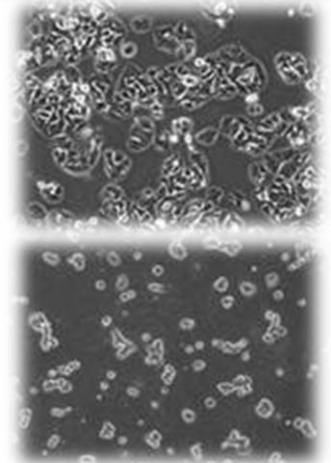
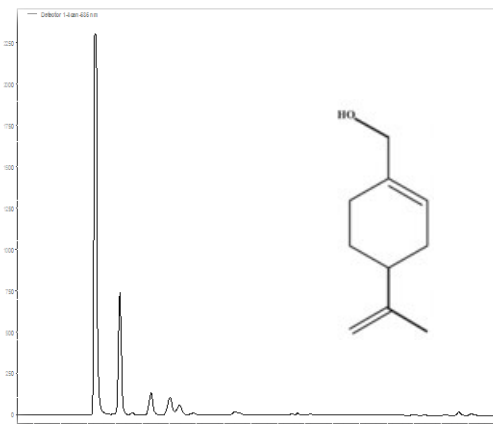


Evaluation of antiproliferative effect and cell cycle arrest on HT29 cells of different plant-derived natural extracts.

Correlation of response with composition



12TH INTERNATIONAL CONFERENCE

FUNCTIONAL FOOD INGREDIENTS AND

NUTRACEUTICALS IN CHRONIC

DISEASE: SCIENCE AND PRACTICE

NOVEMBER 29-DECEMBER 2

DALLAS, TX, USA



Teresa Serra, PhD

Researcher

Nutraceuticals & Controlled Delivery

Unit - IBET/ITQB-UNL

e-mail: tserra@ibet.pt



Cancer

- **In 2008:** 7.5 million people died from cancer worldwide
- **In 2020:** it is estimated that mortality from cancer will increase to more than 10 million

Colorectal cancer is the second most fatal and the third most diagnosed type of cancer worldwide

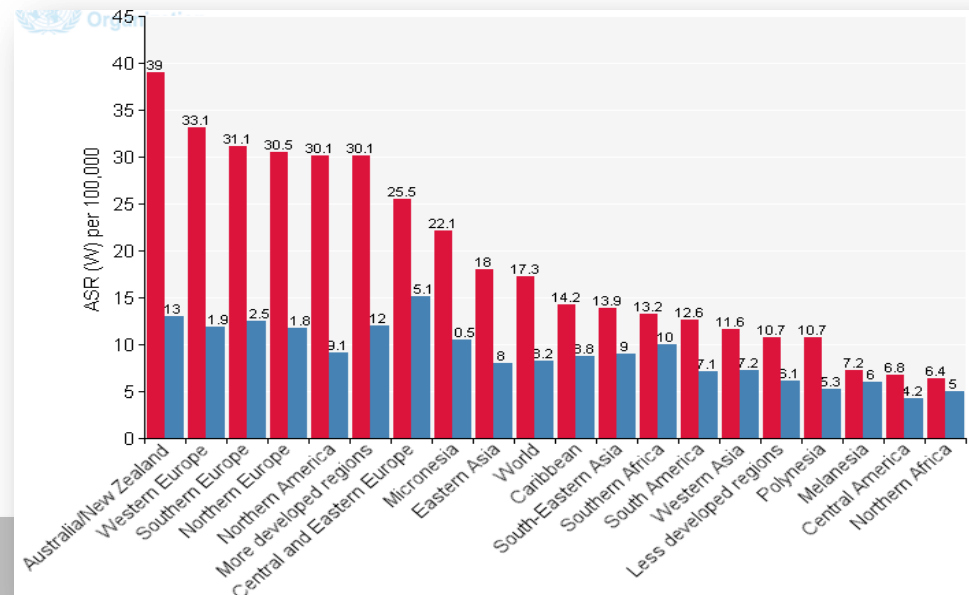
Emerging interest in
chemotherapeutic application of
natural substances



From 1981 to 2002...

Almost 74% of all drugs approved for cancer therapy were:

- natural products
- based on natural products or
- mimicked natural products in one form or another



❑ Evaluation of the antiproliferative effect and cell cycle modulation of several plant derived natural extracts in human colorectal cancer cells

❑ Correlation of the bioactive effect with phytochemical composition of samples



Plant material

SWEET CHERRIES



- **Crop residues**

- **Cherry culls**

(fruit that is not suitable to sell for eating due to small size and weight)

OLIVES



- **Olive oil by-products**

(olive mill semi solid residue)

- **Leaves**

CACTUS PEAR



- **Juice production residues** (peels and seeds)

CORK



- **Cork industry by-products**

Extraction process



High pressure
and
supercritical
fluid
technology



Membrane
Filtration

**Green sustainable
processes**

Reduces or eliminates the
use or generation of
hazardous substances

Conventional
Extraction
(with
biocompatible
solvents)

Adsorption
process (with
macroporous
resins)

Bioactive rich fractions
(e.g. polyphenols, carotenoids,
phytosterols, terpenes,...)



