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The *in vitro* cytotoxicity against human melanoma cells, tyrosinase inhibition and antioxidant activity of *Grewia tenax* leaves extracts

[La citotoxicidad in vitro contra las células de melanoma humano, inhibición de la tirosinasa y actividad antioxidante de los extractos de hojas de *Grewia tenax*]

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Abstract: *Grewia tenax* (Forssk.) Fiori (Malvaceae) grows in the Arabian Peninsula and is used for several medicinal purposes. To characterize the dermatological bioactivities of *G. tenax* in terms of its antimelanoma, antityrosinase and antioxidant activities. Cytotoxicity was assessed by cell proliferation and mitochondrial viability assays. Ability to inhibit mushroom tyrosinase and scavenge free radicals were evaluated by an enzymatic and DPPH scavenging microtiter assay, respectively. Phytochemical analyses were carried out using TLC, HPLC-UV and NMR. The chloroform extract shown significant cytotoxic activity in terms of mitochondrial viability (43 ± 14 µg/mL). We identified lupeol and b-sitosterol as the main active components for the tyrosinase inhibitory activity of the hexane extract. Scavenging activity of the DPPH· radical was confined to the water extract. Extracts from this plant have the potential to be used as a base in the development of cosmeceutical products intended to whiten skin or to combat radical-induced physiopathological processes.

Keywords: Melanoma; Cytotoxicity; Tyrosinase; Antioxidant; Skin; Flora of Saudi Arabia

Resumen: *Grewia tenax* (Forssk.) Fiori (Malvaceae) crece en la Península Arábiga y se utiliza con varios fines medicinales. Para caracterizar las bioactividades dermatológicas de G. tenax en cuanto a sus actividades antimelanoma, antitirosinasa y antioxidante; la citotoxicidad se evaluó mediante ensayos de proliferación celular y viabilidad mitocondrial. La capacidad para inhibir la tirosinasa de hongo y eliminar los radicales libres se evaluó mediante un ensayo de microtitulación enzimático y de eliminación de DPPH, respectivamente. Los análisis fitoquímicos se realizaron mediante TLC, HPLC-UV y NMR. El extracto de cloroformo mostró una actividad citotóxica significativa en términos de viabilidad mitocondrial (43 ± 14 µg/mL). Identificamos lupeol y b-sitosterol como los principales componentes activos para la actividad inhibitoria de tirosinasa del extracto de hexano. La actividad depuradora del radical DPPH· se limitó al extracto acuoso. Los extractos de esta planta tienen potencial para ser utilizados como base en el desarrollo de productos cosmecéuticos destinados a blanquear la piel o combatir procesos fisiopatológicos inducidos por radicales.

Palabras clave: Melanoma; Citotoxicidad; Tirosinasa; Antioxidante; Piel; Flora de Arabia Saudita

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INTRODUCTION

The genus *Grewia* comprises approximately 150 species of small trees and shrubs that grow in subtropical and tropical regions and is the only one that yields edible fruits in the family. It has been recently highlighted by many researchers for its potential food and medicinal uses (Zia-Ul-Haq *et al*., 2013; Sulieman & Mariod, 2019).

Grewia tenax (Forssk.) Fiori (Malvaceae) grows in the Arabian Peninsula, Africa and the Southeast of Asia. The plant has several uses in different ethnopharmacological systems including jaundice and hepatic disorders, the fruits are eaten to treat anaemia and chest diseases. Bark decoction has anthelmintic property, the alcoholic extract ointment accelerates wound healing process, in addition the fruit powder accelerates bone fracture healing and suppresses the swellings (Al-Said *et al*., 2011). The roots are used to make a poultice and the leaves are useful against tonsillitis infections, trachoma and inflammation. A mucilaginous bark preparation is used by women against hair parasites (Orwa *et al*., 2009; Aadesariya *et al*., 2017). The fruits of *G. tenax* are rich in amino acids, mineral elements (K, Ca, Mn, Fe, Cu and Zn), tannins and pectic substances (Elhassan & Yagi, 2010) and have commercial potential in beverages, ice cream, yogurt, and baby food (Gebauer *et al*., 2007) thus endowing this traditional medicinal plant with an enormous economic prospective value both local and regional (Sharma & Vidy, 2012).

Skin cancer is classified into non-melanoma skin cancer (NMSC) and melanoma. Although NMSC is the most common form, melanoma is the most aggressive and is also the major cause of mortality. The death cases caused by malignant melanoma are higher than other cancer types and it currently represents the 5th most common cancer among men and women in the USA (Kuphal & Bosserhoff, 2009). Also, its incidence has increased by 88% in men and 66% in women by 2020 in the UK (Shack *et al*., 2008).

