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Accumulation of phytochemicals at different growth stages of *Cleome gynandra* grown under greenhouse and microplot conditions

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ABSTRACT

Phytochemicals are bioactive non-nutrient plant compounds that accumulate in response to environmental changes and possess medicinal properties. The concentration of these useful phytochemicals is well associated with the stage of the crop and also the management practices adopted. Therefore, a greenhouse and open field microplot experiments were conducted to investigate the accumulation of phytochemicals at different growth stages of *Cleome gynandra*. Seven treatments constituting fifth leaf stage (control), vegetative, flower-bud, flowering, pod initiation, pod filling and physiological maturity stages were arranged in RCBD, with 10 replications. Young leaves and tender shoots were harvested weekly and then subjected to phytochemical analysis. Data on antioxidant activity (AA), total phenolics (TP), total flavonoids (TF) and proanthocyanidins (PAs) were determined prior to analysis of variance through SAS Software. Under greenhouse conditions, relative to the control, accumulation of AA and PAs was the highest (66.84 mg GAE/g and 18.62% DM) at flower-budding stage, whereas the lowest (39.63 mg GAE/g and 2.26% DM) was observed at pod initiation stage, respectively. No significant ($P \leq 0.05$) effect on TP and TF contents were observed. Under microplot conditions, the highest (58.02 mg GAE/g) AA was observed during flower-budding stage and the lowest (–30.14 mg GAE/g) was observed at physiological maturity. In contrast, the accumulation of TP and TF was the highest (20.23 mg GAE/g and 8.11 mg QE/g) during flower-budding stage, whereas the lowest (3.18 mg GAE/g and 0.93 mg QE/g) was observed at pod initiation stage. However, no significant ($P \leq 0.05$) effect was observed on PAs. In conclusion, the phytochemicals evaluated in *C. gynandra* similarly had the highest accumulation at the flower budding stage under greenhouse and open field microplot conditions, however, they started declining at pod initiation stage towards physiological maturity.

Key words : African leafy vegetables, bitterness, defence mechanism, secondary metabolites, spider plant

INTRODUCTION

Traditional edible plants are a significant source of both health and nutritional care known from ancient days to humankind (Barolo *et al.*, 2014; Rinchen *et al.*, 2019; Das *et al.*, 2021). The use of most traditional edible plants had been prevalent in most cultures in the world throughout history and continues to play a significant role in food and nutritional security. It was reported that human populations of disadvantaged rural communities in African countries depends solely on indigenous vegetables for income generation, basic foods and nutritional needs, reducing malnutrition and maintaining biodiversity (Winston, 2019). Certain non-nutrient bioactive phytochemicals that are linked to health protection against cardiovascular and other degenerative diseases, were found to be contained in African leafy vegetables (ALVs) (Kwenin *et al.,* 2011).

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Phytochemicals are bioactive non-nutrient plant compounds in plant foods that have been assumed to reduce the risk of major chronic diseases (Liu, 2004). Generally, ALVs have special groups of phytochemicals with different structures that have similar functions. Among other phytochemicals, there are those that protect plants from environmental stresses using plant defensive metabolites such as phytoalexins, biosynthesised to respond to biotic and abiotic stresses (Schreiner and Huyskens-Keil, 2006).

In plants, phytochemicals accumulate in response to environmental changes and attract beneficial organisms, while repelling harmful organisms (Bala *et al.*, 2010). In leafy vegetables and fruits, phytochemicals have several roles, acting as antioxidant, interfering with DNA replication, destroys bacteria and reduces the development of cancer and heart diseases (Yahia *et al.*, 2019). The accumulations of phytochemicals in vegetable extracts harvested at different growing stages can be due to physiological changes of the plant during growth in response to the plant interaction with the environmental stresses. Genotype along with growth and management conditions influence the concentrations of phytochemicals in plants. Generally, high light intensity, longer days and dry conditions in spring/summer seasons have the highest phytochemicals concentrations especially in plants grown under field conditions (Bian *et al.,* 2015). According to Gololo (2018), under greenhouse conditions, several factors, such as temperature, radiation and precipitation, have an impact on the accumulation of phytochemicals in plants. For instance, light exposure, especially ultraviolet-B rays, can trigger an increment in phytochemicals (Escobar-Bravo *et al.*, 2017). Therefore, growing plants under controlled conditions such as greenhouses, which blocks ultraviolet light, have proved to reduce concentrations of the phytochemicals in plants. However, plants grown outdoors contain more phytochemicals than those cultivated under controlled conditions (Alarcón-Flores *et al.,* 2013). Again, geographical location factors such as altitudes, soil types, seasonal variations and exposure to pollution affect the phytochemical compositions under which plants grow (Liu *et al.*, 2015).

