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Evaluation of phototoxic potential of aerial components of the fig tree against human melanoma

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Abstract

Objectives: To date, *Ficus carica* L. cultivar Dottato (*F. carica*) has not been studied from a phototoxic point of view. In the present work, aerial components of *F. carica* from Italy, were examined to assess their antioxidant and phototoxic activity on human melanoma cells. A relationship between antioxidant, phototoxic activities and chemical composition has also been investigated.

Materials and methods: Coumarin and fatty acid content in *F. carica* leaves, bark and woody parts were examined and compared by capillary GC and GC/MS. Polyphenolic content was also determined. Linoleic acid peroxidation and DPPH test were used to assess antioxidant activities, and MTT assay was used to evaluate anti-proliferative activity, on C32 human melanoma cells, after irradiation with a UVA dose of 1.08 J/cm^2.

Results: Leaves demonstrated the best antioxidant and anti-proliferative activity in comparison to bark and wood. In particular, leaves were shown to possess the highest anti-radical activity and inhibition of peroxidation, with IC_{50} values of 64 and 1.48 μg/ml respectively. The leaves had highest anti-proliferative activity with IC_{50} value of 3.92 μg/ml. The phytochemical investigation revealed different composition between the coumarins, psoralen and bergapten, fatty acids, polyphenols and flavonoid content among plant parts.

Conclusions: Data obtained indicate that this type of fig tree may constitute an excellent source of bioactive compounds, such as phenolics, coumarins and fatty acids. This study offers a new perspective in developing others formulations potentially useful in photodynamic therapy for treatment of non-melanoma skin cancers.

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Introduction

Fig (*Ficus carica*, Moraceae) products are widely used both as food and as medicine in the Middle East. Figs are perhaps the oldest of all cultivated fruit crops and are grown in many areas of the world that have subtropical climates. Latex released on picking the fruits is used to treat skin tumours and warts (1) but the first scientific investigation of activity of fig latex was conducted by Ullman *et al.* in the 1940s and 50s (2–4). High doses of fig latex injected into rats were found to be lethal. Smaller doses injected into mice bearing a benzopyrene-induced sarcoma caused inhibition of growth of tumours and even disappearance of small ones (2). Dialysate of the latex was found to contain the active ingredients. Although isolation of the active components was not pursued further, some pharmacological work has been reported by the same group (3,4). Fig latex has also been tested for its anti-helminthic activity, but was found to cause acute toxicity with haemorrhagic enteritis (5). Leaf decoction affected lipid catabolism in hypertriglyceridemic rats and had hypoglycaemic action in type-I diabetic patients (6,7). Athnasios *et al.* (8) have isolated psoralen, γ-sitosterol, bergapten and taraxasterol from petroleum ether extraction of the leaves and others have isolated triterpenoids (9,10).

Plant-derived natural compounds are an important source for development of cancer-fighting drugs. One of

the most critical risk factors in initiation and development of several skin diseases is exposure to UV solar radiation. As time people spend outside during sports and other activities extends, numbers of skin cancers increase (11).

Malignant melanoma and non-melanoma skin cancers are among the most prevalent cancers in the human population. Free radicals are generated by normal physiological processes, including aerobic metabolism and the inflammatory response, but these may inflict cell damage when their generation is increased and antioxidant defence mechanisms are overwhelmed. Important findings support that the free radical hypothesis in skin carcinogenesis are: (i) reactive oxygen species (ROS) are generated in UVA- and UVB-irradiated skin in excessive doses, (ii) natural cutaneous antioxidant defence is impaired on UV-exposure, (iii) free radicals are involved in all steps of carcinogenesis, (iv) supplementation with antioxidants can inhibit skin carcinogenesis and (v) conditions that increase ROS generation enhance photocarcinogenesis. These findings provide a promising rationale for development of powerful new antioxidant strategies in prevention and therapy of skin cancer (12).