The discovery of anti-cancer agents from nature is one of the most promising strategy to defeat certain types of tumours, it requires continuous screening of natural sources including terrestrial plants seaweeds and marine organisms. Many plant secondary metabolites are either specifically cytotoxic to melanoma cells or inhibit their migratory activity/metastatic potential (AlQathama & Prieto, 2015). The flora of Saudi Arabia is characterized by high biodiversity due to the climatic and geographic variations. Our group has conducted extensive screenings during the last decade aimed to discover anti-cancer agents elucidating the precise mechanism of action including as apoptosis inducer, cell cycle arrest and anti-migratory effect (AlQathama *et al*., 2017; AlQathama *et al*., 2020a; AlQathama *et al*., 2020b; AlQathama *et al*., 2021).

Current experimental efforts against melanoma also encompass the antityrosinase and antioxidant interventions. Tyrosinase expression -and therefore activity- increases during tumorigenesis, which could allow for selective treatment of this tumor type by strategies that target tyrosinase activity (Vargas *et al*., 2011). On the other hand, cancer metabolism generates radicals which together with those generated by the increased activity in the melanosome cause an increased DNA damage and activate cellular signal transduction pathways that prevent cell death (Obrador *et al*., 2019).

In the light of the above evidence, our objective here is to characterize the potential dermatological value of *Grewia tenax* in terms of its *in vitro* antimelanoma, tyrosinase inhibitory and radical scavenging activities.

MATERIALS AND METHODS

Chemicals

Sulforhodamine B (SRB), trichloroacetic acid, Trizma base, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glacial acetic acid, and organic solvents were obtained from Fisher (Leicestershire, UK). TrypLE Express (1×, trypsin, EDTA, phenol red), phosphate-buffered saline (PBS) and trypan blue were purchased from Thermo Fisher Scientific (MA, USA).

Plant materials

The leaves of *Grewia tenax* (Forssk.) Fiori (Malvaceae) were collected in Al-Kurr (21º 21' 38" N 40º 14' 07" E), Saudi Arabia, and identified by Prof. Bader. Sample specimens were deposited in the Pharmacognosy Department at Umm Al-Qura University (SA-UK/2013/9).

Preparation of plant extracts

After powdering the plant materials, extracts were made using different solvents: hexane, chloroform and methanol. The extracts were then dried, and stock solutions were prepared in DMSO at a concentration of 50 mg/mL and lastly filter-sterilized using a 0.2 μm filter before testing on the cell line.

Melanoma cell line

A375 cells were used in this work to evaluate the cytotoxicity properties of the extracts, respectively. They were purchased from Sigma and were obtained from the American Type Culture Collection (ATCC number: CRL-1619™). Both cell lines were maintained in Dulbecco's Modified Eagle Media (DMEM, Gibco, CA, USA) and supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Gibco, CA, US) and 1% penicillin-streptomycin antibiotic, consisting of 10,000 units of penicillin and 10,000 µg/mL of streptomycin (Gibco, CA, USA).

Sulforhodamine B (SRB) assay

This assay determined the ability of the extract to inhibit cellular growth by measuring the cell density and from there estimating cell numbers. The assay was performed according to a previously described method (Vichai & Kirtikara, 2006). A375 cells were seeded at density of 10,000 cells/well in a 96-well plate (Thermo Scientific) and left overnight at 37°C to adhere. Afterwards, cells were treated with a serial dilution of the plant extracts (200, 100, 50, 25, 12.5, 6.25 µg/mL) at several time points. Upon completion of the incubation period, the cells were fixed with trichloroacetic acid solution for 1 h at 4°C. After washing with water, cellular protein was stained with SRB solution and left at room temperature for 1 h, followed by washing the plate four times with 1% acetic acid and flicking to remove unbound dye. Then, Tris base buffer solution was added to each well and the absorbance was measured at 510 nm. Cell growth was calculated using the following equation:

$$\%Cell\ growth = \frac{Absorbance\ (sample) - Absorbance\ (blank)}{Absorbance\ (vehicle\ control) - Absorbance\ (blank)} \times 100$$

