# Effects of varying levels of nitrogen on plant growth, anti-Fusarium oxysporum activity and 3,5-dicaffeoylquinic acid concentration of extracts from hydroponically cultivated Helic...

**Article** *in* Medicinal Plants - International Journal of Phytomedicines and Related Industries · January 2021

DOI: 10.5958/0975-6892.2021.00006.X

CITATIONS
0

READS
127

**4 authors:**

Yonela Matanzima
Cape Peninsula University of Technology
**2** PUBLICATIONS **5** CITATIONS
SEE PROFILE

Charles Petrus Laubscher
Cape Peninsula University of Technology
**52** PUBLICATIONS **425** CITATIONS
SEE PROFILE

Wilfred Mabusela
University of the Western Cape
**45** PUBLICATIONS **643** CITATIONS
SEE PROFILE

Felix Nchu
Cape Peninsula University of Technology
**69** PUBLICATIONS **424** CITATIONS
SEE PROFILE

**Medicinal Plants**
Vol. 13 (1), March 2021, 45-55

doi : 10.5958/0975-6892.2021.00006.X

IndianJournals.com
A product of Diva Enterprises Pvt. Ltd.

**Research Article**

# Effects of varying levels of nitrogen on plant growth, anti-*Fusarium oxysporum* activity and 3,5-dicaffeoylquinic acid concentration of extracts from hydroponically cultivated *Helichrysum cymosum* (L.)

**Yonela Matanzima[1], Charles P. Laubscher[1], Wilfred T. Mabusela[2] and Felix Nchu[1]***

*[1]Department of Horticultural Sciences, Faculty of Applied Sciences, Cape Peninsula University of Technology, P.O Box 1906, Bellville 7535, South Africa*

*[2]Department of Chemistry and South African Herbal Sciences and Medicines Institute, University of the Western Cape, Private Bag X17 Bellville 7535, South Africa*

Received: September 25, 2020; Accepted: February 06, 2021

## ABSTRACT

Experiments were conducted to assess the effects of varied nitrogen (N) concentrations on plant growth, tissue nutrient content, and antimicrobial activity of acetone extract of aerial parts of *Helichrysum cymosum*. Six weeks old rooted cuttings were treated with 52.5, 105.0, 210.0, and 420.0 mg N/L by varying the nutrient N level in plant growth medium. Plant growth parameters, leaf tissue nutrient content, anti-*Fusarium oxysporum* activity, and a well-known bioactive secondary metabolite (3,5-dicaffeoylquinic acid) concentration in the plant extracts were analysed. Leaf number increased with increasing N level, while height increase was highest at the lowest N treatment. Tissue analysis of N content and LC-MS analysis of the acetone extracts of aerial parts indicated that lower N treatment corresponded to lower tissue N and higher accumulation of 3,5-dicaffeoylquinic acid in the extracts, respectively. In conclusion, lower nutrient N level significantly correlated with increased antimicrobial activity and yield of the targeted compound of the crude acetone extract of *H. cymosum*. This study suggests that nitrogen fertilization may be manipulated to optimize bioactivities of crude extracts of medicinal plants.

**Keywords:** Cultivation of medicinal plants, total activity, secondary metabolite, antifungal activity

## INTRODUCTION

Hydroponic plant cultivation has many advantages, including reducing water wastage, increasing crop yield and improving secondary metabolite production in targeted plants. Given these opportunities in soilless cultivation of plants, research focusing on developing optimum hydroponics protocols and increasing bioactive compounds in plants are warranted.

Ambient environmental factors including herbivory, high UV radiation, nutrient availability and, presence of pathogens can trigger plant production of secondary metabolites, such as terpenes, phenolic compounds, nitrogen-alkaloids, and sulphur-containing compounds (Lavola and Julkunen-Tiitto, 1994; Nelson and Kursar, 1999; Van Alstyne and Pelletreau, 2000; Pedneault *et al*., 2002; Crozier *et al*., 2007; Kiferle *et al*., 2011; Mazid *et al*., 2011; Kennedy and Wightman, 2011; Maggini *et al*., 2012a; Sugumaran *et al*., 2013; Lu *et al*., 2018). Nitrogen is one of the important elements needed by plants for growth and many physiological functions. It has also been demonstrated that N availability in plant growth media can

*Corresponding author e-mail: felixnchu@gmail.com

influence the production of secondary metabolites (Beatty *et al*., 2010; Fan *et al*., 2010; Farag 2013). For example, Ibrahim *et al*. (2010) and Deng *et al*. (2019) showed that limited supply of nitrogen was favourable to increase the quantity of flavonoids in tomato, while increased rate of N decreased the concentration of flavonoids in grapefruit. Gayler *et al*. (2007) reported that polyphenols of *Picea sitchensis* (Bong.) Carr. (Family: Pinaceae) were higher in plants exposed to low N concentration. Excessively nitrogen-fertilized plants can depress the activity of superoxide dismutase and peroxidase, increase the accumulation of reactive oxygen species (ROS) and malondialdehyde harm, decrease crop yield, and harm human health if consumed (Liu *et al*., 2014; Kong *et al*., 2017). Inadequate supply of N leads to poor plant growth, increased plant stress as well as increased susceptibility to diseases (Bénard *et al*., 2011; Amanullah and Stewart, 2013). Hence, it is important to determine the optimum N range for cultivation for each medicinal plant species to guarantee high yield of medicinal materials including plant parts and secondary metabolites.

Studies focusing on the hydroponics cultivation of indigenous southern African plants are scarce. *Helichrysum cymosum* subsp. *cymosum* L. (Family: Asteraceae) is an important medicinal plant in southern Africa (Heyman, 2009). Its common English and Xhosa names are gold carpet and Impepho, respectively. *Helichrysum* species are used in traditional medicine for the treatment of stomach-ache, pain, wounds, coughs, and diabetes mellitus (Lourens *et al*., 2008; Demir *et al*., 2009, Albayrak *et al.*, 2010ab; Otang, 2012; Viegas *et al*., 2014). *H. cymosum* grows well in sandy soil and is mostly propagated using cuttings and seeds. Because it is extensively harvested from the wild, identification of optimum conditions for growing *H. cymosum* hydroponically could increase efficacy of the extracts and decrease excessive exploitation of plants from the wild.