Cleome gynandra L., also known as cat's whiskers (English), lerotho (Sepedi), murudi (Venda) and snotterbelletjie (Afrikaans) is an erect leafy vegetable crop from the Capparaceae family (Van Rensburg *et al.*, 2007). *Cleome gynandra* leaves are harvested biweekly mainly for household consumption to allow for regeneration of new shoots and leaves. Frequent harvesting triggers the response of phytochemicals in the harvested leafy vegetable as the antioxidant serve as a defence mechanism against herbivores, pest and insect attacks (Goyal *et al.*, 2012). Harvesting of tender shoots and young leaves by local communities normally begins from 4-6 weeks after seedling emergence and may last up to six weeks till physiological maturity stage, without empirical knowledge of the accumulation of phytochemicals at various growth stages of the leafy vegetable (Kasolo *et al.*, 2018).

However, *C. gynandra* is normally harvested frequently depending on new shoots and leaf development at various growth stages, which triggers the accumulation of phytochemicals. Growing the leafy vegetable under various growth conditions and harvesting it at different growth stages, would be necessary in providing empirical evidence about their minimal or peak concentration of phytochemicals. Therefore, the objective of this study was to determine whether the concentration of phytochemicals in *C. gynandra* grown under greenhouse and microplot conditions and harvested at different growth stages will be similar.

MATERIALS AND METHODS

Description of the Study Area

Parallel studies were conducted at the Green Biotechnologies Research Centre of Excellence (GBRCE), University of Limpopo (23°53′10″S, 29°44′15″E), South Africa during summer (December 2019 to April 2020) and repeated in 2020-2021 at similar seasons, under greenhouse and microplot field conditions. Greenhouse conditions comprised ambient day/night temperatures averaged at 28/21°C, with maximum temperatures controlled using thermostatically-activated fans on the north-facing wall and wet wall on the south-facing side. Microplot open field

conditions site had Hutton soil (65% sand, 30% clay, 5% silt) with 1.6% organic carbon, EC 0.148 dS/m and pH (H_2O) 6.5, with summer rainfall consisting of mean annual rainfall of less than 500 mm, while maximum/minimum temperature averaging 38/19°C.

Treatments and Research Design

Each of the two (2) trials, had seven treatments, namely, 0, 1, 2, 3, 4, 5 and 6 weeks of harvesting times of tender shoots and young leaves, which represented growth stages, namely, seedling (fifth leaf), vegetative, flower-bud, flowering, pod initiation, pod filling and physiological maturity stages, respectively. The fifth leaf stage represented the control. All treatments were arranged in a randomised complete block design (RCBD), with 10 replications (n = 70).

Research Procedures

Steam pasteurised loam soil and compost with nutritional status of 2.5:0.5:2 (N:P:K) at 3:1 (v/v) ratio was used as growing media for the greenhouse and microplot trials.

Greenhouse trial

Pre-chilled *C. gynandra* seeds (Ramphele *et al.*, 2020) were planted in 30 cm diameter plastic pots filled with the prepared growing media mixture. Each pot contained four (4) seeds and were irrigated to full capacity with chlorine-free water at planting. Subsequent irrigation was done using a 500 mL plastic beaker daily. The pots were arranged on greenhouse benches at 0.35 m inter- and intra-row spacing. Manual weeding and pest control were done when necessary following guidelines from *Cleome* production in South Africa (DAFF, 2010).

Microplot Trial

Similar procedures as in the greenhouse trial were used, except that planting was prepared by digging holes befitting 30 cm diameter plastic pots at 0.5 inter- and intra-row spacing.

