Insights into tyrosinase inhibition by compounds isolated from *Greyia radlkoferi* Syzsyl using biological activity, molecular docking and gene expression analysis

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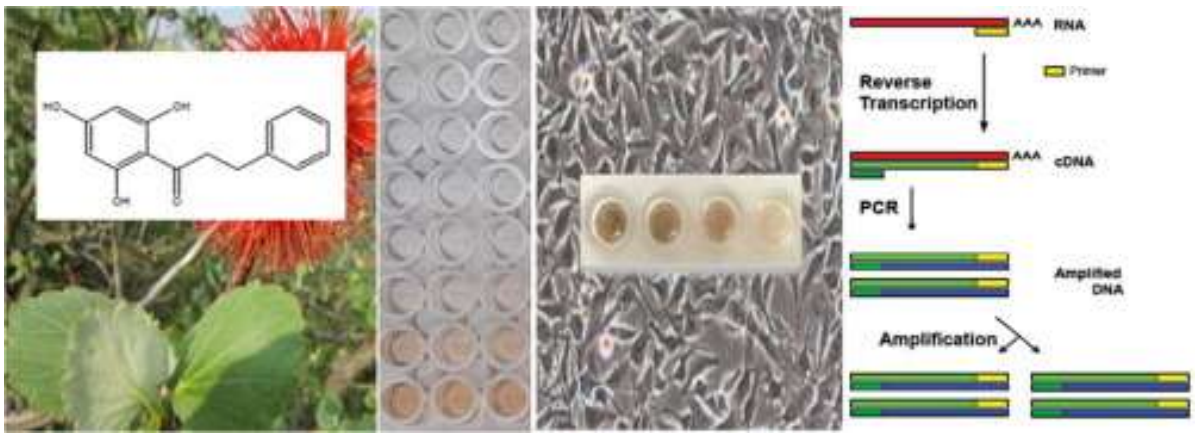
ABSTRACT

Greyia radlkoferi ethanol extract and its five compounds were tested for their inhibitory activity against the mushroom tyrosinase enzyme and melanin production on melanocytes. The crude extract showed significant tyrosinase inhibition with IC_{50} of 17.96µg/ml. This is the first report of the isolation of these 5 compounds from *Greyia radlkoferi*. 2', 4', 6'-trihydroxydihydrochalcone showed the highest tyrosinase inhibition at 17.70µg/ml (68.48µM), with low toxicity when compared with crude extract. This compound is therefore, a key component in the crude extract, which is responsible for tyrosinase inhibitory activity. The RT-qPCR indicated that the mechanism of action is most likely post transcriptional. Further, the molecular docking study showed that tyrosinase inhibitory activity depends on interaction of the compound with Cu^{2+} ions at the active site. This is the first report of the tyrosinase inhibitory activity of the *G. radlkoferi* extract and molecular insights on interaction of its compounds with Cu^{2+} ions as the driving factor for tyrosinase inhibition. These results suggest that the extract of *G. radlkoferi* and the compound 2', 4', 6'-trihydroxydihydrochalcone have great potential to be further developed as pharmaceutical or cosmetic agents for use against dermatological disorders associated with melanin.

Keywords: Greyia radlkoferi; tyrosinase; hyper-pigmentation; melanin inhibition; gene expression, molecular docking

Graphical abstract



1. Introduction

Melanin is the pigment which is responsible for the colour of human skin, hair and eyes. It is produced within melanocyte cells residing in the epidermis of the skin. Tyrosinase is the rate limiting enzyme in the biosynthetic pathway of melanin. [1] Increased activity of the enzyme often results in overproduction of melanin leading to a common skin disorder known as hyper-pigmentation, also known as hypermelanosis. This disorder is more common in women than men and affects all ethnic groups with the highest prevalence observed in the Asian demographic. [2]

Hydroquinone is the gold standard for the topical treatment of skin hyper-pigmentation. There are, however, many adverse effects that accompany the use of this treatment which includes skin irritation, contact dermatitis, and ochronosis in darker skin types (Fitzpatrick IV-VI). Extensive research in the field of skincare has identified a few novel de-pigmenting agents from plant extracts which showed less cytotoxicity and mutagenicity on melanocyte cells. These include active compounds such as kojic acid and arbutin. [3] The Greyiaceae family is rich in flavonoid compounds. [4] These secondary metabolites have been shown to inhibit the tyrosinase enzyme *in vitro*. [5]

Greyia radlkoferi Szyszyl is an attractive garden plant which thrives naturally in misty gullies and along rivers. It is a characteristic shrub that can grow up to 5m long. *Greyia radlkoferi* occurs mainly in the mist-belt mountains of Mpumalanga, in KwaZulu-Natal near Ngome and in Swaziland. It belongs to the *Greyiaceae* family which consists of two other species as well, *Greyia flanaganii* and *Greyia sutherlandii*. [6] The aim of this study was to investigate the potential of *Greyia radlkoferi* a plant indigenous to South Africa and its constituent metabolites for their inhibitory activity against tyrosinase and melanogenesis to determine whether they have an application in the cosmetic and pharmaceutical fields.