Phytochemical characterization of natural extracts

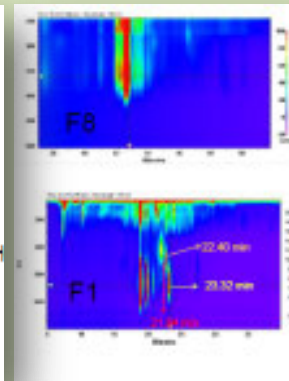
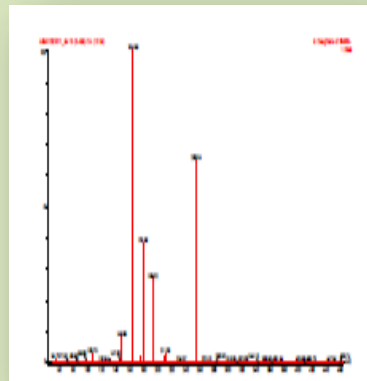
Spectrophotometric assays

- Total polyphenols content
- Total flavonoids content
- Total phenolic acids content
- Total procyanidin content
- Total anthocyanin content
- Total betalain content
- Analysis of carotenoids



Chromatographic techniques

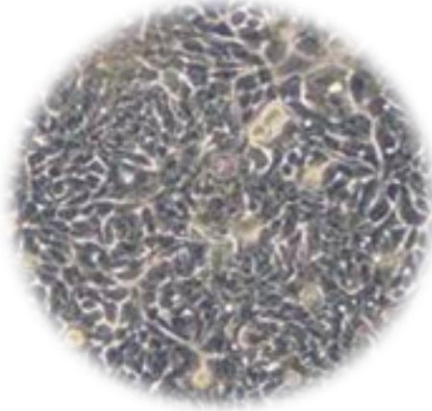
- TLC
- UPLC
- HPLC-DAD-FD-ED
- HPLC-MS/MS
- GC-MS



Anticancer activity evaluation

HT29 cells

(Human colon adenocarcinoma)



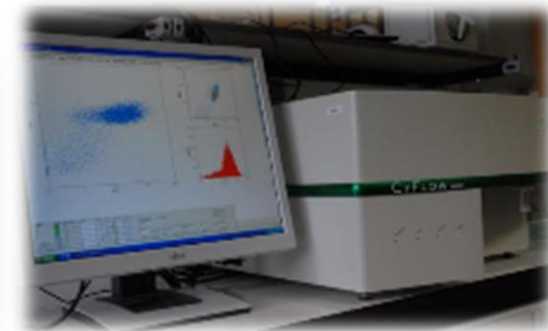
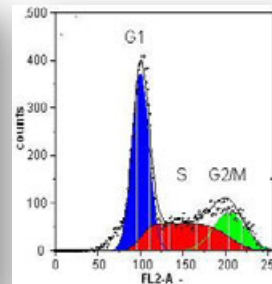
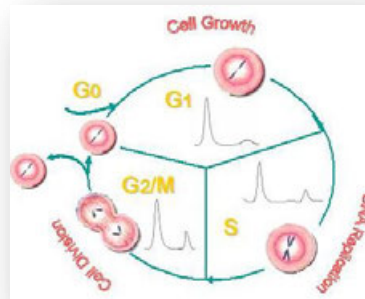
ANTIPROLIFERATIVE EFFECT

- Dose-response curves
- Effective dose values (ED50)



MTT and MTS assays

CELL CYCLE ARREST by Flow Cytometry



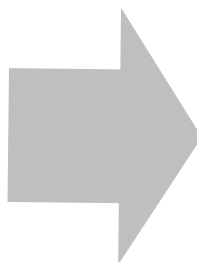
SWEET CHERRIES NATURAL EXTRACTS



Saco cherry culls



Perillyl alcohol



High Pressure Extraction with CO₂ and ethanol



- ✓ Higher selectivity
- ✓ Short extraction times
- ✓ GRAS solvents
- ✓ Supercritical CO₂ has been shown to be efficient in the extraction of perillyl alcohol from natural sources

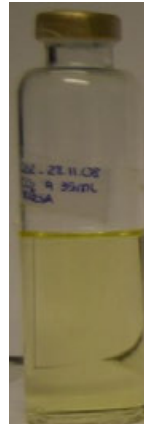
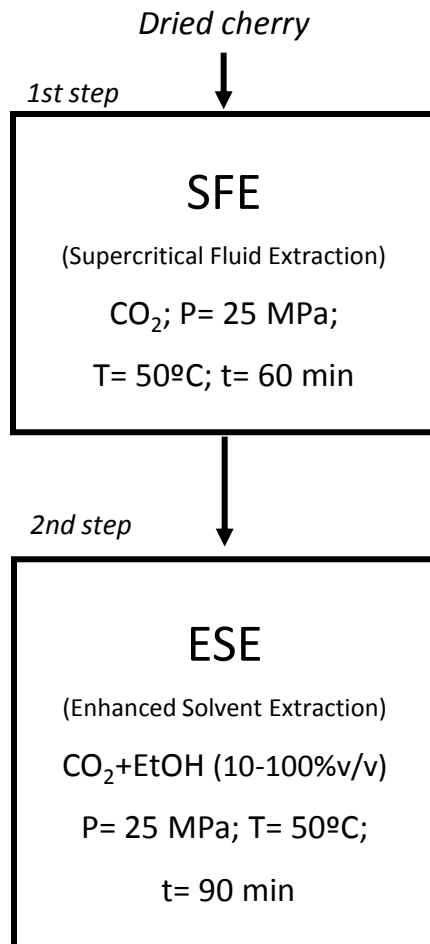
Chemoprevention of Colon Carcinogenesis by Dietary Perillyl Alcohol
Reddy et al., 1997

Chemotherapy of pancreatic cancer with the monoterpene perillyl alcohol
Stark et al., 1995