Plant Extractions

At fifth leave stage, young leaves and tender shoots were harvested and dried at 60°C for 24 hours prior to grinding in a Wiley-mill grinding machine (MF10 Basic, IKA WERKE, USA) to pass through a 1-mm-pore sieve (Makkar, 2000). The procedure was repeated for all treatments (growth stages) for antioxidant activity (AA), total phenolic (TP) content, total flavonoids (TF) content and Proanthocyanidins (PAs).

Chemical Analysis and Data Collection

Quantitative antioxidant activity (AA) assay was quantified using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay as described by Shimada et al. (1992). The AA results were presented as mg of GAE/g of the sample. Total phenolic content was determined using the Folin-Ciocalteu method (Kermiche et al., 2018) and the results were presented as mg of GAE/g of the sample. Total flavonoids content was determined using the aluminium chloride colorimetric assay (Shraim et al., 2021) and the results were expressed as mg of QE/g of extract. Proanthocyanidins content was determined using the butanol–HCl assay as described by Makkar (2000) with modifications. The PAs results were expressed as % DM of extract.

Data Analysis

Data was subjected to analysis of variance (ANOVA) through the Statistical Analysis System software (SAS) version 10.0). When the treatments were significant at the probability level of 5%, the associated mean sum of squares were partitioned to determine the percentage contribution of sources of variation to the total treatment variation (TTV) among the means. Mean separation was achieved using Waller-Duncan Multiple Range Test ($P \leq 0.05$). The variable with significant ($P \leq 0.05$) treatment means were further subjected to lines of the best fit. Unless otherwise stated, only treatment means significant at 5% level of probability were discussed.

RESULTS AND DISCUSSION

Greenhouse Trial

Treatments (plant growth stages) had significant ($P \leq 0.05$) effect on AA and PAs,

contributing 65% and 57% to TTV, respectively. There was no significant (P≤0.05) effect on TP and TF content (data not shown). Relative to the control, accumulation of AA in plant extracts was the highest (66.84 mg GAE/g) at flower budding stage and the lowest (39.63 mg GAE/g) accumulation was observed at pod initiation stage, however the latter was not significantly different from flowering, pod filling, physiological maturity as well as the control (Fig. 1).

Proanthocyanidins accumulation was the highest (18.62% DM) at flower budding stage, but the accumulation was not significantly different from the PAs at vegetative (14.87% DM) and flowering stage (14.20% DM). The lowest (2.26% DM) PAs was observed at pod initiation stage, but the accumulation was not significantly different from the PAs at pod filling (12.70% DM) and physiological maturity stage (9.84% DM) as well as the control (Figure 1). Both AA and PAs over different growth stages exhibited strong positive quadratic relationships with the models explained by 90% and 92%, respectively (Fig. 1).

Microplot Trial

Treatments had high significant (P≤0.01) effect on AA and TP content contributing 71% and 70% to TTV, respectively, whereas treatments had significant (P≤0.05) effect on TF content contributing 63% to TTV. However, no significant (P≤0.05) effect was observed on PAs (data not shown). Relative to the control, the highest (58.02 mg GAE/g) AA was observed during flower-budding stage, but was not significantly different from the accumulation at the control (37.63 mg GAE/g), vegetative (36.50 mg GAE/g) and flowering stage (51.31 mg GAE/g). The lowest (–30.14 mg GAE/g) AA was observed at physiological maturity, but the accumulation was not significantly different from the AA at pod initiation (0.65 mg GAE/g) and pod filling stage (2.20 mg GAE/g) (Fig. 2). The accumulation of TP was the highest (20.23 mg GAE/g) during flower-budding stage, but the accumulation was not significantly different from the one at flowering (17.32 mg GAE/g) and physiological maturity (13.94 mg GAE/g). The lowest (3.18 mg GAE/g) accumulation of TP, relative to the control was observed at pod initiation stage, but the accumulation was significantly different from that at pod filling (4.96 mg GAE/g) and vegetative stages (9.83 mg GAE/g) as well as the control (Fig. 2).

