Thanks to:
Korinna Miliaraki - Director of the Cultural Society of Panormos "Epimenides"
Harry Katerinopoulos - Professor of University of Crete

Carob tree as a source of compounds with bioactive properties

Anabela Romano
The University of Algarve is located in Algarve, the southern part of Portugal and the most touristic region.
This tree is one of the four thickest in the country, with an estimated age of 600 years.

Carob production (t)

Reduction in production of major producing countries
The region of carob cultivation in Portugal is concentrated in the south, Algarve.

* DRAP-Algarve: Repositório de germoplasma com 44 cultivares de Ceratonia siliqua.
At the Plant Biotechnology Lab we conduct research on the use of biotechnological approaches towards the conservation and sustainable use of plant genetic resources and on the biological and chemical characterization of natural compounds from plant origin.
• In vitro propagation of plants

• Germplasm conservation

• Chemical and biological characterization of wild plants and agrifood products

• Biopolymers and surfactant systems

Research on carob tree:

• In vitro studies and micropropagation

• Ecophysiological studies

• Chemical characterisation of the bio compounds

• Study of antioxidant and antiproliferative activity

• Analysis of the volatiles emitted by flowers

• Studies on carob floral biology
Antioxidant activity and in vitro inhibition of tumor cell growth by leaf extracts from the carob tree (Ceratonia siliqua)

Antioxidant and cytotoxic activities of carob tree fruit pulps are strongly influenced by gender and cultivar

Phytochemical profile, antioxidant and cytotoxic activities of the carob tree (Ceratonia siliqua L.) germ flour extracts

In vitro antioxidant and inhibitory activity of water decoctions of carob tree (Ceratonia siliqua L.) on cholinesterases, α-amylase and α-glucosidase

Research on carob tree:

- In vitro studies and micropropagation
- Ecophysiological studies
- Chemical characterisation of the biocompounds
- Study of antioxidant and antiproliferative activity
- Analysis of the volatiles emitted by flowers
- Studies on carob floral biology
Research on carob tree:
• In vitro studies and micropropagation
• Ecophysiological studies
• Chemical characterisation of the biocompounds
• Study of antioxidant and antiproliferative activity

Antioxidant activity and in vitro inhibition of tumor cell growth by leaf extracts from the carob tree (*Ceratonia siliqua*)

Luísa Custódio, Eliana Fernandes, Ana Luísa Escapa, Sandra López-Avilés, Alba Fajardo, Rosa Aligué, Fernando Alberício & Anabela Romano

Leaf extracts from female cultivars, hermaphrodite and male trees were investigated for their contents of phenolic compounds, their in vitro antioxidant activity, and their in vitro tumor growth inhibition on HeLa cells.

• This is the first time that carob tree has been investigated for antitumor activity against human cancer cells.
• This is the first time that carob hermaphrodite and male trees were investigated.
The different cultivars and hermaphrodite and male trees showed high levels of phenols; significant gender-based differences in the antioxidant activity of the extracts were observed, with males and hermaphrodite trees having the highest activities.

Carob extracts significantly inhibited HeLa cell proliferation in a dose dependent manner (except for the female cv. Galhosa, whose extracts had no effect on cell proliferation).

Extracts from male and hermaphrodite trees exhibited higher capacity to inhibit the proliferation of HeLa cells than the female cultivars.
• Extracts from the hermaphrodite tree H2 produced the highest inhibition capacity (IC50 < 25 μg/mL), while extracts from the female Cv. Galhosa were the least effective at preventing the proliferation of HeLa cells (IC50 > 400 μg/mL).

• The maximal inhibition values were high (generally 100%).

Main conclusions

• The different cultivars and hermaphrodite and male trees showed high levels of phenols;

• Gender significantly affected the antioxidant activity, and males and hermaphrodites showed the highest activities;

• Extracts from male and hermaphrodite trees exhibited higher capacity to inhibit the proliferation of cells than the female trees;

• Leaves of carob tree are potential sources of chemopreventive compounds.
Antioxidant and cytotoxic activities of carob tree fruit pulps are strongly influenced by gender and cultivar.

