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# Metabolite Profile of *Athrixia phylicoides* DC. (Bush Tea) and Determination of Inhibitory Mechanism against Tyrosinase Enzyme from Mushroom

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**Abstract:** *Athrixia phylicoides* DC. (Bush tea) is a shrub harvested in the north-eastern mountain regions of South Africa and belongs to the Asteraceae family. Generally, *A. phylicoides* is consumed as a hot tea beverage for its associated health benefits. The use of bush tea extracts for beauty enhancement has not been investigated even though several ethnobotanical reports have indicated its usage against skin imperfections. Therefore, the aim of the study was to assess plant metabolites of *A. phylicoides* for their inhibition of tyrosinase from mushroom and determine their inhibitory mechanism. Methanolic extracts (80% *v*/*v*) of *A. phylicoides* were evaluated using a tyrosinase-based TLC (thin-layer chromatography) autography technique. The inhibitory mechanism of active metabolites against the enzyme was determined using Lineweaver–Burk plots. Quercetin and an unknown metabolite with a retention factor (Rf) value of 0.73 inhibited melanogenesis. However, the $IC_{50}$ value for Quercetin was reported as 51.07 ± 2.43 µg/mL higher than that of kojic acid of 5.22 ± 1.44 µg/mL. Chlorogenic acid was reported to have a similar Rf value as kojic acid, suggesting similarities in polarity and affinity towards the adsorbent material. However, chlorogenic acid with an $IC_{50}$ value of 15.25 ± 1.18 µg/mL and an inhibitory strength of 2.92 could not inhibit melanogenesis with a similar intensity as kojic acid. The inhibitory mechanism for *A. phylicoides* extract was reported to be mixed inhibition (competitive/uncompetitive). The $IC_{50}$ value for *A. phylicoides* was recorded as 20.65 ± 0.14 µg/mL with an inhibitory strength of 3.96. These results suggest that *A. phylicoides* extracts could be used against dark spots associated with scarring and ageing through modulation of tyrosinase activity.

**Keywords:** *Athrixia phylicoides*; competitive inhibition; cosmeceutical; dark spots; inhibitory mechanism; Lineweaver–Burk plots; melanogenesis; tyrosinase



**Citation:** Thibane, V.S.; Ramphinwa, M.L.; Mchau, G.R.A.; Mudau, F.N. Metabolite Profile of *Athrixia phylicoides* DC. (Bush Tea) and Determination of Inhibitory Mechanism against Tyrosinase Enzyme from Mushroom. *Cosmetics* **2022**, *9*, 133. https://doi.org/10.3390/cosmetics9060133

Academic Editors: Adeyemi Oladapo Aremu and Basiru Ajiboye

Received: 25 October 2022
Accepted: 2 December 2022
Published: 5 December 2022



## 1. Introduction

The potential commercialization of *Athrixia phylicoides* DC. (Bush tea), following the success of *Aspalathus linearis* (rooibos tea) and *Cyclopia intermedia* (honey bush tea) in the herbal tea markets, is one that is yet to be fully explored. Bush tea has a traditionally historic usage by the indigenous people of the north-eastern region of South Africa against skin ailments such as sores, eruptions, boils, acne and for wound healing [1–3]. Currently, there are no available skincare products of *A. phylicoides* in the formal market as the plant is mainly consumed by the indigenous people where it is localized. Research on phytochemical content of the plant has reported on the diverse content of metabolites present with some studies further reporting the mineral content to be relatively higher than that of rooibos and *Cyclopia* spp. [4–6]. The apparent bioactivity of phenolic compounds

contributes significantly to the plant's cosmeceutical attributes such as achieving a desired skin complexion, combating skin disorders, and improving skin health. The enzyme tyrosinase is involved in the browning and darkening of food sources and the outer layer of the skin. Modulation of tyrosinase is one of the therapeutic targets for treating the undesired darkening of the skin following scaring or ageing-related dark spots [7].