2. Materials and methods

2.1. Materials

Mushroom tyrosinase, L-tyrosine, kojic acid, arbutin, lipoic acid, Sephadex LH-20, penicillin streptomycin and EMEM were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture reagents including FBS, fungizone and trypsin were purchased from Scientific Group (Randburg, South Africa). The XTT Cell Proliferation Kit II was purchased from Roche (Basel, Switzerland). For RNA extraction the Qiagen RNeasy mini kit was

purchased from WhiteHead Scientific (Pty) Ltd. (Johannesburg, South Africa). For the PCR work, primers were purchased from Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) and the SYBR® Green Master Mix was purchased from Life Technologies (Johannesburg, South Africa).

2.2. Extraction and isolation

The leaves of *Greyia radlkoferi* were collected in January 2011 from the Manie van der Schijff Botanical Garden and the species identity was confirmed by Mr. Jason Sampson and a voucher specimen number was deposited at the H.G.W.J Schwelckerdt Herbarium. The plant material was air dried in a shaded area away from direct sunlight at room temperature. The plant material was then ground to a fine powder and extracted with ethanol (99%). The mixture was left for 48hrs on an orbital shaker and was then filtered through a Whatmann filter system. The filtrate was collected and rotary evaporated under reduced pressure to obtain a crude dried extract of *Greyia ralkoferi*. The isolation of bioactive compounds from *Greyia radlkoferi* was carried out using bioassay guided fractionation. About 59.5g of *Greyia radlkoferi* ethanolic extract was mixed with silica gel (1:4) and added to a silica gel column. Hexane fraction (bp 60-80°C) (Hex): ethylacetate (EtOAc) mixtures of increasing polarity (100:0 to 0:100) were used to yield 40 primary fractions (500 ml). Thin Layer Chromatography was used to check the primary fractions' chemical profiles. Similar fractions were combined to yield eight (8) major fractions. The combined fractions (F1 –F8) were tested for tyrosinase inhibition with L-tyrosine as the substrate. Fractions F4, F5, F6 showed the highest anti-tyrosinase activity (Table 1.). Due to their availability and quantity F4 and F6 were subjected to subsequent separation. Fraction F4 was re-applied to another silica gel column. This fraction was then subjected to series of sephadex columns using 100% MeOH to yield fractions "I – V". Fraction "III" (368mg) was further subjected to a sephadex column using 100% MeOH. Fractions "W-Z" were collected. "Compound 2" (4.6mg) was isolated from fraction "W". Fraction "V" was further subjected to a sephadex column using 100% MeOH and yielded "Compound 3" (8mg). Fraction F6 was subjected to a series of sephadex columns LH-20 (Sigma Chemical Co.) eluted with 100% ethanol (EtOH) and then washed with 100% MeOH to yield "Compound 1" (5mg). Similarly, "Compound 4" and "Compound 5" were isolated.

Table 1. Tyrosinase inhibitory activity, cytotoxicity and molecular docking of *G. radlkoferi* and its isolated compounds

Bioassay	**IC_{50} (µg/ml)/(µM)**							
	***G. radlkoferi* extract**	**Compound 1**	**Compound 2**	**Compound 3**	**Compound 4**	**Compound 5**	**Kojic acid**	**Arbutin**
[a] **Tyrosinase inhibition**	17.96	17.70/ (68.48)	113.60/ (420.37)	>200	>200	>200	3.78/ (26.60)	[e]N/A
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**
	>200	>200	>200	8.20	20.04	11.06	152.03	>200
[b] **Cytotoxicity**	40.89	56.59/ (218.95)	>200	21.42/ (79.21)	8.21/ (30.10)	95.56/ (373.00)	N/A	106.70/ (391.20)
[c] **Docking score (Gold fitness)**		55.58	39.74	41.95	41.97	43.41	47.95	
[d] **Residues involved in H-bond**		His60, Asn260, His263, Met280	Asn260, Met280	His61, Asn260	Met280	Asn260, Met280	His259	

[a] IC_{50} values reported for tyrosinase activity using L-tyrosine as a substrate for major fractions and compounds

[b] IC_{50} values reported for cytotoxicity on B16-F10 cell line

[c] Gold fitness score for compounds in the tyrosinase active site

[d] Number and type of residues where an interaction is observed

[e] N/A – not applicable to bioassay

2.3. Colorimetric tyrosinase inhibition assay

The colorimetric tyrosinase assay was performed using the methods described by Mapunya *et al*. [7] The plant extract and purified compounds were dissolved in DMSO (20mg/ml) and diluted in 50mM potassium phosphate buffer (pH 6.5). Extracts and pure compounds (including kojic acid) were tested from 200µg/ml – 1.56µg/ml. Kojic acid was used as the positive control. [8] In a 96-well microtitre plate, 70µl of sample (at each concentration) and 30µl of tyrosinase (333U/ml) were added in triplicate. The plate was then incubated at room temperature for 5 minutes, after which, 110µl of L-tyrosine (2mM) was added. The optical density (OD) was determined at 492nm using a BIO-TEK PowerWave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa) for 30 minutes. The fifty percent inhibitory concentration was then determined using GraphPad Prism 4.0 software (San Diego, California, USA).