*Helichrysum* species are known to be rich in secondary metabolites, including apigenin, quercetin, kaempferol, helihumulone and quinic acid derivatives (Van Vuuren *et al.,* 2006). Essential oils of *Helichrysum* spp. possess anti-oxidant and antimicrobial activities (Tchoumbougnang *et al*., 2010). According to Van Vuuren *et al*. (2006), *H. cymosum*'s acetone extract is active against fungi, with (MIC) values of 0.313 mg/mL for *Candida albicans* and 0.078 mg/mL for *Cryptococcus* neoformes. *Helicrysum* spp. contain many bioactive dicaffeoylquinic acid derivatives, including 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid that have antifungal activity against plant pathogens, such as *Fusarium solani* (Zhu, 2004; Harrison Jr *et al*., 2008; Heyman *et al.*, 2015). Previously, Clifford *et al*. (2003) used LC-MS to discriminate between isomers of p-coumaroylquinic acid, caffeoylquinic acid, feruloylquinic acid, and dicaffeoyl-quinic acid obtained from methanolic coffee bean extract and commercial cider (hard cider). Plant pathogens, such as *Fusarium* spp. can cause severe diseases of plants, leading to high economic losses (Dean *et al*., 2012). Few studies have been dedicated to the search for anti-infective crude extracts and compounds against plant pathogens in contrast to human pathogens (Shupping and Eloff, 2017).

The objective of this study was to evaluate the effects of nitrogen fertilization on plant growth, secondary metabolites, and anti-*F. oxysporum* activity (growth inhibition) of crude extracts of hydroponically-cultivated *H. cymosum*.

## MATERIALS AND METHODS

### Plant materials

Stem cuttings were made from actively growing *H. cymosum* stock plants on the Cape Town Campus of the Cape Peninsula University of Technology, South Africa. The cuttings were transplanted onto soil mix consisting of 3:1 river sand and compost. The cuttings were placed under mist propagation conditions until they rooted. The rooted cuttings were each transplanted into separate pots (12.5 cm diameter) containing Light Expanded Clay Aggregate (LECA®) as substrate.

### Preparation of nutrient solution

Analytical grade salts were used to prepare the nutrient solutions (Table 1) based on the Hoagland's solution (Table 1). The levels of N in the nutrient solutions were adjusted to obtain four concentrations (52.5, 105.0, 210.0, and 420.0 mg N/L). Care was taken to ensure that both $NO_3$-N and $NH_4$-N forms increased simultaneously with increasing total N. The final nutrient solutions were prepared by dissolving

**Table 1:** Macro- and micro-nutrient concentrations in modified Hoagland's hydroponic solutions; 52.5, 105, 210 and 420 mg N/L

| Nutrients | 52.5 mg N/L | 105 mg N/L | 210 mg N/L | 420 mg N/L |
|---|---|---|---|---|
| $NH_4NO_3$ | 52.5 (N) | 105.0 (N) | 210.0 (N) | 210.0 (N) |
| KCl | 195.5 (K) | 195.5 (K) | 195.5 (K) | 195.5 (K) |
| $CaCl_2$ | 200.0 (Ca) | 200.0 (Ca) | 200.0 (Ca) | 200.0 (Ca) |
| $KH_2PO_4$ | 39. 1 (K)<br>31.0 (P) | 39. 1 (K)<br>31.0 (P) | 39.1 (K)<br>31.0 (P) | 39.1 (K)<br>31.0 (P) |
| $H_3BO_3$ | 0.05 (B) | 0.05 (B) | 0.05 (B) | 0.05 (B) |
| $CuSO_4$ | 0.02 (Cu) | 0.02 (Cu) | 0.02 (Cu) | 0.02 (Cu) |
| $NaMoO_4.2H_2O$ | 0.01 (Mo) | 0.01 (Mo) | 0.01 (Mo) | 0.01 (Mo) |
| $ZnSO_4$ | 0.1 (Zn) | 0.1 (Zn) | 0.1 (Zn) | 0.1 (Zn) |
| $MgSO_4.7H_2O$ | 48.0 (Mg)<br>64.0 (S) | 48.0 (Mg)<br>64.0 (S) | 48.0 (Mg)<br>64.0 (S) | 48.0 (Mg)<br>64.0 (S) |
| Fe-EDTA | 2.0 (Fe) | 2.0 (Fe) | 2.0 (Fe) | 2.0 (Fe) |
| $MnCl_2.4H_2O$ | 0.01 (Mn) | 0.01 (Mn) | 0.01 (Mn) | 0.01 (Mn) |

appropriate amounts of salt and acid combinations in 50 L of sterile deionised water.

**Greenhouse experiment**

The experiment was carried out in a greenhouse. Sixteen white plastic gutters (2 × 0.15 m) were placed on four steel tables. The gutters were held in place by cable ties. Ten plastic pots (12.5 cm) filled with LECA® were placed, spaced evenly, in each gutter**.** Each plastic pot had one six-week old *H. cymosum* rooted stem cutting. Four fish tanks (65 L), each containing a submersible water pump and final nutrient solution, supplied re-circulated nutrient solution through a 20 mL black plastic pipe to one gutter, only. The nutrient solution was continually supplied to the plants by spaghetti tubings at a rate of 4 L/h. Each nitrogen treatment had a total of four gutters and 40 plants. The plants were randomly allocated to the treatments and were arranged using a randomized complete block design with four replicates per treatment. To avoid algae build-up and evaporation, all the gutters were wrapped with black polyethylene sheets. The experimental conditions in the greenhouse were: day temperature 16–36 °C, night temperature 10–18 °C; relative humidity (RH) 35–65%; natural photoperiod; and the pH of growth media was maintained at 5.0–6.5.

**Evaluation of plant growth**

Plant height (cm) and leaf number were recorded at weeks two and nine following commencement of the experiment. Following harvest at nine weeks post treatment, the aerial parts (stem and leaves) were oven-dried and weighed. The differences in leaf numbers and plant height at two weeks and nine weeks were used to measure plant growth over time.

**Plant tissue analysis**

Plants from the treatments were analysed by Bemlab (Pty) Ltd**.**, Somerset West, South Africa, using a Leco N-analyser FP 528 (LECO Corporation, St. Joseph, USA). The leaves were washed and dried, and then ashed and milled at 480 °C. This was followed by extraction in a 50:50 HCl (32%) solution (Campbell and Plank, 1997; Miller, 1998). Total N content of the ground leaves was determined through total combustion in the Leco N-analyser.

**Extraction of plant material**

Fresh aerial plant materials from the greenhouse experimental plants and outdoor plants (field plants) occurring on CPUT property were air-dried at 28 ± 2 °C. The dried plant material were ground into fine powder

using a Jankel and Kunkel Model A 10 mill. Five grams of the powdered foliage material was suspended in 100 mL of acetone in a glass beaker. The beaker was capped, shaken vigorously for 15 min with a vortex mixer, and the supernatant filtered-out using Whatman No. 1 filter papers. The extracted material was left to dry overnight at room temperature of 22 ± 2 °C and the dried acetone extract was weighed to determine yield of extract.

**Antifungal activity of extracts**

To determine the minimum inhibitory concentration (MIC), we used the microdilution assay previously described by Nchu *et al.* (2010) with slight modifications. Spores of *Fusarium oxysporum* f. sp. glycines strain (UPFC no. 21) obtained from stock agar plates were cultured on Potato Dextrose Agar and the mature spores were transferred into Nutrient Broth suspension (Merck, Johannesburg, South Africa). Mancozeb (Agrimark [Pty] Ltd., Cape Town, South Africa) was used as a positive control in the MIC assay at a starting concentration of 80 mg/10 mL. An aqueous acetone solution (water: acetone; 1:1) was used as a negative control. The MIC values (mg/mL) were recorded after 18 h. The MIC assay was replicated three times. The MIC values and yield of extracts obtained after extraction were used to determine the Total Activity (TA). The TA unit is mL/g, and it indicates the degree to which the active compounds in 1 g of plant materials can be diluted and still inhibit the growth of the tested microorganisms (Eloff, 2004).

**Liquid chromatography – mass spectrometry (LC-MS) analysis**

Acetone extracts were subjected to LC-MS analysis in the ESI negative mode using a Waters Synapt G2 quadrupole time-of-flight mass spectrometer coupled to a Waters Acquity ultra-performance liquid chromatograph (UPLC) equipped with an Acquity photo diode array (PDA) detector. LC separation was attained using a Waters UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm particle size) with 0.1% formic acid as mobile phase A and acetonitrile as mobile phase B. A constant flow rate of 0.35 mL/min was used for each analysis. Elution was initiated with 100% solvent A for 0.5 min followed by a gradient to 100% B during the next 24.5 min. The column was washed for 1 min in 100% B, before re-equilibration to the initial conditions for 2 min. Capillary voltage and cone voltage were maintained at 2.5 kV and 15 V, respectively, while the desolvation temperature was set at 275 °C with desolvation gas flowing at 650 L/h. The ion chromatograms and peaks of the molecular masses corresponding to compounds that were previously reported in *H. cymosum* were targeted. These include 3,5-dicaffeoylquinic acid (DCQ) for which a commercial standard was available. DCQ (sourced from Sigma-Aldrich (Pty) Ltd., Kempton Park, South Africa) was used as a chemical standard at three concentrations: 5, 50 and 100 mg N/L. Helihumulone was compared with the library standard. Subsequently, the peaks of the extract for which the molecular mass corresponded to that of dicaffeoylquinic acid and helihumulone were targeted and compared with the eluted standard and the library standard, respectively. The peaks were further analysed using MS-MS and retention time. The area of the peak was compared with the standard to quantify dicaffeoylquinic acid in each eluted extract.

**Statistical analysis**

Plant growth, MIC and total activity data were analysed using one-way analysis of variance (ANOVA). The means were separated using Tukey HSD test. The level of statistical significance was $P < 0.05$. All the analyses were performed using PAST (Hammer *et al.*, 2001).

## RESULTS

**Plant growth**

Plant heights increased and varied significantly (df = 3, 153; F; 4.8; $P < 0.01$) among treatments over time (from week 2 to week 9 post treatment); ranging from 7.1±0.4 cm (210 mg N/L) to 9.0 ± 0.3 cm (52.5 mg N/L) (Table 2). The increase in leaf number was significantly (df = 3, 155; F = 2.8; $p < 0.05$) associated with N treatment, ranging from 305.1± 24.7 (52.5 mg N/L) to 468.0± 45.4 (420 mg N/L) (Table 2). Dry weights of aerial parts of plants obtained in the different treatments did not vary significantly (df = 3, 42; F = 0.2; $P > 0.05$) among treatments at 9 weeks post treatment; it ranged from 0.8 ± 0.2 – 1.0 ± 0.1g/plant (Tables 2). Similarly, there was no significant difference (df = 3, 138; F = 0.5; $P < 0.8$) in the dry weight of roots (0.2 ± 0.0 g/plant) among the different treatments (Table 2).

**Table 2:** Effects of varying nitrogen concentrations in plant growth medium on different growth parameters (Mean ± SE) of *Helichrysum cymosum* recorded at 9 weeks post-treatment.

| Treatment (mg N/L) | Dry weight (*Mean ± SE g/plant) shoots | Dry weight (*Mean ± SE g/plant) roots | Increase in leaf number (*Mean ± SE) between weeks 2-9 | Increase in height (*Mean ± SE cm) between weeks 2-9 |
|---|---|---|---|---|
| 52.5 | 1.0 ± 0.1a | 0.2 ± 0.0a | 305.1± 24.7bc | 9.0± 0.3a |
| 105 | 0.9 ± 0.2a | 0.2 ± 0.0a | 376.7± 4138ab | 7.2± 0.3b |
| 210 | 0.8 ± 0.2a | 0.2 ± 0.0a | 406.1± 46.7ab | 7.1 ± 0.4b |
| 420 | 1.0 ± 0.2a | 0.2 ± 0.0a | 468.0± 45.4a | 7.3± 0.5b |

*Means followed by the same lowercase letters in each column are not significant different at ($P > 0.05$) following comparison using Tukey's test.

### Tissue nutrient content

Plant tissue N content was significantly higher (df = 3, 12; F = 9.7; $P < 0.01$) in plants exposed to the highest nutrient nitrogen level (420.0 mg N/L) (34875 ± 2556 mg/kg) compared with those exposed to lower levels of N treatments (Table 3).

### Minimum inhibitory concentration (MIC) and total activity (TA)

N treatment significantly influenced (df = 5, 12; F = 78.07; $P < 0.01$) MIC level, with the lowest N treatment (52.5 mg N/L) recording the lowest MIC value (0.1 mg/mL) among the greenhouse plants. Furthermore, the antifungal activity in the MIC bioassay showed that the least N concentration was not statistically different ($P > 0.05$) from field plants (0.18 ± 0.0 mg/mL) at 18 H (Table 4). The antifungal activities of the extracts were significantly better ($P < 0.05$) than Mancozeb® (positive control) (Table 4). Although acetone extracts of plants grown hydroponically at the lowest N treatment (52.5 mg N/L) (392.2 ± 99 mL/mg) recorded the highest values of TA against *F. oxysporum* compared to the other N treatments, the differences among treatments were not significantly (df = 4, 10; F = 3.06; $P > 0.05$).

**Table 3:** Effect of varying N concentrations in plant growth medium on tissue nitrogen content in tissues of aerial parts of hydroponically-cultivated *Helicrysum cymosum* at 9 weeks post treatment.

| Treatment (mg N/L) | Tissue N (*Mean ± SE mg/kg) |
|---|---|
| 52.5 | 39725.0 ± 2649.0b |
| 105.0 | 34875.0 ± 2556.0b |
| 210.0 | 37400.0 ± 2017.0b |
| 420.0 | 50050.0 ± 755.0a |

*Means followed by the same in each are not significant different ($P > 0.05$) following comparison using Tukey's test.

**Table 4:** Results on minimum inhibitory concentration (MIC) bioassay and total activities of acetone extracts at 18H obtained from aerial parts of hydroponically-cultivated *Helichrysum cymosum* following exposure to a range of N nutrient levels (52.5–420 mg N/L) for nine weeks.

| Treatment *Mean ± SE mg N/L | MIC *Mean ± SE mg/mL | Total Activity *Mean ± SE mL/g |
|---|---|---|
| 52.5 | 0.1 ± 0.0 b | 392.2 ± 99.0a |
| 105 | 0.4 ± 0.0 d | 142.4 ± 21.8a |
| 210 | 0.2± 0.0 cd | 243 ± 20.1a |
| 420 | 0.2± 0.0 cd | 226.3 ± 17.7a |
| Field plants | 0.18 ± 0.0 bc | 355.5 ± 75.6a |
| +ve control | 0.02 ± 0.0a | - |

*Means followed by the same lowercase letters in each column are not significant different ($P > 0.05$) after comparison using Tukey's test.

### LC-MS

Based on the LC-MS data – extracted mass ion chromatograms and molar mass (MM) values of the compounds – a cluster of helihumulone peaks was detected in all the samples. Two more compounds were detected in variable quantities and these were 2',4',6'-trihydroxy-3'-isoprenylchalcone and 2',6'-dihydroxy-4'-methoxychalcone. A cluster of three peaks that eluted at about $T_R$ 8-9 min were identified as dicaffeoyl quinic acid isomers, and they occurred in detectable quantities at all N levels (Figure 1). Since 3,5-dicaffeoylquinic acid (DCQ) was available as a standard and being one of the bioactives identified, it was

**Table 5:** Effect of N treatment on quantity of 3,5-Dicaffeoylquinic acid in plant extracts of hydroponically cultivated *Helichrysum cymosum* at nine weeks post treatment.

| N Treatment (mg N/L) | Quantity of 3,5-Dicaffeoylquinic acid (DCQ) in extracts (*Mean ± SE µg/L) |
|---|---|
| 52.5 | 15.6 ± 3.2bc |
| 105 | 25.7 ± 6.7ab |
| 210 | 12.9 ± 1.5bc |
| 420 | 5 ± 1.3d |
| Field plants | 17 ± 3.2bc |

*Mean with the same lowercase letters are not significantly different following Tukey HSD test at a $P < 0.05$ level significance.

selected as a reference for monitoring its variation at the different N levels. Interestingly, the least quantity of dicaffeoylquinic acid (DCQ) in extracts (Mean ± SE mg/L) was detected in the samples corresponding to the highest concentration 420.0 mg N/L compared with the lower N concentration samples (DF = 4, 15; F = 3.95; $P < 0.05$) (Table 5).

## DISCUSSION

This study evaluated the effects of N treatment on three important aspects that relates to yield and quality of medicinal materials of *H. cymosum*: plant growth parameters, antimicrobial activity and amounts of a well-known antifungal compound in the acetone extract. Broadly, the results showed that N treatment had mixed effects on plant growth parameters, while antimicrobial activity and quantity of DCQ were negatively associated with higher N treatment.

N treatments had varied effects on the different the plant growth parameters. The most distinctive effect was on plant height and leaf number. The lowest hydroponic N level (52.5 mg N/L) yielded the highest increase in height, while leaf numbers were positively associated with N treatments. It is worth noting that plants exposed to 420.0 mg N/L had the highest tissue concentrations of N. High N fertilization often correlates with high nitrate concentration in plant (Kane *et al*., 2006), which can interfere with the formation and development of leaves (Ma *et al*., 1997). While many studies suggest a positive correlation between nutrient nitrogen level and plant growth (Moniruzzaman *et al*., 2009; Giorgi *et al*., 2009), excessive nitrogen fertilization might actually decrease or have no beneficial effect on plant yield. For example, Miceli and Miceli (2014) showed that the dry matter of Swiss chard (*Beta vulgaris* L. var. cicla L.) was significantly lower after treatment with higher nitrogen fertilization levels compared with the lower nitrogen levels. They also found that higher nitrogen fertilization, beyond 100 kg/ha, did not increase Swiss chard's leaf number per plant. If the rate of N goes beyond the optimum N threshold, growth of plants may be limited (Savvas *et al*., 2006). Our

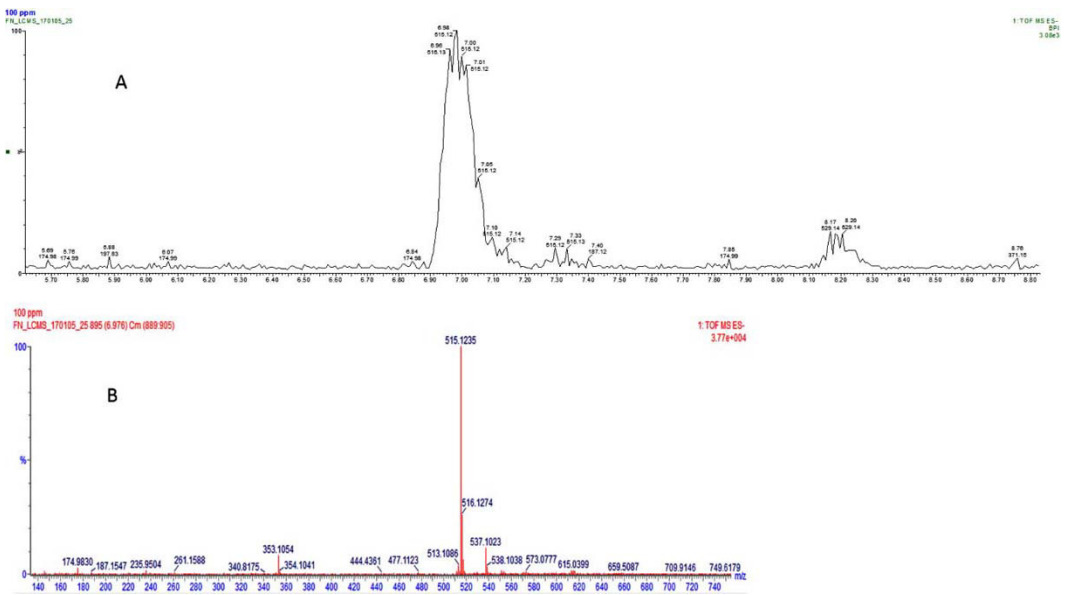


**Figure 1:** LC-MS profile of 3,5-dicaffeoylquinic acid in the plant extract (**A**) and ESI-MS fragmentation pattern of 3,5-dicaffeoyl quinic acid (**B**).

results seem to support this argument because *H. cymosum* did not grow better beyond the lowest concentration of nutrient nitrogen (52.5 mg N/L). Knowing the optimum level of N requirement of medicinal plants is critical for the following reasons: toxic nitrate intake is dangerous to human health, excessive N application is a serious threat to the environment, and mineral fertilizers are costly.

We observed that acetone extracts of *H. cymosum* were, generally, bioactive against *F. oxysporum*. Remarkably, the acetone extract of plants treated with 52.5 mg N/L was the most bioactive against *F. oxysporum* compared to extracts obtained from plants exposed to 105.0 mg N/L, 210.0 mg N/L as well as 420.0 mg N/L treatments. In a previous study, the MICs of crude acetone extracts of *H. cymosum* tested against ten microbial pathogens ranged from 0.156–0.313 mg/mL (Van Vuuren *et al*., 2006). These results are comparable with the current results obtained in our anti-*F. oxysporum* bioassay (Table 3). The bioactivities of extracts are mediated by the bioactive constituents. Fu *et al*. (2016) reported that among ten different solvents, aqueous acetone (70%) had the highest extraction capacity for 3,4-, 3,5- and 4,5- dicaffeoylquinic acids of sweet potato (*Ipomoea batatas* L.) leaves. Dicaffeoylquinic acids are active against the plant pathogen *F. solani* (Radusienë *et al.*, 2019). Previously, bioassay guided fractionation of acetone extracts of *H. cymosum* yielded a highly active antimicrobial compound, helihumulone (Van Vuuren *et al*., 2006). With trans 3-O-caffeoylquinic acid as one of its major compounds, *Eryngium viviparum* extract was more effective than ketoconazole against *Penicillium ochrochloron* (Ayuso *et al.*, 2020).

This negative association between bioactivity of extracts and the high N supply might be explained by the increased production of more bioactive molecules with reduced N supply. For example, in a study conducted by Ibrahim *et al*. (2011), the production of flavonoids and polyphenols in three varieties of *Labisia pumila* reached their peaked under 0 followed by 90, 180 and 270 kg N/ha treatment. They also found that the total flavonoids and total polyphenols correlated with the phenyl alanine lyase activity and the reduced production of soluble proteins.

According to the LC-MS/MS data (Figure 1), the three peaks eluting at 7.79, 7.92, and 8.75 min were due to 3,4-, 3,5- and 4,5-dicaffeoyl quinic acids, respectively. This identification was achieved by comparison with LC-MS/MS data of these compounds as reported by Long *et al*. (2012) and Clifford *et al*. (2003). Thus, although they are isomers of each other, their unique individual fragmentation patterns makes it possible to identify them and distinguish them from each other. Helihumulone also occurred in the extracts of plants from all the treatments. 3,5-dicaffeoylquinic acid concentration was significantly higher at the lower N treatments (Table 5) (Figure 1). Harrison, Jr. *et al*. (2008) reported that 3,5-dicaffeoylquinic acid had growth inhibitory effects against *F. solani*. Nitrogen fertilization can influence the synthesis of carbon based secondary metabolites in plants. In a study involving *Hypericum pruinatum*, increased nitrogen supply significantly reduced the accumulation of major phenolics, such as catechin, chlorogenic acid, hyperoside, quercitrin, and isoquercitrin, as well as total phenolic content and antioxidant activity (Radugienë *et al*., 2019). Bate *et al*. (1994) had demonstrated a quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco. Alves-Silva *et al*. (2020) demonstrated that chlorogenic acid and quercetin derivatives found in the hydrodistillation residual water were the main contributors of the antioxidant activity of *Crithmum maritimum* L. Nani *et al*. (2020) showed that the Brazilian organic propolis (BOP) had anti-inflammatory and anti-*Candida* spp. effects and went on to argue that the bioactivities could be attributed to its chemical constituents, such as caffeoyltartaric acid, 3,4-dicaffeoylquinic acid quercetin, and gibberellins A7, A9, and A20. Dicaffeoylquinic acids have been reported to be present in other Helichrysum species, such as *H. stoechas* (Barroso *et al*., 2014), *H. monizii* (Gouveia and Castilho, 2011) among others. Our findings are in agreement with the wide held view that the enhancement of total flavonoids and phenolics usually occur when a plant is deficient in nitrogen supply; plants deprived of N tend to produce more secondary metabolites (Savvas *et al*., 2006; Le Bot *et al*., 2009). In a study conducted by Ibrahim *et al.* (2010), the total phenolics decreased in the leaves with the increase of N supply, whereas plants that received limited amount of N had an increase in total flavonoids and phenolics. In *Hypericum pruinatum*, increased nitrogen supply significantly reduced the accumulation of major phenolics,

such as catechin, chlorogenic acid, hyperoside, quercitrin, isoquercitrin, and total phenolic content (Radugienë *et al.*, 2019). Interestingly, in this study, the crude acetone extract from the lowest N treatment (52.5 mg N/L) showed the highest anti-*F. oxysporum* activity.

Researchers are investigating the pathways through which nutrient N influences the synthesis of secondary metabolites. For example, Kong *et al.* (2017) reported that the concentrations of some amino acids and amino acids-derived N-acetylleucine and L-lysopine increased strongly under excessive nitrogen conditions, whereas other N-containing molecules, such as γ-aminobutyric acid, choline, acetylpyrrolidine and (4-hydroxybenzoyl) choline, decreased. They also reported that many flavonoids, phenolics, and alkaloids decreased with excessive N fertilization of field-grown wheat (*Triticum aestivum* L.). Nitrogen fertlization affects amino acid phenylalanine and phenylalanine ammonia-lyase activity that are involved in the biosynthesis of 5-O-caffeoylquinic acid (5-CQA) and other related dicaffeoylquinic acids (diCQAs) (Ibrahim *et al*., 2011; Clifford *et al*., 2017). Based on the data obtained in the LC-MS analysis, growing the subject species under low nutrient N level favoured the accumulation of 3,5-dicaffeoylquinic acid and that of helihumulone.

It should be noted that helihumulone elutes as part of the second cluster alongside 2',4',6'-trihydroxy-3'-isoprenylchalcone and 2',6'-dihydroxy-4'-methoxychalcone and other unidentified constituents. Previously, a bioassay-guided fractionation of the acetone extract of *H. cymosum* by Van Vuuren *et al*. (2006) led to the identification of helihumulone. However, they also noted that it belongs to a class of air-and light-sensitive compounds which compromises its relative stability. For this reason, DCQ would be a better choice to target as a marker for quality control purposes where this may be required. Furthermore, the higher polarity of the DCQ's as reflected by the LC elution pattern, suggests that they should be more aqueous soluble than helihumulone and therefore more bioavailable in physiological media. DCQ can also be considered as one of the important markers for chemotaxonomic investigations of *Helicrysum* spp. However, the inclusion of chemometrics in future studies can enhance chemotaxonomic distinction among the *Helicrysum* spp. (Santos *et al*., 2020).

In conclusion, this study showed that 52.5 mg N/L nutrient content in the growth medium yielded the most bioactive acetone extracts of *H. cymosum* and 420.0 mg N/L yielded the highest increase in leaf number, suggesting that the effect of nutrient N on medicinal plants is not straightforward. Further studies on the effects of nitrogen on the metabolomics of *Helichrysum* spp. are warranted.

## ACKNOWLEDGMENTS

The authors thank Mr Fletcher Hiten (University of Stellenbosch) for performing the LC-MS analyses. This research was funded by Cape Peninsula University of Technology through the University Research Funds (URF), grant number R166.

### Conflicts of Interest

The authors declare no conflict of interest.

## REFERENCES

Albayrak S, Aksoy A, Sagdic O and Budak U (2010a). Phenolic compounds and antioxidant and antimicrobial properties of Helichrysum species collected from eastern Anatolia, Turkey. *Turk J. Biol*., 34: 463-473.

Albayrak S, Aksoy A, Sagdic O, Hamzaoglu E and Sagdic O (2010b). Compositions, antioxidant and antimicrobial activities of Helichrysum (Asteraceae) species collected from Turkey. *Food Chem.*, 119: 114–122. https://www.semanticscholar.org/paper/Phenolic-compounds-and-antioxidant-and-properties-Albayrak-Aksoy/35c2fb7cac8d4e65c66ecd4a8077630082f91555

Alves-Silva JM, Guerra I, Gonçalves MJ, Cavaleiro C, Cruz MT, Figueirinha A and Salgueiro L (2020). Chemical composition of *Crithmum maritimum* L. essential oil and hydrodistillation residual water by GC-MS and HPLC-DADMS/MS, and their biological activities. *Ind. Crops and Prod.* 149: 112329, doi: 10.1016/j.indcrop.2020.112329

Amanullah Jr and Stewart BA (2013). Dry matter partitioning, growth analysis and water use efficiency response of oats (*Avena sativa* L.) to excessive nitrogen and phosphorus application. *J. Agric. Sci. Tech*., 15: 479-489. https://jast.modares.ac.ir/article-23-3038-en.pdf .

Ayuso M, Pinela J, Dias MI, Barros L, Ivanov M, Calhelha RC, Sokoviæ M, Ramil-Rego P, Barreal ME, Gallego PP and Ferreira ICFR (2020). Phenolic composition and biological activities of the *in vitro* cultured endangered *Eryngium viviparum* J. *Gay Industrial Crops and Products,* 148: 112325, doi: 10.1016/j.indcrop.2020.112325

Barroso M, Barros L, Dueñas M, Carvalho AM, Santos-Buelga C, Fernandes I.P, Barreiro MF, Ferreira IC and Dueñas M (2014). Exploring the antioxidant potential of *Helichrysum stoechas* (L.) Moench phenolic compounds for cosmetic applications: chemical characterization, microencapsulation and incorporation into a moisturizer. *Ind. Crop. Prod.*, 53: 330–336.

Bate NJ, Orr J, Ni W, Meromi A., Nadler-Hassar T, Doerner PW, Dixon RA, Lamb CJ and Elkind Y (1994). Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. *Proc. Nat. Acad. Sci.,* 91(16): 7608-7612. doi: 10.1073/pnas.91.16.7608

Beatty P, Anbessa Y, Juskiw P, Carroll RT, Wang J and Good A (2010). Nitrogen use efficiencies of spring barley grown under varying nitrogen conditions in the field and growth chamber. *Ann. Bot.,* 105: 1171–1182. doi: 10.1093/aob/mcq025

Bénard C, Bourgaud F and Gautier H (2011). Impact of temporary nitrogen deprivation on tomato leaf phenolics. *Int. J. Mol. Sci.,* 12: 7971–7981. doi: 10.3390/ijms12117971

Campbell CR and Plank CO (1997). Preparation of plant tissue for laboratory analysis. In: H*andbook of reference methods for plant analysis*; Informa UK Limited, pp. 37–49.

Clifford MN, Jaganath IB, Ludwig IA and Crozier A (2017). Chlorogenic acids and the acyl-quinic acids: discovery, biosynthesis, bioavailability and bioactivity. *Nat. Prod. Rep.*, 34: 1391-1421. https://doi.org/10.1039/C7NP00030H

Clifford MN, Johnson KL and Kuhnert N (2003). Hierarchical scheme for LC-MS identification of chlorogenic acids. *J. Agric. Food Chem.,* 51(10): 2900-2911. doi: 10.1021/jf026187q

Conn S., Hocking B, Dayod M, Xu B, Athman A, Henderson S, Aukett L, Conn V, Shearer MK, Fuentes S, *et al.* (2013). Protocol: optimising hydroponic growth systems for nutritional and physiological analysis of *Arabidopsis thaliana* and other plants. *Plant Methods*, 9: 4. https://doi.org/10.1186/1746-4811-9-4

Crozier A, Jaganath IB and Clifford M (2007) *Phenols, Polyphenols and Tannins: An Overview*. In: Crozier A, Clifford MN, Ashihara H (eds) Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet: Blackwell Publishing, Glasgow, UK 2007, pp. 1–24.

Dean R, Van Kan JAL, Pretorius Z A, Hammond Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J and Foster GD (2012). The top 10 fungal pathogens in molecular plant pathology. *Mol Plant Pathol.,* 13(4): 414–430.https://doi.org/10.1111/j.1364-3703.2011.00783.x

Demir A, Taban BM, Aslan M, Yesilada E and Aytac SA (2009) Antimicrobial effect of *Helichrysum plicatum* subsp. plicatum. *Pharm. Boil.*, 47: 289–297. https://doi.org/10.1080/13880200802590434

Deng B, Li Y, Xu D, Ye Q and Liu G (2019). Nitrogen availability alters flavonoid accumulation in *Cyclocarya paliurus* via the effects on the internal carbon/nitrogen balance. *Sci. Rep.*, 9: 2370. https://doi.org/10.1038/s41598-019-38837-8

Eloff JN (2004). Quantification the bioactivity of plant extracts during screening and bioassay guided fractionation. *Phytomedicine*, 11: 370–371. doi: 10.1078/0944711041495 218

Fan J, Hao M and Malhi SS (2010). Accumulation of nitrate N in the soil profile and its implications for the environment under dryland agriculture in northern China: A review. *Can. J. Soil Sci.*, 90: 429–440. doi: 10.4141/CJSS09105

Farag AAA, Abdrabbo MAA and Abd-Elmoniem EM (2013). Using different nitrogen and compost levels on lettuce grown in coconut fiber. *J. Hort. For.,* 5: 21-28. doi: 10.5897/JHF09.041.

Fu Z, Tu Z, Zhang L, Wang H, Wen Q and Huang T (2016). Antioxidant activities and polyphenols of sweet potato (*Ipomoea batatas* L.) leaves extracted with solvents of various polarities. *Food Bioscience,* 15: 11-18. https://doi.org/10.1016/j.fbio.2016.04.004

Gayler S, Grams TE, Heller W, Treutter D and Priesack E (2007). A dynamical model of environmental effects on allocation to carbon-based secondary compounds in juvenile trees. *Ann. Bot.*, 101: 1089–1098. doi: 10.1093/aob/mcm169.

Giorgi A, Mingozzi M, Madeo M, Speranza G and Cocucci M (2009). Effect of nitrogen starvation on the phenolic metabolism and antioxidant properties of yarrow (*Achillea collina* Becker ex Rchb.). *Food Chem.*, 114: 204–211. doi: 10.1016/j.foodchem.2008.09.039

Gontier E, Clement A, Tran T, Gravot A, Lièvre K, Guckert A and Bourgaud F (2002). Hydroponic combined with natural or forced root permeabilization: a promising technique for plant secondary metabolite production. *Plant Sci.*, 163: 723–732. doi: 10.1016/S0168-9452(02)00171-1

Gouveia S and Castilho PC (2011). *Helichrysum monizii* Lowe: Phenolic composition and antioxidant potential. *Phytochem. Anal.*, 23: 72–83. doi: 10.1002/pca.1326.

Hammer O, Harper DAT AND Ryan PD (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontol. Electron.,* 4(1): 9, Available via DIALOG. https://folk.uio.no/ohammer/past/. Accessed April 23, 2020.

Harrison Jr' HF, Mitchell TR, Peterson JK, Wechter WP, Majetich GF and Snook ME (2008). Contents of caffeoylquinic acid compounds in the storage roots of sixteen sweet potato genotypes and their potential biological activity. *J. Amer. Soc. Hort. Sci.,* 133(4): 492-500. doi: 10.1021/jf026187q

Heyman H (2009). Metabolic comparison of selected Helichrysum species to predict their antiviral properties. Unpublished Master's Thesis, University of Pretoria, Pretoria, South Africa. https://www.researchgate.net/profile/Heino_Heyman/publication/266350677_Metabolomic_comparison_of_

selected_Helichrysum_species_to_predict_their_antiviral_properties/links/54bcc4d70cf253b50e2d5d26.pdf,

Heyman HM, Senejoux F, Seibert I., Klimkait T, Maharaj J, Johannes J and Meyer M (2015). Identification of anti-HIV active dicaffeoylquinic- and tricaffeoylquinic acids in *Helichrysum populifolium* by NMR-based metabolomic guided fractionation. *Fitoterapia,* 103: 155-164, https://doi.org/10.1016/j.f

Hussain A, Anjum F, Rab A and Sajid, M (2006). Effect of nitrogen on growth and yield of Asparagus (*Asparagus officinalis*). *J. Agric. Biol Sci*., 1: 41-46.

Ibrahim MH, Jaafar HZE, Rahmat A and Rahman ZA (2011). Effects of nitrogen fertilization on synthesis of primary and secondary metabolites in three varieties of *Kacip Fatimah* (*Labisia Pumila* Blume). *Int. J. Mol. Sci.,* 12(8): 5238–5254. doi: 10.3390/ijms12085238

Ibrahim MH, Jaafar HZE, Rahmat AB and Rahman ZA (2010). The relationship between phenolics and flavonoids production with total nonstructural carbohydrate and photosynthetic rate in *Labisia pumila* Benth. under high $CO_2$ and nitrogen fertilization. *Mol.*, 16: 162–174. doi: 10.3390/molecules16010162.

Kane CD, Jasoni RL, Peffley EP, Thompson LD, Green CJ, Pare P and Tissue DT (2006). Nutrient solution and solution pH influences on onion growth and mineral content. *J. Plant Nutr.*, 29: 375–390. https://doi.org/10.1080/01904160500477028

Kennedy DO and Wightman EL (2011). Herbal extracts and phytochemicals: Plant secondary metabolites and the enhancement of human brain function1. *Adv. Nutr.*, 2: 32–50. doi: 10.3945/an.110.000117

Khan A, Shah SNM, Rab A, Sajid M, Ali K, Ahmed A and Faisal S (2014). Influence of nitrogen and potassium levels on growth and yield of chillies (*Capsium annuum* L.). *Int. J. Farm. Allied Sci.,* 3: 260-264. http://ijfas.com/wp-content/uploads/2014/03/260-2642.pdf.

Khan S, Singla RK and Abdin MZ (2011). Assessment of phytochemical diversity in *Phyllantus* using HPTLC fingerprints. *Indo-Global, J. Pharma Sci.,* 1: 1-12. https://doi.org/10.1021/jf3046686

Kiferle C, Lucchesini M, Mensuali-Sodi A, Maggini R, Raffaelli A and Pardossi A (2011). Rosmarinic acid content in basil plants grown *in vitro* and in hydroponics. *Open Life Sci.,* 6: 946–957. https://doi.org/10.2478/s11535-011-0057-1

Kiferle C, Maggini R and Pardossi A (2013). Influence of nitrogen on growth and accumulation of rosmarinic acid in sweet basil (*Ocimum basilicum* L.) grown in hydroponic culture. *Aus. J. Crop Sci.,* 3: 321-327. http://www.cropj.com/kiferle_7_3_2013_321_327.pdf.

Kong L, Xie Y, Hu L, Si J and Wang Z (2017). Excessive nitrogen application dampens antioxidant capacity and grain filling in wheat as revealed by metabolic and physiological analyses. *Sci. Rep.,* 7: 43363; doi: 10.1038/srep43363.

Lavola A and Julkunen-Tiitto R (1994). The effect of elevated carbon dioxide and fertilization on primary and secondary metabolites in birch, *Betula pendula* (Roth). *Oecologia*, 99: 315–321. doi: 10.1007/BF00627744

Le Bot J, Bénard C, Robin C, Bourgaud F and Adamowicz S (2009). The 'trade-off' between synthesis of primary and secondary compounds in young tomato leaves is altered by nitrate nutrition: experimental evidence and model consistency. *J. Exp. Bot.*, 60: 4301–4314. doi: 10.1093/jxb/erp271

Liu C-W, Sung Y, Chen B-C and Lai H-Y (2014). Effects of Nitrogen Fertilizers on the Growth and Nitrate Content of Lettuce (*Lactuca sativa* L.). *Int J Environ Res Public Health*, 11(4): 4427–4440. doi: 10.3390/ijerph110404427.

Long HS, Stander MA and Van Wyk B-E (2012). Notes on the occurrence and significance of triterpenoids (asiaticoside and related compounds) and caffeoylquinic acids in *Centella species*. *S A J Bot.,* 82: 53-59, https://doi.org/10.1016/j.sajb.

Lourens A, Viljoen A and Van Heerden F (2008). South African Helichrysum species: A review of the traditional uses, biological activity and phytochemistry. *J. Ethnopharmacol.*, 119: 630–652. doi: 10.1016/j.jep.2008.06.011.

Lu L, Fu S, Feng J and Wei J (2018). Growth and accumulation of five main bioactive components in the roots of *Salvia miltiorrhiza* at different growth stages and using different culture systems. *J. Dis. Med. Plants,* 4(3): 59-68. http://www.sciencepublishinggroup.com/journal/paperinfo?journalid=320&doi=10.11648/j.jdmp.20180403.11

Ma Q, Longnecker N and Dracup M. (1997). Nitrogen deficiency flows leaf development and delays flowering in narrow-leafed Lupin. *Ann. Bot.*, 79: 403–409. https://doi.org/10.1006/anbo.1996.0361

Maggini R, Kiferle C, Guidi L, Pardossi A and Raffaelli A (2012a). Growing medicinal plants in hydroponic culture. *Acta Hortic.,* 952: 697–704. https://doi.org/10.17660/ActaHortic.2012.952.88

Maggini R, Tozzini L, Pacifici S, Raffaelli A and Pardossi A (2012b). Growth and accumulation of caffeic acid derivatives in *Echinacea angustifolia* DC. var. angustifolia grown in hydroponic culture. *Ind. Crop. Prod.*, 35: 269–273. doi: 10.1016/j.indcrop.2011.07.011

Mazid, M, Khan TA and Mohammad F (2011) Role of secondary metabolites in defence mechanisms of plants. *Biol. Med.,* 3: 232-249. https://www.longdom.org/open-access/role-of-secondary-metabolites-in-defense-mechanisms-of-plants-0974-8369-3-128.pdf

Miceli A and Ciceli C (2014). Effect of nitrogen fertilization on the quality of Swiss chard at harvest and during storage as minimally processed produce. *J. Food Qual.,* 37: 125–134. doi: 10.1111/jfq.12073

Miller RO (1998). High-temperature oxidation: dry ashing. In: Kalra YP (ed.) Handbook of reference methods for plant analysis: CRC Press, Boca Raton, FL, pp. 53-56.

Moniruzzaman M, Islam M, Hossain M, Hossain T and Miah M (2009). Effects of shade and nitrogen levels on quality Bangladhonia production. *Bangladesh J. Agric. Res.,* 34: 205–213. doi: https://doi.org/10.3329/bjar.v34i2.5791

Nani BD, Sardi JCO, Lazarini JG, Silva DR, Massariolli AP, Cunha TM, de Alencar SM, Franchin M and Rosalen PL (2020). Anti-inflammatory and anti-Candida effects of Brazilian Organic Propolis, a promising source of bioactive molecules and functional food. *J. Agric. Food Chem*., 1168(10): 2861-2871.

Nchu F, Aderogba M, Mdee L and Eloff JN (2010). Isolation of anti-*Candida albicans* compounds from *Markhamia obtusifolia* (Baker) Sprague (Bignoniaceae). *South Afr. J. Bot.*, 76: 54–57. doi: 10.1016/j.vetpar.2011.06.017

Nelson AC and Kursar TA (1999). Interactions among plant defense compounds: a method for analysis. *Chemoecology*, 9: 81–92. doi:10.1007/s000490050037

Otang WM (2012). Ethnobotanical survey of medicinal plants used in the management of opportunistic fungal infections in HIV/AIDS patients in the Amathole District of the Eastern Cape Province, South Africa. *J. Med. Plants Res.*, 6: 2071–2080. DOI: 10.5897/JMPR11.069

Pedneault K, Leonhart, S, Gosselin A, Papadopoulos A, Dorais M and Angers P (2002). Variations in concentration of active compounds in four hydroponically-and field-grown medicinal plant species. *Acta Hortic.,* pp 255–262.

Radusiene J, Marksa M, Ivanauskas L, Jakstas V, Caliskan Ö, Kurt D, Odabas MS and Cirak C (2019). Effect of nitrogen on herb production, secondary metabolites and antioxidant activities of *Hypericum pruinatum* under nitrogen application, *Ind. Crop and Prod.,* 139: 111519, https://doi.org/10.1016/j.indcrop.2019.111519.

Santos I.S, Filho EGA, Ribeiro PRV, Zocolo GJ, Silva SM, de Lucena EMP, Alves RE and de Brito ES (2020). Chemotaxonomic evaluation of different species from the Myrtaceae family by UPLC-qToF/MS-MS coupled to supervised classification based on genus. *Biochem. System Ecol.,* 90: 104028. doi: 10.1016/j.bse.2020.104028

Savvas D, Passam H, Olympios C, Nasi E, Moustaka E, Mantzos N and Barouchas P (2006). Effects of ammonium nitrogen on lettuce grown on pumice in a closed hydroponic system. *Hort. Sci.*, 41: 1667–1673. doi: 10.21273/HORTSCI.41.7.1667

Shailajan S, Sayed N and Tiwari B. (2013). Impact of regional variation on lupeol content in *Carissa carandas* Linn. fruits: evaluation using validated high performance thin layer chromatography. *J. Adv Sci Res*., 4: 21-24. https://www.researchgate.net/deref/http%3A%2F%2Fwww.sciensage.info%2Fjasr .

Shuping DSS and Eloff JN (2017). The use of plants to protect plants and food against fungal pathogens: a review. *Afr. J. Tradit. Complement Altern Med*., 14(4)*:* 120-127. doi: 10.21010/ajtcam.v14i4.14.9.

Sugumaran P, Kowsalya N, Karthic R and Seshadri S (2013). Biomass production and antibacterial activity of *Justicia gendarussa* Burm. f. - A valuable Medicinal plant. *J. Trop. Life Sci.*, 3: 8–13. doi: 10.11594/jtls.03.01.02

Tchoumbougnang F, Sameza ML, Jazet DPM, Nkouaya MEG, Fekam BF, Ngoko Z, Amvam ZP H and Menut C (2010). Composition, radical scavenging and antifungal activities of essential oils from three Helichrysum species growing in Cameroon against *Penicillium oxalicum* a yam rot fungi. *Afr. J. Agric. Res*., 4: 121-127. https://doi.org/10.1016/j.sajb.2005.07.007

Van Alstyne KL and Pelletreau KN (2000) Effects of nutrient enrichment on growth and phlorotannin production in *Ficus gardneri* embryos. *Mar. Ecol. Prog. Ser.,* 206: 33-43. doi: 10.3354/meps206033

Van Vuuren S, Viljoen A, Van Zyl R, Van Heerden F and Baser KHC (2006). The antimicrobial, antimalarial and toxicity profiles of helihumulone, leaf essential oil and extracts of *Helichrysum cymosum* (L.) D. Don subsp. *cymosum*. *South Afr. J. Bot.*, 72: 287–290. https://core.ac.uk/download/pdf/82176863.pdf

Viegas DMAC, Palmeira-De-Oliveira A, Salgueiro L, De Oliveira JM and Palmeira-De-Oliveira R (2014). *Helichrysum italicum*: From traditional use to scientific data. *J. Ethnopharmacol.*, 151: 54–65. doi: 10.1016/j.jep.2013.11.005

Zhu X, Zhang H and Raymond L (2004). Phenolic compounds from the leaf extract of Artichoke (*Cynara scolymus* L.) and their Antimicrobial Activities. *J. Agric. Food Chem*., 52(24): 7272-7278 https://doi.org/10.1021/jf0490192