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Temperature and water stresses on antioxidant activity of selected medicinal plants have implications for sustainable use and global warming

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ABSTRACT

Some traditional healers believe that herbal medicines derived from wild populations have higher activity than cultivated medicinal plants. This has important implications for the conservation of medicinal plants if traditional healers do not want to use cultivated plants. We have previously shown that water and temperature stresses have little effect on antimicrobial activity acetone leaf extracts of *Leonotis dysophylla* Benth., *Bulbine frutescens* (L.) Willd. and *Tulbaghia violacea* Harv. Antioxidant activity of plant extracts may be important in treating several different diseases. We have therefore investigated the effect of water and temperature stresses on the quantitative and qualitative antioxidant activity of these species in plants with limited genetic variation using 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and L-ascorbic acid as positive controls. Activity was measured against two free radicals, 2,2′-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and 2, 2-diphenyl-1-picryl hydrazyl (DPPH). All plant extracts had some antioxidant activity following 182 to 240 days of exposure to temperature (15 °C and 30 °C) and water (50–500 ml/pot every second day) treatments. The IC_{50} values across water treatments did not show any clear patterns, which suggests that water stress had little influence on the antioxidant activity. On the other hand, the IC_{50} values under high temperature of 30 °C were statistically significantly higher than those under the temperature of 15 °C, which indicates that high temperature led to a reduced antioxidant activity. The effect of fluctuating temperatures higher than 30 °C because of prevailing global warming is more likely to lower the antioxidant activity of many important medicinal plants in nature. The results also indicate that plants in nature do not necessarily have higher activity to address concerns of traditional healers. Using cultivated plants may affect the quality and/or safety of medicinal properties to be used to treat different illnesses. As such, the establishment of optimal growth regimes for *ex situ* cultivation for important and especially threatened medicinal plants is recommended. It may help yield consistent supply of quality and quantity of bioactive material for self-medication and commercialisation. Cultivation may also contribute to conservation of species biodiversity.

1. Introduction

The use of medicinal plants is important for the health care of most of the people in the world. They form the primary materia medica for 70–95 % of people in most developing countries and people in wealthy countries are increasingly using medicinal plants (Applequist et al., 2020). The unsustainable use of medicinal plants can lead to local extinction, especially with plants species that have high activity. In several communities the local leader understands the threat and some local traditional healers are sensitive to not to overexploit the plants. There is however a major problem with collectors from urban areas that collect these plants to serve the needs of people who have migrated to the cities. In such a case the main driving force is not sustaining the availability of the resource but rather in making a profit by selling these plants in markets. One possible solution is to cultivate the plants under good growing conditions that would lead to a high yield of plant material with reproducible biological activity.

Some traditional healers, however, believe that plants growing in nature have higher activity than cultivated plants and prefer to collect plants in nature for use (Keirungi and Fabricius, 2005). It is known that in some cases stress imposed by e.g. pathogens or

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herbivores leads to defence responses that changes the chemical composition of plants and may affect the medicinal properties. We have shown previously that water stress and temperature stress under controlled conditions did not lead to increased antibacterial activity against four important nocosomial bacteria of three widely used medicinal plants with limited genetic variation (Netshiluvhi and Eloff, 2016, 2019). These results indicate that with these three medicinal plants water or temperature stress did not lead to a change in antibacterial activity and that cultivating highly active medicinal plants is a good strategy that should not lead to lower antimicrobial activities. This is also in line with the call of international organizations that cultivation of medicinal plants could resolve the problem of local extinction by non-sustainable collecting. This approach has already been followed by major producers of medicinal plant products with guaranteed activity in Europe. Because plants are used for much more than just protecting against nosocomial bacteria, we decided to determine the antioxidant activity of plants growing under water and temperature stress conditions. Many diseases are correlated with the presence of free radicals in human and animal bodies. The oxidative damage caused by free radical has been implicated in several chronic human diseases such as diabetes mellitus, cancer, arthritis, and aging process (Patel et al., 2010). We therefore decided to investigate to what degree water and temperature stress influenced the antioxidant activity of plant extracts. We used the same plants and stress conditions as reported earlier (Netshiluvhi and Eloff, 2016, 2019).

The concern about climate change and its effect on the conservation, availability and efficacy of medicinal plants is an important matter that requires urgent attention (Applequist et al., 2020). This is so because water and temperature are the two factors that have a major effect on plant yield and productivity, which will be affected by global warming. These factors may also influence the growth survival and efficacy of medicinal plants. This aspect will also be discussed in this paper.

