

THERAPEUTIC POTENTIAL OF *GNIDIA CAPITATA* L. F.: INVESTIGATIONS ON ITS ANTI-TYROSINASE, ANTIBACTERIAL, ANTIOXIDANT AND ANTICANCER ACTIVITIES

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Abstract

Background: *Gnidia capitata* L. F. belongs to the family Thymelaeaceae, and has been widely reported for its ethnobotanical uses, especially for the treatment of several human ailments which include skin conditions. However, there is limited information about the pharmacological properties of this plant as a potential cosmetic agent or pharmaceutical. The aim of this study was to evaluate the therapeutic potential of *G. capitata* for its anti-tyrosinase, antibacterial, antioxidant, anticancer and anti-mycobacterial properties.

Materials and methods: *G. capitata* was extracted with methanol (MeOH), ethyl acetate (EtOAc), dichloromethane (DCM) and hexane (n-Hex). All extracts were tested *in vitro* for activities against *Propionibacterium acnes* (ATCC 11827) and *Mycobacterium tuberculosis* H37Rv (ATCC 27294). Tyrosinase inhibitory activity was screened using tyrosinase from *Agaricus bispor*. Antioxidant activity was investigated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.

Results: EtOAc, DCM and n-Hex extracts of the plant showed antibacterial activity against *P. acnes* with MICs of 125 µg/ml. The DCM and n-Hex extracts showed anti-mycobacterial activity with MICs of 500µg/ml. The methanolic extract showed the highest antioxidant activity with an IC₅₀ of 41.83µg/ml.

Conclusion: The findings presented in this study may explain the potential use of *G. capitata* for the treatment of certain skin conditions. The potent antioxidant activity could help control the negative effects associated with inflammatory mediators that are produced during the immune response in people that are affected by skin conditions.

Keywords: Anti-acne, Anti-tyrosinase, Antibacterial, Antioxidant, Anti-TB, *Gnidia capitata*

Introduction

The human skin is the largest organ in the body and forms the first-line of defence against any harmful stimuli. There are three distinctive layers of the skin which include the epidermis, dermis and hypodermis (subcutaneous) which have unique functions of protection and in maintaining homeostasis (Tabassum and Hamdani, 2014). A number of factors including hypersensitivity reactions may trigger skin and subcutaneous tissue disorders (Voie et al., 2012). Some individuals suffer several dermatological conditions such as skin dryness, itching, redness, scaly patches and lichenified thick plaques with excoriation (Tay et al., 2002; Worm, 2002). Other forms of dermatological conditions include mild to severe acne, rashes, lesions, fungal dermatitis, irritations and nail disorders. Adverse dermatological conditions may also include skin ulcers, skin nodules, herpes simplex, herpes zoster, and pustular rash (Pharmacare, 2003; Stevens et al., 2005). These may cause serious discomfort and post inflammatory hyper-pigmentation.

Acne is one of the most common multifactorial chronic inflammatory diseases of the pilosebaceous follicles involving androgen induced sebaceous hyperplasia, altered follicular keratinisation, hormonal imbalance, immune hypersensitivity and bacterial colonisation by *Propionibacterium acnes* (Coenye et al., 2007; Williams et al., 2012). The development of inflammatory acne occurs through the activation of complement and metabolizing sebaceous triglycerides into fatty acids that irritate the follicular wall and surrounding dermis. *P. acnes* also produces exoenzymes which chemotactically attract neutrophils (Webster, 2002). The clinical manifestations of acne include

seborrhoea (excess grease), non-inflammatory lesions (open and closed comedones), inflammatory lesions (papules and pustules), and various degrees of scarring due to cyst formation (Williams et al., 2012). Another severe skin condition is cutaneous tuberculosis (TB) of the skin which is caused by *Mycobacterium tuberculosis*. A recent survey showed a broader spectrum of skin conditions among patients hospitalized with TB in South Africa and these conditions could be exacerbated due to co-infection of TB and HIV (McLachlan et al., 2016). The problem of antibiotic resistance and high treatment cost for skin conditions such as acne have led to the search for alternative remedies using medicinal plants.