Alamar Blue (AB) assay

An AB assay was conducted to investigate the effect of the extracts on mitochondrial viability. AB dye is converted to a highly fluorescent substance by metabolically active mitochondria in a live cell. A375 cells were processed in the same way as described in Section 2.6. After incubation, 100 µL of diluted AB reagent was added and incubated for 2 h at 37°C. The fluorescence intensity (FI) was measured at 560 nm excitation and 590 nm emission and the percentage of cell viability was calculated using the following equation:

$$\%Viable\ cells = \frac{FI\ (sample) - FI\ (blank)}{FI\ (vehicle\ control) - FI\ (blank)} \times 100$$

Phytochemical analyses

TLC (Thin Layer Chromatography) analyses were made using Silica gel 60F254 analytical plates. HPLC-UV (High-Performance Liquid Chromatography-Ultra-Violet) conditions were as described by Giner *et al.* (Giner *et al.*, 1993). Chromatograms were obtained in an Agilent 1100 Series (gradient quaternary pump, online degasser, photodiode array detector) using ChemStation software. Elution for phenols and flavonoids was achieved using a Phenomenex® C18 column (250 × 4.6 mm id, 5 µm). Solvents A (H_2O + Acetic Acid 0.2% aq) and B (Methanol + Acetic Acid 0.2 % aq) were mixed in gradient mode as follows: 0 min 90% A, 0-5 min 80% A, 5-45 min 50% A, 45-55 min 20% A; flow rate 0.8 mL/min. The injection volume, column temperature, and UV wavelengths were set at 80 µL (50 mg/mL), 30°C and 254/360 nm, respectively. NMR (Nuclear Magnetic Resonance) spectra were obtained on Bruker AVANCE 500 MHz spectrometer equipped with a CP QNP multinuclear cryoprobe head using the standard ^{1}H sequence pulse (254 scans). The TOPSPIN v1.3 software was used for spectra acquisition and processing.

Radical scavenging assay

The method described by (Prieto *et al.*, 2012) was used. Briefly, 100 µL of a 0.1 mM DPPH ethanolic solution was added to 100 µL of each diluted extract or reference standard antioxidant in 96-microwell plates. After 30 min of incubation in the dark at room temperature the absorbance was measured at 517 nm against a blank.

$$\%DPPH\ scavenging = \frac{Absorbance\ (sample + DPPH) - Absorbance\ (\text{sample}\ blank)}{Absorbance\ (DPPH) - Absorbance\ (\text{solvent}\ blank)} \times 100$$

Tyrosinase inhibitory activity

Qualitative inhibition was assessed by TLC autobiography spraying tyrosinase (10,000 U/ml) and L-tyrosine (0.05 µM) and incubated at 37°C until development of a uniform brownish background (Wangthong *et al*., 2007).

Quantitative inhibition was assessed according to the method described by (Masuda *et al*., 2005). 2.5 mM L-tyrosine in buffer and the sample in DMSO are mixed in a total volume of 160 µL, to which 40 µL of mushroom tyrosinase (46 units/mL) is added. After 20 minutes the absorbance was measured for each well at 475 nm, then the % of Tyrosinase inhibition calculated against the control.

$$\%Tyrosinase\ activity = \frac{Absorbance\ (sample) - Absorbance\ (blank)}{Absorbance\ (vehicle\ control) - Absorbance\ (blank)} \times 100$$

Statistical analysis

Results are expressed as mean ± standard deviation (SD) from at least three independent experiments. All data were analysed using unpaired two-tailed student's t-test with a *p*-value of <0.05 taken as significant to find the statistical significance between treated groups and controls using InStat v.3 (GraphPad, San Diego, USA).

RESULTS

Table No. 1 summarizes the GI_{50} values of the tested plant extracts. According to the criteria of the American National Cancer Institute, crude plant extracts which show an GI_{50} of less than 100 µg/mL are considered to be cytotoxic, while the most promising have an GI_{50} of < 30 µg/mL (Nor Aini *et al*., 2008). Only the chloroform extract showed some cytotoxic effect.

Table No. 1
Half-maximal inhibitory concentrations (IC_{50}) of plant extracts and reference compounds upon cell proliferation (SRB assay, 48h), mitochondrial viability (AB assay at 48h), radical scavenging (DPPH assay) and tyrosinase activity (enzymatic assay)

Treatment	**Proliferation assay** (A375 cells)	**Mitochondrial viability** (A375 cells)	**Radical Scavenging**	**Tyrosinase Inhibitory Activity**
Hexane extract (µg/mL)	> 200	95 ± 9	> 100	60 ± 3
Chloroform extract (µg/mL)	83 ± 6	43 ± 14	> 100	> 100
Methanol extract (µg/mL)	> 200	> 200	10 ± 2	> 100
Reference compound (µM)	9.4 ± 2 [1]	8.5 ± 3 [1]	2 ± 0.8 [2]	11 ± 0.6 [3]

(1) Paclitaxel; (2) Kojic acid; (3) Caffeic acid

Inhibition of tyrosinase activity below 100 µg/mL was only shown by the hexane extract ($IC_{50} \cong$ 60 µg/mL). Table No. 2 shows that a Solid Phase Extraction fractionation (silica gel 60) afforded 12 fractions. A similarly active fraction was eluted with hexane-ethyl acetate 75:25.

Table No. 1
Inhibition of tyrosinase activity by the fractions of the hexane extract of *G. tenax* leaves

Fraction	Hexane-Ethyl Acetate	Tyrosinase IC_{50} (µg/mL)
1	(100:0)	> 200
2	(90:10)	103 ± 32
3	(75:25)	59 ± 11
4	(50:50)	111 ± 43
5-12	(215:75 – 0:100)	> 200

Fraction 3 was analysed by TLC. Figure No. 1 shows tyrosinase inhibition, which is easily distinguished as white spots on the brownish background (Wangthong *et al*., 2007).

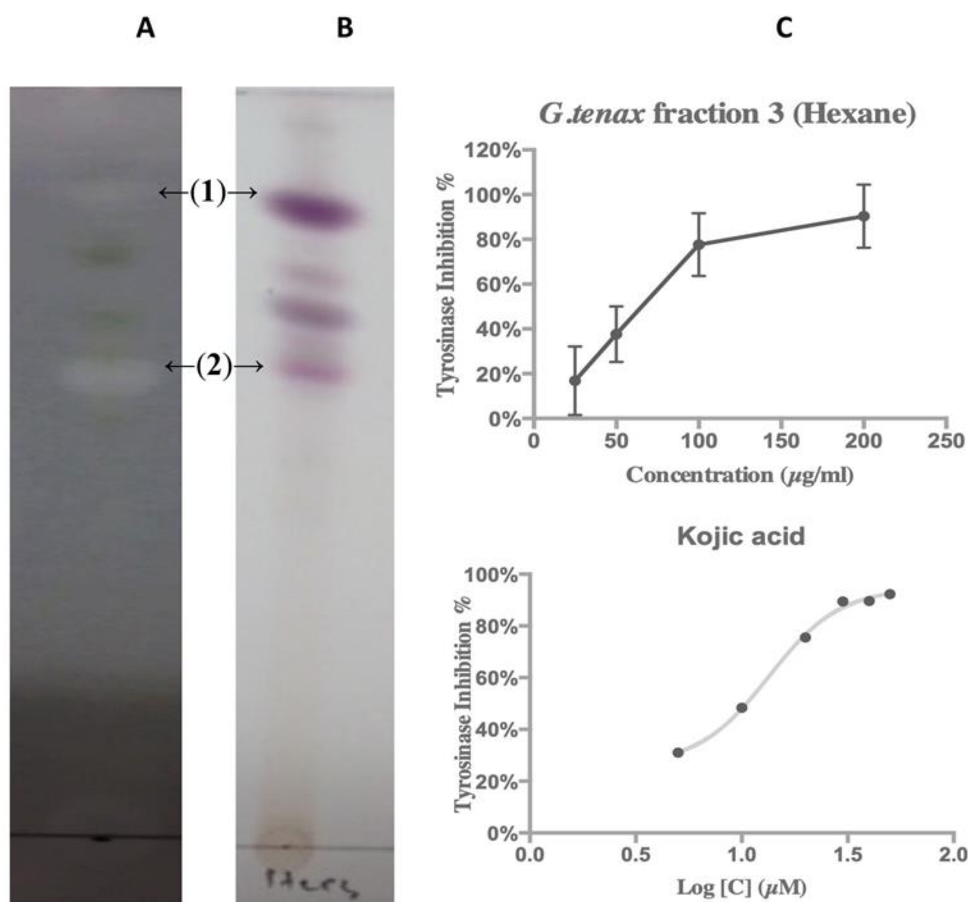

Figure No. 1
TLC of the hexane fraction of leaves of *G. tenax* sprayed with (A) tyrosinase (10,000 U/ml) and L-tyrosine (0.05 µM) and incubated at 37°C; (B) with anisaldehyde/H_2SO_4 and heated (100°C). (C) Graph shows concentration-dependent inhibitory activity of the most active fraction of the hexane extract of leaves of *G. tenax* and kojic acid (positive control)

The bands were identified as (**1**) β-sitosterol and (**2**) lupeol (Figure No. 2) by TLC against standards and the Rf and colour were matched under the same conditions described above. To further confirm its identity, Band **2** was isolated by preparative TLC using the same conditions (2 runs) and subjected to NMR analysis. It appeared as a white amorphous powder and HR ESI-MS

established the molecular formula as $C_{30}H_{50}O$ giving the $[M+H]^+$ ion at m/z 427.3930. ^{1}H-NMR signals identified it as lupeol (Burns *et al*., 2000) (Data not shown).

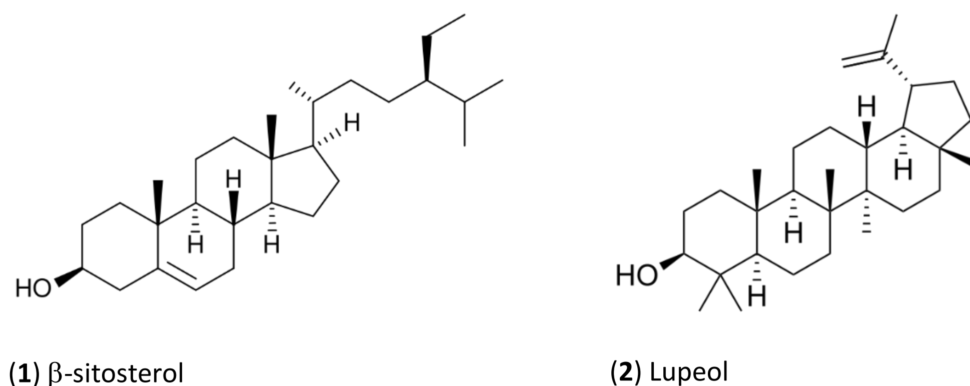

Figure No. 1
Chemical structures of β-sitosterol and lupeol

Antioxidant activity in terms of DPPH radical scavenging is absent in non-polar extracts ($EC_{50} > 100$ µg/mL) and retained by the water extract ($EC_{50} = 10 \pm 3$ µg/mL). Caffeic acid ($EC_{50} = 2 \pm 0.8$ µg/mL) as used as antioxidant of reference. The water extract was fingerprinted by HPLC-UV showing that major components conform with the Rt region and UV spectra of glycosylated flavonoids (Figure No. 3).

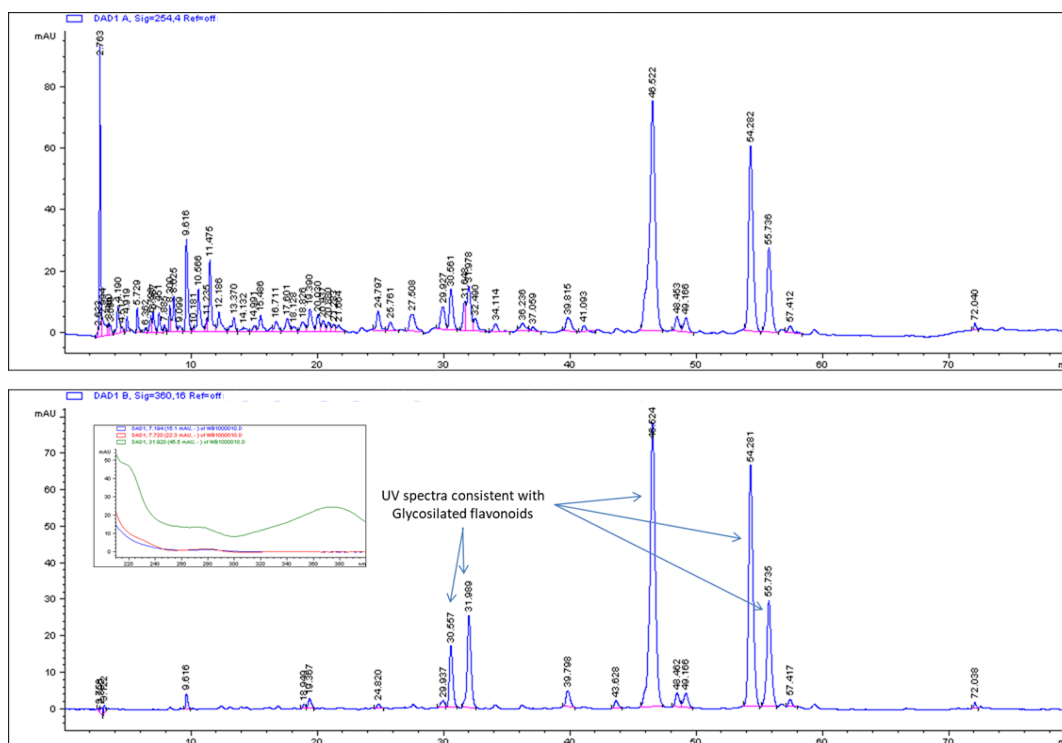

Figure No. 2
HPLC-UV of the water extract of G. *tenax* leaves at 254 and 360 nm.
Insert shows UV spectra of the major peaks

DISCUSSION

We report here for first time on the active principles of *G. tenax* leaves extracts responsible for its cytotoxic effects in human melanoma cells, its antioxidant and tyrosinase inhibitory activities. Previous similar work on *G. tenax* fruit extracts failed to show any antioxidant or tyrosinase inhibitory activity (Muddathir *et al*., 2017).

Promising cytotoxic effects were concentrated in the chloroform extract. Its mechanism is primarily the impairment of the mitochondrial viability with the proliferation rate being less affected. Moreover, the hexane extract demonstrates an inhibition of tyrosinase activity (IC_{50} = 60 µg/mL). We accomplished a bioguided isolation using SPE fractionation. TLC bioautographic analysis of the active fraction showed two different bands with inhibitory activity identified as (**1**) β-sitosterol and (**2**) lupeol as compared against standards. Their identity was further confirmed by their isolation by preparative TLC and analysed by NMR analysis and HR ESI-MS.

We here identify lupeol for the first time as present in *G. tenax*. It was previously found in *G. bicolour, G. tiliaefolia, G. damine* (Ullah *et al*., 2012) and *G. asiatica* (Zia-Ul-Haq *et al*., 2013). Lupeol is endowed with known cytotoxic activity against melanoma cells (AlQathama *et al*., 2020) as well as antityrosinase activity (Azizuddin *et al*., 2011) so its presence may explain these biological activities of the non-polar extracts. Both activities seem to be acting synergistically as lupeol induces the gene expression of microphthalmia-associated transcription factor, tyrosinase, and tyrosinase-related protein-2, which are markers of pigment cell differentiation, in canine oral malignant melanoma cells, and the agent markedly inhibited tumor progression in canine melanoma-bearing mice (Ogihara *et al*., 2014). The mechanism for the direct inhibition of tyrosinase by lupeol is still under study, although *in silico* studies revealed that it interacts with the active tyrosinase site with favorable docking energy. However, its non-competitive kinetics suggests it also binds to another site different from the active one (Omotoyinbo, 2020).

The *in vivo* effects of β-sitosterol in topical inflammation and allergy include a significant reduction of the oedema induced by oxazolone in a model of delayed-type hypersensitivity (DTH) contact dermatitis was tested. These effects indicate that this compound can modulate cell-mediated oedema without any short-term in vitro effect on the arachidonate pathway of intact cells. The ubiquity of this compound can explain, and predict, the beneficial effect of this and many other plant extracts in dermatological processes. (Prieto *et al*., 2006). More recently, other authors reported that β-sitosterol effectively reduced mitochondrial respiratory capacity, mediated by an inhibition of mitochondrial complex I. The net result of this action was increased oxidative stress that led to apoptosis. This effect was only seen in tumor cells, and not in normal cells. Large-scale analyses of human melanoma brain metastases indicated a significant role of mitochondrial complex I compared to brain metastases from other cancers. Moreover, it completely abrogated BRAF inhibitor resistance when was combined with vemurafenib thus representing a promising adjuvant to BRAF inhibitor therapy in patients with, or at risk for, melanoma brain metastases (Sundstrøm *et al*., 2019).

Scavenging activity of the DPPH• radical was confined to the water extract (IC_{50} = 10 ± 3 µg/mL). The water extract was fingerprinted by HPLC-UV-DAD showing that major components conform with the Rt region and UV spectra of glycosylated flavonoids. Previous studies indicated that other species of *Grewia* had good antioxidant profile where *G. tenax* possesses the highest reducing potential as well as antioxidant capacity (Sharma *et al*., 2016).

CONCLUSION

Lupeol and β-sitosterol are active constituents of *G. tenax* and have a favorable toxicological profile with good evidence-based records. *G. tenax* therefore has potential as an active ingredient in dermatological products for skin whitening or prevention of radical-induced ageing. It could also be safely used in the chemoprevention of malignant nevi.

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