Over the last few decades, natural compounds have attracted considerable attention as cancer chemopreventive agents, as well as in cancer therapeutics. Research on natural products has recently regained prominence due to greater understanding of their biological significance and growing recognition of origins and functions of their structural diversity. It has been estimated by the World Health Organization that approximately 75–80% of the world's population, uses either in part or solely, plant medicines, either in part or entirely. For many people, this is out of necessity, as they cannot afford high costs of pharmaceutical drugs. In this article, we describe evaluation of phototoxicity of various phytocomplexes obtained from different components of the fig tree. Leaves, bark and wood of *F. carica* cultivar Dottato (from here on referred to simply as *F. carica*), from Italy were examined to assess their antioxidant activity on linoleic acid peroxidation, their free radical-scavenging activity with 1,1-diphenyl-2 picrylhydrazyl (DPPH) and their phototoxicity, on C32 melanoma cells. In view of potential pharmaceutical applications, the relationship between antioxidant, phototoxic activities and chemical composition has also been investigated.

Materials and methods

Reagents

The following were obtained from Sigma-Aldrich Spa. (Milano, Italy): β-carotene, quercetin, chlorogenic acid, NaCl, ascorbic acid, propyl gallate, DPPH radical, linoleic acid, Tween 20, Folin-Ciocalteu reagent, RPMI 1640 medium, foetal bovine serum, L-glutamine, penicillin/streptomycin, trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hanks' Balanced Salt Solution and Amelanotic melanoma C32 (ATCC No.: CRL-1585) cells. All other reagents, of analytical grade, were supplied by VWR International srl. (Milan, Italy).

Plant materials

Leaves, bark and woody parts of *F. carica* were collected in September 2009 in Calabria (Italy) from the 'Miracco Lucia' (Lat.: 16°16′14″; Long.: 39°32′56″) farm, from mature plants. Identification was performed by Dr Uzunov of the Botanic Garden, University of Calabria, Italy, and plants were deposited at the Herbarium CLU of the Natural History Museum of Calabria. Components of the *Ficus* samples were extracted from the different aerial elements, using a maceration technique with hydroalcoholic solution (70% ethanol) at room temperature. Extraction was repeated three times for 48 h. Hydroalcoholic solutions were combined and dried to obtain total extracts with 9.7, 8.2 and 6.4 of yield per cent of extraction for leaves, bark and woody parts respectively.

GC and GC-MS analysis

Analysis of lipophilic compounds was performed using a Hewlett-Packard gas-chromatograph, model 5890, equipped with a mass spectrometer, model 5972 series II, and controlled by HP software with capillary column 30 m × 0.25 mm, static phase SE30, using programmed temperatures from 60 to 280 °C (rate 16°/min); detector and injector were set at 280 and 250 °C respectively (split vent flow 1 ml/min). Compound identification was verified according to relative retention time and mass spectra with those of Wiley 138 library data of the GC-MS system (Hewlett-Packard Co., Milan, Italy). Extracts were analysed, also using the Shimadzu GC17A gas chromatograph system (Columbia, MD, USA). An SE-30 capillary column (30 m, internal diameter 0.25 mm and film thickness 0.25 μm) was used with nitrogen as the carrier gas. GC oven temperature and conditions were as described above. Percentages utilized for composition of samples analysed were computed by the normalization method from GC peak areas related to GC peak area of external standards, injected into the GC equipment in isothermal conditions, at 180 °C. Percentage of their total area was obtained by their addition. All determinations were performed in triplicate and averaged.

Determination of total phenolic and flavonoid content

Total phenolic content of *Ficus* samples was assessed using Folin-Ciocalteau reagent (13). The extract (100 μl) was mixed with 0.2 ml Folin-Ciocalteau reagent (Sigma-Aldrich) and 2 ml of distilled water, and flasks were shaken vigorously. Subsequently, 1.0 ml of 15% sodium carbonate solution was added and combinations were mixed thoroughly again. Mixtures were allowed to rest for 2 h, protected from light. Absorbance of blue colouration produced was measured using a spectrophotometer (UV-Vis Jenway 6003, Staffordshire, UK) at 765 nm. Levels of total phenolic contents were determined in triplicate. Chlorogenic acid was used as standard and total phenolic contents was expressed as chlorogenic acid equivalents in mg/g of dried material.