2.4. Determination of cytotoxicity and melanogenesis in B16-F10 cells

The mouse melanocyte cell line, B16-F10, was cultured in complete EMEM supplemented with 10% FBS, 15.g/L $NaHCO_3$, 2mM L-glutamine, 10µg/ml penicillin, 10µg/ml streptomycin and 0.25µg/ml fungizone at 37°C in a humidified atmosphere with 5% CO_2. B16-F10 cells in EMEM were seeded in sterile 96-well plates at a cell density of 10^4 cells per well. The plates were then incubated for 24hrs at 37°C in a humidified atmosphere with 5% CO_2 to allow for adherence. Arbutin is a known inhibitor of melanin and was therefore used as a positive control for melanogenesis. The crude extract and arbutin were tested at 400µg/ml-6.25µg/ml. Pure compounds were tested at concentrations ranging from 100µg/ml-1.56µg/ml. Toxicity was determined 72h after the addition of samples using the XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide) Cell Proliferation Kit II.

The effect of the plant extract and compounds on melanin synthesis was determined by washing the melanocytes cells in the 24-well plate with PBS, and lysing with 200µl of sterile NaOH. Optical densities were then determined at a wavelength of 405 nm. The effect on melanin production was determined by comparison with the control sample (medium with DMSO). The IC_{50} values were determined using GraphPad Prism 4 (San Diego, California, USA).

2.5. Mechanism of action of *G. radlkoferi* extract

2.5.1. Cell treatments, RNA extraction and reverse transcription

B16-F10 cells cultured in complete EMEM were seeded in a 24-well plate (1 x 10^6 cells). Cells were treated with 25µg/ml of *G. radlkoferi* extract in triplicate. Controls included a solvent control of DMSO (2%) and a negative control of untreated cells. The solvent control was used to account for the effect of DMSO which was used to dissolve the plant extract. Plates were then incubated at for 72 hours at 37ºC at 5% CO_2. After incubation, total RNA from cells was extracted using the Qiagen RNeasy Mini Kit. A NanoDrop™ 2000 spectrophotometer (Thermo scientific, Minnesota, USA) was then used to determine the $OD_{260/280}$ and $OD_{260/230}$ ratios of all samples. Gel electrophoresis was performed to evaluate the quality of RNA. Extracted RNA (500ng) was used to prepare cDNA using the cDNA synthesis kit (Thermo Scientific, South Africa). Lipoic acid, a known transcription inhibitor of the tyrosinase gene, was used as a positive control. [9]

2.5.2. PCR Primers

Specific primers for the three genes (mouse tyrosinase (P11344), mouse β- actin (NC_000071) and mouse carnatine palmitoyltransferase (NC_000070)) were designed with the aid of Primer3 (version 0.4.0) software Selected primers for each gene were as follows: Tyrosinase forward primer: 5’ CCAGTGCCTTGTATATGC 3’, Tyrosinase reverse primer: 5’ CCTTGAACCGCTAGAGAA 3’, β-Actin forward primer: 5’ AAATCGTGCGTGACATCAAA 3’, β-Actin reverse primer: 5’ TCTCCAGGGAGGAAGAGGAT 3’, Carnitine palmitoyltransferase 2 (Cpt2) forward primer: 5’ CCTGCCAGAAGTGACACAGA 3’, Cpt2 reverse primer: 5’ ATCCAGGGGATATGCATTGA 3’.

2.5.3. Real Time-quantitative PCR (RT-qPCR)

Real time-quantitative PCR was conducted on the Biorad CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad Laboratories, Johannesburg, South Africa) using SYBR® Green Master Mix (Life technologies, Johannesburg , South Africa). The experiment consisted of three biological replicates and three technical replicates for each sample. Concisely, every 10µl reaction comprised of 1 x SYBR® Green Master Mix, 0.2M forward primer, 0.2M of reverse primer, 3.6µl water and 1µl cDNA template. The PCR was

programmed as follows; 95°C for 3min, followed by 40 cycles of 95°C for 10sec, 59°C for 30sec, 95°C for 10sec. A melting curve was incorporated at the end of the program which was from 65°C to 95°C with increments of 5sec. Melting curves and amplification plots with CP values were generated automatically by the CFX Manager™ software. Biogazelle qBasePlus 2.0 was used to analyse the expression results and generate standard curves for all three genes.

2.6. Molecular docking study

Molecular docking was performed using the GOLD program. It uses a genetic algorithm which considers ligand conformational flexibility and partial protein flexibility i.e. side chain residues. The default docking parameters were employed for the docking study. It includes 100 000 genetic operations on a population size of 100 individuals and mutation rate of 95. The crystal structure of mushroom tyrosinase (isolated from *Agaricus bisporus*) was taken from Protein Data Bank (PDB ID: 2Y9X). It has a crystal structure resolution of 2.78Å and contained an inhibitor; tropolone and two Cu^{2+} atoms in the active site. The structures of the small compounds were sketched using Chemdraw3D and minimized considering RMSD cut-off of 0.1Å. The docking protocol was set by extracting and re-docking tropolone in the tyrosinase crystal structure with RMSD <1.0Å. This was followed by docking of all compounds; including the known inhibitor, kojic acid, in the active site defined as 6Å regions around the co-crystal ligand in the tyrosinase protein. Furthermore, all compounds were evaluated for possible molecular interactions with tyrosinase active site residues using PyMol Molecular Graphics System.

2.7. Statistical analysis

The results were analysed statistically using one-way analysis of variance (ANOVA) and the least significant differences were determined using a Duncan's t-test. Results were considered significant where $P < 0.05$.

3. Results and discussion

3.1. Identification of compounds 1-5

All the isolated compounds from *G. radlkoferi* are known substances and were identified by directly comparing their spectroscopic data with previously identified known

compounds. Compound 1, a known chalcone has been previously isolated from the leaves of *Lindera umbellata* and *Boesenbergia pandurata*. [10, 11] Compound 2 (Galangin), a known flavonol, has previously been isolated from *Helichrysum aureonitens* and is a constituent of *Alpina officinarum* and the rhizomes *Alpina galanga*. [12, 13] Compound 3 (Genistein) has been reported in *Genista tinctoria*. [14] Compound 4 (Pinocembrin) is a flavonoid, reported in high concentrations in both *Turnera diffusa* and *Glycyrrhiza glabra* L. [15, 16] Compound 5 has been isolated from a number of *Piper* species including *hostmannianum, dennisii* and *aduncum*. [17-19] Compound 1, 4 and 5 have also been isolated from another Greyiaceae species, *G. flanaganii*. [7] This finding is not surprising as the B-ring deoxyflavonoids of each of the species belonging to the Greyiaceae family which are found on the leaf surface are almost identical. [4]

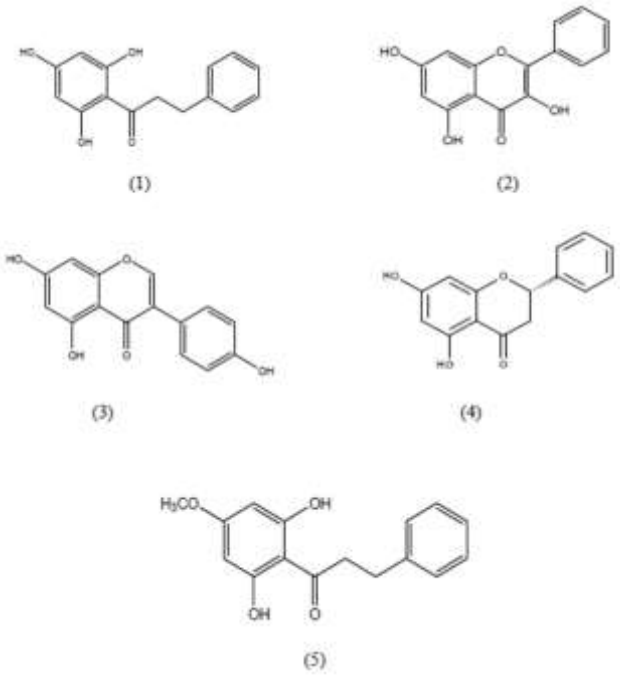


Figure 1. The chemical structures of the isolated compounds of *G. radlkoferi* (1) 2′,4′,6′ trihydroxydihydrochalcone (2) 3,5,7-trihydroxyflavone (3) 4′,5,7-trihydroxyisoflavone (4) 5,7-dihydroxyflavanone (5) 2′,6′-dihydroxy-4′-methoxydihydrochalcone.

3.2. Tyrosinase inhibitory activity

The ethanolic leaf extract of *Greyia radlkoferi* was tested for its tyrosinase inhibitory activity using L-tyrosine (monophenolase inhibition) and L-DOPA (diphenolase inhibition) as substrates. The crude extract showed an IC_{50} of 17.96µg/ml for monophenolase activity

and was comparable with a known inhibitor, kojic acid, the positive control which showed an IC_{50} of 3.78µg/ml. When L-DOPA was used as a substrate, however, there was no significant inhibition by both the extract and the positive control, kojic acid. The IC_{50} for the extract using L-DOPA as a substrate was greater than the highest concentration tested (>200µg/ml). The effect of the substrate also decreased the kojic acid activity from 3.78µg/ml to 107.30µg/ml. These results compared well with that of another species from the family Greyiaceae, *Greyia flanaganii*. The ethanol extract of *G. flanaganii* exhibited an IC_{50} of 32.62µg/ml, almost twice than that of *G. radlkoferi*. In a similar study, the inhibition of tyrosinase was investigated using methanol, acetone and dichloromethane extracts of the stem bark of *Sideroxylon inerme* which showed an IC_{50} of 82.10, 63.00 and >400µg/ml respectively. High levels of inhibition were also observed for kojic acid with an IC_{50} of 1.14µg/ml which correlated well with this study. [20] The *G. radlkoferi* extract compared well with the methanolic fruit rind extracts of various cultivars of pomegranate. The Ganesh, Molla de Elche, Ruby and Wonderful cultivars showed IC_{50} of 25.38, 25.56, 20.33 and 23.67µg/ml, respectively against monophenolase activity. The inhibition of these extracts against diphenolase activity showed similar patterns observed in this study. [21] The tyrosinase enzyme has a much higher affinity for dihydroxyphenols such as L-DOPA, catechol and catechin and in most instances when L-DOPA is used as a substrate it is able to bind competitively which could explain low levels of diphenolase inhibitory activity by both the extract and the kojic acid. [22]

