Ficus carica SSP Dottato Buds by Intercropping Different Species: Metabolites, Antioxidant Activity and Endogenous Plant Hormones (IAA, ABA)

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Ficus carica is one of the most common tree crops in the Mediterranean basin. Its ethnobotanic use has been extensively studied to evaluate its biological activity in relation to the presence of specific secondary metabolites. In this paper, the extract of the gemstones of the ficus carica ssp dottato di Cosenza was studied with respect to different vegetation habitats (intercrops) and two different extraction techniques. Buds, in fact, are used in gemotherapy as macerated glycerides obtained by long extraction processes (21 Days).The use of a Dynamic extractor (Naviglio® Extractor) has allowed not only to reduce the extraction time (10 h) but to obtain a qualitatively and quantitatively enriched extract withactive ingredients to which the specific biological activity is reported. In fact, the total polyphenolic and total flavonoid components were determined, of which Quercetin-3O-Glucoside and 3-O-Rhamnoside were dosed, and the resulting anti-oxidant activity. IAA and ABA have also been quantified.

Keywords: meristematic, gemmotherapic, Ficus carica, total phenolic, IAA, AB

Ficus carica L. belongs to the *Moraceae* family, is a temperate species, native to southwest Asia and the Mediterranean region (from Afghanistan to Portugal) and has been widely cultivated from ancient times for nutritional value of the its fruits. Figs are perhaps the oldest of all cultivated fruit crops and are grown in many areas of the world that have subtropical climates.

Plant growth and development, as well as the responses to environmental factors, are highly regulated by the complex and coordinated action of the five classical hormones: gibberellins (GAs), abscisic acid (ABA), cytokinins (CKs), auxins (IAA) and ethylene. In addition, some other molecules, such as brassinosteroids (BRs), polyamines (Pas), jasmona-tes (JAs) and salicylic acid (SA), and some polypeptide hormones have shown to be involved, both directly or indirectly, in such processes [1].

In particular, Abscisic acid (ABA) and auxin, indole-3-acetic acid (IAA), are endogenous plant hormones known to regulate myriad aspects of plant growth and development.

Regulation of the initiation of axillary meristems is important for controlling the overall plant form [2, 3] and several studies have confirmed the roles of endogenous ABA in seed and lateral root development, dormancy versus germination, transpiration, and stress responses, and IAA in cell division, cell elongation, pattern formation, differentiation, and tropisms [4]. Studies of abscisic acid (ABA) and auxin have revealed that these pathways impinge on each other [4-6].

Biomolecules from plants have attracted a great deal of attention, mainly concentrated on their role in preventing diseases. Epidemiological studies have shown that there is a clear signiûcant positive association between intake of these natural products and reduced rate of heart disease mortalities, common cancers and other degenerative diseases [7,8].

Infact, hormone abscisic acid (ABA) is released from glucose-challenged human pancreatic â cells and stimulates insulin secretion [9] and presents antitumorogenenesis activity [10], the plant hormone abscisic acid increases in human plasma after hyperglycemia and stimulates glucose consumption by adipocytes and myoblasts [9].

From an agronomic point of view, figs have a high commercial value, also invites attention of the researchers worldwide for its biological activities, primary and secondary metabolites, enzyme profile, nutritive value and a significant genetic diversity that guarantees a interesting pharmacological activity [11-15].

In traditional medicine systems such as Ayurveda, Unani and Siddha [16] is used as a diuretic, mild laxative, expectorant and also in the diseases of the liver and spleen as a deobstruent and anti-inflammatory agent [14].

For this reason, are commercially available Ficus supplements and cosmetics. In Mediterranean traditional medicine, the fruits, which are sweet, have antipyretic, purgative, aphrodisiac properties and are used in inflammation and paralysis. The juice of the fruit with honey was prescribed for controlling hemorrhages [16]. Fruit of *F. carica* shows spasmolytic activity, mediated through the activation of K+-ATP channels along with anti platelet activity. Hence, it can be used in gut motility and inflammatory disorders [17].

There is a general agreement in the scientific community that its properties are due to the presence of phytochemicals that control the right cell and metabolic functionality [18-20].

