflorescences	0.9	0.9	7.9
Rhizomes	0.7	4.6	8.4
Roots	0.5	2.9	9.4

These were cut into smaller pieces and left to dry at room temperature in a well-ventilated room, before being ground into powder. Extracts of the different plant parts were prepared by successive maceration with hexane, dichloromethane (DCM) and methanol (MeOH). Ten grams of the powdered material was extracted with 20 vol of the appropriate solvent for 20 h at ambient temperature. The filtrates were subsequently concentrated under reduced pressure. The concentrated extracts were then transferred to separate 10 mL vials where they were dried in a nitrogen atmosphere (Table 1). The dried extracts were then stored at −20 °C.

2.4. In vitro acetylcholine esterase inhibition assay

The in vitro AChE inhibition assay was performed on the different extracts by measuring optical densities (ODs) at different time intervals to monitor the change in acetylcholine concentration, using a method adapted from the one used by Hendricks and Pool (Hendricks and Pool, 2012). The assay was performed in triplicate. Phosphate buffer (0.1 M; pH 7.5) was prepared using sodium hydrogen phosphate. Stock solutions of the extracts were prepared in dimethyl sulphoxide (DMSO), at concentrations of ca. 10 mg/mL. A dilution of each extract was prepared in phosphate buffer to obtain final concentrations of 0.1 mg/mL. The positive control consisted of 0.1 mM chlorpyrifos, an organophosphate insecticide that inhibits acetylcholinesterase, prepared in phosphate buffer (Hendricks and Pool, 2012; Rhee et al., 2013). The negative control, 10% DMSO, was also prepared in phosphate buffer, and serial dilutions of both the positive and negative controls were performed. Blood was collected at the University of the Western Cape from a healthy male volunteer who was not on any medication, using sterile heparin vacutainer tubes (Lasec, South Africa) and stored at room temperature. Blood samples were used for AChE inhibition assays within 30 min of collection. An AChE extract was prepared by mixing the heparinised blood, distilled water and phosphate buffer (in a ratio of 3:20:97). A volume of 25 μL of each plant extract solution was added in triplicate to a flat bottom 96-well microtiter plate followed by 25 μL of the AChE extract. A DMSO blank was also prepared which contained 0.1% DMSO with no sample, and was used to compare AChE enzyme activity. The plate was subsequently left to incubate for two hours. Thereafter, 50 μL of substrate solution was added to each well containing sample solution as well as those containing the controls. The substrate solution was prepared by mixing 200 μL of 0.01 M 5,5-dithiobis-(2-nitro-benzoic acid), 6 μL phosphate buffer and 40 μL of 0.075 M freshly prepared acetylthiocholine iodide. Immediately after adding the substrate solution the OD was measured at 405 nm (BMG Labtech, FLUOstar Omega microtiter plate reader) and then every 15 min for one hour. The AChE enzyme activity was calculated as a percentage of the DMSO blank and the data was expressed as the percentage AChE enzyme activity ± standard deviation (SD), using the following equation.

$$\text{Percentage AChE activity} = \left(\frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{DMSO blank}}}\right) \times 100$$

2.5. Statistical analysis

Systat SigmaPlot (v12.2) was used for statistical analysis and figures of statistical relevance were constructed using Graphpad Prism (v5.01). Data was analysed by One Way Analysis of Variance (ANOVA) with the Holm-Sidak test as a multiple comparison test compared to the DMSO blank sample. An overall value of $P < 0.05$ was considered statistically significant.

2.4. Chemical composition

2.4.1. Phytochemical screening

Qualitative chemical tests were carried out on the powdered leaves, inflorescences, rhizomes, roots and flowers of *T. laxa* to determine the presence or absence of selected classes of compounds. Tests were performed in duplicate, based on the methods described by Harborne (Harborne, 1998). The phytochemical screens were also carried out with negative controls in order to compare colour changes. Weak coloration was assigned *a* + while strong coloration was indicated by +++.