Flavonoid content was determined spectrophotometrically using a method based on formation of flavonoid-aluminium complex (14). One millilitre of extract was added to a 10 ml volumetric flask. Distilled water was added to produce a volume of 5 ml. At time zero, 0.3 ml of 5% (w/v) sodium nitrite was added to the flask. After 5 min, 0.6 ml 10% (w/v) $AlCl_3$ was added, then at 6 min, 2 ml of 1 M NaOH was also added to the mixture, followed by addition of 2.1 ml distilled water. Absorbance at 510 nm was measured immediately. Quercetin was chosen as standard and levels of total flavonoid content were determined in triplicate and expressed in mg/g of extract.

Free radical scavenging activity assay

Free radical scavenging activity was rapidly evaluated using a TLC screening method based on reduction of a methanolic solution of the coloured free radical DPPH. After application of hydroalcoholic solution of extracts, developing (mobile phase $CHCl_3$/MeOH 8:2) and drying, TLC plates were sprayed with a 0.2% DPPH solution in MeOH and examined after 30 min. Samples with antioxidant activity yielded yellow spots against a purple background; positive samples were assayed for their radical scavenging potency as described by Wang *et al.* (15). Two hundred microlitres of test sample solution (1–1000 μg/ml) was added to 800 μl of 10^{-4} M ethanolic solution of DPPH; reaction mixtures were vigorously shaken and kept in the dark for 30 min. Absorbance of resulting solutions was measured in 1 cm cuvettes using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer (Milan, Italy) at 517 nm against blank (without DPPH). All tests were run in triplicate and mean values were calculated. Ascorbic acid was used as positive control.

β-Carotene bleaching-linoleic acid assay

Antioxidant activity was determined using the β-carotene bleaching test (16) with modifications. Briefly, 1 ml of chloroform β-carotene solution (0.2 mg/ml) was added to 0.02 ml of linoleic acid and 0.2 ml of 100% Tween 20. After evaporation of chloroform and dilution with 100 ml of water, 5 ml of the emulsion was transferred into different test tubes containing 0.2 ml of samples, in 70% ethanol, at different concentrations. Propyl gallate at the same concentration was used as positive control. Tubes were then gently shaken and placed in a water bath at 45 °C for 60 min. Absorbances of the samples, standard and control, were measured at 470 nm using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer against a blank, consisting of emulsion without β-carotene. Measurement was carried out at initial time ($t = 0$) and successively at 30 and 60 min. All samples were assayed in triplicate and mean value was calculated. Antioxidant activity was measured in terms of successful prevention of β-carotene bleaching, using the following equation:

$$\text{Antioxidant activity} = [1 - (A_0 - A_t)/(A^\circ{}_0 - A^\circ{}_t)] \times 100$$

where A_0 and $A^\circ{}_0$ are absorbance values measured at initial incubation time for samples/standard and control, respectively, while A_t and $A^\circ{}_t$ are absorbance values measured in samples/standard and control respectively, at $t = 30$ min and $t = 60$ min.

Phototoxicity

The phototoxicity test was adapted from Barraja *et al.* (17) with modifications. Amelanotic melanoma cells (C32) were grown in RPMI-1640 medium (Sigma-Aldrich) supplemented with 1% L-glutamine, 1% of penicillin/streptomycin and 10% foetal calf serum. Individual wells of a 96-well tissue culture microtitre plate (Falcon BD, Milan, Italy) were inoculated with 100 μl of complete medium containing 1×10^4 cells. Plates were incubated at 37 °C in a humidified 5% incubator for 24 h prior to experiments. After medium removal, 100 μl drug solution, dissolved in DMSO and diluted with Hanks' balanced salt solution (HBSS, pH 7.2), was added to each well and incubated at 37 °C for 30 min, then irradiated. HPW 125 Philips lamps, mainly emitting at 365 nm, were used for irradiation experiments. Spectral irradiance of the source was 4.0 mW/cm^2 as measured at sample level, by a Cole-Parmer Instrument Company radiometer (Niles, IL, USA), equipped with a 365-CX sensor. Cells were irradiated for 1 h at a dose of 1.08 J/cm^2. After irradiation, the solution was

replaced with medium, and plates were incubated for 48 h. Cell viability was assayed by MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide)] test, as previously described (18).