There have been several challenges in commercializing *A. phylicoides* as a herbal tea beverage. However, other alternative uses of the plant such as for cosmeceutical product formulation can overcome some of these challenges. Recently, researchers have started looking at the potential cosmeceutical application of *A. phylicoides* extracts when agronomic practices of the plant are altered [8,9]. The cutting of dried plant material has been reported to result in a sticky residue developing on the cutting blades, which impeded further processing [9]. Slurry and paste preparations from the plant material may prove to be an alternative processing method since cosmeceutical extracts can be prepared from infusion of the leaves [10]. Maybe the most challenging aspect is the reported variation in the phenolic content of different *A. phylicoides* samples. These could be attributed to the genetic variation in wild harvested plants and different agronomic conditions the plant can be cultivated under [11–13]. Plant metabolites commonly found in *A. phylicoides* extracts can elucidate the mechanisms with which the cosmeceutical attributes of the plant are achievable. Furthermore, pharmacological studies on enriched samples of *A. phylicoides* revealed extracts to have potential in natural product research for the nutraceutical and cosmeceutical markets [6,14]. Additional products other than traditional tea beverages can assist in fast tracking the cultivation and commercialization of tea plants due to the possible increased demand of the raw material. No in-depth studies have been conducted on active metabolites responsible for administering reported ethnobotanical usage against skin imperfections. Therefore, the aim of the study was to assess plant metabolites of *A. phylicoides* for their inhibition of tyrosinase using a tyrosinase-based TLC (thin-layer chromatography) autography technique, and to determine the inhibitory mechanism of active metabolites against mushroom tyrosinase using Lineweaver–Burk plots.

## 2. Materials and Methods

### 2.1. Sample Preparation

Plant samples of *A. phylicoides* were collected from field trials at the University of Venda, Thohoyandou, Limpopo, at GPS coordinates 22°58′44.6″ S 30°26′23.0″ E. Collected plant material was identified by Prof F.N Mudau from the School of Agriculture, Earth and Environmental Science, University of KwaZulu-Natal. A voucher specimen (*Athrixia phylicoides*-9055000) was prepared and stored at the South African National Biodiversity Institute (SANBI), National Herbarium, Pretoria. Mature leaves and twigs of *A. phylicoides* were dried under shade (~25 °C) until constant dry weight. Samples were ground to a fine powder using a benchtop grinder and stored at room temperature in brown bags until extraction. Crude extracts were prepared by extracting samples of 20 mL/g (*v*/*w*) with 80% aqueous methanol (*v*/*v*) in an orbital shaker at 25 °C for 24 h. Crude extracts were then filtered through Whatman No. 1 filter paper and dried under vacuum to near dryness using a Gene Vac EZ-2 plus evaporator (SP Industries, Warminster, PA, USA).

### 2.2. Inhibition of Mushroom Tyrosinase Activity Assay

#### 2.2.1. Thin-Layer Chromatography (TLC) Autography Assay

Prepared methanolic crude extracts and standards at a concentration of 1 mg/mL were spotted on TLC (silica gel 60 $F_{245}$, 20 × 20 cm; Merck, Modderfontein, South Africa) aluminum plates at 6 mm bands, 5 mm from the lower edge. Plates were separated using ethyl acetate/methanol eluent systems and their inhibition of tyrosinase was evaluated. Chromatograms were developed in a closed tank with the atmosphere saturated with eluent vapor. The plates were dried overnight, or until there were no traces of solvent smell, at room temperature under a stream of air to remove excess solvent. The dried plates were sprayed with a vanillin-sulfuric solution (0.1 g vanillin, 28 mL methanol, 1 mL sulfuric

acid) and placed in an oven at 105 °C for 5 min for optimal color development. Separated compounds were viewed under visible light [15]. Two identical TLC plates were prepared, with Plate 1 as the reference chromatogram used to identify the presence of compounds and determine their Rf (retention factor) values. Plate 2 was used for the autography assay to identify inhibitors of tyrosinase from mushroom, *Agaricus bisporus* (LGE.) SING.

#### 2.2.2. Tyrosinase-Based TLC Autography

The assay was performed on the plate (Plate 2) with metabolites separated to identify inhibitors of the tyrosinase [16]. Briefly, a volume of 10 µL of tyrosinase from mushroom (100 units/mL—in 0.1 M potassium phosphate buffer, pH 6.5; Merck, South Africa) was spotted on each separated compound and the plate was incubated for 30 min at room temperature. L-3,4-dihydroxy-L-phenylalanine (L-DOPA; 10 µL, 10 mM) was added to each spot. A spot with everything except for the substrate served as a negative control and kojic acid (Merck, South Africa) served at the positive control. The prevalence of a dark spot/patch on the TLC plate was indicative of melanin formation while a clear spot indicating inhibition of tyrosinase. The Rf values of the inhibition zones on the plate were compared with those determined from the reference chromatograms (Plate 1). Plates were prepared in duplicate, and the experiment was repeated three times.

#### 2.2.3. Determination of Tyrosinase Inhibition Using Lineweaver–Burk Plots

The competitive, uncompetitive, and non-competitive inhibition of mushroom tyrosinase was determined using L-DOPA as a substrate [17]. Briefly, plant extracts and standards were re-suspended in 0.5% dimethyl sulfoxide (DMSO; Merck, South Africa) and diluted to a final concentration of 50, 100, 200 and 400 µg/mL in potassium phosphate buffer (0.1 M, pH 6.5) and two-fold serially diluted down the plate. In a 96-well microtiter plate, 70 µL of the plant extract was added followed by 30 µL of the mushroom tyrosinase enzyme (300 units/mL in potassium phosphate buffer, 0.1 M; pH 6.5). The reaction mixture was incubated for 10 min at room temperature. After incubation, 110 µL L-DOPA at concentrations of 25, 50, 100 and 200 mM was added to each well to initiate the reaction. Extracts with all compounds except for the substrate were used as negative controls and kojic acid served as the positive control. The microtiter plate was further incubated for 45 min at room temperature and the absorbance was read at 492 nm on a microplate reader. The inhibitory mechanism of tyrosinase was determined on Lineweaver–Burk plots. The experiment was performed in duplicate and was repeated three times.

### *2.3. Statistical Analysis*

Data was subjected to one-way analysis of variance (ANOVA) using GenStat® (ver. 11.1, VSN, Rothmsted, UK) and means were separated using Fischer's least significant difference (LSD) test and differences were considered significant at $p < 0.05$. The experiments were performed in duplicate and were repeated three times.

## 3. Results and Discussion

### *3.1. Tyrosinase-Based TLC Autography*

The results for the Tyrosinase-based TLC autography activity assays are presented in Figure 1. Column A of the plate demonstrates the separated metabolites of *A. phylicoides* extracts on an ethyl acetate/methanol eluent system. The intensity of the bands was directly proportional to the concentration of the extract spotted on the TLC plate. The potential of the separated metabolites in inhibiting melanogenesis through modulation of tyrosinase activity is depicted by column B. Formation of a dark spot/patch on the spotted area corresponding to a separated metabolite was indicative of melanogenesis. The results meant that the metabolite was unable to inhibit the activity of tyrosinase in converting L-DOPA to the dopachrome [7]. The results for kojic acid, a standard inhibitor of tyrosinase, are displayed in column C. The inhibition of melanogenesis was characterized by formation of clear to lightly shaded patches. This was further coupled with the presence of a shaded

ring towards the edges of the spotted areas. The persistence of the rings could be attributed to reduced concentrations towards the edges of the spotted areas.