Perillyl Alcohol Inhibits Human Breast Cancer Cell Growth in vitro and in vivo
Yuri et al., 2004

Effects of Perillyl alcohol on Melanoma in the Tpras Mouse Model
Lluria-Prevatt et al., 2002

SWEET CHERRIES NATURAL EXTRACTS

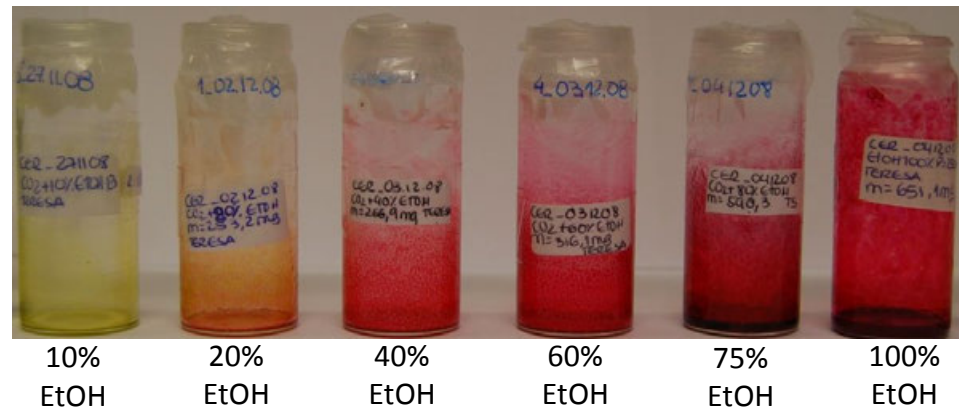


Methodology:

Fractionated High Pressure Extraction



To remove unwanted compounds

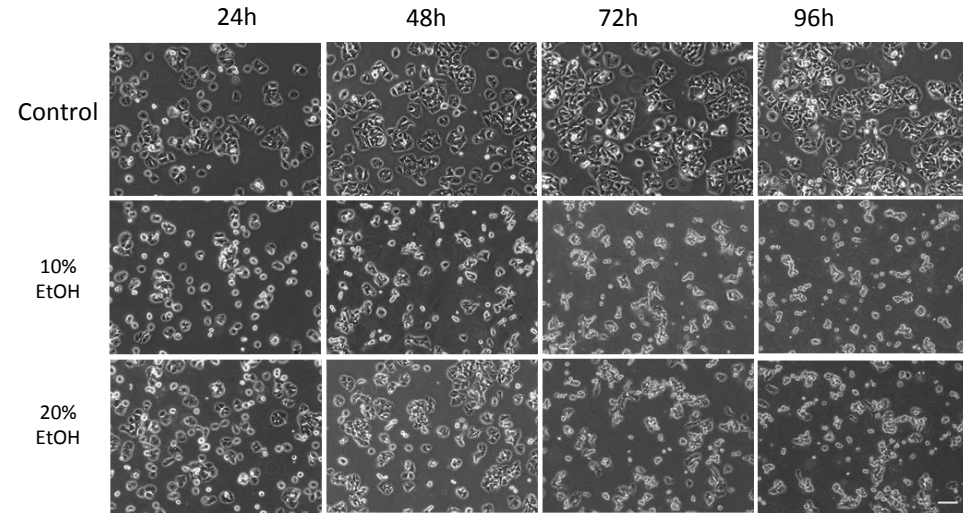
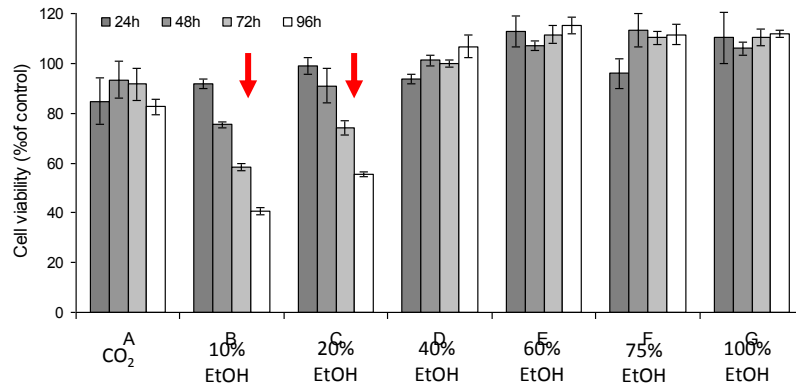


SWEET CHERRIES NATURAL EXTRACTS

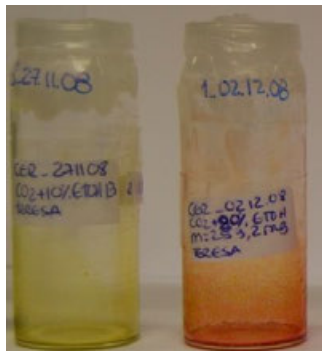


• Antiproliferative effect

Tested concentration: 0.5mg/mL



100µm



✓ Only two extracts (**10%EtOH** and **20%EtOH**) inhibited cancer cell growth in a time dependent manner and this effect is related with the presence of Perillyl Alcohol

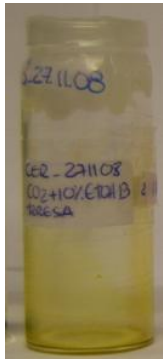
✓ The extract obtained with 10% EtOH exhibited the highest antiproliferative effect (ED₅₀=0.20 mg/mL) being 150 times more effective than fresh fruit