Statistical analysis

Data are expressed as means ± SD. Statistical significance was assessed with one-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* testing. Differences were considered significant at $P \leq 0.05$. Inhibitory concentration 50% (IC50) was calculated from the dose–response curve obtained by plotting percentage of inhibition versus concentration using Prism statistical software (www.graphpad.com).

Results and discussion

Phytochemical analysis

Chemical composition varies from plant to plant and within different parts of the same plant. To identify non-polar components of *F. carica* extracts, GC-MS and GC analyses were carried out. Coumarins and fatty acids were identified and quantified as major constituents in all samples. Among coumarins, psoralen and bergapten are two of the major active components identified in *F. carica* extracts. Psoralen concentrations were significantly different between plant parts ($P < 0.05$), following the order bark>leaves>woody components (mean values of 23.3%, 19.2% and 8.6% respectively) (Fig. 1). In the case of bergapten, concentrations also were different ($P < 0.05$), following the order: leaves>bark>woody components (mean values of 8.2%, 6.6% and 2.6% respectively).

Composition of fatty acids of *F. carica* extracts is reported in Table 1. Extracts were characterized by linolenic acid methyl and ethyl ester and linoleic acid ethyl ester, as the most abundant components. Leaf extracts were characterized by linolenic acid methyl ester as the most abundant constituents of leaves (12.4%), while linolenic acid ethyl ester and linoleic acid ethyl ester were the most abundant compounds in bark and woody parts samples.

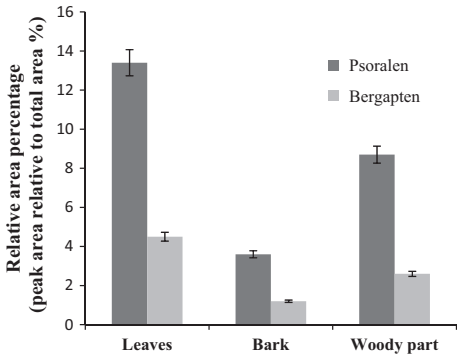

Figure 1. Coumarin composition of *F. carica* cv. Dottato extracts. Data, mean ± SD ($n = 3$).

Table 1. Composition of fatty acids of *F. carica* cv. Dottato extracts

Compound[a]	Leaves	Bark	Woody part
Palmitic acid methyl ester	0.5	ND	0.5
Palmitic acid ethyl ester	1.9	0.5	5.6
Palmitic acid	3.7	2.1	6.9
Linolenic acid methyl ester	12.4	2.4	tr
Linoleic acid	1.6	0.3	5.4
Linolenic acid ethyl ester	ND	9.8	9.7
Linoleic acid ethyl ester	6.5	7.9	7.5
Stearic acid ethyl ester	ND	5.2	0.6

tr, trace; ND, not detected.
[a]Relative area percentage (peak area relative to total peak area%).

Ethyl esters of fatty acids were artefacts formed during extraction with hydroalcoholic solution (70% ethanol).

Total phenolic content was significantly different among the three plant parts ($P < 0.05$) (Table 2). In particular, leaves possessed higher quantities of phenolic compounds. Leaves also were characterized by major content of total flavonoid. Some studies have described presence of several phenolic compounds in *F. carica* (19–22) and their antioxidant activity (23). Composition and antioxidant activities of leaves from two Portuguese *F. carica* varieties have also been evaluated (24), however, metabolic profiles of bark and woody components had not been compared.

Antioxidant activity

Many studies have shown that natural antioxidants are closely related to their bio-functionalities, such as reduction in chronic conditions such as mutagenesis, carcino-

Table 2. Polyphenol and flavonoid content of *F. carica* cv. Dottato extracts

Sample	Polyphenols[a] (mg/g)	Flavonoids[b] (mg/g)	Flavonoids/ polyphenols
Leaves	3.6 ± 0.18	1.0 ± 0.05	0.3
Bark	2.8 ± 0.14	1.3 ± 0.06	0.5
Woody parts	1.4 ± 0.07	0.8 ± 0.04	0.6

[a]Chlorogenic acid equivalents/g of dried material.
[b]Quercetin equivalents/g of dried material.

genesis, DNA damage and more. Thus, antioxidant capacity is widely used as a parameter to characterize food or medicinal plants and their bioactive components. Antioxidant properties, especially free radical scavenging activities, are very important due to the deleterious role of free radicals in foods and biological systems. Reduction in DPPH absorption is indicative of capacity of the extracts to scavenge free radicals, independently of any enzymatic activity.