Ficus species contain flavanoid glycosides, alkaloids, phenolic acids, steroids, saponins, coumarins, tannins, triterpinoids – oleanolic acid , rusolic acid , á- hydroxy ursolic acid, protocatechuic acid, maslinic acid. The nonenzymatic constituents include phenolic compounds, flavanoids, vitamin C [18-20] all responsible for established biological activities.

Our target is the evaluation of new extraction techniques in the field of gemmotherapy so as to maximise the procedure of extraction. And also, an evaluation of the different phytochemical compositions between *Ficus*

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carica L.cultivar dottato buds cultivated in different habitats (intercropping).

Experimental part

Ficus buds were harvested in mid-April (2016) in experimental fields near Sila (Southern Italy).

The samples were divided into categories according to the origin:

-Fig buds from fig trees with Olea Europaea (BOE)

-Fig buds from fig trees with Vitis vinifera (BVV)

-Fig buds from only Fig trees (BFC)

All have been harvested and stored at 4°C for 12 hours and then subjected to extraction.

Samples extraction

Two kinds of extraction were choosen:

- Glycerine macerate →20 g of buds in glycerin: ethanol (1:10:10) . Fresh buds collected are left to macerate 5 days in ethanol and then, another 21 g in a mixture of water and glycerin. After 21 days the buds are decanted and filtered, the residue is squeezed and left to rest for 48 h, then filtered again before being diluted (1:10) with a new mixture of water-alcohol and glycerin.

- Hydroalcoholic solution with Extractor Naviglio®→100 grams of buds in ethanol and water 50:50; each cycle works in two phases: dynamic and static phase. In the dynamic phase, pistons pressings on the plant material 30 times, while the static phase last 10 min for a total of 13 min per cycle that repeats 20 times.

Then, the yield has been determined as a percentage of extraction, through the following formula:

Yield (%)= [(peso estratto (g)/peso fresco (g))]*100

Phytochemical tests

Determination total poliphenol content

0.75 mL of Folin-Ciocalteau have been added to our extract (concentration of 1 mg/mL. The solution has been left to stand at 22°C and later mixed with a sodium hydrogen carbonate solution. The samples have been left to stand for 90 min at 22°C and then with a spectrophotometer at 765 nm it has been measured the absorbance, using a blank solution as comparison.

Straight line calibration: $y = 0.0091x - 0.0155$, $R^2=0.999$

Determination total flavonoid content

The total flavonoid content of crude extract was determined on the same extracts used for total phenols determination by the $AlCl_3$ colorimetric method [21]. In brief, 1 mL of EtOH was added to 2 mg of crude extract. After 5 min of incubation, 1 mL di 2% $AlCl_3$ aqueos solution was added and the mixture was allowed to stand for 15 min. The calibration curve was determined with eight standard concentrations, ranging from 25 to 900 µg/mL. The absorbance was measured at 430 nm. Total flavonoid content was expressed as mg quercetin equivalent (QE) per g of fresh material (FW).

Biological tests

DPPH Assay

1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay was adapted from Marrelli and others [7] to examine antioxidant properties. In an ethanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (final concentration = 1.0×10"4 M), extracts at different concentrations were added. The reaction mixtures were shaken vigorously and then kept in the dark for 30 min. The absorbance of the resulting solutions was measured at 517 nm, against a blank with DPPH. Decreasing absorbance values of the DPPH solutions indicated an increase of DPPH radical scavenging activity. The DPPH solution without sample solution was used as control. Ascorbic acid was used as positive control. This activity was given as % DPPH radical scavenging, calculated by the following equation: % DPPH radical scavenging = (absorbance control – absorbance sample)/ absorbance control × 100.

Preparation of the samples

For both samples (glycerin macerate and hydroalcoholic solution) we used Sep-Pak C18 Classic Cartridge, a silica-based bonded phase with strong hydrophobicity; used to adsorb analytes of even weak hydrophobicity from aqueous solutions; similar in behavior to reversed-phase HPLC columns. Then, the filtrate has been dried (Rotavapor).