SWEET CHERRIES NATURAL EXTRACTS

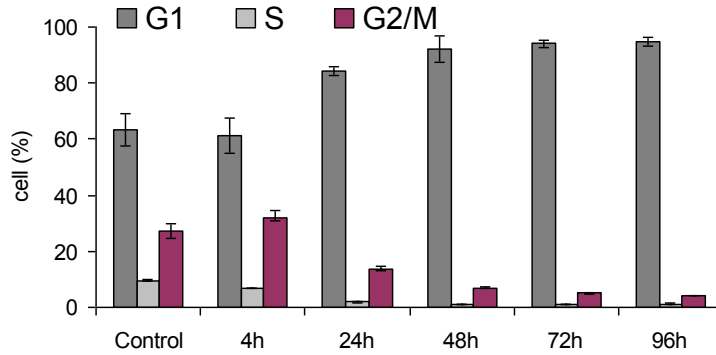


• Induction of cell cycle arrest

10%EtOH

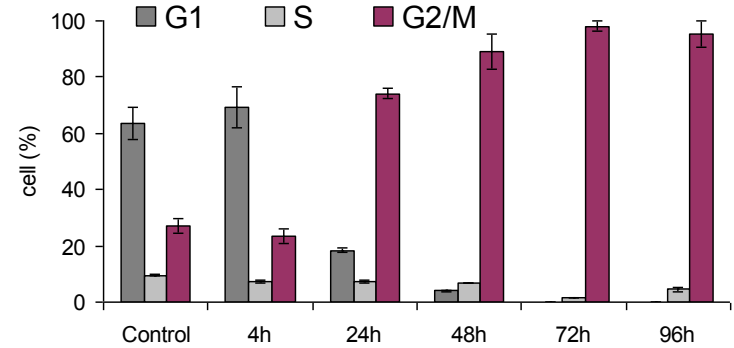


Cherry extracts (0.5mg/mL)



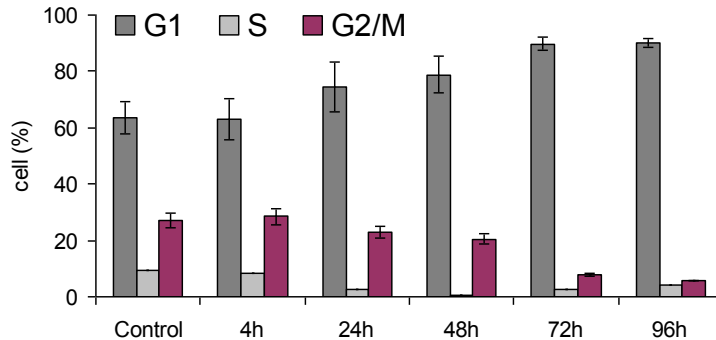
Cell cycle arrest in **G1**

Doxorubicin (125nM)



Cell cycle arrest in **G2/M**

20%EtOH



✓ Cherry extracts induced cell cycle arrest in a different cell cycle checkpoint than doxorubicin suggesting that they can be used in combination with the drug in chemotherapy

Serra et al. (2011), *J Superfluids*, 55, 1007-1013

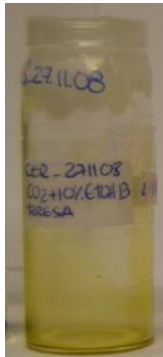
some studies showed that potent inhibition of tumour survival is achieved when combining drugs with different cell cycle checkpoints

SWEET CHERRIES NATURAL EXTRACTS

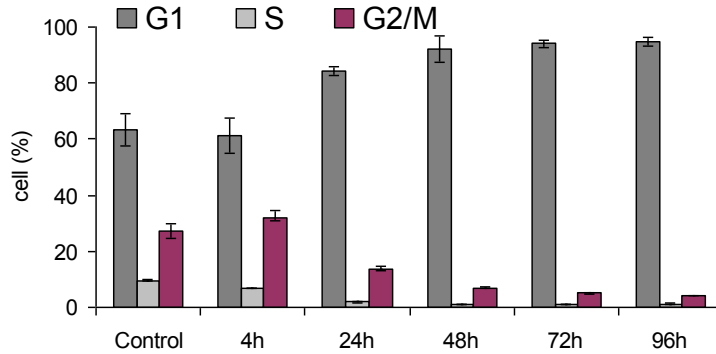


• Induction of cell cycle arrest

10%EtOH

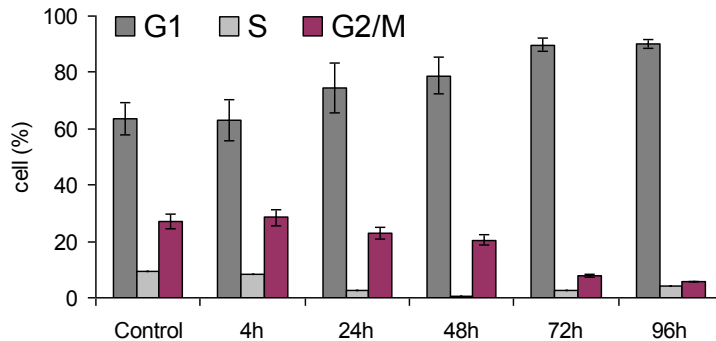


Cherry extracts (0.5mg/mL)