2.4.2. GC–MS analysis

GC–MS analysis were performed on a Thermo Scientific Focus GC coupled to an ITQ 700 MS. The flow rate of the carrier gas, helium, was kept constant at 1.0 mL/min and a split ratio of 10 was used. A SGE BP5MS capillary GC column (30 m x 0.25 mm i.d), with a 5% phenyl, 95% polysilphenylene-siloxane stationary phase (0.25 μm film thickness) was used for the separations. The GC injector temperature was maintained at 220 °C. Ion source and interface temperatures of 200 and 250 °C, respectively, were used for the analysis. The oven was programmed at a rate of 2 °C/min from 40 to 300 °C. Electron ionization-Mass Spectrometry (EI-MS) data was acquired at 70 eV and a mass range of *m/z* 25 to 600 was scanned. Data acquisition and processing was performed using Xcalibur Software, version 2.1. A mixture of *n*-alkanes, C_7 and C_{10}–C_{40} (all even numbers of carbons), was analysed under the same conditions for experimental retention index (RI) determination. Samples were prepared for GC–MS analysis by dissolving each extract in DCM at concentrations of ca. 10 mg/mL. A volume of 1 μL of each solution was analysed. To estimate the relative quantities of the compounds that are present in the extracts, a semi-quantitative internal normalisation approach was used, where the relative amounts of the compounds were calculated as percentages of the detected peaks in the total ion chromatogram (TIC) of the GC–MS analysis, by calculating the percentage of each compound's peak area of the total peak area (of all detected peaks) and assuming all response factors to be unity.

3. Results and discussion

3.1. In vitro acetylcholine esterase inhibition activity determination

The AChE inhibition assay of the different extracts of the leaves, roots, inflorescences, rhizomes and flowers of *T. laxa* revealed high enzyme activities in the DMSO blank as well as most of the extracts, indicating that the DMSO blank and most of the extracts do not inhibit AChE activity (Fig. 2A-C). However, compared to the DMSO blank, a significant decrease in the AChE enzyme activity was observed in the DCM extracts of the leaves ($P < 0.001$), inflorescences ($P < 0.001$), and rhizomes ($P < 0.001$), and hexane extracts of the roots ($P = 0.019$) and rhizomes ($P = 0.016$), suggesting that these extracts inhibited AChE activity the most (Fig. 2A and B). No statistically significant decrease in AChE activity was observed in the methanolic extracts, nor in any of the extracts of the flowers (Fig. 2A-C). The positive control, chlorpyrifos, was able to inhibit between 3 and 80% of AChE activity within the range of 1.5–100 μM, with a good