Phytochemical profiling

Samples have been analyzed through Liquid Chromatography. Our chromatograph is a VWR-Hitachi, a liquid chromatograph fitted with a pulse-free pump and a suitable detection device (model L-2455)

We used the following eluents:

-(A) 0.05% aqueous formic acid,

-(B) 100% metanolo.

We have taken 20 µL of each sample for single injection.

Qualitative analysis have lasted 40 min, plus 5 min post-analysis, with a total of 45 min for each sample. It was a steady stream (1.0 mL/min) but the percentage of the eluents varied according the following gradient:

Gradient program: 0-5 min, B=10%; 5-30 min B=10-90%, 30-40 min B=90%.

Quantitative analysis: isocratic procedure (concentration v/v 65:35).

Each sample has been measured at a wave length of 200-400 nm.

Quantitative analysis by GC/MS

IAA and ABA were derivitized by the addition of 50 µL of pyridine solution of m-methylbenzoic acid internal standard at a concentration of 20 µg/ mL and 50 µL of the derivatization reagent BSTFA + TMCS with heating at 60 °C silylated in a sealed 2-mL minivial for 30 min. The silylated samples were analyzed by GC-MS. The large excess of BSTFA + TMCS ensured that the derivatization was complete. Care was taken to ensure anhydrous conditions during the preparation and derivatization process because of the high sensitivity of trimethylsilyl (TMS) derivatives toward moisture.

Samples diluted by EtOAc were injected into gas chromatography (GC) equipped with mass spectrometry (MS) (Hewlett-Packard Co.) gas chromatograph, model 5890 equipped with a mass spectrometer, model 5972 series II, and controlled by HP software equipped with capillary column 30 m×0.25mm, static phase SE30, using programmed temperature from 60 to 280°C (rate 16°/min); the detector and the injector have been set to a temperature of 280 and 250°C, respectively (split vent flow 1 mL/min"1). Compounds 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.).

Statistical analysis

Data were analyzed using SPSS r.11.0.0 statistical software (SPSS, Inc., Chicago, IL, USA). All measurements were carried out in replicates (n=5). Significant differences

were calculated at Pd"0.05 level among means by one-way ANOVA, using Tukey's test. The values of IC50 (half maximal inhibitory concentration) for each measured parameter was calculated by means of scatter charts (where the X-axis indicates the concentration and the Y axis is the % activity or % inhibition).

Trend lines were plotted and IC50 calculated by a linear trendline (Y = a X + b) by the formula IC50 = (0.5 – b) / a.

Results and discussion

The aim of this work is to compare an innovative tecnique of dynamic extraction (Extractor Naviglio®) with a traditional tecnique used for glycerine macerate's production.

For this reason, we have studied not only secondary metabolites content but also endogenous hormones (IAA e ABA) content, in *Ficus carica spp dottato* buds that are grown in a fig field and in *Ficus carica spp dottato* buds grown in consociation with Olea Europaea and Vitis vinifera.

Ficus is a very widespread plant in the Mediterranean basin, highly resistant to drought conditions and capable of creating different interactions with other tree species, such as olive and vines.

The results show how a hydroalcoholic solution obtained through 10 hours of dynamic extraction (Extractor Naviglio®) can have a better metabolomics fingerprinting than buds-extract obtained with a 21 days glycerine maceration.

Poliphenols content

The variability of phenolic content is due to the intercropping; not only the geopedological characteristics of the habitat play a significant role, but also the interactions between microorganisms, micorrize and roots.

This mainly affects secondary metabolism, and justifies the quantitative differences in the ratio of total polyphenolic content to total flavonoid.

BBV sample obtained through EN has a higher poliphenolic content compared to the samples BEO e BFC obtained with the same tecnique, while BFC has a higher flavonoidic content compared to BEO e BVV.

Polyphenols content (as phytochemical marker) in *Ficus carica spp Dottato* buds is 30.12 ± 2.14 mg/g in BFC; 36.12 ± 4.22 mg/g in BEO; 38.24 ± 5.32 mg/g in BVV, when BFC, BEO and BVV are obtained with EN; while, when the same samples are obtained with the classic GM the content is respectively 20.12 ± 8.54; 16.12 ± 6.34; 30.24 ± 9,36 mg/g .