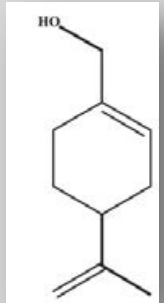
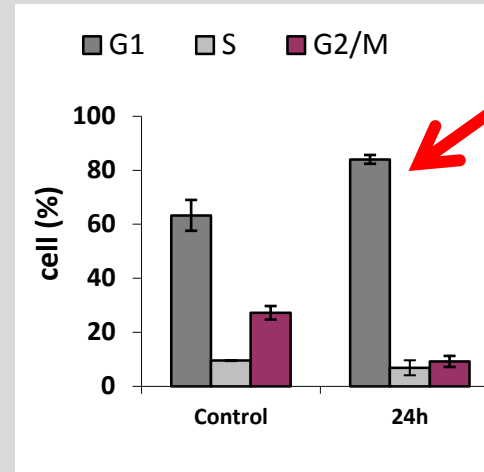


Cell cycle arrest in **G1**

20%EtOH



Perillyl alcohol (130 µg/mL)



Cell cycle arrest in **G1**

✓ Cell cycle modulation of cherry extracts was related with the presence of perillyl alcohol

OLIVE - BASED NATURAL EXTRACTS

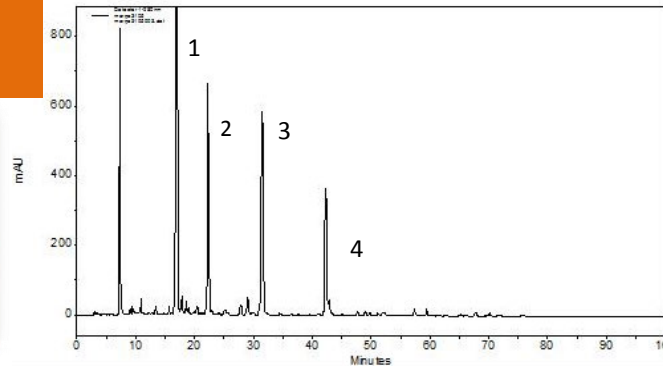


Membrane separation process

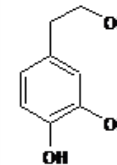
EP1910257, 2008.

Olive oil by-products
(olive mill semi solid residue)

HT rich extract



1- Hydroxytyrosol



C=220mg/g extract (dw)

2- Tyrosol

3- Caffeic acid

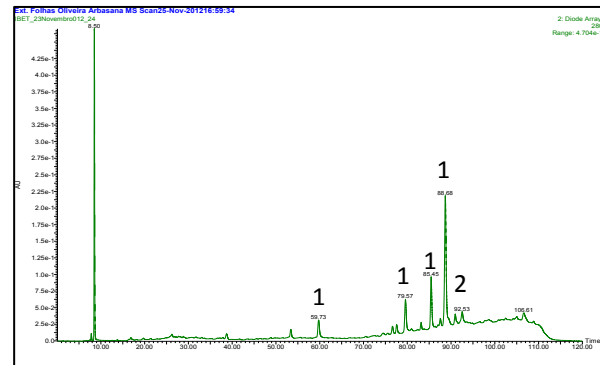
4- p-coumaric acid



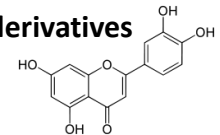
Conventional solvent extractor

Dried leaves

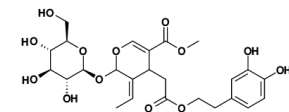
LT+OL rich extract



1- Luteolin derivatives



2- Oleuropein



✓ Olive natural extract derived from olive oil by-products is rich hydroxytyrosol – it contained 22% w/w of HT

Matias, AA (2009), PhD thesis

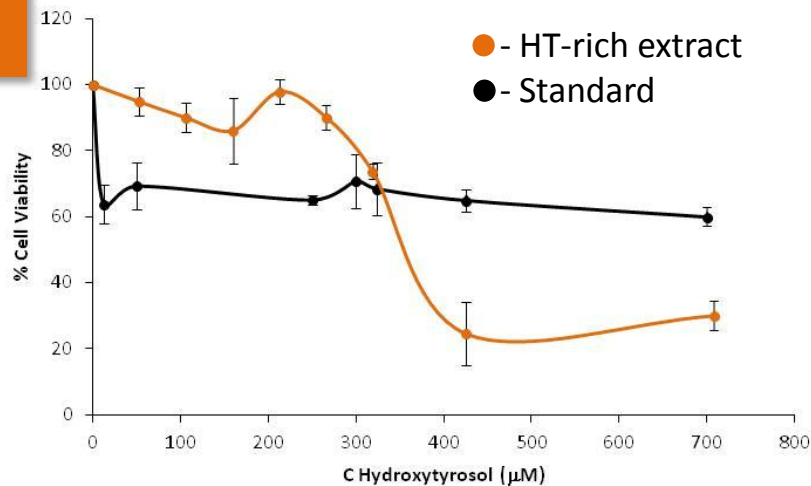
✓ Olive natural extract derived from dried leaves contains mainly luteolin derivatives and oleuropein