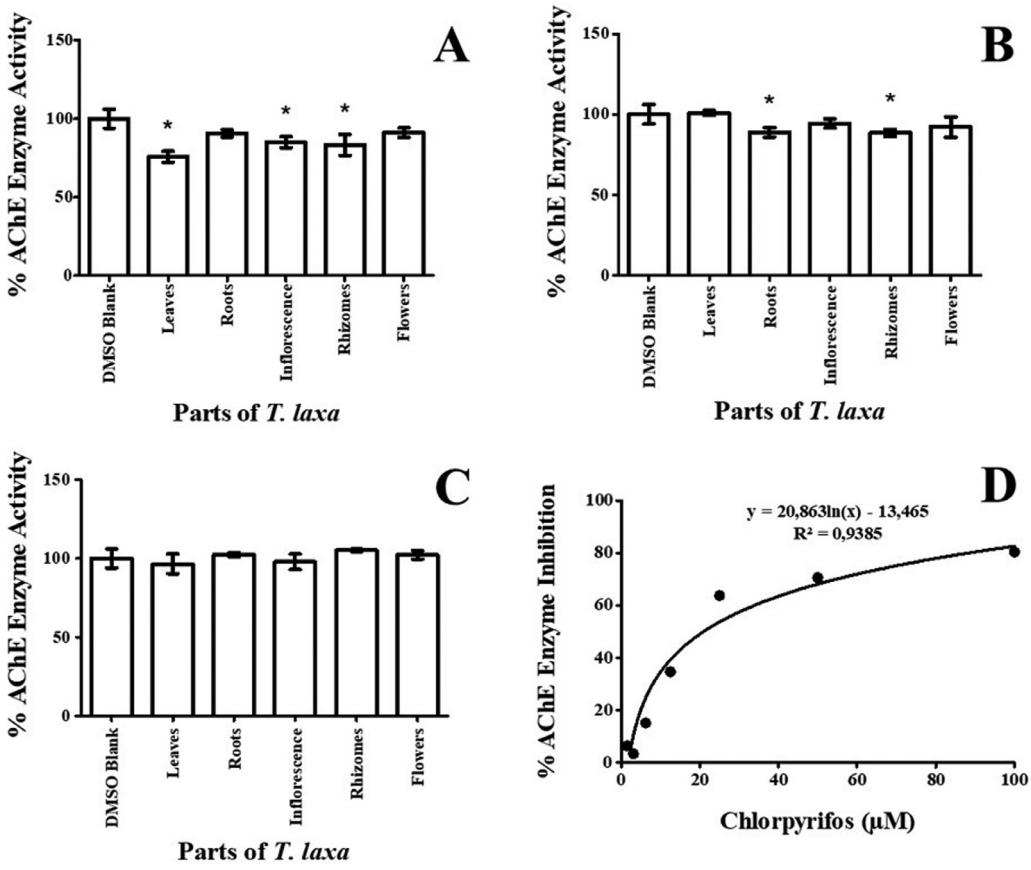

Fig. 2. Percentage AChE enzyme activity demonstrated by the extracts of the different parts of *Trachyandra laxa* var. *rigida* (leaves, roots, inflorescences, rhizomes, and flowers). (A) Dichloromethane (DCM) extracts. (B) Hexane extracts. (C) Methanol (MeOH) extracts. (D) A non-linear regression plot of chlorpyrifos inhibition and the percentage AChE enzyme inhibition. The DMSO blank contained all components, except the sample. Data is expressed as the% AChE enzyme activity ± standard deviation (SD). * indicates a significant ($P < 0.05$) difference compared to DMSO blank.

correlation (R^2 = 0.9385) between the concentration of chlorpyrifos and the percentage of AChE inhibition (Fig. 2D).

In a recent review of acetylcholinesterase inhibitors from southern African plants it was reported that for most plants the root and bulb extracts exhibit the highest AChE inhibitory activity (Masondo et al., 2019). Interestingly, in our study, the DCM extract of the leaves displayed the best AChE inhibitory activity, with an AChE inhibition of ca. 25% at an extract concentration of 0.05 mg/mL. One example of a plant of the Asphodelaceae family that exhibit AChE inhibitory activity, in addition to its anti-inflammatory and antioxidant properties, is *Aloe ferox* Mill. It was reported that the effective inhibition concentration (EC_{50}) of the 50% methanol, petroleum ether and DCM extracts of *A. ferox* leaves against AChE was 84.0, 37.7 and 62.6 μg/mL, respectively (Fawole et al., 2010).

3.2. Chemical composition

3.2.1. Phytochemical screening

Phytochemical screening was performed to detect the classes of compounds that are present in the different parts of the plant (Table 2). All the plant parts tested positive for polyphenols and tannins. Saponins were detected in all the plant parts except the flowers, while anthraquinones were found to be present in all the plant parts except for the roots. The leaves were the only plant part where flavonoids could be detected. The presence of anthraquinones have been reported to be a common characteristic of the Asphodelaceae family (previously known as the Xanthorrhoaceae family) (Chien et al., 2015). In addition, Wink and Van Wyk (Wink and van Wyk, 2008) reported that most saponins and anthraquinones are toxic as well as certain tannins. Significant acetylcholine esterase (AChE) inhibition was demonstrated for flavonols isolated from the methanol leaf extract of *Maytenus robusta* (Celastraceae) (Sousa et al., 2017) and flavonoids from the ethyl acetate extracts of whole plants of *Agrimonia pilosa ledeb* (Rosaceae) (Jung and Park, 2007). Alkaloids, anthranoids, cardenolides, terpenes and leucoanthocyanins were not detected in any plant parts.

Table 2
Classes of phytochemicals present in the different parts of *Trachyandra laxa (N.E.Br.) Oberm.* var. *rigida*.

Compounds classes	Plant parts				
	Leaves	Inflorescences	Rhizomes	Roots	Flowers
Alkaloids	–	–	–	–	–
Saponins	+++	+++	++	+++	–
Anthraquinones	+	++	++	–	+
Anthranoids	–	–	–	–	–
Cardenolides	–	–	–	–	–
Terpenes	–	–	–	–	–
Polyphenols	+	+	++	++	+++
Flavonoids	+	–	–	–	–
Leucoanthocyanins	–	–	–	–	–
Tannins	++	+	+++	++	+++

- not detected.
+ present in small quantities.
++ present in medium quantities.
+++ present in large quantities.

Table 3
Compounds identified in the DCM extracts of *Trachyandra laxa (N.E.Br.) Oberm.* var. *rigida* leaves and inflorescences.

Compound number[a]	t_R(min)	RI (exp)[b]	RI (lit)[c]	Compound	Identification methods[d]	Molecular formula	Relative quantity[e] (%)	
							Leaves	Inflorescences
1	16.11	1019	–	Branched C_{11} alkane	A	$C_{11}H_{24}$	2.0	1.3
2	21.23	1106	–	Unknown alcohol or alkene	A	–	5.5	7.3
3	48.30	1516	1525[f]	Dihydroactinidiolide	A, B	$C_{11}H_{16}O_2$	1.9	1.1
4	61.61	1751	1758[g]	Hydroxy dihydroactinidiolide	A, C	$C_{11}H_{16}O_3$	4.3	1.0
5	66.31	1841	–	Unknown alcohol or alkene	A	–	1.8	0.6
6	70.47	1927	1921	Methyl palmitate	A, B	$C_{17}H_{34}O_2$	6.8	0.1
7	72.82	1974	1973[h]	Palmitic acid	A, B	$C_{16}H_{32}O_2$	32.3	17.5
8	78.07	2090	2085	Methyl linoleate	A, B	$C_{19}H_{34}O_2$	2.4	0.1
9	78.30	2095	2098[i]	Methyl linolenate	A, B	$C_{19}H_{32}O_2$	4.3	0.1
10	79.03	2111	2111[h]	Phytol	A, B	$C_{20}H_{40}O$	2.3	0.3
11	80.42	2142	2143[j]	Linolenic acid	A, B	$C_{18}H_{30}O_2$	8.4	2.4
12	115.70	3102	3100	Hentriacontane	A, B	$C_{31}H_{64}$	1.6	27.3
13	121.32	3289	–	β-sitosterol	A, C	$C_{29}H_{50}O$	4.9	2.7

[a] Compounds are numbered in order of elution from the GC column.
[b] RI values determined experimentally on an HP-5 equivalent column.
[c] Kovátz RI values reported for authentic reference standards on an HP-5 equivalent column by Adams (Adams, 2007), except where other publications are cited.
[d] A: Comparison of mass spectrum with NIST MS database and published data, B: RI matches published value (within 10 RI units), C: mass spectrum interpretation.
[e] Estimated relative amounts of the compounds were calculated as percentages of the detected peaks in the TIC of the GC–MS analysis.
[f] RI value determined on an HP-5 equivalent column (identified by Pino et al. (Pino et al., 2005) based on comparison with literature MS and RI data only).
[g] Estimated value based on RI group contribution calculations (Stein et al., 2007).
[h] RI value reported for authentic reference standard on an HP-5 equivalent column (Maggi et al., 2009).
[i] RI value reported for authentic reference standard on an HP-5 equivalent column (Edris et al., 2008).
[j] RI value reported for authentic reference standard on an HP-5 equivalent column (Radulovic et al., 2010).