This paper reports the antioxidant and the in vitro cytotoxic activities of fruit pulp extracts from six female cultivars (Mulata, Galhosa, Aida, Canela, Gasparinha, and Preta de Lagos) and two hermaphrodite trees.

A phytochemical analysis of the phenolic fraction of the extracts was made, and the effect on cancer cell viability was evaluated on a panel of human cancer cell lines (prostate: DU-145, breast: MDA-MB-231 and colon cell line: HCT-166).

Inside the pods there are seeds (5-7 mm), ovate, flat, smooth and hard.

The seeds represent 10% of the weight of the fruit.

Composition of carob seed.

<table>
<thead>
<tr>
<th>Part</th>
<th>Proportion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Husk</td>
<td>30–33</td>
</tr>
<tr>
<td>Endosperm</td>
<td>42–46</td>
</tr>
<tr>
<td>Germ</td>
<td>23–25</td>
</tr>
</tbody>
</table>

The seed embryo has a protein content of 50% and is used for human and animal feeding.
Carob pulp composition

- **Sugars (40-60%)**
  - Galactose (40-70% total sugars)
- **Fiber (7.7%)**
- **Protein (7%)**
- **Vitamins (A, B1, B2, D)**
- **Low fat (0.6%)**
- **Polyphenols (448 mg/kg)**
  - Derived from flavons-3,4-diol,ambondisol,flavon-3,4-diol
  - Condensed Tannins (53 mg/kg)
  - Myricetin (113 mg/kg)
  - Ellagitanin (9 mg/kg)
  - Quercetin (113 mg/kg)

**Minerals**
- K > P > Ca > Mg > Na
- K (2000 mg/kg), P (200 mg/kg), Ca (79 mg/kg), Mg (2.1 mg/kg), Na (0.45 mg/kg)
The phenolic compound found in the largest amount in carob pulp is gallic acid:

- Strong antioxidant;
- Displays antimutagenicity;
- Used in processed foods, cosmetics and food packaging to avoid rancidity induced by lipid peroxidation and deterioration.

Extracts were rich in phenolic compounds and exhibited strong antioxidant activity; the gender and cultivar significantly influenced the contents of phenolics and antioxidant activity.
Morphological alterations of HeLa cells following exposure to extracts of carob pulps.

- The extracts had a concentration- and time-dependent effect on cell viability, and, as expected from the antioxidant assays, there was a significant variation in bioactivity between genders.
- Hermaphrodite trees exhibited the strongest activity at concentrations below 10 mg/mL.
- Among females, cv. Gasparinha the strongest cytotoxic activity on HeLa cells.
- The decrease in cell viability was associated with apoptosis on HeLa and MDA-MB-231 lines (arrows indicate chromatin condensation and nuclear fragmentation).

(prostate: DU-145, breast: MDA-MB-231 and colon cell line: HCT-166)

Main conclusions:

- Extracts from fruit pulps exhibited strong antioxidant activity and were rich in phenolic compounds;
- The extracts decreased the viability of different human cancer cell lines;
- Gender and cultivar significantly influenced the chemical content and the biological activities;
- Extracts from hermaphrodite trees had a higher content of phenolic compounds, and exhibited higher antioxidant and cytotoxic activities;
- Cv. Aida had the highest radical scavenging activity and total content of phenolics, Mulata the highest capacity to inhibit lipid oxidation and Gasparinha the strongest cytotoxic activity on HeLa cells.
This study aimed to characterize the phytochemical profile and to determine the antioxidant and the in vitro cytotoxic properties of germ flour extracts from different female cultivars and hermaphrodite trees.

- Germ flour extracts were rich in phenolic compounds, had considerable antioxidant activity.
- The chemical content and the antioxidant activity of the extracts were significantly affected by gender and cultivar.
- Female cv. Galhosa had the highest levels of phenolic compounds, and the highest antioxidant activity.
Germ flour extracts from the female cultivars Galhosa and Costela/Canela and an hermaphrodite tree exhibited the highest cytotoxic activity.

Effect of treatment with germ flour extracts on HeLa cells viability after 24 h (a), 48 h (b) or 72 h (c) of incubation.