OLIVE - BASED NATURAL EXTRACTS



• Antiproliferative effect

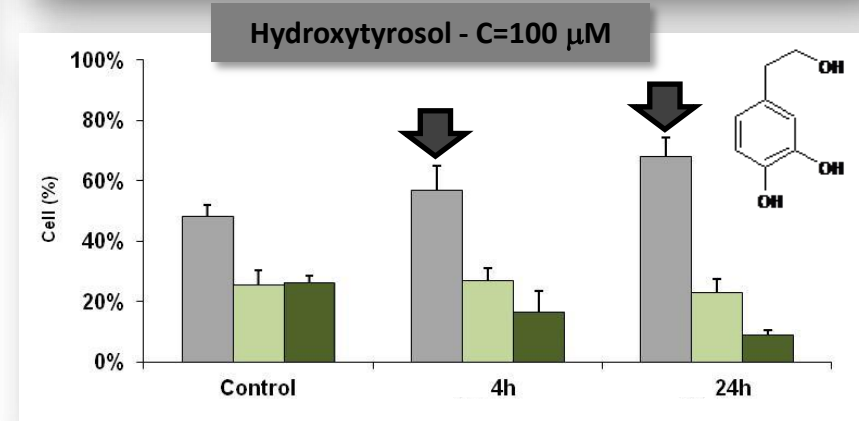
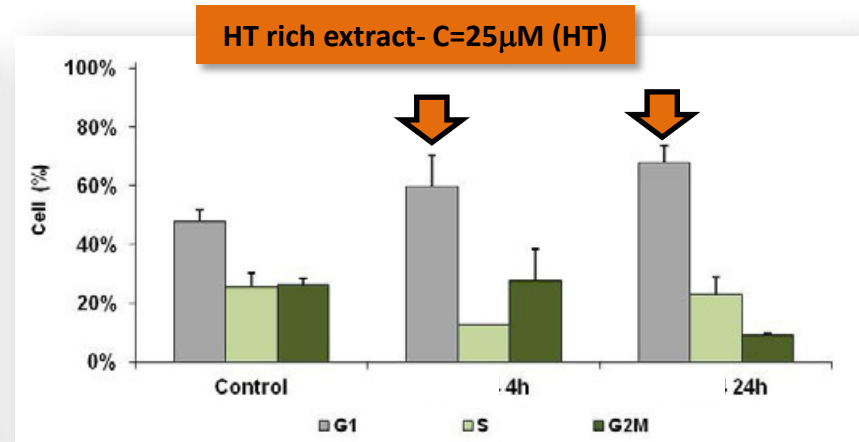
HT rich extract



Note: Cells were incubated with extract/compound for 4h and cell proliferation was evaluated after 24h

✓Olive HT-rich extract was more effective in inhibiting cancer cell growth and inducing cell cycle arrest in the G1 phase than standard compound

• Cell cycle arrest

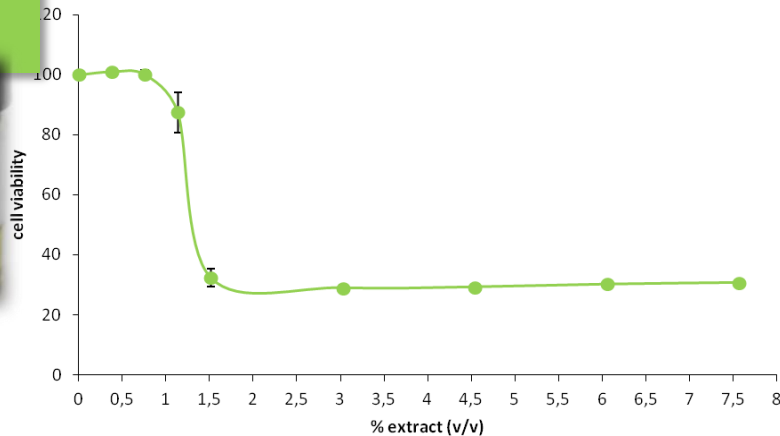


OLIVE - BASED NATURAL EXTRACTS



• Antiproliferative effect

LT+OL
rich
extract

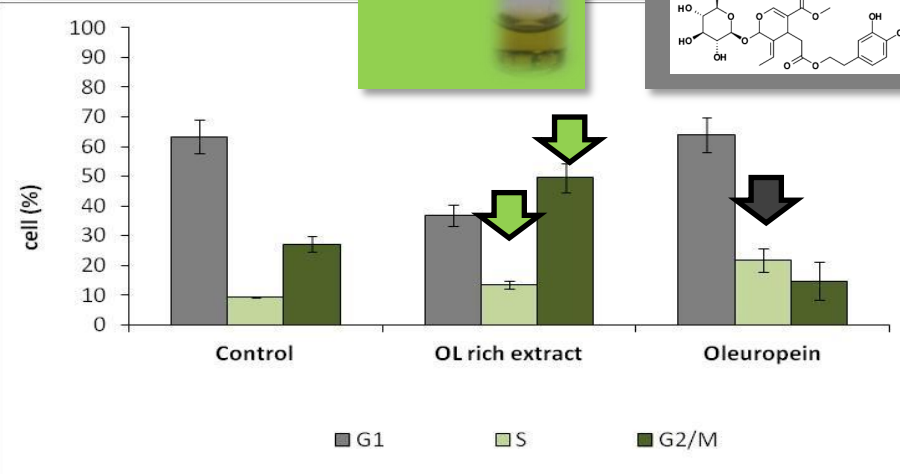
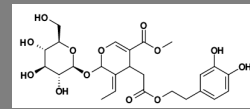


• Cell cycle arrest

LT+OL
rich
extract



Oleuropein
C=50 μ M



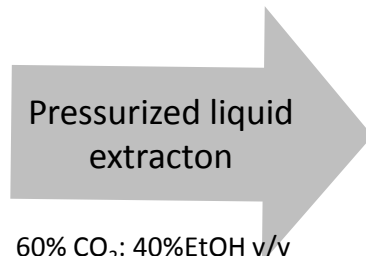
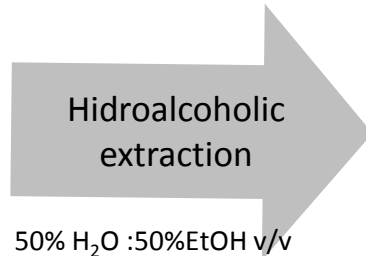
Note: Cells were incubated with extract/compound for 24h

- ✓ LT+OL rich extract induced cell cycle arrest in both S and G2 phases
- ✓ Oleuropein induced cell cycle arrest of HT29 cells in S phase
- ✓ Luteolin was shown to induce cell cycle arrest in G2/M phase (*Lim et al., 2006*)

OPUNTIA - BASED NATURAL EXTRACTS



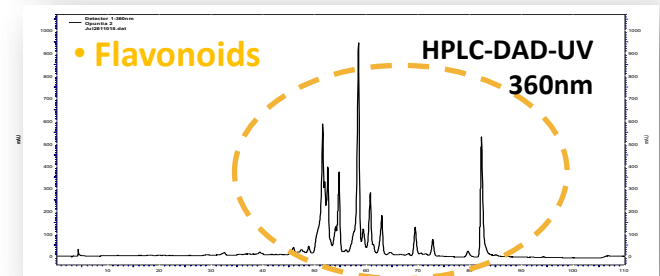
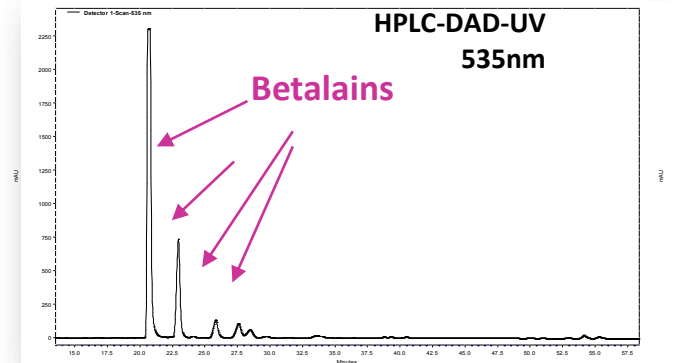
Opuntia rubusta
fruit juice residues
(seeds and peels)



Opuntia ficus indica
fruit juice residues
(seeds and peels)

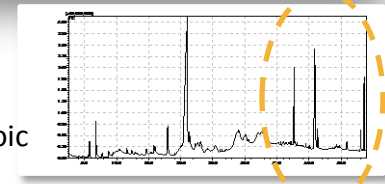


Phytochemical composition



• Fatty acids

- palmitic acid
- octadecadienoic acid



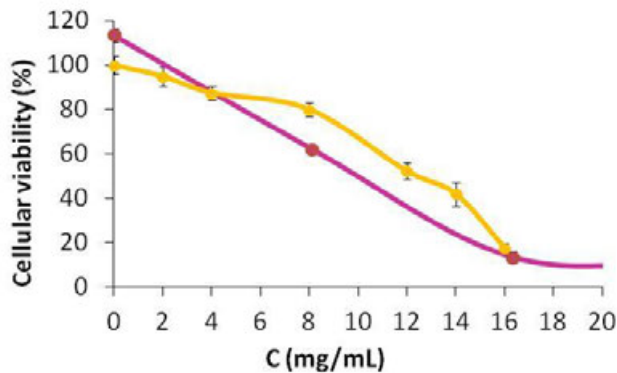
✓ *Opuntia rubusta* extract contains betalains pigments whereas *Opuntia ficus indica* extract present flavonoids (isorhamnetin and quercetin glycosides), terpenes and fatty acids (palmitic acid, octadecadienoic acid)