Flower-budding stage had the highest accumulation of TF content (8.11 mg QE/g), however the accumulation was not significantly different from the one at vegetative (7.71 mg QE/g) and flowering stage (5.94 mg QE/g) as well as the control. The lowest (–0.93 mg QE/g) accumulation was observed at pod initiation and physiological maturity stage, however the accumulation was not significantly different from the TF at pod filling stage (–0.64 mg QE/g) (Fig. 2). Antioxidant activity, total flavonoids content and total phenolic content over different growth stages exhibited positive quadratic relations. The relationship model was explained by 98, 78 and 85%, respectively (Fig. 2).

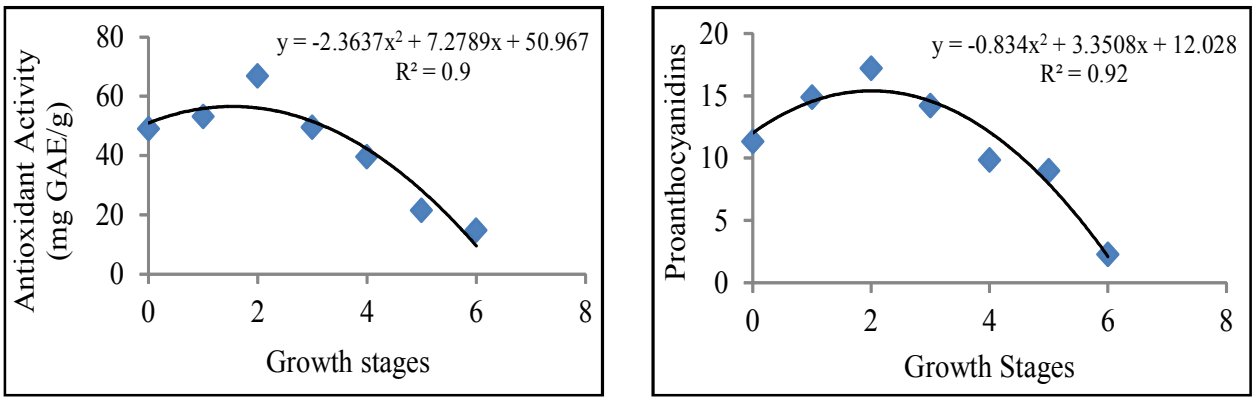

Fig. 1. Quadratic relationships of antioxidant activity (AA) and proanthocyanidins (PAs) over different growth stages of *Cleome gynandra* under greenhouse growth conditions (n = 70), where 0 = fifth leaf stage, 1 = vegetative stage, 2 = flower budding stage, 3 = flowering stage, 4 = pod initiation stage, 5 = pod filling stage and 6 = physiological maturity stage.