Flavonoid contentin *Ficus carica spp Dottato* buds is 22.07 ± 3.18mg/g in BFC, 15.32 ± 2.07mg/g in BEO, 25.35 ± 4.24mg/g in BVV, when BFC, BEO and BVV are obtained with EN; while, when the same samples are obtained with the classic GM the content is respectively 12.12 ± 2.25mg/g ,07.68 ± 1,38mg/g ,11.59 ± 3.15mg/g

Total phenolics are expressed as chlorogenic acid equivalents (CAE) per g of fresh material (FW). Total flavonoids are expressed as quercetin equivalents (QE) per g of fresh material (FW). Means (n = 5) ± SD with different letters within the same column are significantly different at $p< 0.05$.

Among the main flavonoids, through HPLC we have been identified Quercetin 3-O-glucoside (Q3-OG) e Quercetin 3-O-rhamnoside (Q3-OR)

Quali-quantitative determination of Q3-OG and Q3-OR has been made using an external standard.

Our results are calculated according to the calibration curve.

Q3-OG's content in samples BFC, BEO and BVV obtained with MG is respectively 1,1 ± 0.05 mg/g, 1.22 ± 0.14mg/g , 2.04 ± 0.06 mg/g.

Q3-OG's content in samples BFC, BEO and BVV obtained with EN is respectively 2.12± 0.15 mg/g, 2.12 ± 0.22 mg/g, 2.24 ± 0.12 mg/g.

Q3-OR's content in samples BFC, BEO and BVV obtained with MG is respectively 1.02 ± 0.25 mg/g, 1.68 ± 0.08 mg/g, 2.39 ± 0.15 mg/g.

Q3-OR's content in samples BFC, BEO and BVV obtained with EN is respectively 1.98 ± 0.08 mg/g, 2.25 ± 0.16 mg/g, 2.95 ± 0.24 mg/g.

Looking at the results of DPPH's assay we can see that all the samples obtained witn EN have a higher antiradical activity than samples obtained with the classic method:

IC50 calculated for the samples BFC, BEO and BVV obtained with MG is, respectively: 1.07 ± 0.38μg/mL , 5.32 ± 1.07μg/mL, 2.09 ± 1.28μg/mL.

IC50 calculated for the samples BFC, BEO and BVV obtained with EN is respectively:0.91 ± 0.18μg/mL , 2.21 ± 0.99 μg/mL , 1.05 ± 0.24μg/mL.

To complete the metabolomic fingerprinting we have determined the main primary metabolites present. Gemmotherapy, infact, is based on many products that work thanks the synergism of secondary and primary metabolites.

Quantitative determination of ABA e IAA is consistent with our results: the ratio of IAA e AB in the samples obtained witn EN is significantly higher compared to the samples obtained in GM.

It's important to notice the significant variation of ABA and IAA's content in the samples, which makes it clear that BVV > BOE> BFC .

Infact IAA's content in BVV (obtained with EN) is 18.34 ± 0.12μg/g and (obtained with GM) is 17.04 ± 0.26μg/g

IAA's content in BOE (obtained with EN) is 16.02 ± 0.02 μg/g and (obtained with GM) is 16.32 ± 0.14 μg/g

IAA's content in BFC (obtained with EN) is 13.12 ± 0.55 μg/g and (obtained with GM) is 12.07 ± 0.55μg/g

While, ABA's content in BVV (obtained with EN) is 155.35 ± 0.24μg/g and (obtained with GM) is 105.39 ± 0.15μg/g

Table 1
TOTAL POLYPHENOL AND FLAVONOID CONTENT IN *FICUS CARICA SPP. DOTTATO* BUDS EXTRACT

BUDS EXTRACTS	Total polyphenols (mg CAE / g FW)		Total flavonoids (mg QE / g FW)	
	MG	Naviglio®	MG	Naviglio®
BFC	20.12 ± 8.54[a]	30.12 ± 2.14[b]	12.12 ± 2.25[a]	22.07 ± 3.18[b]
BOE	16.12 ± 6.34[a]	36.12 ± 4.22[b]	07.68 ± 1.38[a]	15.32 ± 2.07[b]
BVV	30.24 ± 9.36[a]	38.24 ± 5.32[b]	11.59 ± 3.15[a]	20.35 ± 4.24[b]

Total phenolics are expressed as chlorogenic acid equivalents (CAE) per g of fresh material (FW).Total flavonoidsare expressed as quercetin equivalents (QE) per g of fresh material (FW).Means (n = 5) ± SD with different letters within the same column are significantly different at $p< 0.05$.