- Germ flour extracts were rich in phenolic compounds, had considerable antioxidant activity, and reduced the viability of cervical (HeLa) cancer cells;
- The chemical content and the biological activities of the extracts were significantly affected by gender and cultivar;
- Female cv. Galhosa had the highest levels of phenolic compounds, and the highest antioxidant activity;
- Extracts from the hermaphrodite trees and from the female cvs. Galhosa and Canela exhibited the highest cytotoxic activity.

Main conclusions:
This work reports the *in vitro* inhibitory activity of water decoctions of leaves, germ flour, pulp, locust bean gum and stem bark of carob tree on α-amylase, α-glucosidase, acetylcholinesterase and butyrylcholinesterase.

- **Leaves** and **stem bark** had the highest total phenolics content (TPC).
- **Pulps** were rich in tannins (TCT).
- The maximum levels of flavonoids (TFC) were observed in **leaves**.
- The **major compounds** were identified by HPLC analysis as **gallic acid** (GA) in the leaves and **gentisic acid** in the stem bark.
Leaves and stem bark decoctions had significant antioxidant activity.

Leaves and stem bark decoctions strongly inhibited all the enzyme tested.

---

Table 2. RSA on DPPH and ABTS radicals and FRAP of decoctions from leaves, stem bark, pulp, stem flour, and LBG of the cashew tree.

<table>
<thead>
<tr>
<th>Samples</th>
<th>RSA on DPPH*1</th>
<th>RSA on ABTS*2</th>
<th>FRAP (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>0.5 ± 0.2</td>
<td>28 ± 0.2</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Stem bark</td>
<td>49 ± 0.2</td>
<td>27 ± 1.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>Pulp</td>
<td>1.5 ± 0.1</td>
<td>15 ± 0.1</td>
<td>nd</td>
</tr>
<tr>
<td>Stem flour</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>LBG</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>BTT**</td>
<td>77 ± 2.5</td>
<td>50 ± 0.2</td>
<td>3.0 ± 0.0</td>
</tr>
</tbody>
</table>

Table 3. Inhibitory activity (%) of decoctions from leaves, stem bark, pulp, stem flour, and LBG of the cashew tree applied at the concentrations of 1 and 10 mg/mL on SCMS, RbCN, α-amylase and α-glucosidase.

<table>
<thead>
<tr>
<th>Samples</th>
<th>SCMS 1 mg/mL</th>
<th>SCMS 10 mg/mL</th>
<th>RbCN 1 mg/mL</th>
<th>RbCN 10 mg/mL</th>
<th>α-Amylase 1 mg/mL</th>
<th>α-Amylase 10 mg/mL</th>
<th>α-Glucosidase 1 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>73 ± 1</td>
<td>80 ± 6</td>
<td>68 ± 1</td>
<td>81 ± 6</td>
<td>74 ± 8</td>
<td>96 ± 10</td>
<td>99 ± 10</td>
</tr>
<tr>
<td>Stem bark</td>
<td>52 ± 7</td>
<td>87 ± 9</td>
<td>80 ± 4</td>
<td>88 ± 9</td>
<td>77 ± 7</td>
<td>92 ± 10</td>
<td>99 ± 5</td>
</tr>
<tr>
<td>Pulp</td>
<td>27 ± 9</td>
<td>87 ± 9</td>
<td>88 ± 9</td>
<td>99 ± 7</td>
<td>65 ± 10</td>
<td>66 ± 10</td>
<td>67 ± 10</td>
</tr>
<tr>
<td>Stem flour</td>
<td>15 ± 2</td>
<td>21 ± 2</td>
<td>18 ± 2</td>
<td>24 ± 2</td>
<td>21 ± 2</td>
<td>22 ± 2</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>LBG</td>
<td>94 ± 2</td>
<td>83 ± 3</td>
<td>81 ± 3</td>
<td>83 ± 3</td>
<td>nd</td>
<td>nd</td>
<td>93 ± 10</td>
</tr>
</tbody>
</table>

Note: Values are presented as mean ± SEM (n = 3). For each enzyme, statistical analysis was made between plant tissues by Duncan’s new multiple range test (95% confidence interval) and differences were considered significant at P < 0.05.