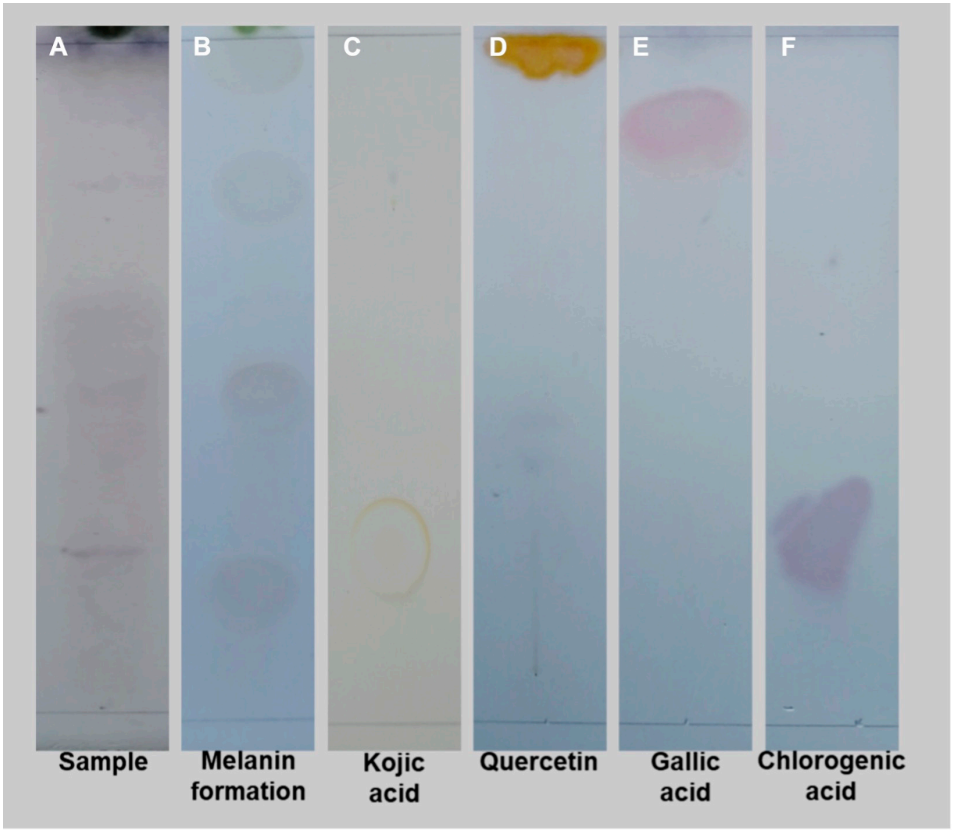


**Figure 1.** Tyrosinase-based TLC autography activity assay. Column A (untreated sample), column B (melanin formation), column C (kojic acid), column D (quercetin), column E (gallic acid) and column F (chlorogenic acid).

Quercetin, gallic acid and chlorogenic acid have been reported as some of the biomarkers for quality control of bush tea [18,19]. Column D, E and F represent quercetin, gallic acid and chlorogenic acid standards, respectively. Four separated metabolites with intense bands were tested for their inhibition of melanogenesis. The Rf values of the standards were correlated with the Rf values of the separated metabolites, and two could be correlated to quercetin and chrologenic acid (Table 1). Quercetin (column D) inhibited melanogenesis as it was characterized by formation of a clear to lightly shaded patch. The inhibition of tyrosinase by quercetin has been previously reported with the phenolic compound known to induce its inhibitory activity by binding to the active site of the enzyme and thus prevent interaction with the substrate [20]. A metabolite with an Rf value of 0.73 was also shown to inhibit melanogenesis. Chlorogenic acid (column F) had an Rf value of 0.19, similar to that of kojic acid, but could not inhibit melanogenesis similar to the positive control. Similar Rf values could indicate similarities of the metabolite to the standard in polarity and affinity towards the adsorbent material [21]. Other present metabolites (Rf value 0.44) were unable to inhibit the melanogenesis process. Gallic acid was shown to be present in lower concentrations as evident by a fainted band corresponding to the standard. The results from this study indicate that certain metabolites present in *A. phylicoides* extracts can modulate tyrosinase activity. These can contribute to the achievement of the desired skin tone and complexion through modulation of melanogenesis in scarred and ageing skin. The levels of the target metabolites could be increased by selecting plants from a geographic location, promoting the prevalence of the phenolic compound [19]. The content

of the metabolite can further be enhanced by alterations of agronomic practices during cultivation as it has previously been reported by [22].

**Table 1.** Retention factor (Rf) of *Athrixia phylicoides* metabolites and kojic acid.

| Compound | Retention Factor (Rf) |
|---|---|
| Quercetin [a] | 0.94 |
| Gallic acid [a] | 0.85 |
| Unknown 1 [a] | 0.73 |
| Unknown 2 [a] | 0.44 |
| Chlorogenic acid [a] | 0.19 |
| Kojic acid [b] | 0.19 |

[a] *A. phylicoides* metabolite; [b] tyrosinase inhibitor.

### *3.2. Determination of Tyrosinase Inhibition Using Lineweaver–Burk Plots*

Quality control and quality assurance are critical determinants in identifying active metabolites present in *A. phylicoides* that can have a long-lasting contribution to the cosmeceutical industry. Tyrosinase inhibitors from natural products still attract research interest due to the significant role of the melanogenesis process in the pharmaceutical and cosmeceutical industry. There are still safety concerns over the prolonged use of tyrosinase inhibitors such as kojic acid and hydroquinone to the skin. Competitive, uncompetitive, mixed type (competitive/uncompetitive) and noncompetitive inhibitors of tyrosinase are generally recognized [23]. The types of inhibition mechanism for the interaction of mushroom tyrosinase with active metabolites from *A. phylicoides* are presented using Lineweaver–Burk plots. The results of the maximum initial rate of an enzyme-catalyzed reaction ($V_{max}$) and the substrate concentration at which the initial reaction rate is half of the maximum reaction ($K_m$) for bush tea extract are represented in Figure 2. *Athrixia phylicoides* extract was composed of active metabolites that were able to increase the $K_m$ in the presence of 400 µg/mL plant extract. An increase in $K_m$ coupled with an unchanged $V_{max}$ indicates a competitive inhibitor, as the inhibitor has a higher affinity for the active site of the enzyme. There was a mixed inhibition by *A. phylicoides* extracts. The plant was notably composed of active metabolites that were able to increase $K_m$ while further decreasing $V_{max}$. The mixed inhibition results suggest that the inhibitory metabolite from the plant can bind the active site of the enzyme at the same time as the enzyme–substrate complex [23]. The $IC_{50}$ (100 units/mL tyrosinase; 100 mM L-DOPA) for *A. phylicoides* was significantly higher at 20.65 ± 0.14 µg/L when compared to that of the positive control (Table 2). The inhibitory strength is a measure of how effective an inhibitor is relative to kojic acid. The inhibitory strength value is achieved by dividing the $IC_{50}$ value of an inhibitor to that of kojic acid [24]. Therefore, an inhibitory strength of less than 1 is more desired, with extracts of *A. phylicoides* only recording a value of 3.96.

**Table 2.** $IC_{50}$ values for inhibition of mushroom tyrosinase by *Athrixia phylicoides* metabolites and kojic acid.

| Compound | $IC_{50}$ (µg/mL) | Inhibitory Strength ($IC_{50}^{I}/IC_{50}^{KA}$) |
|---|---|---|
| *A. phylicoides*[a] | 20.65 ± 0.14 | 3.96 |
| Quercetin [a] | 51.07 ± 2.43 | 9.78 |
| Chlorogenic acid [a] | 15.25 ± 1.18 | 2.92 |
| Kojic acid [b] | 5.22 ± 1.44 | NA |

[a] *A. phylicoides* metabolite; [b] tyrosinase inhibitor; NA—Not applicable.

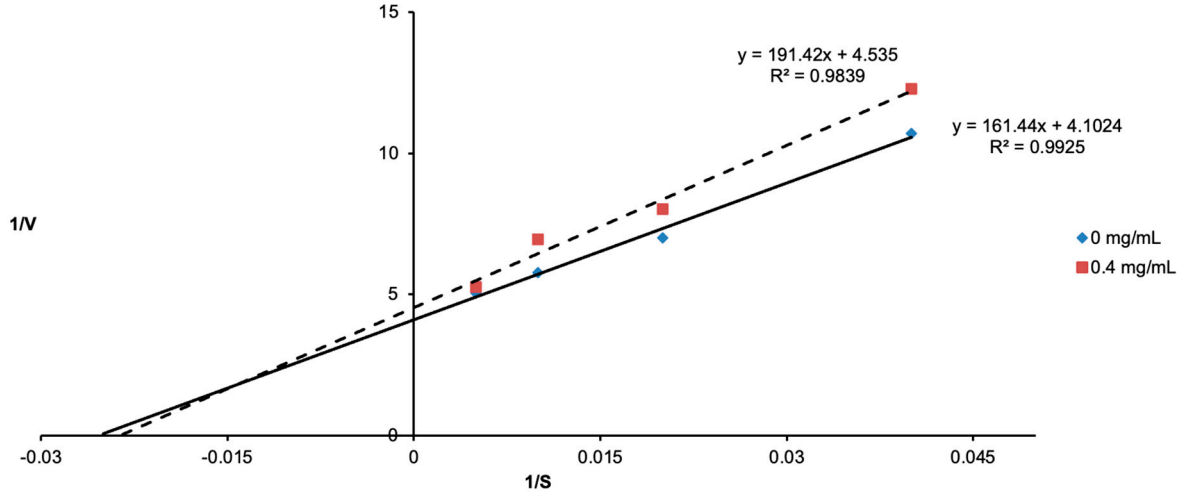


**Figure 2.** Lineweaver-Burk plot of conversion of L-DOPA to dopachrome by tyrosinase in the presence and absence of bush tea extract.

Kojic acid is the most intensively studied inhibitor of tyrosinase and the inhibitory mechanism is well understood. Kojic acid shows a competitive inhibitory effect on the monophenolase activity and a mixed inhibitory effect on the diphenolase activity of mushroom tyrosinase [25]. Kojic acid has further been reported to be a slow-binding inhibitor of the diphenolase activity [26].

The present study was able to confirm the mixed type of inhibition exhibited by kojic acid on mushroom tyrosinase. There was a reduction of $V_{max}$ coupled to an increase in $K_m$ in the presence of 400 µg/mL kojic acid (Figure 3). The value of $K_m$ indicates the affinity of tyrosinase towards L-DOPA, thus a reduction in $K_m$ signals a greater affinity of tyrosinase towards the substrate.

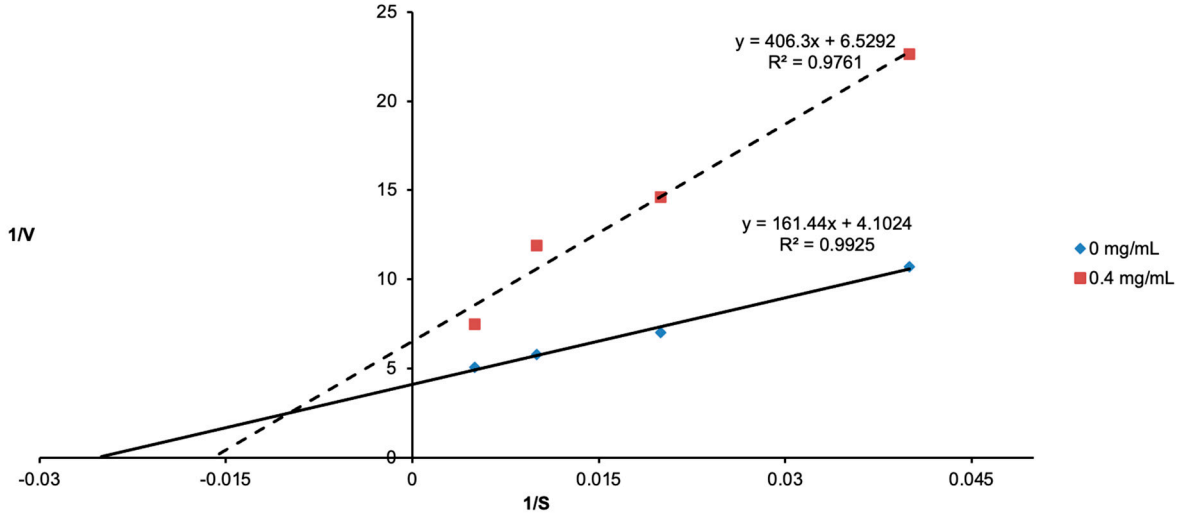


**Figure 3.** Lineweaver-Burk plot of conversion of L-DOPA to dopachrome by tyrosinase in the presence and absence of kojic acid.

The inhibition of mushroom tyrosinase by quercetin has previously been reported to be competitive [27]. The results from this study were able to present this known phenomenon by reporting an apparent increase in $K_m$ while $V_{max}$ remained unchanged (Figure 4). The anti-tyrosinase activity of flavonol has been reported, with quercetin reported to be the most active inhibitor [27]. The $IC_{50}$ and inhibitory strength for quercetin were recorded as 51.07 ± 2.43 µg/mL and 9.78, respectively. However, the inhibitory strength of quercetin is

significantly lower than that of kojic acid and thus the search for natural anti-tyrosinase products is still relevant.

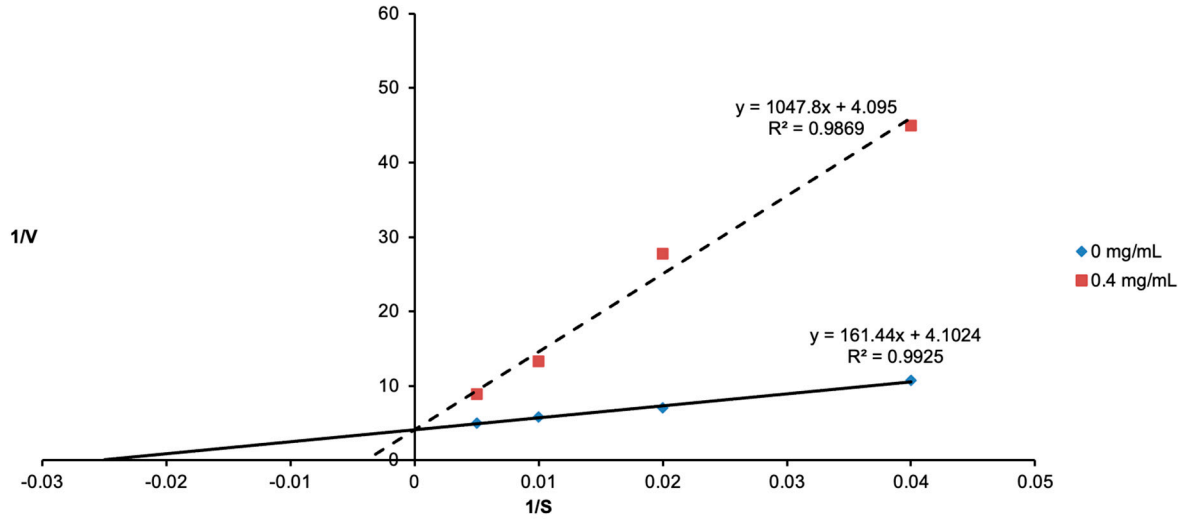


**Figure 4.** Lineweaver-Burk plot of conversion of L-DOPA to dopachrome by tyrosinase in the presence and absence of quercetin.

Chlorogenic acid was a competitive inhibitor of the mushroom tyrosinase with an apparent increase in $K_m$ while $V_{max}$ remained unchanged (Figure 5). Chlorogenic acid was reported to have the same Rf value as kojic acid, suggesting similarities in polarity. However, the notable difference in their inhibitory mechanism could be attributed to their structural and stereochemical arrangement differences. Chlorogenic acid could be acting only on the active site of mushroom tyrosinase, while kojic acid can bind on both the enzyme active site and the enzyme–substrate complex. The inhibitory strength of chlorogenic acid was also lower than that of kojic acid, with an $IC_{50}$ value of 15.25 ± 1.18 µg/mL.

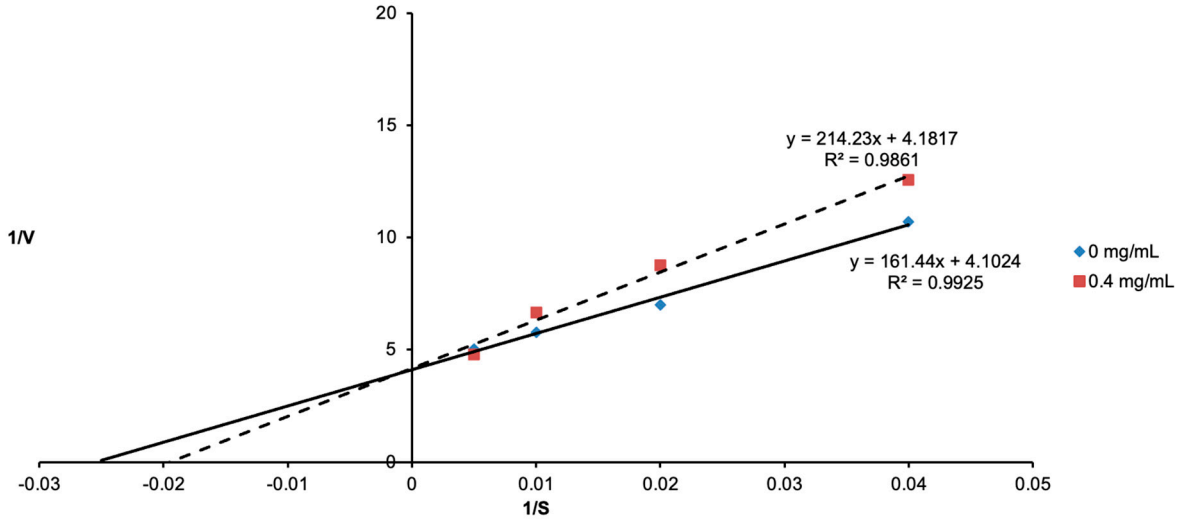


**Figure 5.** Lineweaver-Burk plot of conversion of L-DOPA to dopachrome by tyrosinase in the presence and absence of chlorogenic acid.

## 4. Conclusions

The potential of plants' metabolites to inhibit tyrosinase activity is a perfect target when searching for novel anti-tyrosinase compounds. New compounds should present their potential without compromising the skin as with other known inhibitors. The skin plays a pivotal role in maintaining the barrier function and providing protection against

photo irradiation. Tyrosinase is heavily involved in the post scarring and ageing process, resulting in the prevalence of dark spots. Extracts of *A. phylicoides* have demonstrated their potential to modulate the melanogenesis process without any potential compromise to the skin. The results on the inhibitory mechanism and strength demonstrated that the use of extracts will not result in skin bleaching. The bleaching of the skin is undesirable and exposes the skin to photo damage. Metabolites of *A. phylicoides* can interact with the active site of the enzyme or the enzyme–substrate complex and thus maintain the total number of melanocytes. Furthermore, the reproducibility of the results for the standards suggests high accuracy of the methodologies used in this study. Future studies on how metabolites from *A. phylicoides* affect tyrosinase gene level expression using cell culture models should be investigated. Computational studies such as molecular docking, molecular dynamic simulation and deep learning models should also be investigated to exploit how specific metabolites can interact with the active site of the enzyme tyrosinase.

**Author Contributions:** Conceptualization: V.S.T. and F.N.M., data collection and analysis: V.S.T. and M.L.R., writing original draft: V.S.T., review and editing: V.S.T., F.N.M., G.R.A.M. and M.L.R., funding: F.N.M. and G.R.A.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Research Foundation of South Africa, grant number 116775.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

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