ABA's content in BOE (obtained with EN) is 123.25 ± 0.56 μg/g and (obtained with GM) is 103.68 ± 0.38μg/g

ABA's content in BFC (obtained with EN) is 104.98 ± 0.18μg/g and (obtained with GM) is 100.02 ± 0.25μg/g

In addition to phytosociological aspects related to the intercropping, it should be noted that, among environmental factors which affect primary and secondary metabolism, there are pedological differences in different vegetation habitats.

In fact, vineyards grow prevalently on sandy soils, while the olive groves grow on clay soils . The microbial interactions in the subsoil depend on the physical-chemical characteristics of the soil itself and on the abiotic stress conditions, which are able to influence the metabolism of the plant.

Conclusions

The most important result was to accelerate the extraction executability times compared to the traditional GM extraction system.

Moreover, the extractive capacity of the method used (EN) is evaluated positively against GM for what concern the content of phytochemical markers analyzed.

This allows to obtain a hydroalcoholic extract that can be used in gemmotherapy and, thanks to the absence of the glycerol fraction, in nutraceutical and cosmetological formulations.

References

1.BOTTON A., ZIOSI V., BREGOLI A.M., COSTA G. E RAMINA A. Il controllo ormonale dell'attivita vegetativa e produttiva negli alberi da frutto. Review N. 1 – Italus Hortus 14 (1), 2007, p. 24 – 36

2.SUSSEX IM, KERK NM. The evolution of plant architecture. Curr opin plant biol. 4(1), 2001, p. 33-37

3.COUDERT Y., PALUBICKI W., LJUNG K., NOVAK O., LEYSER O., HARRISON CJ, Three ancient hormonal cues co-ordinate shoot branching in a moss. Elife. 2015; 4: E06808. PUBLISHED ONLINE 2015 MAR 25. DOI: 10.7554/ELIFE.06808 PMCID: PMC4391503

4.ROCK C.D., SUN X. Crosstalk between ABA and AUXIN signaling pathways in roots of Arabidopsis thaliana (L.) Heynh' Planta 2005, 222, p. 98–106. DOI 10.1007/S00425-005-1521-9

5.HAGEN G, GUILFOYLE TJ, GRAY WM AUXIN SIGNAL TRANSDUCTION. IN: DAVIES P (ED) PLANT HORMONES-BIOSYNTHESIS, SIGNAL TRANSDUCTION, ACTION 2004

6.FINKELSTEIN RR, GIBSON SI, ABA and sugar interactions regulating development: CROSS-TALK OR VOICES IN A CROWD? Curr opin plant biol 2002, 5, p. 26–32

7.MARRELLI M, CRISTALDI B, MENICHINI F, CONFORTI F. Inhibitory effects of wild dietary plants on lipid peroxidation and on the proliferation of human cancer cells FOOD CHEM TOXICOL 2015, 86, p. 16-24

8.DUKE JA, BUGENSCHUTZ-GODWIN MJ, DU COLLIER J, DUKE PK. HAND BOOK OF MEDICINAL HERBS.2ND EDITION. BOCA RATON, FLA, USA: CRC PRESS; 2002.

9.AMERI P, BRIATORE L, MANNINO E, BASILE G, ANDRAGHETTI G, GROZIO A, MAGNONE M, GUIDA L, SCARFI S, SALIS A, DAMONTE G, STURLA L, NENCIONI A, FENOGLIO D, FIORY F, MIELE C, BRUZZONE S, RUVOLO V, BORMIOLI M, COLOMBO G, MAGGI D, MURIALDO G, CORDERA R, DE FLORA A, ZOCCHI E., The plant hormone Abscisic acid increases in human plasma after hyperglycemia and stimulates glucose consumption by adipocytes and myoblasts'. FASEB JOURNAL 2012, 26(3), p. 1251-1260.