3.2.2. *Identification of the major volatile constituents in the DCM extracts of* T. laxa *plant material by gC–MS*

Based on the results of the acetylcholine inhibition assays, the DCM extracts of the leaves, inflorescences and rhizomes were considered for further investigation. However, since it is only the aerial parts of the plants that are consumed by livestock, the chemical composition of only the DCM extracts of the leaves and inflorescences were determined using GC–MS (Fig. S1). Thirteen compounds, were identified by comparing their mass spectra and RIs to those reported in the literature and those in the National Institute of Standards and Technology (NIST) 11 MS and RI databases (Table 3). In cases where the experimentally determined RIs of the compounds did not differ by more than 10 index units from those reported in the literature, the identity of such compounds were considered to be positively confirmed (provided that the authors reporting the values determined the RIs experimentally using authentic reference standards) (Bicchi et al., 2018). It was found that the extracts comprised a diverse mixture of compounds including α,β-unsaturated lactones, carboxylic acids and esters, a diterpenoid, phytol, and a phytosterol, β-sitosterol. Palmitic acid (**7**) and hentriacontane (**12**) are the major compounds in the DCM extracts of the leaves and inflorescence, respectively.

Compound **3** was tentatively identified as dihydroactinidiolide (DHA) (Fig. 3). This α,β-unsaturated lactone is commonly found in nature as a degradation product of carotenoids. When β-carotene is cleaved it gives rise to various compounds like β-ionone, 5,6-epoxy-β-ionone and DHA (Bosser et al., 1995). In a study conducted by Das *et al.* (Das et al., 2018) the anti-Alzheimer's disease potential of DHA was investigated and it was found that it is a potent AChE inhibitor with a IC_{50} value of 34.03 nM. In a study of the leaves of *Vallisneria. spiralis* (family Hydrocharitaceae), DHA was also found to be an active allelochemical which could strongly inhibit the growth of algae and other aquatic macrophytes (Xian et al., 2006).

The mass spectrum of compound **4** (Fig. S3) has a similar fragmentation pattern to that of compound **3** (Fig. S2), but with prominent ions at e.g. *m/z* 178 and *m/z* 135, instead of *m/z* 180 and *m/z* 137, respectively. If it is assumed that the ion observed at *m/z* 196, is the molecular ion, then the ion at *m/z* 178 is the $[M-H_2O]^{+\cdot}$ ion and hence it may be inferred that compound **4** is a hydroxylated analogue of DHA (**3**). The ion observed at *m/z* 163 will then correspond to a $[M-CH_3-H_2O]^+$ ion, while the base peak, observed at *m/z* 135, will then be formed by the loss of CO from the ion observed at *m/z* 163 (Chen et al., 1970). The RI of compound **4** was experimentally determined to be 1751. Using the group contribution method for the

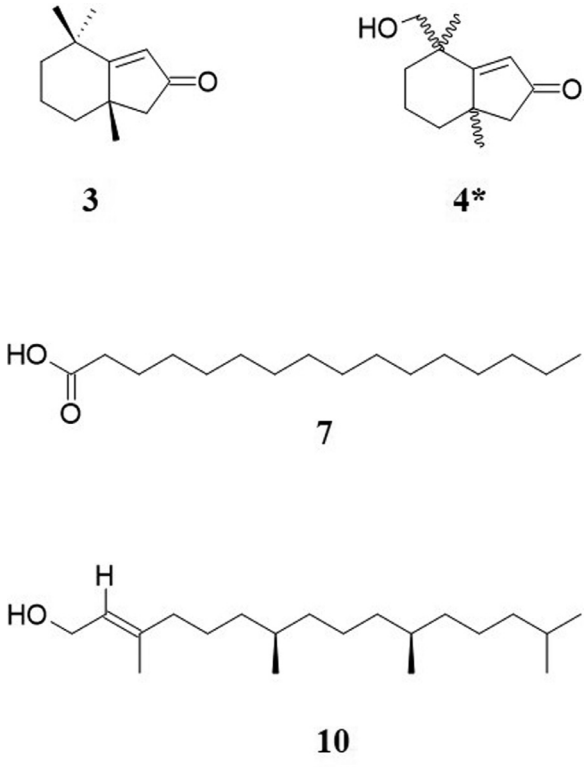

Fig. 3. Structures of the compounds identified in the DCM extracts of *Trachyandra laxa* var. *rigida* leaves and inflorescences that could contribute to the neurotoxicity of the plant (*one of the possible structures).