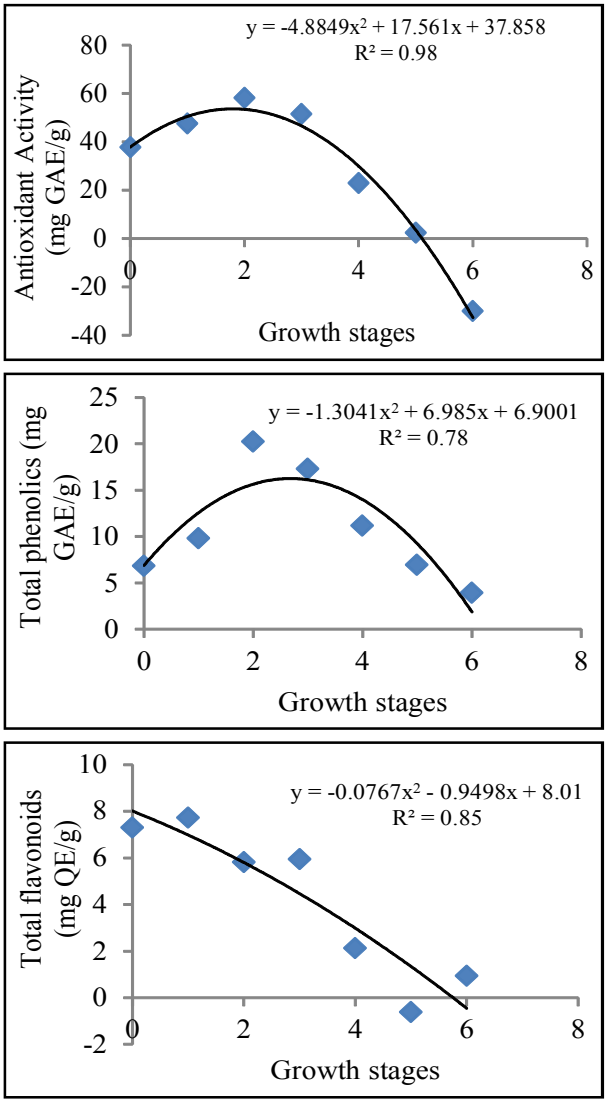

Fig. 2. Quadratic relationships of antioxidant activity (AA), total flavonoids (TF) content and total phenolics (TP) content to different growth stages of *Cleome gynandra* under microplot growth conditions (n = 70), where 0 = fifth leaf stage, 1 = vegetative stage, 2 = flower budding stage, 3 = flowering stage, 4 = pod initiation stage, 5 = pod filling stage, 6 = physiological maturity stage.

Effect of Growth Stages on Antioxidant Activity of *Cleome Gynandra* Grown Under Greenhouse and Microplot Conditions

Antioxidant activity (AA) of C. gynandra plant extracts was determined using DPPH radical scavenging assay. The assay is based on hydrogen/electron transfer from a given antioxidant to DPPH. The DPPH has a strong absorption band at λ_{max} = 517 nm with deep purple colour, whereas the redacted product is yellowish without any absorption band (Lu et al., 2017). Previous studies have established the abundance of antioxidants in C. gynandra plant leaves and that there was a general trend towards increased AA with increased TP content (Khandaker et al., 2008). In this current study, observations revealed that accumulations of AA of C. gynandra under greenhouse and microplot growth conditions depended on the growth stage at which the plant was harvested. The results from AA of the plant extracts of C. gynandra from both greenhouse and microplot conditions revealed that the radical scavenging of the material was the highest at the vegetative stage and the lowest at physiological maturity. The antioxidant potential of the extract established that extracts at flower budding stage have the ability to scavenge free radical at different concentrations providing scientific credence for its therapeutic usage in folklore medicine as reported by Sowunmi and Afolayan (2015). Karamac *et al.* (2019) also reported that AA increases with plant maturity. It has been assumed that AA tends to vary in response to phenolic changes. Several studies have reported that an increase of phytochemical content results in an increase of AA (Proteggente *et al.*, 2002; Scalzo *et al.*, 2005). A study by Stangeland et al. (2009) reported that high levels of AA of C. gynandra were attributed to the significant interaction of the plant age and the growth temperature of the plant. According to Smirnoff and Wheeler (2000), antioxidants are responsible for photoprotection and provide resistance to environmental stresses. In this study, higher AA was observed under controlled environment during the early stages of C. gynandra growth.

Effect of Growth Stages in Proanthocyanidins of *Cleome Gynandra* Grown Under Greenhouse and Microplot Conditions

Growth stages of *C. gynandra* influenced PAs concentration significantly. The accumulation of PAs at various stages of *C. gynandra* was the highest in the plant extracts at the vegetative (14.87% DM), flower budding (22.62% DM) and flowering stage (14.20% DM) under greenhouse growth conditions, whereas under microplot conditions, in all the tested growth stages, the accumulation was similar statistically.

According to Mbaebie *et al.* (2012), high concentration of PAs serves as a potential source of bioactive components in the treatment and prevention of cancer and other radical related ailments. Generally, PAs, commonly known as condensed tannins contribute to the bitter flavour and astringency in many crops and are widely distributed in foods of plant origin (Ramphele *et al.*, 2020). In *C. gynandra* shoots and leaves, PAs concentration levels tend to accumulate with the age of the plant as it grows, and reduction commences when the plant's photosynthetic rate decreases due to aging of the plant. A study conducted under greenhouse conditions demonstrated that the PAs concentration in tender leaves and shoots of *C. gynandra* was observed to increase with harvesting time (vegetative stage), until a saturation level was reached at 10 weeks (flowering stage) and then, the concentration level started declining with physiological maturity (Ramphele *et al.*, 2020). In the current study, similar observations were made under greenhouse conditions. Zhang *et al.* (2009) has observed a decline in PAs with advances in the age of the leaves. The authors reported higher levels of PAs in young leaves than in matured leaves in their study, which could be explained by the fact that the primary role of tannins in plant material is defending against herbivory (Heil *et al.*, 2002). Higher levels of PAs in early growth of the plant confers protection because defoliation at a young age would likely tend to be more destructive than at a later growth stage and this is, therefore, a nature's way of the plant to ensure continuation of its life cycle (Ncube *et al.*, 2015).