---

*Significant differences between the plant tissues by Duncan’s new multiple range test (95% confidence interval).
Main conclusions:

- Leaves and stem bark decoctions had significant antioxidant activity and the highest total phenolics content;
- The major compounds were identified as gallic acid in the leaves and gentisic acid in the stem bark;
- Leaves and stem bark decoctions strongly inhibited all the enzymes tested;
- These results suggest a scientific support for the traditional uses of the carob tree as antidiabetic and sedative.

Summary:

- Extracts from leaves, fruit pulps and germ flour have high levels of phenolics, antioxidant activity and in vitro inhibition of tumor cells growth;
- Extracts from hermaphrodite trees had a higher content of phenolic compounds, and exhibited higher antioxidant and cytotoxic activities;
- Decoctions made from the leaves and stem bark strongly inhibited the α-amylase, α-glucosidase, AChE and BuCHE, had significant antioxidant activity and the highest total phenolics content;
- The phenolic content was correlated to both antioxidant and cytotoxic activities;
- Gender and cultivar significantly influenced the chemical composition and the biological activities of the extracts;
- Advantages of hermaphrodite trees over female cultivars as a source of compounds with biological interest, which may represent an increase of their agronomic interest.
Micropropagation of the Mediterranean tree *Ceratonia siliqua*

A. Romano1,*, S. Barros1 & M.A. Martins-Loução2

An *in vitro* propagation protocol based on axillary bud proliferation has been developed for mature female trees of *Ceratonia siliqua* L. ‘Galhosa’ and ‘Mulata’.

Shoot culture initiation was greatly influenced by explanting season, with the highest survival percentage observed in spring.

The cultivar, cytokinin type and concentration were the most important factors affecting shoot multiplication.

Rooting was achieved on growth-regulator-free medium after basal dipping of shoots in indole-3-butyric acid (4.9 mM).

Plantlets were successfully acclimatized (80-85%) under high relative humidity and then moved to the glasshouse. A field trial was established to follow their agronomic behaviour.
Micropropagation of Carob tree


1. In vitro initiation
2. Proliferation
3. Rooting
4. Acclimatization
5. Field reintroduction
After 2 years

After 7 years

Carob trees (*Ceratonia siliqua* L.) regenerated in vitro can acclimatize successfully to match the field performance of seed-derived plants.

The use of in vitro regenerated plants in forestry and orchard depends ultimately on the development of efficient transplantation protocols, ensuring high survival rates and successful establishment under field conditions.

Here we describe in detail how micropropagated carob trees respond to new environmental conditions encountered during the acclimatization process in terms of survival, growth, gas exchange rates, and physiological traits, and we compare their behavior in the field to conventionally propagated seed-derived and mother plants.

We discuss whether our micropropagation technique is suitable for largescale propagation as an alternative to traditional methods.
The mean number of new leaves differentiated on main shoot per micropropagated plant increased gradually during acclimation until 12 months after field transplantation.

After 12 months, both micropropagated and seed derived plants had yielded a considerable number of new axillary shoots.

Plant height of both micropropagated and seed-derived plants was similar following 12 months in field.
The photosynthetic performance of carob tree leaves is flexible throughout acclimatization in the face of a rapidly changing environment.

This flexibility enables the species to cope with the environmental conditions after transfer to the field without a noticeable decline in photosynthetic competence and growth.

Photosynthetic rate (PN); stomatal conductance (gs) and intrinsic water use efficiency (WUE) in leaves of micropropagated plants in vitro, during acclimatization and 1 year after field establishment, in comparison with seed-derived and mother plants.

The effects of drought stress under high temperature were investigated (variations of photosystem II activity, energy partitioning, photosynthetic pigments and lipid peroxidation).

*C. siliqua* revealed a great capability for photosynthetic recovery 36 h after rewatering, which suggests that the species can cope with predicted climate changes.

Two thermal regimes (LT: 25/18°C; HT: 32/21°C) and three soil water conditions (control, water stress, and rewetting) were considered.
The field trial was 100% successful.

We found no major differences between micropropagated, seed-derived and mother plants in terms of growth rate, height, number of leaves, photosynthetic efficiency, chlorophyll fluorescence, chlorophyll content and soluble protein content.

These micropropagation and acclimatization protocols can provide a suitable alternative to traditional mass propagation techniques for the carob tree.

Main conclusions: