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Biological Activities and Nutraceutical Potentials of Water Extracts from Different Parts of *Cynomorium coccineum* L. (Maltese Mushroom)

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Maltese Mushroom *(Cynomorium coccineum* L.) is a non-photosynthetic plant that has been used in traditional medicine for many centuries. In this paper, water extracts from the whole plant, external layer and peeled plant were studied to determine the main components responsible for its biological activities, *i.e.*, its antimicrobial, antioxidant, and anti-tyrosinase activities; its cytotoxicity against mouse melanoma B16F10 cells; and its pro-erectile activity in adult male rats. The results of electron transfer and hydrogen transfer assays showed that the antioxidant activity was mainly due to anthocyanins in the external layer, whereas the external layer and peeled plant extracts both inhibited the microbial growth of several Gram-positive strains. In contrast, the whole plant extract had the highest anti-tyrosinase activity and exhibited pro-erectile activity when administered subcutaneously. Overall, this study elucidated which parts of Maltese Mushroom are responsible for its antimicrobial, antioxidant, and anti-tyrosinase activities and thus which extracts have potential for use in nutraceutical formulations.

ABBREVIATIONS

EL – water extract from the external layer of *C. coccineum*; PP – water extract from the peeled *C. coccineum* plant; and WP – water extract from the whole *C. coccineum* plant.

INTRODUCTION

Cynomorium coccineum L. is a non-photosynthetic, parasitic plant of the Cynomoriaceae family that is ubiquitous in the Mediterranean basin and Arabian Peninsula [Dharmananda, 2011]. It is commonly known as "tarthuth" in Arabic countries or Maltese "Mushroom" in the western world [Cui *et al.,* 2013; Dharmananda, 2011] because it does not have any chlorophyll (Figure 1, part (a)). The *Cynomorium* genus also includes *C. songaricum*, which is found in western Asia.

Of the two *Cynomorium* species, *C. songaricum* is the most well-known and has been widely studied because of traditional Chinese medicine. A comprehensive ethnopharmacological and phytochemical review on the *Cynomorium* genus in China was recently published [Cui *et al.,* 2013]. The pharmaceutical and ethnobotanical properties of Maltese Mushroom are also known and were recorded centuries ago. As even reported in the Bible [Duke, 2008], it has been utilized in antihemorrhoidal, aphrodisiac, hypotensive, antiemetic, and antitumor applications and in the stimulation of spermatogenesis [Dharmananda, 2011; Wang *et al.,* 2010; Yang *et al.,* 2010; Yu *et al.,* 2010]. During famines, it was eaten by humans, and it is currently used in herbal infusions and other food preparations [Cui *et al.,* 2013; Jin *et al.,* 2014].

Some of the biological activities of Maltese Mushroom have been studied in animal and *in vitro* models [Jin *et al.,* 2014; Shi *et al.,* 2011] and were recently reviewed [Cui *et al.,* 2013; Jin *et al.,* 2014; Meng *et al.,* 2013]. In previous works, the lipid composition of the Maltese Mushroom fixed oil was detailed, and its biological activity towards intestinal epithelial cells was determined [Rosa *et al.,* 2012, 2015]. Moreover, aqueous and methanolic extracts from Maltese Mushroom stems exhibited significant antifungal [Gonçalves *et al.,* 2015] and antioxidant activities as demonstrated by several antioxidant assays and *in vitro* biological models [Zucca *et al.,* 2013a]. In these extracts, the main polyphenols detected were gallic acid (3,4,5-trihydroxybenzoic acid, CAS 149–91–7) and cyanidin 3-*O*-glucoside (chrysanthemin or kuromanin, CAS 7084–24–4). Both of these compounds are well-known, promising food supplements [Jayamani & Shanmugam, 2014; Mulabagal *et al.,* 2012] because the biological activities of the polyphenolic fractions of the plant extracts

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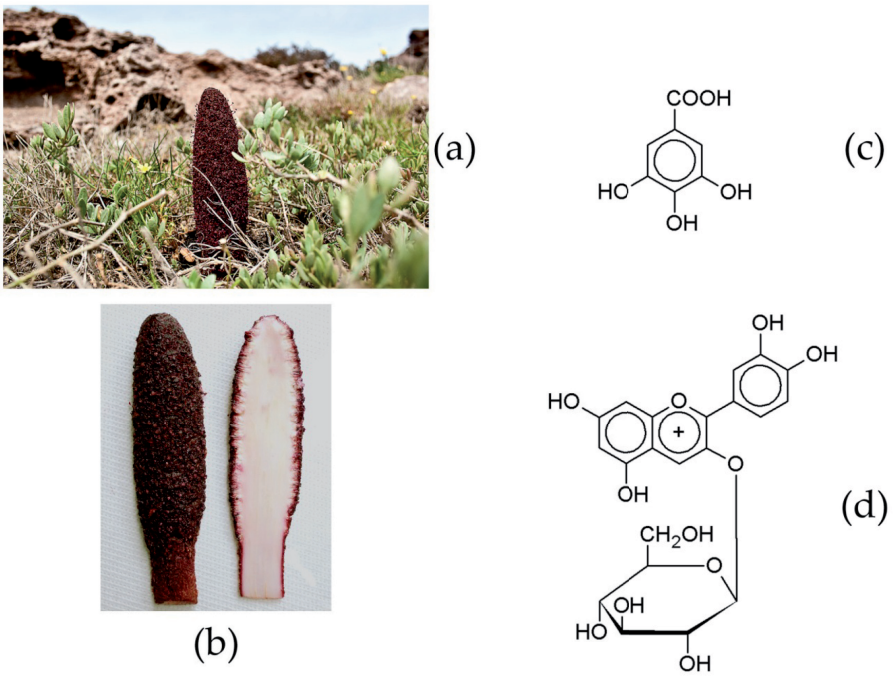

FIGURE 1. (a) A whole plant (WP) growing in a coastal area of Sardinia, Italy. (b) Slices of Maltese Mushroom (*C. coccineum*) showing the vividly colored external layer (EL) and the nearly colorless internal peeled part (PP). The two main constituents of the extracts were determined to be gallic acid (c) and cyanidin 3-*O*-glucoside (d); the former is nearly equally distributed between the external layer (EL) and peeled plant (PP), whereas the latter is nearly absent in the PP and highly concentrated in the EL, which is consistent with the intense color of anthocyanins.

are currently widely studied [Chahmi *et al.,* 2015; Sanjust *et al.,* 2008; Traka & Mithen, 2011]. In particular, several studies focused on the composition of edible and medicinal plant extracts to determine their beneficial health effects and their potential use as a food supplement [Traka & Mithen, 2011; Zucca *et al.,* 2013a]. These reports demonstrate that Maltese Mushroom is a promising nutraceutical source.

Maltese Mushroom has a red-brown external layer (EL), whereas the peeled plant (PP) is nearly colorless (see Figure 1, part (b)). Gallic acid is nearly equally distributed between these two parts, whereas cyanidin 3-*O*-glucoside is nearly absent in the PP and highly concentrated in the EL [Zucca *et al.,* 2013a], which is consistent with the intense color of anthocyanins.

Based on these observations, the water extracts from the whole plant (WP), external layer (EL) and peeled plant (PP) were studied to elucidate the roles of the main components. The biological activities (*i.e.*, the cytotoxicity and antimicrobial, antioxidant, anti-tyrosinase and pro-erectile activities) of the extracts were tested to determine the specific plant parts that give rise to them. This work could potentially help in the formulation of Maltese Mushroom-based nutraceuticals; therefore, only aqueous extracts were included in this study.

MATERIALS AND METHODS

Chemicals and instrumentation

All reagents were of the best commercial grade available and were used without further purification. Analytical grade methanol, ethyl acetate, acetonitrile and 85% w/w phosphoric acid solvents were obtained from Sigma-Aldrich, Fluka (Milan, Italy). A cyanidin-3-*O*-glucoside standard was purchased from Extrasynthese (Genay, France). Ultrapure water (18 mΩ) was obtained with a Milli-Q Advantage A10 system (Millipore, Milan, Italy). Spectrophotometric measurements were performed using an UltroSpec 2100 pro instrument (Amersham Bioscience, Milan, Italy). Fatty acid methyl ester standards were purchased from Sigma–Aldrich (Milan, Italy). All solvents were of the highest available purity and were also purchased from Sigma-Aldrich. Methanolic HCl (3 N) was purchased from Supelco (Bellefonte, PA). The fatty acid methyl ester content was measured using a Hewlett-Packard (HP) 6890 gas chromatograph (Hewlett-Packard, Palo Alto, USA) equipped with a flame ionization detector (FID) and a cyanopropyl methylpolysiloxane HP-23 FAME column (30 m x 0.32 mm x 0.25 μm) (Hewlett-Packard).

Plant materials

Maltese Mushroom was collected in April 2014 in Portoscuso (southwestern Sardinia, Italy), where it is known as "cagalloni strantaxiu". Stem reference materials (AR-CC-2014/4/1) were deposited in the collection of the Department of Biomedical Sciences at the University of Cagliari. The samples were carefully handled as described elsewhere [Zucca *et al.,* 2013a]. In part of the samples, red-brown external layer (EL), colorless peeled plant (PP) (Figure 1, part (b)) were carefully separated from whole plant (WP), and the three samples were freeze-dried using Telstar LyoQuest -55 (Milan, Italy).

Preparation of Maltese Mushroom extracts for antioxidant, anti-tyrosinase, antimicrobial and cytotoxicity experiments

Freeze-dried Maltese Mushroom plant material was ground to a powder and extracted at room temperature.

The material (1 g) was soaked in 5 mL of distilled water and mildly agitated for 60 min. The supernatant was collected after centrifugation at 8000×*g* for 10 min at 4°C. The procedure was repeated seven times until the supernatant was colorless. The total volume of the extracts was lyophilized and stored at 4°C.

Preparation and purification of Maltese Mushroom extracts for pro-erectile activity testing

A slightly different extraction procedure was followed: 1 g of powdered plant material was dissolved in 10 mL of H_2O. After vortexing the mixture overnight, the resulting suspension was centrifuged at 8000×*g* for 10 min at 4°C. The supernatant was removed and stored at 4°C. The remaining powder was dissolved in 10 mL of H_2O. After the mixture was vortexed for 1 h and then centrifuged at 8000×*g* for 15 min at 4°C, the supernatant was removed, combined with the first supernatant and stored at 4°C until use (Extract Solution 1, ES-1).

ES-1 (10 mL) was passed twice through a Sep-Pak C18 cartridge (Waters Corporation, Milford, MA, USA) that had been washed with 10 mL of methanol and 10 mL of H_2O. Then the extract solution was collected and stored at 4°C until use (Extract Solution 2, ES-2). It should be noted that ES-2 was completely colorless. To some extent, ES-2 could be similar to the PP extract. The pigmented material adsorbed in the Sep-Pak C18 was then eluted with 10 mL of methanol, which was collected and evaporated under a nitrogen stream. The remaining material was then dissolved in 5 mL of saline to give a colored solution, which was stored at 4°C until use (Extract Solution 3, ES-3, that to some extent resembles the composition of the EL extract).

Animal experiments were performed according to the guidelines of the European Communities Directive of September 22, 2010 (2010/63/EU) and Italian legislation (D.L. March 4, 2014, n. 26) and were approved by the Ethical Committee for Animal Experimentation of the University of Cagliari.

Nutritional facts label

The nutritional facts were determined according to current legislation (Regulation EU No. 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers) at the facilities of "Laboratorio Chimico Merceologico della Sardegna" (Cagliari, Italy).

Total phenolic, flavonoid, and anthocyanin content determination

The total soluble phenolic content was determined using the Folin-Ciocalteu reagent as described in a previous report [Zucca *et al.,* 2013a]. Gallic acid was used as the standard (linearity range 0.05–0.6 mmol/L), and the results were expressed in gallic acid milliequivalents (mGAE).

A spectrophotometric method was used to quantify the total flavonoid content [Zucca *et al.,* 2010a]. Briefly, 0.25 mL of the sample, 1.25 mL of H_2O, and 0.075 mL of $NaNO_2$ (5% w/v) were incubated for 5 min at 25°C. Then, 0.15 mL of $AlCl_3$ (10% w/v) was added to the mixture. After 6 min, 0.5 mL of 1 mol/L NaOH and 0.275 mL of H_2O were also added, and the absorbance was measured at 510 nm. Catechin was used as the standard (linearity range 0.1–0.6 mmol/L), and the results were expressed in catechin milliequivalents (mCE) using a standard curve.

The total anthocyanin content was determined by a spectrophotometric assay based on differential pH absorbance [Delazar *et al.,* 2010]. Briefly, the absorbances at 510 nm and 700 nm were measured at both pH 1.0 and 4.5 using 0.2 mol/L HCl/KCl and 1 mol/L sodium acetate buffers, respectively. The final absorbance was calculated using Equation 1.

$$A_{fin} = [(A_{510}–A_{700})_{pH1.0}–(A_{510}–A_{700})_{pH4.5}] \quad (1)$$

The molar extinction coefficient of cyanidin 3-*O*-glucoside (29,300 $M^{-1}\cdot cm^{-1}$) was then used in the Lambert-Beer equation to calculate the total anthocyanin concentration (Equation 2, l = optical path).

$$[total\ anthocyanin] = \frac{A_{fin}}{29{,}300\ M^{-1}cm^{-1}\cdot l} \quad (2)$$

Antioxidant assays

The 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radical scavenging assay was performed according to a well-known protocol [Huang *et al.,* 2005]. The results were expressed in Trolox milliequivalents (mTE, linearity range 5–50 μmol/L) and as IC_{50} values.

The ferric reducing antioxidant power (FRAP) assay was performed as previously described [Huang *et al.,* 2005]. Trolox was used for the calibration curve, and the results were expressed in Trolox milliequivalents (mTE) and in mmol of Fe(II) per gram of dry material (mmol FeII/g).

The Trolox equivalent antioxidant capacity (TEAC) assay was performed using the 2,2'-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS) cationic radical as previously described [Zucca *et al.,* 2013a]. The results were expressed in Trolox milliequivalents (mTE, linearity range 0.1–0.8 mmol/L) and as IC_{50} values.

The oxygen radical absorbance capacity-pyrogallol red (ORAC-PYR) assay was performed according to a recently described method [Zucca *et al.,* 2010a]. The results were expressed in Trolox milliequivalents (mTE, linearity range 0.1–0.8 mmol/L).

Extraction and saponification of Maltese Mushroom oil

Oil was extracted from the external layer (EL) and internal peeled part (PP) using the procedure of Folch *et al.* [1957] in which 12 mL of a 2:1 (*v/v*) $CHCl_3$:MeOH solution was added to the powder sample. After filtration, 4 mL of H_2O was added to the mixture, which was then centrifuged at 900×*g* for 1 h to separate the $CHCl_3$ fraction (lipophilic extract) from the MeOH/H_2O mixture. Aliquots of the dried $CHCl_3$ fractions were subjected to mild saponification [Rosa *et al.,* 2015]. Aliquots of the dried fatty acids were then methylated with 1 mL of methanolic HCl (3 N) [Rosa *et al.,* 2015] for 30 min at room temperature. After *n*-hexane and H_2O were added to the samples, they were centrifuged for 20 min at 900×*g*. The hexane phase containing the fatty acid methyl

esters was collected, and the solvent was subsequently evaporated. Then, the residue was dissolved in *n*-hexane, and aliquots of the samples were subjected to gas chromatography analysis (GC). All solvent evaporation procedures were performed under vacuum.

GC analysis of the fatty acid methyl esters

The fatty acid methyl ester content was measured using an HP 6890 system. Nitrogen was used as the carrier gas at a flow rate of 2 mL/min. The oven, injector, and detector temperatures were set to 175°C, 250°C, and 300°C, respectively. The fatty acid methyl esters were identified by comparing the observed retention times to those of standard compounds. The amounts of the individual fatty acids were calculated as percentages of the total amount of fatty acids (g %) using the Hewlett-Packard A.05.02 software.

Antimicrobial activity

All bacterial strains used in this study belong to the Leibniz Institute DSMZ (German Collection of Microorganism and Cell Cultures). In particular, four Gram-negative and five Gram-positive reference bacterial strains were used: *Staphylococcus aureus* DSM 1104, methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis* DSM 1798, *Enterococcus faecalis* DSM 2570, *Escherichia coli* DSM 1103, *Enterobacter cloacae* DSM 30054T, *Pseudomonas aeruginosa* DSM 1117, *Acinetobacter baumannii* DSM 30007T, and *Klebsiella pneumoniae* DSM 681.

Inoculum suspensions were prepared using the direct colony suspension method according to CLSI procedures [Institute, 2012]. Saline suspensions of isolated colonies, which were selected from an 18–24 h Luria-Bertani agar plate, were used to achieve a turbidity equivalent to a 0.5 McFarland standard.

The antibacterial activities of the Maltese Mushroom extracts were determined using the disc diffusion method according to CLSI procedures [Institute, 2012]. Petri plates were prepared with 25 mL of Mueller-Hinton agar (MHA). The inoculum suspensions were swabbed on the MHA plate surface, and 1 mg of dry extract was added to each disk separately. A negative control was prepared by placing a specific amount of Milli-Q water on the control disk surface. Gentamicin and oxacilline were used as positive controls.

Tyrosinase inhibition

Tyrosinase from *Agaricus bisporus* was purified as described previously [Sollai *et al.,* 2008]. Experiments were performed to check for possible contaminant enzyme activities [Rescigno *et al.,* 2007]. In particular, syringaldazine was used as the substrate [Zucca *et al.,* 2011] to rule out laccase activity (<0.001 E.U./mL [Zucca *et al.,* 2010b]) because contaminant laccases might share substrates, *i.e.*, *o*-diphenols, with tyrosinase, leading to ambiguous results.

The tyrosinase activity was measured by monitoring the absorbance at 625 nm (ε_{625} = 11,120 $M^{-1}\cdot cm^{-1}$) after the formation of an adduct between 4-*tert*-butyl-1,2--benzoquinone (TBBQ) and 4-amino-*N*,*N*-diethylaniline (ADA), as previously described [Rescigno *et al.,* 1999; Zucca *et al.,* 2013b]. The assay mixture, which had a final volume of 1 mL, contained a 50 mmol/L sodium phosphate buffer at pH 7.0, 5 mmol/L 4-*tert*-butylcatechol (TBC), 0.75 mmol/L ADA and 2 E.U. of the enzyme (1 tyrosinase E.U. was defined as the amount of enzyme capable of producing 1 μmol of the adduct between TBBQ and ADA per minute at pH 7 and 25°C). The tyrosinase inhibition by the test samples was quantified by the concentration necessary to achieve 50% inhibition (IC_{50}).

Cell line and culture conditions

The B16F10 mouse melanoma cell line (ICLC ATL 99010) was purchased from the National Institute for Cancer Research c/o CBA (Genoa, Italy). Subcultures of the cell line were grown in a 75 cm^2 culture flask in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 1% non-essential amino acids, 1 mmol/L Na-pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO_2.

MTT assay for cell viability

The cytotoxic effects of the Maltese Mushroom water extracts (WP, PP, and EL) were evaluated by conducting the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, CAS 298–93–1) assay, in which the tetrazolium salt is cleaved by mitochondrial dehydrogenases in viable cells [Rosa *et al.,* 2012; Schiller *et al.,* 1992], on the B16F10 mouse melanoma cells. MTT is a yellow, water-soluble tetrazolium salt. Metabolically active cells can convert the dye into a dark blue, water-insoluble formazan dye by reductively cleaving the tetrazolium ring. In brief, 3×10^3 cells/mL in 100 μL of medium were seeded into a 96-well plate and incubated at 37°C. After a 48 h incubation period, the aqueous extracts were added to the cultures in concentrations ranging from 25 to 500 μg/mL, and the cells were incubated for an additional 24 h at 37°C. The MTT solution (8 μL, 5 mg/mL in H_2O) was then added to the culture, which was maintained at 37°C for 4 h. The cells were lysed with 100 μL of DMSO, and the color was measured at 570 nm with an auto microplate reader (Infinite 200, Tecan, Austria). The absorbance was proportional to the number of viable cells.

In addition, melanin content in the B16F10 cell cultures was determined by a previously described method [Soddu *et al.,* 2004].

Pro-erectile activity in adult male rats

The effects of the Maltese Mushroom extracts (ES-1, ES-2, and ES-3) on penile erection in male rats were studied as described in the literature [Sanna *et al.,* 2011, 2012]. Briefly, adult male Sprague-Dawley rats (225–250 g, Harlan Nossan, Italy) were housed at 24°C and 60% humidity and given water and standard laboratory food *ad libitum*. Aliquots of ES-1, its dilutions with saline, or ES-2 or ES-3 were administered subcutaneously (SC) in volumes of 0.1 mL/80 g of rat body weight. Rats injected with the same volume of saline were used as controls. After treatment, each rat was placed in a Plexiglas cage (30 × 30 × 30 cm) by itself and observed for 60 min to count the number of penile erection episodes and monitor other gross behavioral changes.

Statistical analysis

GraFit 7 (Erithacus Software, London, UK) and the R 2.5.1 software (R Foundation for Statistical Computing, Vienna) were used for the statistical analysis. All analyses were performed at least in triplicate unless otherwise stated. The statistical significance of differences was evaluated by analysis of variance (one-way ANOVA). Statistical evaluation of the penile erection experiments was performed using one-way ANOVA followed by Tukey's multicomparison test. For the B16F10 cell experiments, the statistical significance of differences was evaluated using one-way ANOVA followed by the Bonferroni post-test; the differences were considered to be significant at $P<0.001$, $P<0.01$, and $P<0.05$.

RESULTS AND DISCUSSION

Nutritional facts of Maltese Mushroom

Previous reports ascribe several biological activities to Maltese Mushroom [Rosa *et al.,* 2012; Wang *et al.,* 2010; Zucca *et al.,* 2013a], suggesting that its extract has a potential for use in nutraceutical formulations. Based on the possibility that Maltese Mushroom might be used for human nutrition, the nutritional facts for the whole plant were collected. The results are reported in Table 1. Carbohydrates, including 27% dietary fibers, were the principal component (approximately 50%). A significant portion of protein (approximately 9%) was also detected, whereas only a small amount of lipids (1%) were detected. Most of the lipids were unsaturated fats. Particularly, polyunsaturated fats, which are thought to be beneficial for many human pathological conditions [Roncaglioni *et al.,* 2013], constituted nearly 40% of the total lipids. Overall, the nutritional profile of Maltese Mushroom appears to be compatible with human consumption as described in traditional medicine [Cui *et al.,* 2013; Jin *et al.,* 2014], supporting the aim of this study.

TABLE 1. Nutritional information panel for lyophilized Maltese Mushroom. The data are for 100 g of the dried material.

Specification	Value
Calories	281 kcal 1176 kJ
Proteins	8.69 g
Total carbohydrate	45.5 g
sugars	29.6 g
Total fat	1.0 g
saturated fat	27.36 %
polyunsaturated fat	39.57 %
monounsaturated fat	33.07 %
Sodium	765 mg
Total dietary fiber	27.7 g

Phenolic composition and antioxidant power

Phenolic compounds are the principal plant constituent associated with antioxidant activity. Accordingly, their contents in the WP, EL and PP extracts were quantified first (Table 2). Based on the Folin-Ciocalteu assay results, the WP extract had a phenol concentration of approximately 3.3 mmol/L GAE (gallic acid equivalents) per gram of dry extract, which is on the same order of magnitude as that measured for other *Cynomorium* specimens [Jin *et al.,* 2014; Rached *et al.,* 2010; Wong *et al.,* 2006] and for typical antioxidant plants (such as green tea, tomatoes, and oranges) [Cieślik *et al.,* 2006]. The phenolic compounds were unevenly distributed between the EL and PP; their concentrations were higher (more than 2-fold) in the red-brown external layer. The flavonoid distribution also followed this trend, and anthocyanins were only detected in the EL. HPLC analysis revealed that cyanidin 3-*O*-glucoside accounted for the majority of the total anthocyanin content [Zucca *et al.,* 2013a]. The UV-Vis spectra of both the WP and EL extracts supported this conclusion: two main absorption maxima were observed at 277 nm and 521 nm. The addition of 5% $AlCl_3$ led to a 5 nm bathochromic shift in the visible band, which is consistent with the presence of *ortho*-hydroxyl groups on the cyanidin-3-*O*-glycoside B-ring [Delazar *et al.,* 2010].

To fully assess the activities of the extracts, the total antioxidant power was determined by both the ET and HAT assays because only hydrogen atom transfer methods (such as ORAC-PYR) can evaluate the antioxidant capacity in addition to the reducing power [Zucca *et al.,* 2010a]. The results are listed in Table 2. All the assays showed that the EL extract had a higher antioxidant power ($P<0.05$ for each method), indicating that the antioxidant capacity is mainly due to anthocyanins. However, both the ET and HAT assays showed that the PP extract also exhibited significant activity (on average, its antioxidant power was slightly less than half that of the EL extract), suggesting that the colorless component of Maltese Mushroom could also be used in nutraceutical formulations.

TABLE 2. Total antioxidant capacity of the Maltese Mushroom extracts from the external layer (EL), peeled plant (PP) and whole plant (WP). (n.d.) not detectable.

Assay	WP extract	EL extract	PP extract
ORAC-PYR (mTE/g)	6.81±0.41	8.13±0.70	2.39±0.24
DPPH (mTE/g)	1.16±0.04	1.82±0.11	0.76±0.03
DPPH· (IC_{50} μg/mL)	10.70±0.39	9.07±0.29	25.09±1.54
TEAC (mTE/g)	3.86±0.23	5.92±0.61	1.83±0.10
TEAC (IC_{50} μg/mL)	188±40	109±7	266±86
FRAP (mTE/g)	2.66±0.01	3.54±0.02	1.39±0.01
FRAP (mmol Fe^{II}/g)	3.12±0.01	4.73±0.01	2.37±0.01
Total phenolics (mGAE/g)	3.34±0.01	4.97±0.22	2.06±0.02
Total flavonoids (mCE/g)	1.12±0.01	1.86±0.02	0.87±0.01
Total anthocyanin (mg cyanidin 3-*O*-glucoside/g)	6.6±0.3	13.7±1.2	n.d.

Gallic acid was found to be one of the main components of the WP by HPLC analysis and was nearly equally distributed between the EL and PP [Zucca *et al.,* 2013a]. These results indicated that gallic acid makes a significant contribution to the total antioxidant activity of the Maltese Mushroom extracts, which is consistent with several reports of the high antioxidant activity and healthy effects of gallic acid and suggests that it has potential for use in clinical and industrial applications [Jayamani & Shanmugam, 2014].

TABLE 3. Fatty acid composition (% of total fatty acids) determined by GC analysis of the lipid extracts obtained from the external layer (EL) and internal peeled part (PP) of Maltese Mushroom using the procedure of Folch *et al*. [1957].

Fatty acids	EL extract	PP extract
12:0	0.34±0.11	0.21±0.11
14:0	1.28±0.27	1.08±0.12
16:0	25.02±2.49	24.28±0.83
16:1 *n-7*	1.96±0.62	1.45±0.09
18:0	4.34±1.06	2.81±0.73
18:1 *n-7*	1.41±0.14	1.39±0.09
18:1 *n-9*	21.70±1.27	22.79±0.25
18:2 *n-6*	33.34±4.17	36.66±0.43
18:3 *n-3*	3.84±1.20	2.81±0.15
20:0	0.43±0.21	0.24±0.08
20:1 *n-9*	5.51±0.53	2.77±0.25
SFA	31.84±3.61	28.69±1.42
MUFA	30.05±1.38	28.38±0.35
PUFA	34.89±3.83	39.47±0.29

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Mean and standard deviation of 4 samples.

Composition of the Maltese Mushroom oil extracts

Qualitative and quantitative information on the individual fatty acids in the Maltese Mushroom EL and PP oil extracts was obtained by GC analyses. The fatty acid compositions of the oil extracts, expressed as % of the total fatty acids, are reported in Table 3. The EL oil was composed of approximately 32% saturated fatty acids (SFA, mainly palmitic acid 16:0 and 18:0, 25 and 4%, respectively), 30% monounsaturated fatty acids (MUFA, mainly oleic acid 18:1 n-9 and 20:1, 22 and 5%, respectively), and 35% polyunsaturated fatty acids (PUFA, mainly composed of the essential fatty acids linoleic acid 18:2 n-6 and a-linolenic acid 18:3 n-3, 33 and 4%, respectively). The PP oil extract had a profile similar to that of the EL oil, with a higher level of PUFA (39%) and lower amounts of MUFA (28%) and SFA (29%). It is interesting to note that both oils were characterized by a high proportion of PUFA. The fatty acid profile of the Maltese Mushroom oil obtained in this study differed from those obtained in previous studies [Rosa *et al.,* 2012, 2015], possibly because the plant collection, extraction procedure, and plant metabolic activity were performed at a different time.

However, the striking similarities between the oil compositions of the different plant parts, PP and WP indicates that the whole plant could be used in nutraceutical applications.

Antibacterial activity

Antibacterial activity is a crucial feature of plant extract-based formulations. Accordingly, the effects of the WP, PP and EL extracts on several Gram-positive and Gram-negative bacterial strains, including the clinical isolate *S. aureus* MRSA, were evaluated by the disc diffusion method. The results are summarized in Table 4.

Based on the parameters suggested by Alves and coworkers [Alves *et al.,* 2000] (inhibition zones <9 mm, inactive; 9–12 mm, less active; 13–18 mm, active), the inhibition zones (1 mg/disc) of the aqueous Maltese Mushroom extracts against the Gram-positive strains, particularly those belonging to the *Staphylococcus* genera, were considerable. Of these

TABLE 4. Antibacterial activity of the aqueous Maltese Mushroom extracts determined by the disc diffusion method (inhibition zone in mm) (1 mg/disc). Gentamicin and oxacilline were used as the reference antibiotic controls. (–) no activity.

Bacteria	WP extract	EL extract	PP extract	Antibiotic control
Gram positive				
S. aureus DSM 1104	11.3±0.6	11.0±0.1	8.3±0.6	Ox 26.0±0.2
S. epidermidis DSM 1798	12.3±0.6	12.7±0.6	10.7±0.6	Ox 23.0±0.1
E. faecalis DSM 2570	9.3±0.6	8.7±0.6	7.7±0.6	Gent 12.5±0.6
Staphylococcus MRSA	10.3±0.6	10.3±0.6	10.0±0.1	–
Gram negative				
E. coli DSM 1103	–	–	–	Gent 21.8±1.3
E. cloacae DSM 30054^T	–	–	–	Gent 19.5±0.6
P. aeruginosa DSM 1117	–	–	–	Gent 18.3±0.5
A. baumannii DSM 30007^T	8.7±0.6	8.3±0.6	–	Gent 16.5±1.0
K. pneumoniae DSM 681	–	–	–	Gent 21.5±1.0

bacterial strains, the clinical isolate MRSA *S. aureus*, a methicillin-resistant strain, is of particular clinical interest. These results are consistent with previous studies that showed that a crude acetone–water extract of *C. songaricum* had a moderate effect on a methicillin-resistant *S. aureus* strain [Jin *et al.,* 2014]. Several mechanisms are known explaining the antimicrobial activity of anthocyanins, including interference with permeability of cell membranes and enzyme inhibition by their oxidized compounds [Marin *et al.,* 2015]. However the effect of cyanidins on MRSA is actually quite controversial [Hatano *et al.*, 2005], suggesting that the antimicrobial activity of Maltese Mushroom is not due to anthocyanins. This assumption is corroborated by the fact that the EL and PP extracts exhibited similar antibacterial activities for all the tested strains (Table 4), indicating that the antimicrobial activity might be due to gallic acid, which is consistent with previous reports [Kang *et al.,* 2008] and well-known antimicrobial activity of gallates (possibly due to their prooxidant activity [Badhani *et al.,* 2015]). Accordingly, the PP or WP should be preferred for microbiologically active formulations. Conversely, the extracts did not substantially inhibit the growth of Gram-negative strains. Only *A. baumannii* was inhibited by the WP and EL extracts, although it was not inhibited by the PP extract, strongly suggesting that anthocyanins are responsible for this specific biological activity.

Anti-tyrosinase activity

Tyrosinase (or polyphenol oxidase, PPO) is a copper monooxygenase involved in melanogenesis and food browning [Marongiu *et al.,* 2007; Rescigno *et al.,* 2011]. This enzyme catalyzes the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones in the presence of molecular oxygen. It is desirable to inhibit this enzyme in food formulations to increase the shelf life of some food products, especially plant-based ones.

The WP, PP and EL extracts, even dialyzed samples, did not exhibit monophenolase or diphenolase activity for several well-known tyrosinase substrates [Olianas *et al.,* 2005; Zucca *et al.,* 2013b]. This finding supports the hypothesis that the PPO in plants is associated with the chloroplasts [Mayer, 2006]. It would be interesting to investigate whether the apparent absence of PPO corresponds to the absence of an encoding gene or to the lack of PPO gene expression.

Both the EL and PP extracts inhibited tyrosinase activity, although the EL extract was approximately twice as active as the PP extract (IC_{50} of EL=30.4±1.0 μg, IC_{50} of PP=70.6±3.6 μg). It should be noted that the WP extract exhibited a stronger inhibition effect than both of its component extracts (IC_{50}=15.6±0.5 μg), suggesting that secondary metabolites derived from interactions between the EL and PP play a crucial role in its anti-tyrosinase activity [Jin *et al.,* 2014]. This finding is further supported by the IC_{50} value determined for the main EL component cyanidin-3-*O*-glycoside (23.6±2.4 μg), which suggests that this compound is only responsible for a small fraction of the global anti-tyrosinase activity.

Overall, the anti-tyrosinase activity data suggest that the WP is a possible candidate for use in food formulations to prevent browning.

Cytotoxic effects of the aqueous extracts on B16F10 cells

Melanoma is the most malignant skin cancer, and its occurrence has remarkably increased over the past few decades. The B16F10 mouse melanoma cell line is highly metastatic and is commonly used to screen antitumor agents. The cytotoxicity of the aqueous Maltese Mushroom extracts against the B16F10 cells were tested (MTT assay) and compared. Figure 2 shows the viability, expressed as % of the control, of the B16F10 cells after incubation in the presence of different concentrations of the EL, PP, and WP extracts (25–500 μg/mL) for 24 h. This concentration range was chosen based on similar experiments performed with other plant extracts. Treatment with a 100 μg/mL EL extract solution significantly reduced the cell viability compared to the control, and the viability was 49% at EL extract concentrations of 250 and 500 μg/mL. The cell viability decreased by 41% and 68% when they were treated with 250 and 500 μg/mL WP extract solutions, respectively. Although the PP extract was less effective than the EL and WP extracts, it reduced the cell viability by 46% at a concentration of 500 μg/mL. The poten-

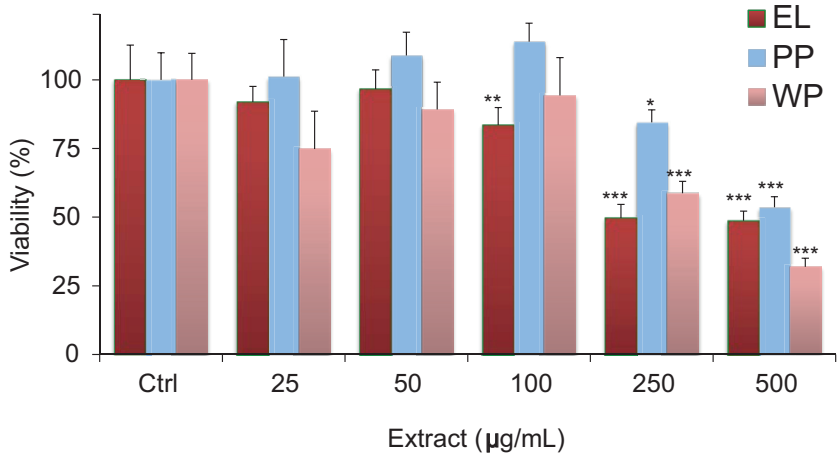

FIGURE 2. Viability (expressed as % of the control) (MTT assay) after incubating melanoma B16F10 cells with the Maltese Mushroom extracts obtained from the external layer (EL), internal peeled part (PP), and whole plant (WP). ***=P<0.001; **=P<0.01; *=P<0.05 *vs* control; a'=P<0.001 *vs* control for both cell lines.

tial anti-cancer properties of natural extracts obtained from plants/herbs used in traditional medicine have received much attention. Evaluating the cytotoxic activities of the aqueous Maltese Mushroom extracts revealed their ability to reduce the viability of B16F10 cancer cells. Recently, gallic acid was shown to induce cellular apoptosis in B16F10 melanoma cells [Liu *et al.,* 2014], probably due to the complex prooxidant action of gallate compounds [Badhani *et al.,* 2015]. A proteomic approach revealed that the gallic acid-induced cellular apoptosis in these cells is associated with metabolic glycolysis. Therefore, the cytotoxic effect observed in this study could be attributed to gallic acid because it is one of the major components in the Maltese Mushroom extracts.

In a previous study, the fixed oil obtained from the whole plant by SFE extraction also significantly inhibited the growth of melanoma and colon cancer cells, thus demonstrating the potential of Maltese Mushroom [Rosa *et al.,* 2015].

Interestingly, the extracts did not affect the cells' ability to produce melanin.

Pro-erectile activity in adult male rats

Many studies of *C. songaricum* have shown that its extracts affect the reproductive system; therefore, many products containing these extracts have been marketed for the treatment of erectile dysfunction, premature ejaculation, spermatorrhea and general sexual health [Cui *et al.,* 2013]. Only a few studies of the effects of Maltese Mushroom on the reproductive system have appeared in the literature. Abd El-Rahman and colleagues found that a plant water extract induced a significant increase in the sperm count of rats, increased the percentage of live sperm and their motility and decreased the number of abnormal sperm [Abd El-Rahman *et al.,* 1999].

A synergistic combination of an aqueous *Withania somnifera* extract and Maltese Mushroom extract was found to have a direct spermatogenic influence on the seminiferous tubules of immature rats, presumably by exerting a testosterone-like effect [Abdel-Magied *et al.,* 2001].

To our knowledge, the effect of Maltese Mushroom extracts on penile erection in laboratory animals or humans has not been studied. Our results show for the first time that aqueous Maltese Mushroom extracts of the whole plant exhibited pro-erectile activity when administered subcutaneously in adult male rats (see Figure 3). Because the pro-erectile activity persisted after the colored component(s) were removed from the extract and the colored component(s) did not exhibit pro-erectile activity, it was concluded that the pro-erectile activity was due to one or more colorless, highly hydrophilic components, which might be present in both the internal and external parts of the plant and whose structures are currently unknown. Indeed, the plant components that have been identified (*i.e.*, gallic acid, cyanidin 3-*O*-glucoside, flavonoids, *etc.*) have not been reported to exhibit pro-erectile activity, although they exert a general protective effect on penile tissues due to their antioxidant activity [Cui *et al.,* 2013]. Approximately 10 mg of the WP extract exhibited a pro-erectile activity similar to that induced by 20 μg of apomorphine, a dopamine receptor agonist well known for its ability to induce penile erection in laboratory animals and humans by acting systemically [Argiolas, 2005; Sanna *et al.,* 2011]. It is currently unknown whether the Maltese Mushroom extracts induced penile erection by acting locally (*i.e.,* in the penile tissues), systemically or both. These results are consistent with a US patent claiming that a *C. songarium* L. Rupr. extract exhibits anti-phosphodiesterase activity, potentiating the relaxation of isolated cavernous smooth muscle strips by cGMP, which makes the extract suitable to treat erectile dysfunction (US Patent Ref. No. US20030157208 A1 [Wang *et al.,* 2003]).

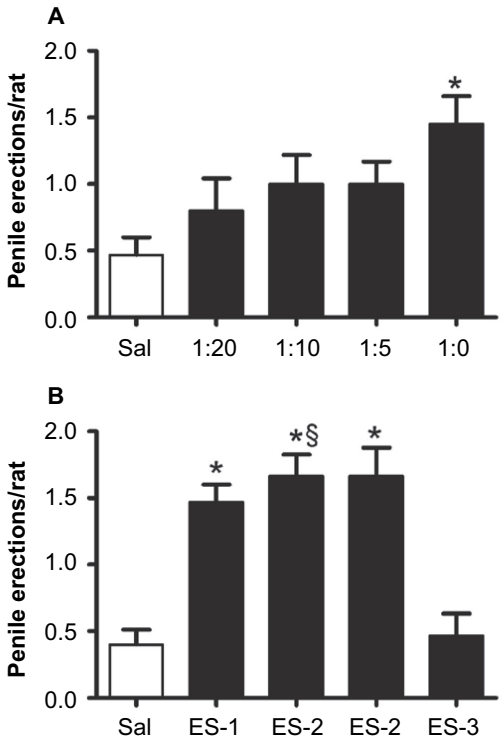

FIGURE 3. Effect of Maltese Mushroom extract solutions 1, 2 and 3 on penile erection in male rats. ES-1 (1:0) and its dilutions (1:5; 1:10; 1:20) (A), ES-2 and ES-3 (B) were administered SC in aliquots of 0.1 mL/80 g of rat body weight. At the doses used, no gross behavioral changes were observed. *P< 0.01 with respect to saline-treated rats (one-way ANOVA followed by Tukey's multicomparison test). § This group was treated with 0.05 mL of ES-2 instead of 0.1 mL/80 g rat body weight.

SUMMARY AND CONCLUSIONS

In the past, medicinal properties of Maltese Mushroom were held in high regard [Dharmananda, 2011]. As time passed, its use in traditional medicine waned except in some populations of the Arabian peninsula.

In this study, some healthful properties of Maltese Mushroom were verified, and other biological activities of this plant (cytotoxicity against B16F10 mouse melanoma cells and anti-tyrosinase, antibacterial, and pro-erectile activities) were described for the first time. Despite being results about only partially differentiated plant materials, needing some additional in-depth analysis, the reported data highlight the potential of Maltese Mushroom as a functional food. Accordingly, additional studies of toxicity in normal cell lines and *in vivo* model will be necessary prior to human application. In fact, therapeutic effects of plant extracts could

be associated with the presence of substances present in low amount in the extract or synergistic performance of many components.

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