2. Materials and methods

2.1. Preparation of plant material

Three widely used medicinal plants *Tulbaghia violacea* Harv., *Bulbine frutescens* (L.) Willd. and *Leonotis dysophylla* Benth. were selected for this study. The selection criteria were fast growth, ethno-botanical use, known antimicrobial activities, availability, and ease of identification and limited genetic variation by using plant material from one plant or clump.

B. frutescens and *T. vioalcea* plantlets were generated through the division of mother clumps at the Vegetable and Ornamental Plant Institute (VOPI) of the Agricultural Research Council (ARC). Seeds of a single L. *dysophylla* plant were collected from the wild population at the Akasia Municipality located in the north of Pretoria. Voucher specimens of *T. violacea* (117,131), L. *dysophylla* (117,130) and *B. frutescens* (117,129) plants were prepared. Mrs Elsa van Wyk, the curator, verified the identity of and kept specimens in the H.G.W.J. Schweickerdt Herbarium situated at the University of Pretoria. Plantlets of *T. violacea* and *B. frutescens*, and seeds of L. *dysophylla*, were established and germinated in growth trays filled with vermiculite under similar room temperature (25 °C), light, and irrigation in the greenhouse before trial experiments began, respectively. Plantlets or seedlings of 10 to 15 cm height with at least two leaves were transplanted from growth trays into pots (27 cm diameter × 25 cm height, volume *c.* 14 L) filled with potting-mix described in Netshiluvhi (1999). Some plantlets and seedlings were subjected to different watering regimes of 50, 100, 200, and 500 ml of distilled water per pot that was given every second day (Netshiluvhi and Eloff, 2016). Others were subjected to temperature regimes of 15 and 30 °C in the growth chambers (Netshiluvhi and Eloff, 2019). Such temperatures were selected on the assumption that they might induce stress without killing the plant. Growth chambers fitted with 24 fluorescent (215 W) and incandescent (60 W) bulbs were set to provide 12 h of light and 12 h of darkness. Seedlings under temperature treatments were watered with 500 ml distilled water per pot every second day before midday.

After 182–240 days of growth, recently mature leaves from vegetative and flowering plants were harvested, air-dried, and finely ground before extraction. A known mass of each of the powdered material was then extracted with ten volumes of acetone at room temperature for 24 h and filtered. Acetone was used as an extractant because it has been found to extract large quantities of bioactive plant material (Eloff, 1998). The extracts obtained were concentrated under vacuum at 40 °C using a rotary evaporator (Buchi®, Switzerland) to give the crude extracts of each plant material. The dry extracts were stored in sealed vials in the refrigerator prior to evaluation of antioxidant activity.

2.2. Growth and application of stress

The methods used were the same as those applied in Netshiluvhi and Eloff (2016, 2019).

2.3. Chemicals

Chemicals used were l-ascorbic acid (Merck), potassium persulphate (Sigma), 2,2′-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Sigma), 6-hydroxy-2, 5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®) (Fluka), 2,2-diphenyl-1-picryl hydrazyl (DPPH) (Sigma) and absolute ethanol (Merck).

2.4. Qualitative and quantitative determination of antioxidant activity

Qualitative screening for antioxidant activity was determined by using 2, 2-diphenyl-1-picryl hydrazyl (DPPH) (Takao et al. (1994) as a spray reagent after TLC of extracts. The DPPH (0.2 %) in methanol was sprayed on the thin layer chromatograms (TLC) of extracts developed in EMW (ethyl acetate/methanol/water (10/1.35 /1) solvent system. Antioxidant activity was detected on the chromatograms when the initially purple DPPH background turns yellow in bands where an antioxidant compound is present.

Quantification of antioxidant activity (AOXA) was determined spectrophotometrically using two radicals, ABTS and DPPH and a Versa-max® microplate reader (Labotec). In one method, use was made of the Trolox equivalent antioxidant capacity (TEAC) assay based on the scavenging of the ABTS radical into a colourless product (Re et al., 1999). The absorbance was read at 734 nm. Trolox (6-hydroxy-2, 5,7,8-tetramethylchromane-2-carboxylic acid) is a Vitamin-E analogue. If an extract had antioxidant activity equivalent to Trolox, its TEAC value would be 1 and if the extract were more active its TEAC would be greater than 1.

The second method employed the DPPH free radical assay (Mensor et al., 2001). Different concentrations of the extracts were prepared between 1.0 and 200 μg/ml. A volume of 10 μL of 0.4 mM DPPH in ethanol was added to 25 μL of each concentration of extract tested and allowed to react at room temperature in the dark for 30 min. Blank solutions were prepared with each test sample solution (25 μL) and 10 μL ethanol only while the negative control was DPPH solution, 10 μL plus 25 μL ethanol. l-ascorbic acid was the positive control. The decrease in absorbance was measured at 518 nm. Values obtained were converted to percentage antioxidant activity (AOXA%) using the formula: -

$$AOXA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\}$$

Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and $Abs_{control}$ is the absorbance of the control.

L-ascorbic acid (vitamin C) was used as a positive control (antioxidant agent). The antioxidant activity is expressed as inhibitory concentration (IC_{50}) values. The lower the IC_{50} value the more effective

antioxidant activity. The IC_{50} value, defined as the concentration of the sample leading to 50 % reduction of the initial DPPH concentration, was calculated from the linear regression of plots of concentration of the test extracts (μg/mL) against the mean percentage of the antioxidant activity obtained from three replicate assays. The IC_{50} is half maximal (50 %) inhibitory concentration (IC) of a substance. For statistical analysis, the results were expressed as mean ± SEM (standard error of mean) and the IC_{50} values obtained from the linear regression of plots of concentration of the test compounds (μM) against the mean percentage of the antioxidant obtained from the three replicate assays. Such plots show a good coefficient of determination, with most values being $r^2 \geq 0.910$ (SigmaPlots^R 2001, SPSS Science).

2.5. Statistical analysis

Data were statistically analysed using GenStat ® for Windows ® (2003) and SA® PROC GLM. The results for antioxidant activity of all plant species were reported as means ± standard error (SE). Significant differences for comparisons were determined by a one-way analysis of variance (ANOVA) procedure. The results with 5 % level of confidence ($P \leq 0.05$) were regarded as statistically significant.

3. Results and discussion

The results with the qualitative determination did not show convincing differences in terms of antioxidant compounds and related activity (data not shown). Even the most polar solvent system used (EMW) did not separate well the different antioxidant compounds present in extracts of all three species. In the case of *T. violaceae*, the extract from water treatment of 500 ml every second day per 14 pots did not show an antioxidant compound when separated with EMW while other treatments (50, 100, and 200 ml) had. We could not therefore determine if the water or temperature (15 and 30 °C) treatments led to a change in the composition of the antioxidant compounds in any of the extracts. It appears that the antioxidants present in these species, must be polyphenolic or tannin-like compounds that adhere very strongly to silica gel on the TLC plates.

The DPPH free radical assays of acetone leaf extracts under temperature and water treatments were determined, and the quantitative antioxidant results are presented in Table 1. The IC_{50} values based on water (0.10–0.64 mg/ml) and temperature (0.03–0.42 mg/ml) treatments across different plant extracts were considerable, but much higher than those of positive controls (Trolox [0.002 mg/ml] and ascorbic acid [0.004 mg/ml]). The tested plant species had by far much lower antioxidant capacity than of the positive controls, which is to be expected seeing that the extracts contained many compounds without antioxidant activity.

The aim of the study was to see how different environmental conditions (water and temperature) influence the antioxidant activity of plant extracts to address the contention of traditional healers that cultivated plants are not as active as plants collected in nature. It is well-known that stress can affect the composition of metabolites in plants. Our first task was to determine if plants under water stress were more active than plants growing under good agricultural conditions. The IC_{50} values across treatments in Table 1 showed that water stress did not have a statistically significant effect on antioxidant activity of L. *dysophylla* and *B. frutescens* except for *T. violacea* where water stress led to a decreased antioxidant activity. This finding corroborates other studies based on different plant species (Albergaria et al., 2020; Payment and Cvetkovska, 2023; Unal and Okatan, 2023). A reduction in the antioxidant activity of *T. violacea* might have been attributed to a long-term exposure to water stress as was the case in Morteza (2012). Our second task was to determine if plants under temperature stress were more active than those that were not. The IC_{50} values of plants grown at 30 °C of all plant extracts were significantly higher ($P \leq 0.05$) than those of similar plants grown at 15 °C (Table 1). This means that extracts of the tested plant species under high temperature of 30 °C had lower antioxidant activity than that of similar plant species under low temperature of 15 °C. This finding too is consistent with other studies where temperature stress (at 25–39 °C) reduced the accumulation of some bioactive compounds of certain plant species (Islam et al., 2021; Payment and Cvetkovska, 2023; Punetha et al., 2022). The enhanced activities of antioxidant enzymes of plants affected by temperature stress are usually not adequate for stress tolerance in plants, especially in susceptible genotypes (Hasanuzzaman et al., 2013). The reduced antioxidant activity seems to correlate with poor tolerance to temperature stress in plants. Apart from the influence on (antioxidant) phytochemical compounds, high temperature of 30 °C had also significantly reduced dry mass, leaf area and stomatal conductance of the tested plants, especially L. *dysophylla* and *T. violacea* (Netshiluvhi and Eloff, 2019; Table 1). Similarly, stomatal conductance, leaf area, and dry weight (aboveground biomass) of *Artemisia sieberi alba* that grew under heat stress (high temperature) for about 10 days were also significantly reduced (Alhaithloul, 2019). Like other environmental stresses, high temperature adversely affects growth, development, and productivity (biomass) of plants. The reduced biomass of the harvestable products of medicinal plants for self-medication or income generation may result in economic harm and increased unsustainable harvest levels (Applequist et al., 2020). Our results indicate that it is not only the lower biomass produced but also the antioxidant activity per dry mass of plant material that plays a role. Biomass of *B. frutescens* was however not affected by the temperature stress (Table 1) as it thrived well under high temperature treatment probably because it is one of crassulacean acid metabolism (CAM) plants that are adapted to arid conditions and high temperatures(Drennan and Nobel, 2000).

With most living organisms a temperature of 30 °C would not lead to a decrease in enzyme activity. The effect of temperature on antioxidant activity cannot easily be ascribed to inhibiting enzyme activity but may be due to a cumulative effect. The effect of temperatures higher than 30 °C because of prevailing global warming may lower the antioxidant activity of many important medicinal plants in nature. This may possibly affect the biomass production and activity or chemical content of medicinal plants to be used in the fight against

Table 1
Effects of temperature and water (volume/pot/week) treatments on dry leaf biomass and DPPH radical scavenging activity of acetone leaf extracts of plant species. Values (means ± standard error; $n = 4$) with different (a & b) and similar (a & a or b & b) superscripts in the same column at 5 % confidence level ($P \leq 0.05$) are significantly and not significantly different, respectively.

Plant species	Treatments	Dry leaf biomass (g)	Antioxidant activity (IC_{50} in mg/ml)
L. *dysophylla*	Water 500 ml	142 ± 43^a	0.10 ± 0.02^a
	Water 200 ml	97 ± 27^b	0.12 ± 0.05^a
	Water 100 ml	95 ± 38^b	0.14 ± 0.06^a
	Water 50 ml	77 ± 18^b	0.12 ± 0.06^a
	Temp 15 °C	74 ± 33^a	0.03 ± 0.01^a
	Temp 30 °C	52 ± 27^b	0.13 ± 0.04^b
T. violacea	Water 500 ml	185 ± 38^a	0.24 ± 0.08^a
	Water 200 ml	164 ± 40^a	0.38 ± 0.29^a
	Water 100 ml	84 ± 16^b	0.76 ± 0.31^b
	Water 50 ml	86 ± 41^b	0.64 ± 0.31^b
	Temp 15 °C	164 ± 40^a	0.24 ± 0.01^a
	Temp 30 °C	83 ± 18^b	0.42 ± 0.14^b
B. frutescens	Water 500 ml	1618 ± 272^a	0.19 ± 0.01^a
	Water 200 ml	930 ± 51^b	0.18 ± 0.05^a
	Water 100 ml	586 ± 74^c	0.20 ± 0.05^a
	Water 50 ml	333 ± 121^d	0.25 ± 0.06^a
	Temp 15 °C	549 ± 37^a	0.19 ± 0.12^a
	Temp 30 °C	711 ± 74^b	0.34 ± 0.22^b

Positive controls: Trolox = 0.002 mg/ml; Ascorbic acid = 0.004 mg/ml.

oxidative stress (free radicals and reactive oxygen species [ROS]), and onset of chronic diseases such as neurodegenerative and cardiovascular diseases, hypertension, hyperglycaemia, obesity, diabetes, and cancer.

Cultivation will ensure conservation of species biodiversity and prevent possible misidentification in nature. Genetically engineered medicinal plants with desirable traits, growing under good agricultural conditions, may lead to larger biomass quantities, possibly at lower costs and have better proof of activity as used by large companies in the herbal medicinal market.

4. Conclusion

These results do not support the general perception of some traditional healers. Many different factors may influence the effect of water or temperature stress. It would therefore be presumptuous to state that water stress does not influence antioxidant activity of all plants. At least in this study, plants under water or temperature stress did not have a higher antioxidant activity.

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.

CRediT authorship contribution statement

Thiambi R. Netshiluvhi: Writing – original draft, Visualization, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jacobus N. Eloff:** Writing – review & editing, Validation, Supervision, Software, Resources, Methodology, Funding acquisition.

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