Figure 2 illustrates DPPH free radical scavenging ability (IC_{50} values) of different parts of plants. Ascorbic acid was used as free radical scavenger reference. Leaves, bark and woody component extracts exhibited DPPH scavenging capacity, in a concentration-dependent manner, leaves being the most effective material, with IC_{50} value of 100 μg/ml. Thus the samples were revealed to have a protective effect for lipid peroxidation, the effect being concentration-dependent (Fig. 3). As happened for DPPH, leaves exhibited the strongest capacity with IC_{50} value of 1.48 μg/ml after 30 min incubation (Fig. 3a).

Overall, results obtained in the two assays revealed that leaves possess the strongest antioxidant potential and bark, the weakest. These results may be partially explained due to the highest amounts of phenolic compounds occurring in leaves. Antioxidant capacity of phenolic compounds is based on their ability to scavenge free radicals, chelate pro-oxidant metal-ions and to inhibit some enzymes (25,26). Nevertheless, the contribution of organic acids cannot be ignored. Previous studies have shown the antioxidant activity of *F. carica* leaf extract (10 mg/ml), which had approximately 50% free radical scavenging activity, using the DPPH test (27). Results obtained in our study however, reveal that leaf extract was very potent. Oliveira *et al.* (24) compared anti-free radical activity of pulp, peel and leaves of two Portuguese *F. carica* varieties and the authors showed that leaf extract, obtained by boiling in water, was the most effective material.

Phototoxicity

Phototoxicity of *F. carica* samples was investigated here, for the first, time on the human tumour cell line C32 (amelanotic melanoma). Table 3 shows IC_{50} value, concentration that induces 50% inhibition of cell population growth, of leaves, bark and woody part extracts, after irradiation at the specific UVA dose of 1.08 J/cm^2. Control experiments using UVA light and parallel

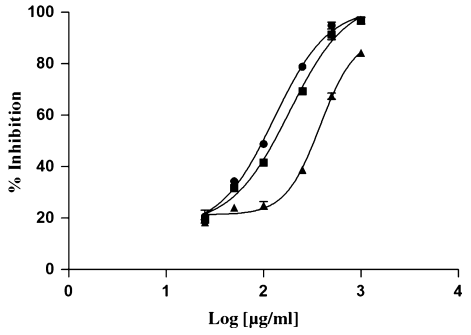

Figure 2. Free radical scavenging activity on DPPH of *F. carica* cv. Dottato extracts. ●: leaves; ■: bark; ▲: woody parts. Data, mean ± SEM (n = 3). Ascorbic acid (IC_{50} value of 2 ± 0.01 μg/ml) was used as positive control.

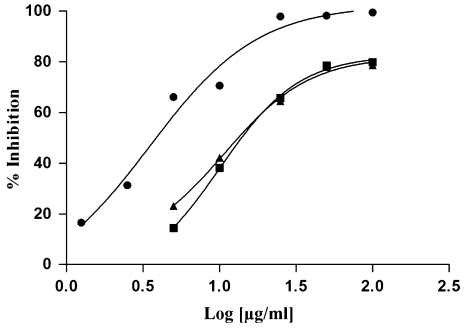

Figure 4. Phototoxic effects exerted by total extracts and fractions of *F. carica* cv. Dottato extracts on UVA-induced A375 cells. ●: leaves; ■: bark; ▲: woody parts. Data represent mean ± SEM (n = 4). Bergaptene (IC_{50} = 0.0416 ± 0.008 μg/ml) was used as positive control.

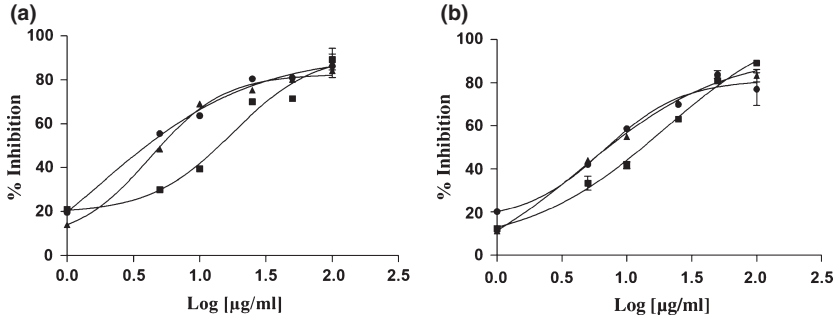

Figure 3. Lipid peroxidation inhibition activity using β-carotene-linoleic acid system after 30 and 60 min of incubation of *F. carica* cv. Dottato extracts. ●: leaves; ■: bark; ▲: woody parts; a: 30 min; b: 60 min. Data represent mean ± SEM (n = 3). Propyl gallate (IC_{50} = 1 ± 0.02 μg/ml) was used as positive control.

Table 3. IC_{50} values (μg/ml) of antioxidant and phototoxic activities of *F. carica* cv. Dottato extracts

Sample	DPPH	Lipid peroxidation inhibition		Phototoxicity	
		30 min	60 min	With UV	Without UV
Leaves	100 ± 2.51^{c}	1.48 ± 0.07^{d}	6.61 ± 0.33^{b}	3.92 ± 0.19^{c}	100 ± 2.75^{a}
Bark	161 ± 3.04^{b}	18.36 ± 0.92^{a}	18.9 ± 0.94^{a}	10.03 ± 0.57^{b}	ND
Woody part	360 ± 3.89^{a}	4.02 ± 0.21^{c}	4.92 ± 0.25^{bc}	10.69 ± 0.53^{b}	ND

For antioxidant activities, data are expressed as means ± SD ($n = 3$). For photodynamic activity, data are expressed as mean ± SD ($n = 4$). Different letters along column (DPPH test), or within the two columns (β-carotene test and MTT) indicate statistically significant differences at $P < 0.05$ (Bonferroni test).
ND, not detectable.

treatment groups of non-irradiated cells, were carried out without significant cytotoxic effects.

Ficus carica leaves had best anti-proliferative activity in comparison with bark and woody components, with IC_{50} values of 3.92, 10.0 and10.69 μg/ml, respectively.

The reason for this is in their composition. All extracts contain polyphenolic compounds such as flavonoids, however, individual components and their concentrations differ. Huang *et al.* came to similar conclusions when they studied effects of extracts of black raspberries, strawberries and blueberries on UV-induced damage, to a mouse epidermal cell line (28).

Anti-proliferative activity may be attributed also to presence of furanocoumarins, which represent a novel class of potentially effective natural drugs for treatment of several types of cancer and skin disease (29).

A modern approach in chemoprevention is to formulate a mixture of active compounds or prepare extracts/fractions from plant material with protective activity. A mixture of various phytochemicals has a number of advantages, for example, extracts and fractions are cheaper and easier to prepare than pure formulations, as individual components may provide for each other additive and/or synergistic effects, and lower concentration of constituents lessens their possible toxic effects.

Conclusion

This is the first study comparing *F. carica* leaves, bark and woody parts. Strong relationships observed between psoralen and bergapten within and between different plant structures support the idea that these are probably metabolically dynamic compounds with a cycle of synthesis, translocation and transformation in the plant (30).

Concentrations of coumarin compounds found in *Ficuc carica* cv. Dottato reaffirm early findings on presence of psoralen and bergapten in different structures of this species. Utilization of the leaves may contribute to prevention of diseases in which homeostasis is impaired by oxidative features. In addition, the leaves were characterized as having higher quantities of psoralen and bergapten.

This study confirms that plant-derived natural compounds are an important source for development of anticancer drugs. *F. carica* cv. Dottato leaves could provide a new perspective in developing new formulations, potentially useful in PUVA therapy for treatment of malignant melanoma and other diseases. Further experiments are necessary to examine bioavailability of the active components, to exclude any possible toxic effects they may have and to clarify their photoprotective activity *in vivo*.

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