Compound 1 and Compound 5 were identified as chalcones. The presence of the A and B rings are typical characteristics of the chalcone base structure, however, for these two compounds the enone double bond seems to have been reduced. [23] Compound 1 showed an IC_{50} similar to that of the total extract (17.96µg/ml and 17.70µg/mlµµg/ml, respectively) and is most likely responsible for the inhibitory activity of the crude extract. Jun *et al* [24] investigated the monophenolase inhibitory activity of 2', 4', 6'- trihydroxychalcone and found this compound to exhibit an IC_{50} of 120.00µM. Compound 1 in this study had an IC_{50} of 69.15µM, which could be due to the absence of the double bond at the α, β position. Loizzo *et al* [25] reported that the IC_{50} for two chalcones, namely, 2, 4, 2', 4'-tetrahydroxychalcone and Morachalcone A were 0.06µM and 0.14µM, respectively. Takahashi *et al* [26] reported the activity of 2, 4, 2', 4'-tetrahydroxychalcone with an IC_{50} of 0.21µM. Flavonoids containing the resorcinol moiety on their aromatic rings, particularly at positions 2, 4 or 2',4' are known to have enhanced inhibitory activity due to the interaction of

the resorcinol hydroxyl groups have with the copper atoms at the active site. Lee *et al* [27] reported the activity of a cyclohexenyl chalcone against melan-a tyrosinase with an IC_{50} of 8.2µM. The activity is said to be linked to the presence of the 2, 4-hydroxyl resorcinol structure, specifically on the B-ring of chalcones. This could explain the IC_{50} of Compound 1 which also lacks the two hydroxyl groups at the A ring of the chalcone. Compound 5 was completely inactive at 200µg/ml which could be due to these differences. Compound 1 did show better activity when compared with two synthetic chalcones 4', 4-dihydroxychalcone and 4'- Amino-4-hydroxychalcone which showed inhibitory activity at an IC_{50} of 4.80mM and 8.30mM, respectively.

The present study is the first report of the isolation of Compound 2 from *G. radlkoferi*. This compound is, however, not soluble in water-based test solutions at concentrations higher than 50µM (13.51µg/ml). Therefore the IC_{50} of 113.60µg/ml (420.37µM) could be due to precipitation of the compound. However, Kubo & Kinst-Hori [28] showed that Galangin was able to significantly lengthen the lag phase of the tyrosinase enzyme at a concentration of 0.02mM and therefore, inhibits monophenolase activity. Kubo *et al* [29] reported that galangin significantly inhibited the oxidation of L-tyrosine by tyrosinase. This study also showed that Galangin was able to decrease the formation of dopachrome by 20% after 30min due to the formation of adducts with *o*-dopaquinone. Xie *et al* [30] observed that Galangin has inhibitory effects on diphenolase activity with an IC_{50} of 101.00µM.

The isolation of Compound 3 (Genistein) from *G. radlkoferi* has been reported for the first time in this study. This isoflavone compound showed no significant inhibition of tyrosinase activity, at the highest tested concentration of 200µg/ml (740µM). Chang *et al* [31] reported the IC_{50} of Genistein against monophenolase activity to be 0.822mM (822µM) similar to the findings of the present study. Park *et al* [32] also found activity of this compound on tyrosinase isolated from melan-a cells to be >500µM (>135µg/ml).

Compound 4 (2*S*-Pinocembrin) is a flavanone compound. This compound was not active at the highest concentration tested. The activity is comparable to that of a very similar compound, Naringenin, which showed only 2.8% inhibition at 100µM. Two similar compounds were reported by Peralta *et al* [33], namely (*2S*)-8-prenylpinocembrin and (2*S*)-6, 8-dimethylpinocembrin, which showed monophenolase inhibition at an IC_{50} of 80.60µM and 97.6µM, respectively. The lack of activity in Naringenin and Pinocembrin, can be explained

due to the absence of the 4-substituted phloroglucinol moiety with 2 free hydroxyl groups in the A ring of flavanones, which are a dominant feature for tyrosinase inhibition.

3.3. Cytotoxicity on B16-F10 cells and effect on melanogenesis

The IC_{50} of the *Greyia radlkoferi* extract was found to be 40.89µg/ml on the B16-F10 cell line, showing moderate toxicity. [34] The extract was able to inhibit the production of melanin at a concentration of 12.5µg/ml by 50%, while cell viability remained above 50%. The cytotoxicity of Compound 1, 4 and 5 showed an IC_{50} of 219µM, 373µM and 8.21µM, respectively on the B16-F10 cell line. 2', 4', 6'- trihydroxydihydrochalcone (Compound 1) inhibited 25% of melanin content at 12.5µg/ml and 25µg/ml where cell viability remained above 50%. 2*S*-Pinocembrin (Compound 4) showed an 8% inhibition of melanin content at 12.5µg/ml. 2', 6'-dihydroxy-4'-methoxydihydrochalcone (Compound 5) was able to inhibit 10% of melanin content at a concentration of 6.25µg/ml while cell viability remained above 80%, however at higher concentrations this compound showed toxic effects (Table 1).

Compound 2 (Galangin) showed no toxicity on B16-F10 cells. Melanin content was inhibited by 40% and 30% at a concentration of 25µg/ml and 12.5µg/ml, respectively. Horibe *et al* [35] reported the activity of two compounds on melanin induction, Kaempferide and Kaempferol, which contain a 4'-O-methyl and 4'-OH fuctional group, respectively. These features make Galangin different from Kaempferide and Kaempferol. The 4'-O-Methyl flavonoid Kaempferide resulted in an increase in melanin content whereas the 4'-OH flavonoid Kaempferol, showed no significant changes in melanin content. The absence of the 4'-Methyl functional group could explain the activity of Galangin.

Compound 3 exhibited an IC_{50} of 21.42µg/ml on B16-F10 cells. This compound is considered to be cytotoxic against B16-F10 cells. The decrease in melanin content at concentrations 21.42µg/ml can therefore, be attributed to the cytotoxicity and not due to inhibition. Melanin content was not reduced even at non-lethal concentrations. Yan *et al* [36] tested the cytotoxicity of this compound on B16-BL6 cells, which are similar to B16-F10 cells with some genetic differences and using the MTT assay, the IC_{50} was found to be 4.18µg/ml. [37] This correlates well with the results observed in the current study. Yan *et al* [36] also tested the effects of this compound on melanogenesis in the B16-BL6 cell line and observed an increase in melanin content by 2.4 times at concentrations as low as 10µM and 4

fold increases at 20µM and 30µM after a 72 hour incubation. Park *et al* [32] reported that for isoflavone compounds to inhibit melanin content the C8 or C-3' hydroxyl groups are important, which are absent in Genistein and it is assumed that tyrosinase is not target with regards to the compound inhibiting melanin. This compound could, however, target the inhibition of the glycosylation of tyrosinase and suppress cell growth.

Arbutin was used as the positive control and was non-toxic to the B16-F10 melanocytes with 50% of the cells remaining viable, even at a concentration of 100µg/ml. Arbutin was able to inhibit melanin content up to 75-80% at the range of concentrations tested without having cytotoxic effects. These results compared well with that of Roh *et al* who showed that a concentration of 33.21µg/ml (122µM) is required to inhibit 50% of melanin production in B16-F10 melanoma cells. [38] These results are expected considering that Arbutin is currently used in anti-blemish cosmetic products and is known to decrease melanin synthesis in melanocytes stimulated with α-Melanocyte Stimulating Hormone (α-MSH). It also inhibits tyrosinase activity in cell-free systems. The α-MSH is generally overproduced with increased sun exposure which is one of the environmental factors influencing the progression of skin hyper-pigmentation. [39]

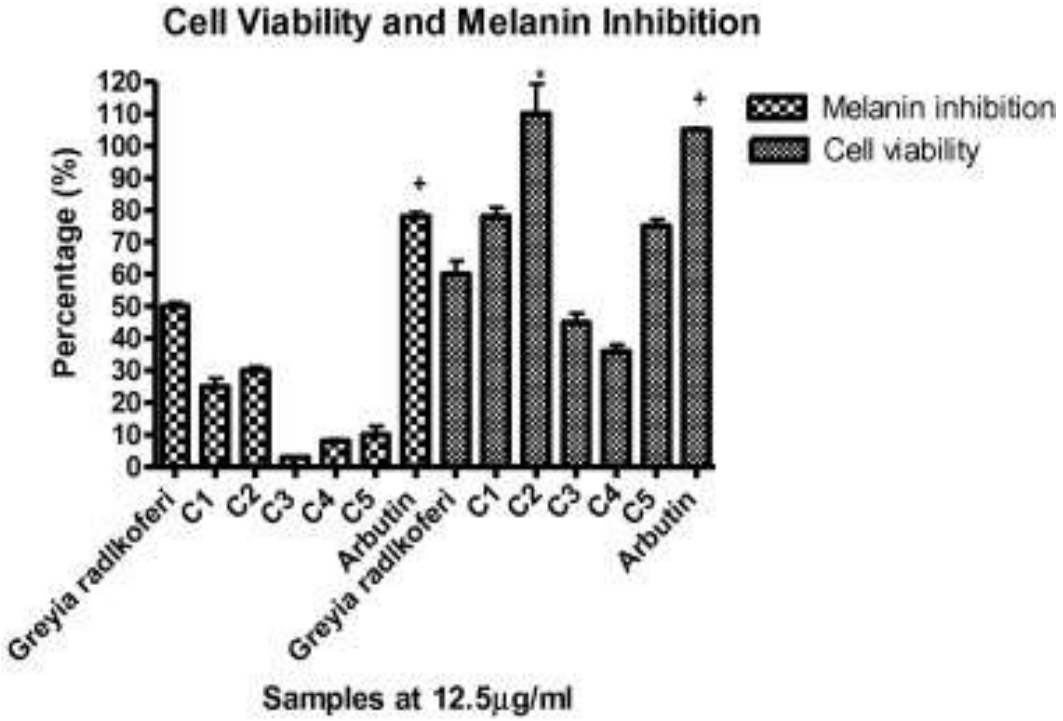


Figure 2. Inhibition of melanogenesis by the extract and compounds at 12.5 µg/ml.

3.4. Effect of the extract on tyrosinase gene expression in B16-F10 cells

Based on the significant tyrosinase inhibitory activity of the *G. radlkoferi* extract it was assessed whether the observed inhibition of the mushroom tyrosinase activity and overall melanin production inhibition in treated mouse melanocytes was due to decreased transcription of the tyrosinase gene. In this regard, expression of tyrosinase mRNA in treated cells was measured by using qRT-PCR. The β-Actin and Cpt2 genes served as the housekeeping genes. Tyrosinase mRNA in B16F10 mouse cells was found to be up-regulated in response to *G. radlkoferi* treatment as compared to the untreated controls where expression was increased by 2.4 fold at 25μg/ml (Fig. 2).

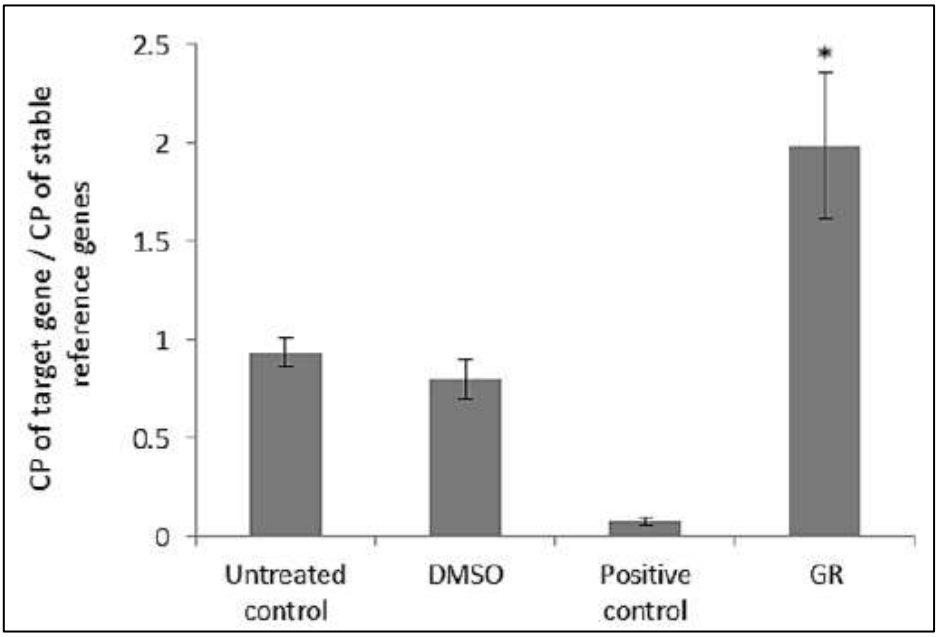


Figure 3. Expression of TYR mRNA in treated B16-F10 melanocyte cells. Expressions of tyrosinase were normalized to both the expressions of Actin-B and Cpt2 mRNAs ($P < 0.05$).

The results suggest that the inhibitory effect of the *G. radlkoferi* extract on tyrosinase is post-transcriptional. Compounds in this extract therefore inhibit melanin biosynthesis through a mechanism other than tyrosinase gene expression. For example, hydroquinone, functions by interacting directly with the tyrosinase enzyme by binding to the active site and soy bean extracts were shown to decrease tyrosinase production post-transcriptionally. [40, 41] The study therefore, lays foundation for the understanding of how *G. radlkoferi* constituents inhibit the production of melanin.

3.5. Molecular docking study of kojic acid and purified compounds

The molecular docking was performed to predict the binding pose and orientation of molecules at the active sites of tyrosinase. GOLD program successfully docked kojic acid and purified molecules into the active sites of tyrosinase enzyme. Gold fitness scores of kojic acid and the molecules are summarized in Table 1. The comp **1** showed highest fitness score of 55.58 and was followed by the standard molecule i.e. kojic acid with fitness score of ~48. However, rest of the molecules showed fitness score below ~45 depicting a fitness score difference of ~10 as compared to comp **1.** It indicated that binding mode of comp **1** and kojic acid reasonably differed from other at the active site of tyrosinase. The analysis of binding pose showed that OH group of kojic acid penetrates in deep of active site, making polar interactions with Cu^{2+} ions. The van der waals distances between Cu^{2+} ions and heavy atom of OH group were observed to be 2.17Å and 2.53Å (Fig. 3). Similarly, OH group bonded to C4 position in comp **1** showed the interactions with Cu^{2+} ions with distances 2.24 Å and 2.31 Å. In addition to this, π-π interaction was also found between Phe264 and phenyl ring of comp **1** protruding outside the cavity. Despite similar interactions to comp **1**, comp **3** showed low biological activity. However, absence of π-π interaction between Phe264 with protruding moiety of comp **3** is supposed to diminish the binding affinity, and hence, its biological activity. In contrast to these molecules, comp **2**, **4** and **5** showed large distances between nearest heavy atoms to Cu^{2+} and Cu^{2+} ions. In addition to this, comp **1** is observed to make four H-bond interactions while comp **2**, **3** and **5** were observed with two H-bond interactions (Table 1.). The kojic acid and comp **4** were observed to make only one H-bond, however, interactions pattern shows that affinity of molecules depends more on interaction with Cu^{2+} ions. The observed interactions in these molecules are in agreement of their observed biological activity. Thus, this study indicated that interactions of molecules with Cu^{2+} ions emphasized a critical phenomenon that controls the tyrosinase inhibitory activity of molecules.

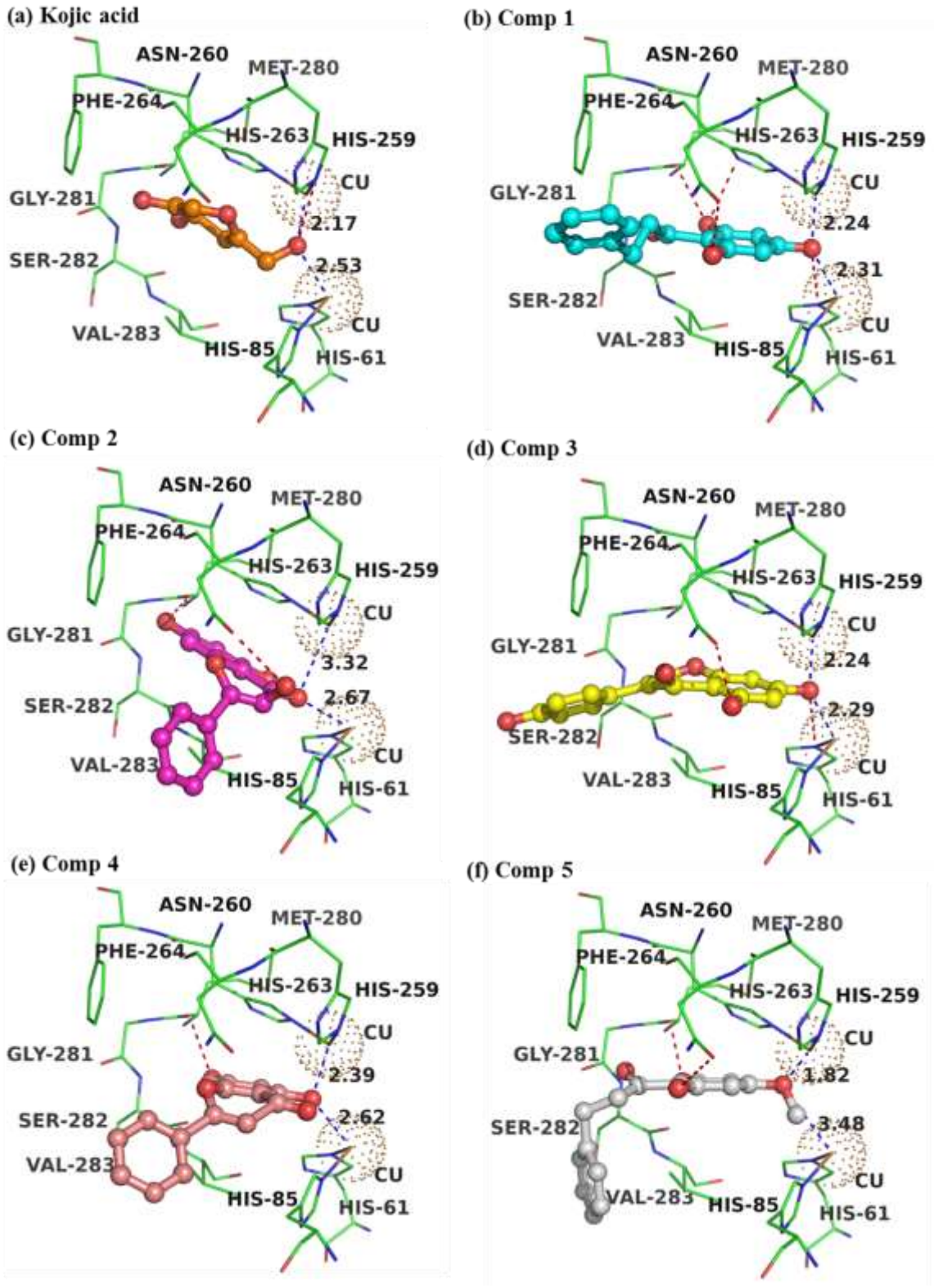


Figure 4. The bound molecules in the active site of tyrosinase show H-bond (red) and polar interactions (blue) with residues and Cu^{2+} ions, respectively. The distance of the O atom from Cu^{2+} is given in angstrom (Å).

4. Conclusion

The molecular docking study correlated well with the biological activity of the compounds *in vitro*. Compound 1 (2', 4', 6'-trihydroxydihydrochalcone) has similar tyrosinase inhibitory activity to that of the extract and should be considered as the biological marker compound. This compound is most likely responsible for the extract activity as illustrated by the interactions between this compound and Cu^{2+} ions at the active site of the tyrosinase enzyme. These results suggest that the extract of *G. radlkoferi* and the compound 2', 4', 6'-trihydroxydihydrochalcone have great potential to be further developed as pharmaceutical or cosmetic agents for use against dermatological disorders associated with melanin. The study also paves the way for future work which could include the extract and the biological marker in an appropriate vehicle or formulation for efficacy testing in clinical studies.

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