Effect of Growth Stages on Total Phenolic Content of *Cleome gynandra* Grown Under Microplot Growing Conditions

Growth stages influenced TP of *C. gynandra* significantly. Highest significant increase when compared to fifth leaf stage was observed at bud initiation stage (20.23 mg GAE/g) and flowering stage (17.32 mg GAE/g). Under open field microplot conditions, plants are exposed to multiple biotic stress such as insect attack and pathogen infection and abiotic stresses such as light, temperature, water availability, nutrient supplies and UV radiation. These factors affect the accumulations of TP in ALVs (Mazid *et al.*, 2011). An increase of TP content in plants can be explained by aging of the plant and effects of elevated temperatures, day length and the season in which the plant is grown. Ramani and Jayabaskaran (2008) findings, assumed that phytochemicals tend to vary in amount and content depending on the age of the plant. Study done by Kirigia *et al.* (2019) on nightshade showed that TP increased with plant age and the authors concluded that it was irrespective to the growing environment. In contrast, both plant growing stage and the environment of the growing plant; play a significant role in accumulation TP content. The findings of this study showed an increase of TP content as the plant grows and declined at reproduction stage. Jorgensen (1994) also, reported that light and temperature plays a significant role in biosynthesis of total phenolics. A decrease in TP could be attributed to the fact that plant metabolism is minimised by concentrating and retrieving metabolites from dying leaves and transporting them to seeding or fruiting organs (Waterman and Mole, 1994).

Effect of Growth Stages on Total Flavonoids Content of *Cleome gynandra* Grown Under Microplot Growing Conditions

Total flavonoids were also influenced significantly by *C. gynandra* growth stages in this study. At different growth stages, TF were highest from vegetative stage (7.71 mg QE/g) to flowering stage (8.11 mg QE/g), showing no significant differences. However, a significant decline was observed as from pod initiation, pod filling and physiological maturity stage. The growing stages with a significant decline occurred after flowering towards the reproduction stages of the plant. Riipi *et al.* (2002) reported that flowering stage is the stage in plants whereby differentiation dominates over the synthesis of phytochemicals. The higher reduction for TF in older leaves is often associated with a greater accumulation of metabolites for recycling, to protect the plant from adverse conditions such as solar radiation effects and they are more needed in development stages for degeneration of tissue cell processes (Grace and Logan, 2000). Moreover, with this study, flavonoids were highly produced in the early stages of growth in large concentrations. This was also

supported by Gobbo-Neto *et al.* (2017) findings and it was also emphasised that as a natural process of plant development, leaves ages and phytochemicals get diluted (Hendriks *et al.*, 1996). Also, an observed decrease from flowering stage to physiological maturity can again be explained by resource allocation for flowering protection and development of new branches (Gobbo-Neto and Lopes, 2007). Study conducted by Azeez *et al.* (2017) reported high contents of phytochemical at pre-flowering stage. Jimoh *et al.* (2019) emphasised that the chemical composition of the plant decreases as the plant approaches reproductive stage.

CONCLUSION

Results from this study suggest that environmental conditions and growth stages affect phytochemicals accumulation in *C. gynandra* ALV. All the phytochemicals evaluated were highest, mainly at the pre-flowering stage when flower buds were initiated, which is the bud initiation stage. This occurred as a response of the leafy vegetable to protect itself during this crucial stage of its life cycle in preparation to seed bearing for the next generation of plants. Antioxidant activity of *C. gynandra* for both greenhouse and microplot growing conditions were highest when compared to TP, TF and PAs, indicating the plant's ability to scavenge free radicals. *Cleome gynandra* showed significant amounts of the analysed phytochemicals, which are of high medicinal value. Therefore, future studies on the accumulation of phytochemicals in open field grown *C. gynandra* is recommended.

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