10.ALVAREZ PALOMO A.B, SAMUEL MCLENACHAN, JORDI REQUENA OSETE, CRISTINA MENCHÓN, CARME BARROT, FRED CHEN, SERGI MUNNÉ-BOSCH, MICHAEL J. Edel Plant Hormones Increase Efficiency of Reprogramming Mouse Somatic Cells to Induced Pluripotent Stem Cells and Reduce Tumorigenicity. Stem Cells Dev. 2014, 23(6), p. 586–593.

11.CHAWLA A, KAUR R, SHARMA AK. Ficus carica linn, a review on its pharmacohnostic, pytochemical and pharmacologica aspects. Int J Pharam Phytopharmacol Res 2012, 1, p. 215–232.

12.JOSEPH B, RAJ SJ. Pharmacognostic and phytochemical properties of Ficus carica linn – an overview. Int J Pharmtech Res 2011, 3, p. 8–12. KALASKAR MG, SHAH DR, RAJA NM, ET AL. Pharmacognostic and phytochemical investigation of Ficus carica linn. Ethnobot leaflets 2010, 14, p. 599–609.

13.ASADI F, POURKABIR M, MACLAREN R, SHAHRIARI A. Alterations to lipid parameters in response to Fig tree (FICUS CARICA) leaf extract in chicken liver slices. Turkish Journal of Veterinary and Animal Sciences, 2006, 30(3), p. 315–318.

14.ETERAF-OSKOUEI T., ALLAHYARI S., AKBARZADEH-ATASHKHOSROW A., ABBAS DELAZAR A., PASHAII M., HUA GAN S., NAJAFI M. Methanolic extract of Ficus carica linn. Leaves exerts antiangiogenesis effects based on the rat air pouch model of inflammation. Evid based complement alternat med. DOI: 10.1155/2015/760405

15.PEREZ C, DOMINGUEZ E, RAMIRO JM, ROMERO A, CAMPILLO JE, TORRES MD. A study on the glycaemic balance in streptozotocin-diabetic rats treated with an aqueous extract of FICUS CARICA (FIG TREE) leaves. PHYTOTHERAPY RESEARCH. 1998, 10(1), p. 82–83.

16.PRASAD PV, SUBHAKTHA PK, NARAYANA A, RAO MM. Medicohistorical study of ASVATTHA (SACRED FIG TREE). Bull Indian Inst History Med (HYDERABAD) 2006, 36, p. 1–20.

17.GILANI AH, MEHMOOD MH, JANBAZ KH, KHAN AU, SAEED SA. Ethnopharmacological studies on atispasmodic and antiplatelet activities of FICUS CARICA'. J Ethnopharmacol. 2008, 119(1), p. 1-5.

18.SOLOMON A, GOLUBOWICZ S, YABLOWICZ Z, GROSSMAN S, BERGMAN M, GOTTLIEB HE, ALTMAN A, KEREM Z, FLAISHMAN MA. Antioxidant activities and anthocyanin content of fresh fruits of common Fig (FICUS CARICA L.). J AGRIC FOOD CHEM. 2006, 54(20), p. 7717-7723.

19.SIRISHA N., SREENIVASULU M., SANGEETA K., MADHUSUDHANA CHETTY C., Antioxidant properties of FICUS SPECIES – A REVIEW''. Internationa Journal of Pharmtech Research Coden (USA): IJPRIF ISSN: 0974-4304 VOL.2, NO.4, p. 2174-2182

20.PATIL, V.V., PATIL, V.R., FICUS CARICA LINN. AN OVERVIEW. RES. J. MED. PLANT. 2011, 5, p. 246–253

21.MARRELLI M, CONFORTI F, TONIOLO C, et. al., Hypericum perforatum: influences of the habitat on chemical composition, photo-induced cytotoxicity, and antiradical activity. Pharm Biol 2014, 52, p. 909-918.

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