The use of traditional medicinal plants to treat skin conditions is a common practice in many cultures, although the precise mechanism of cure is not always understood (Grierson and Afolayan, 1999). The topical application of many plant medicines for treatment of various skin conditions has proven effective due to the presence of plant secondary metabolites that have potent antimicrobial and antioxidant properties (Quave et al., 2008). These secondary metabolites can either eliminate or inhibit the activity of the infectious pathogens (Samy and Gopalakrishnakone, 2008). Antioxidants from plants protect against the damaging effects of free radicals generated during metabolic processes (Adedapo et al., 2008). The use of plants with substantial antioxidant activity and with the potential to afford protection against ultra-violet radiation is receiving increasing attention (Katiyar et al., 2001). Some plant extracts are used as sunscreen against UV rays, to improve skin texture and complexion (Bhat, 2013). Also, certain phytochemicals have shown to restrict diffusion of toxins and reduce adverse reactions caused by insect bites and stings, calm itching and skin irritation (Finn, 2004).

The Xhosa people in the Eastern Cape Province, use medicinal plants for the treatment of diseases and in rituals. One of the common ritual practices among the Xhosas is the initiation and circumcision of young men every year and plant extracts are used to dress and treat the wounds.

The genus *Gnidia* belongs to the family *Thymelaeaceae* and comprises of approximately 152 species; several members of which are frequently utilized for treating a variety of ailments. Leaves of *G. capitata* are used as snuff or applied as a poultice for treating toothache, boils, sores, snake bites, bruises and burns (Hutchings and van Staden, 1994, Philander, 2011). Other uses of *Gnidia* species include the induction of blistering, treating backache, coughs, epilepsy, headache, influenza, fevers, measles, pulmonary tuberculosis, smallpox, snake bites, sprains and tonsillitis among others (Bhandurje et al., 2013). This study was carried out to investigate the pharmacological properties of *G. capitata* to treat conditions associated with the skin.

Materials and methods

Plant collection and preparation

Plant material (bark) was obtained from the Bizana area in the O.R. Tambo municipality district, Eastern Cape Province, South Africa. Plants were identified by Dr. Kathleen Immelman of the Kie Herbarium at Walter Sisulu University. A voucher specimen (Mahachi 012) was deposited in the herbarium for future reference. Plant material was oven dried at 40°C for 5 days and ground into powder. Thereafter, plant sample was extracted with methanol, ethyl acetate and hexane and then filtered using Whatman No. 1 filter paper. Plant extracts were concentrated using a Buchi rotatory evaporator R124 and then air-dried under a stream of cold air. Crude extracts were kept at 4°C in the dark until analysis.

Antibacterial activity against *Propionibacterium acnes*

The minimal inhibitory concentration (MIC) of the *G. capitata* extracts were determined by microdilution assay. This assay was done using methods as described by McGaw and Eloff (2005) and Sahin et al. (2003) with slight modifications. The antibacterial activity was investigated against *Propionibacterium acnes* (ATCC 11827). The bacteria were cultured from a Kwik-Stick on nutrient agar and incubated at 37°C for 72h under anaerobic conditions before the assay. The 72h culture was re-suspended in sterile nutrient broth and adjusted to 0.5 McFarland standard turbidity. Tetracycline was used as the positive control (Lim et al., 2007). The plates were incubated at 37°C for 72h in anaerobic conditions. The MIC was determined by observing the colour change in the wells after addition of *p*-iodonitrotetrazolium violet salt (INT) and was defined as the lowest concentration that showed no colour change.

Colorimetric tyrosinase inhibition assay

The colorimetric tyrosinase assay was performed using methods described by Curto et al. (1999) and Momtaz et al. (2008). The source of tyrosinase enzyme used in the experiments was *Agaricus bisporus*, an edible mushroom species which is native to grasslands in Europe and North America. Extracts from *G. capitata* were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 20mg/ml (stock solution). This stock solution was then diluted to 600µg/ml in 50mM potassium phosphate buffer (pH 6.5). Kojic acid was used as the positive control (Lee et al., 1997). In a 96 well microtitre plate, 70 µl of each sample solution of different concentrations were combined with 30µl of tyrosinase (333Units/ml in phosphate buffer, pH 6.5) in triplicates. After incubation at room temperature for 5min, 110 µl of substrate (2mM L-tyrosine or 12mM L-DOPA) was added to each well. Final concentrations of the extract

ranged from 200µg/ml-1.56µg/ml. Microtitre plates were incubated for 30 min at room temperature in a BIO-TEK power Wave XS multi-well plate reader and optical density was then determined at 492nm over 30min (A.D.P., Weltevreden Park, South Africa). The fifty percent inhibition concentration (IC₅₀) value was determined by the use of GraphPad Prism v4.0 software.

Antioxidant activities of *G. capitata* extract and purified compounds

Antioxidant activity of the various extracts of *G. capitata* were investigated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, South Africa) antioxidant assay. Following the procedures as described by Du Toit et al. (2001) for each sample, a series of 8 dilutions was prepared in a 96-well ELISA plate by adding distilled water as a diluent. Final concentrations of the samples ranged from 0.78 to 100µg/ml. Each concentration was tested in triplicate. Vitamin C was used as the positive control. The radical scavenging capacities of the samples were determined using a BIOTEK plate reader to measure the disappearance of DPPH at 550nm. The radical scavenging activity was measured based on the amount of antioxidant necessary to decrease the initial DPPH absorbance by 50% (IC₅₀) (Du Toit et al., 2001). The IC₅₀ value for each sample was determined graphically by plotting the absorbance of DPPH as a function of the sample concentration in µg/ml. The results are expressed as the mg Vit C equivalents/g dry weight and are calculated as follows: VitEAC (mg AA/100g) = [IC₅₀ (Vit c)/ IC₅₀ (sample)] x 1000.

***In vitro* anti-mycobacterial assay**

The susceptibility of *M. tuberculosis* H37Rv (ATCC 27294) to plant extracts was investigated using the BACTEC system as described by Lall and Meyer (2001). Plant extracts were re-suspended in DMSO to stock concentration of 100 mg/ml. Subsequent dilutions were prepared in DMSO (20, 15, 10, 5, 2.5, 0.5 and 0.2 µg/ml) and added to BACTEC 12B vials containing 4ml 7H12 medium PANTA (Becton Dickinson & Company), an antimicrobial supplement. The solvent control consisted of a final concentration of DMSO (1%). Drug susceptibility was also performed for the standard anti-TB drugs, Streptomycin, Isoniazid (INH) and Ethambutol (Sigma Chemical Co, South Africa), at concentrations of 0.2, 6.0 and 7.0µg/ml respectively were used as the positive controls. A homogenous culture (0.1ml) of the strains of *M. tuberculosis*, yielding 1x10⁴ to 1x10⁵ CFU/ml, were inoculated in the vials containing the plant extracts as well as in the control vials (Heifets et al., 1985). Two extract-free vials were used as controls (medium+1% DMSO): one vial (V1) was inoculated in the same way as the vials containing the extracts, and the other (V2) was inoculated with a 1:100 dilution of the inoculum (1:100 control) to produce an initial concentration representing a bacterial population of 1x10² to 1x10³ CFU/ml found in the vials containing extracts. The MIC was defined as the lowest concentration of the extract that inhibited more than 99% of the bacterial population. When mycobacteria grow in 7H12 medium containing ¹⁴C-labeled substrate (palmitic acid), they utilize the substrate and ¹⁴CO₂ is produced. The amount of ¹⁴CO₂ detected reflects the rate and amount of growth occurring in the sealed vial, and is expressed in terms of the Growth Index (GI). Inoculated bottles were incubated at 37°C and each bottle was assayed every day to measure GI, at about the same hour until cumulative results were obtained. The difference in the GI values of the last two days is designated as ΔGI. The GI reading of the vials containing the test compound was compared with the control vial (V2). Readings were taken until the control vials, containing a 100 times lower dilution of the inoculum than the test vials, reached a GI of 30 or more. If the ΔGI value of the vial containing the extract was less than the control, the population was reported to be susceptible to the extracts. Each test was replicated three times.

Anticancer activity against the human malignant melanoma cells

The human malignant melanoma cell line, A375, was cultured in complete DMEM supplemented with 10% FBS, 15.g/L NaHCO₃, 2mM L-glutamine, 10µg/ml penicillin, 10µg/ml streptomycin and 0.25µg/ml fungizone at 37°C in a humidified atmosphere with 5% CO₂. A375 cells in DMEM were seeded in sterile 96-well plates at a cell density of 10⁴ cells per well. The plates were then incubated for 24 h at 37°C in a humidified atmosphere with 5% CO₂ to allow for adherence. The extracts were tested at concentrations ranging from 6.25 - 100µg/ml. Actinomycin D was used as the positive control as it shows toxic effects against the cell line. Toxicity was determined 72h after addition of samples using the XTT Cell Proliferation II Kit.

Results and discussion

Antibacterial activity of *G. capitata* extracts

The results presented in Table 1 showed the antibacterial properties of *G. capitata* extracts against *P. acnes*. Based on the MIC values, good antibacterial activity against *P. acnes* was recorded in DCM, EtOAc and n-Hex extracts (Table 1). In comparison with the positive control all the crude extracts had good MIC values ranging from 125 -500 µg/ml. Contrary to a previous study, McGaw et al. (2008) who reported that the methanolic extract of *G. capitata* had good MIC values of 1.6mg/ml against *S. aureus* however, in the present study a MIC of 500µg/ml was recorded in methanolic extract against *P. acnes*. Gowrish et al. (2016), also reported a MIC (1.5 mg/ml) against *Bacillus subtilis* and *Vibrio cholerae* in pet-ether extracts of *Gnidia glauca*. The antibacterial activity against *P. acnes* in the present

study provided an insight for the potential use of *G. capitata* in the treatment of skin related conditions. Phytochemical screening has shown that *Gnidia* species possess a wide range of compounds including polyphenols, daphnane diterpenes, lignans and neolignans dimers. These compounds have been implicated in several pharmacological properties such as antibacterial, antifungal, antitumor and antimitotic (MacRae and Towers, 1984; Bhandurje et al., 2013). Perhaps, the inherent phytochemical composition of *G. capitata* such as polyphenols could be responsible for the good antibacterial activity recorded in this study. The results also suggest that potential active compounds could be found in the intermediate and non-polar fractions as opposed to those that are soluble in more polar solvents.

Anti-tyrosinase activity of *G. capitata* extracts

The mushroom tyrosinase inhibitory activity of *G. capitata* extracts was tested. The anti-tyrosinase inhibitory activity showed that all the extracts of *G. capitata* exhibited $IC_{50} > 200 \mu\text{g/ml}$ against the enzyme at the highest concentration tested. Tyrosinase is known to be a key enzyme in melanin biosynthesis. When the skin is exposed to ultraviolet radiation the secretion of melanin is enhanced leading to rapid proliferation of melanocytes (Yasui and Sakurai, 2003). Acne condition can also lead to abnormal secretion of melanin which leads to hyperpigmentation of the skin. Hence, inhibition of antityrosinase activity could help control the post inflammatory hyperpigmentation associated with acne. Tyrosinase inhibitory potential of *G. capitata* extracts should perhaps be considered using in-depth screening to further elucidate the mechanism of inhibition. The activity of this extract may not show inhibitory activity on the enzyme but could potentially inhibit the transfer of melanin to the epidermis or possibly inhibit tyrosinase gene expression.

Anti-mycobacterial activity of *G. capitata* extracts

Anti-mycobacterial activity of *G. capitata* is presented in Table 1. In the present study, good anti-mycobacterial activity against drug-susceptible reference strain *M. tuberculosis* H37Rv was recorded in the DCM and n-Hex extracts. However, Gowrish et al. (2016) in a similar experiment reported excellent anti-TB activity (25 $\mu\text{g/ml}$) in pet-ether extract of *G. glauca* against *M. tuberculosis* H73Rv strain using the Microplate Alamar Blue Assay. Perhaps, different solvents used for extraction could be one of the possible explanations for the differences in anti-TB activities recorded in the present study. The H37Rv isolate was earlier found to be resistant against some reference drugs including rifampicin and isoniazid, in addition to some other first line and second line drugs (Bergval et al., 2009). Growth inhibition of H37Rv strains by some of the *G. capitata* extracts reported is an indication that this species could be used as a good anti-TB agent against drug resistant strains of *M. tuberculosis*. However, further studies are needed using more isolates/strains of *M. tuberculosis* as well as the identification of bioactive compounds responsible for anti-TB activity.

Table 1: Anti-TB, antioxidant, anti-tyrosinase, anti-acne and anticancer properties of *G. capitata* extracts.

Extract and positive controls	Anti-mycobacterial MIC ¹ ($\mu\text{g/ml}$)	Antioxidant		Anti-tyrosinase IC_{50} ³ ($\mu\text{g/ml}$)	Anti-acne MIC ($\mu\text{g/ml}$)	Anti-cancer IC_{50} A375 cells ($\mu\text{g/ml}$)
		DPPH ($\mu\text{g/ml}$)	IC_{50} ²			
Methanol	>1000	41.83		>200	500	>100
Ethyl acetate	1000	69.66		>200	125	>100
DCM	500	88.22		>200	125	>100
Hexane	500	138.20		>200	125	>100
Isoniazid	0.20	n/a		n/a	n/a	n/a
Vitamin C	n/a	1.98		n/a	n/a	n/a
Kojic acid	n/a	n/a		3.16	n/a	n/a
Tetracycline	n/a	n/a		n/a	3.1	n/a
Actinomycin D	n/a	n/a		n/a	n/a	4.5×10^{-6}

¹Lowest concentration required to inhibit *M. smegmatis* growth

²Lowest concentration required to scavenge 50% of the DPPH free radical

³Lowest concentration required to inhibit 50% of the tyrosinase enzyme activity

⁴Lowest concentration required to inhibit the growth of *P. acnes*

⁵Lowest concentration required to inhibit 50% of A375 cell viability

Antioxidant activity of *G. capitata* extracts

The antioxidant DPPH scavenging activity of *G. capitata* extracts is presented in Table 1. DPPH is a relatively stable nitrogen centred free radical that easily accepts an electron by reacting with suitable reducing agents. As a result, the electrons become paired off and the DPPH solution loses its violet colour depending on the number of electrons

taken up (Blois, 1958). The highest quenching activity was recorded in methanolic extract with an IC_{50} value of 41.83 $\mu\text{g/ml}$ followed by ethyl acetate extract then the DCM extract. The lowest DPPH activity was recorded in n-Hex extract. The Xhosa people in the Eastern Cape Province use extracts of *G. capitata* as sun screen indicating that this plant species has the capacity to prevent the pernicious effects of ultraviolet radiation from the sunlight which is the most common exogenous to the skin. Perhaps, the extracts of *G. capitata* not only act as sunscreen but might also maintain the linearity of dermal elastic fibres of the skin, reduce collagen degeneration which induces wrinkling and sagging, resulting in aging appearance of the skin. However, further studies are required to understand the exact mechanism of action by using fractions, semi pure and pure compounds of *G. capitata* in different assays.

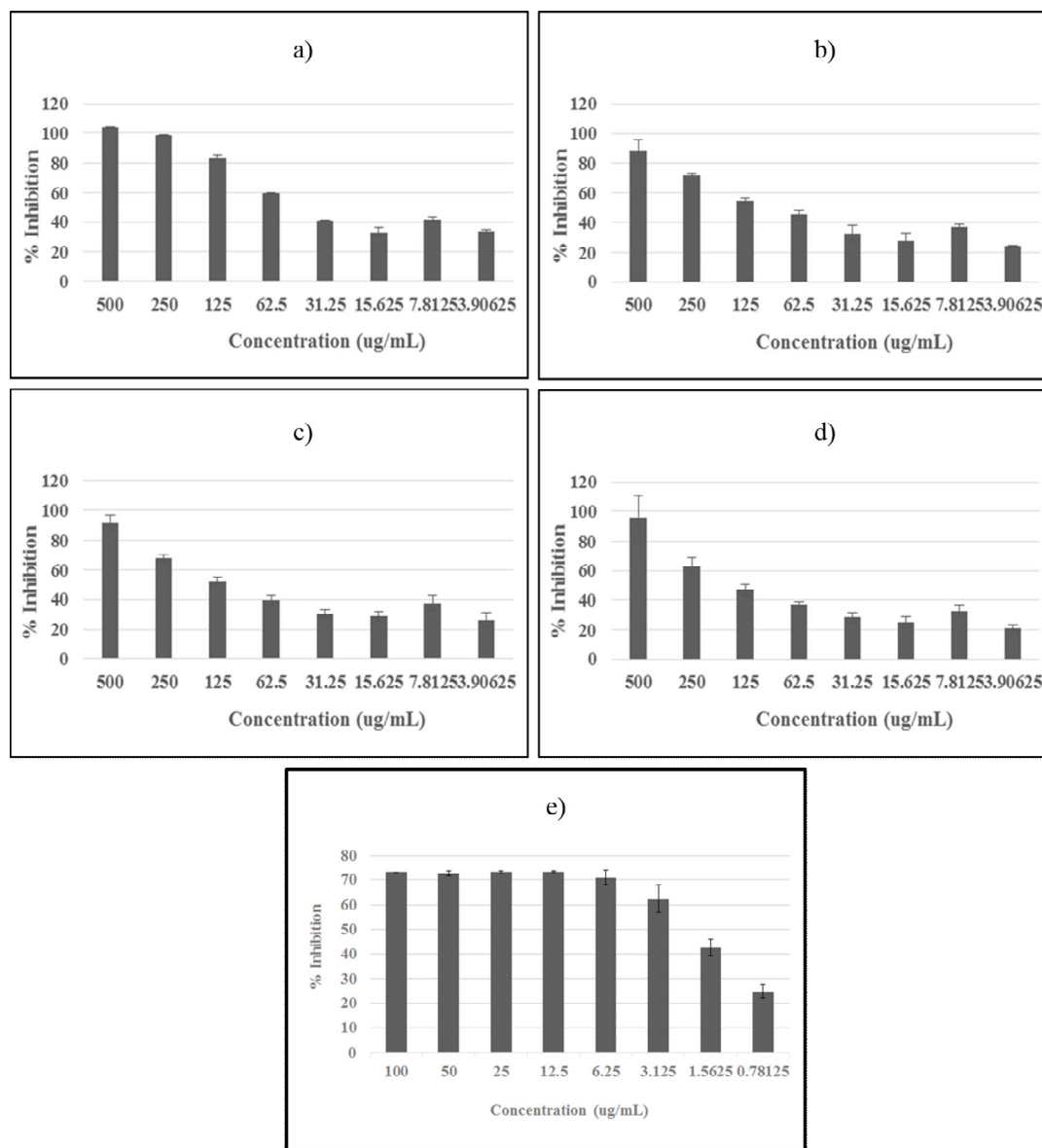


Figure 1: The dose-response curves of the inhibition of DPPH free radicals for the a) methanol b) ethyl acetate c) dichloromethane and d) hexane extracts tested at different concentrations compared with e) Vitamin C, a known scavenger of DPPH free radicals

Anti-cancer activity

Melanoma is defined as a tumour formed in melanocytes (melanin-forming cells). This is often a malignant tumour associated with skin cancer. The cytotoxic activity of *G. capitata* against epidermal malignant melanoma A375 is presented in Table 1. All the extracts tested in the present study showed no activity at the highest concentration tested (100 $\mu\text{g/ml}$) against the proliferation of melanoma cells.

Conclusion

The results of this study may explain the use of *G. capitata* for the treatment of skin related conditions among the Xhosa taking into account the fact that extracts exhibited low to moderate activity when the values obtained are compared to the positive standards in each model. The various extracts of *G. capitata* showed the best potential as either an anti-acne agent using non-polar solvent extraction and potentially as a sun protection factor (SPF) product using more polar solvents such as methanol. The bioassay-guided isolation and structure elucidation could be beneficial to determine which types of compounds could be responsible for the antibacterial and antioxidant activity and whether activity of these is better alone or within the crude extract. Many other species of *Gnidia* have been tested, however, *G. capitata* is relatively understudied.

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Conflicts of Interest: Authors declare that this research presents no conflict of interests.

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