estimation of Kováts RIs (Stein et al., 2007), the experimental RI value of compound **4** can be explained by using the experimentally determined RI value of DHA (**3**) and subtracting the group increment of a methyl group (112 index units (iu)), and adding the group increments of a methylene group (99 iu) and a primary hydroxyl group (225 iu). This brings the estimated RI value to 1758, which is within 10 units of the experimentally determined value of compound **4**. This provides corroborating evidence that compound **4** is a hydroxylated analogue of DHA (**3**), and it also suggests that the hydroxyl group is located at a terminal position (Fig. 3). The compounds loliolide, DHA and actinidiolide are structurally similar C11-terpene lactones which arise from biological or oxidative degradation of carotenoids. Loliolide, a hydroxylated analogue of DHA with the hydroxyl group situated on the molecule's six membered ring, is found in a number of plants, including tobacco and tea, while DHA and actinidiolide have been identified as flavour compounds in tea and tobacco (Eidman and MacDougall, 2006). Interestingly, it has been reported that certain α,β-unsaturated sesquiterpene lactones are toxic to animals (Van Wyk et al., 2002). Although compounds **3** and **4** are not sesquiterpenoids, it is conceivable that they could contribute to the toxicity of *T. laxa*. due to the α,β-unsaturated lactone moeity in their structures.

Two other compounds that may also contribute to the neurotoxicity of the *T. laxa* extracts is phytol (**10**) and palmitic acid (**7**). Phytol (**10**) (Fig. 3) is reported to have a wide range of biological activities including anxiolytic, metabolism-modulating, cytotoxic, antioxidant, autophagy- and apoptosis-inducing, antinociceptive, anti-inflammatory, immune-modulating, and antimicrobial effects (Islam et al., 2018). In two recent studies, phytol was found to exhibit significant AChE and butyrylcholinesterase inhibitory potential (Sathya et al., 2020, 2018). The saturated fatty acid, palmitic acid (**7**) (Fig. 3), was implicated in neuronal loss through lipid-induced enteric nervous system neuropathy (Voss et al., 2013). No reports could be found of any potential neurotoxic effects of the remaining compounds identified in the *T. laxa* extracts (according to PubChem data). It is noteworthy that since the hexane extracts of the rhizomes and roots also exhibit inhibitory activity, the plant contains additional inhibitors with lower polarities than those identified here.

4. Conclusion

In this study, the AChE inhibition properties and chemical composition of *T. laxa* was investigated for the first time. Interestingly, only the DCM and hexane extracts of certain parts of the plant displayed significant AChE inhibitory activity. Qualitative phytochemical screen tests provided an overview of the classes of phytochemicals present in the plant. Subsequently, the detailed determination of the chemical composition of the DCM extracts of the aerial parts (leaves and inflorescences) of the plant by GC–MS led to the discovery that known AChE inhibitors, DHA (**3**) and phytol (**10**) are present in the extracts. These compounds are therefore expected to contribute to the AChE inhibitory activity of the DCM extracts and possibly the neurotoxicity of *T. laxa*. In subsequent studies, hydroxy-DHA (**4**) should be isolated for full characterisation and AChE inhibitory activity testing. In addition, the DCM and hexane extracts of the rhizomes, as well as the hexane extracts of the roots, which also exhibited AChE inhibition activity, should be chemically characterised. In particular, the hexane extracts are expected to contain non-polar inhibitors not found in the DCM extracts investigated in this study.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.sajb.2021.12.013.

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