OPUNTIA - BASED NATURAL EXTRACTS

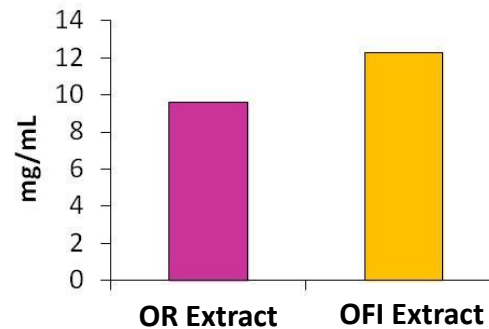


• Antiproliferative effect

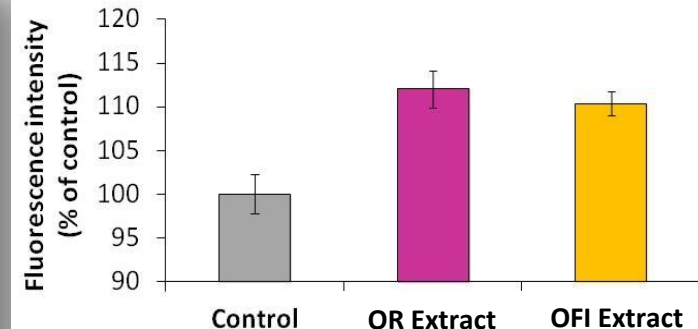
Dose response curves



ED50 values





ROS generation



Note: Incubation time- 24 hours

Note: Extracts concentration- 0.5mg/mL

 - *Opuntia rubusta* extract

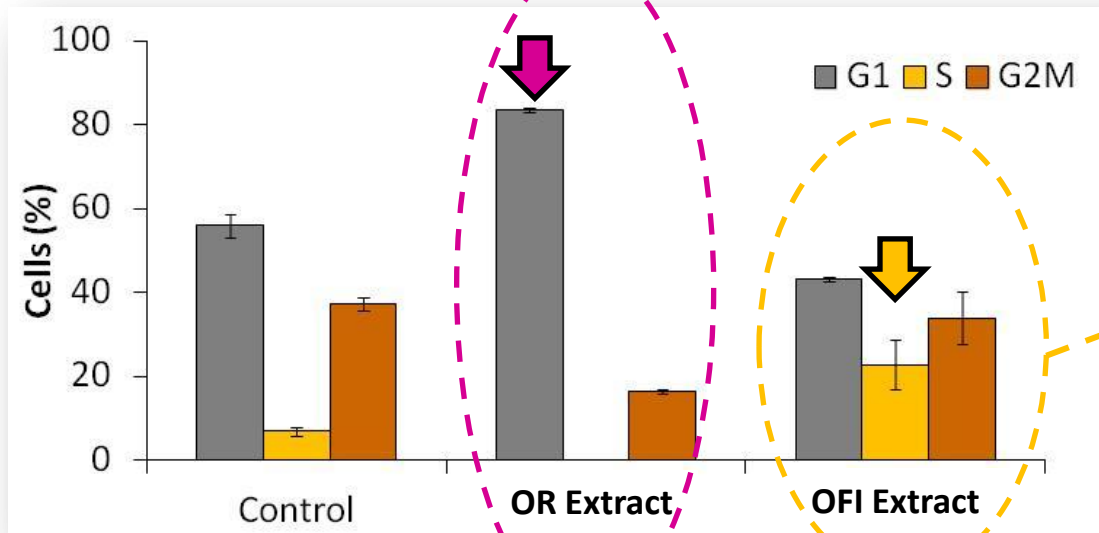
 - *Opuntia ficus indica* extract

- ✓ Both *Opuntia* extracts inhibited HT29 cell growth in a dose dependent manner.
- ✓ *Opuntia rubusta* extract was the most effective in inhibiting HT29 cell growth – lowest ED50 value.
- ✓ The antiproliferative effect of *Opuntia* extracts is probably related with the ROS generation at a cellular level

OPUNTIA - BASED NATURAL EXTRACTS



• Cell cycle arrest



Note: Incubation time- 24 hours; extracts concentration-ED50 value

Effect of betalains on cell cycle arrest

- **Betanin**– G0/G1 phase
(myeloid leukemia cell line- K562)

(Sreekanth et al., 2007)

Effect of phytochemicals on cell cycle arrest

- **Isorhamnetin**– G2 and S phase
(colon cell line, HCT-116) (Jaramillo et al., 2010)
- **Quercetin**–S phase
(colon cell line, SW480) (Richter et al., 1999)

✓ Opuntia extracts exhibited different responses on cell cycle arrest, which could be related with the distinct composition of samples:

- *Opuntia robusta* extract induced cell cycle arrest into G1
- *Opuntia ficus indica* extract induced cell cycle arrest in S phase

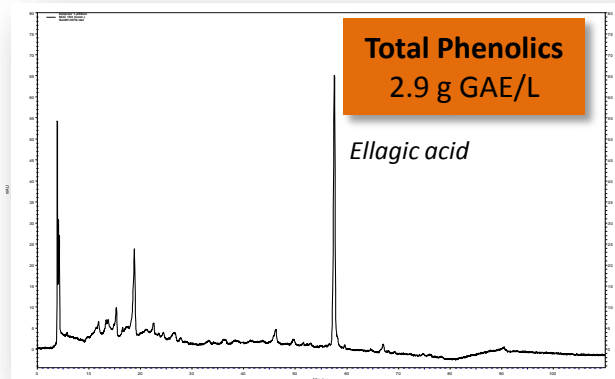
CORK - BASED NATURAL EXTRACTS

natural extracts provided by Corticeira AMORIM, S.G.P.S., S.A
(NutraCork project, QREN)

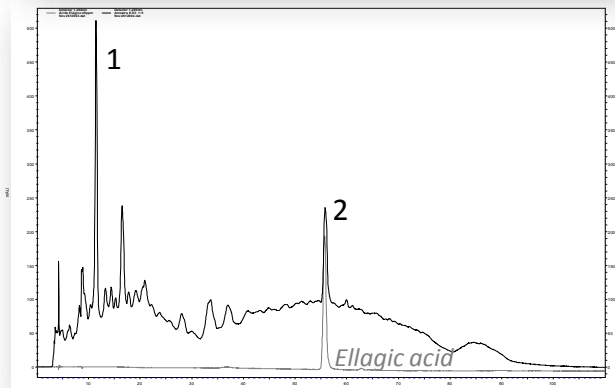
Phytochemical composition



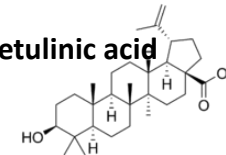
CORK E1



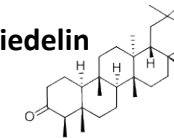
CORK E2



Betulinic acid

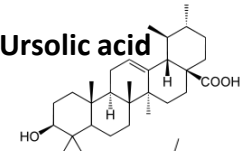


Friedelin

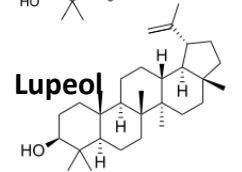


Triterpenes

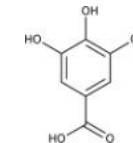
Ursolic acid



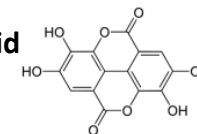
Lupeol



1- Gallic acid



2- Ellagic acid



- Ellagitanins

Total Phenolics
116 mg GAE/g

✓ Cork extracts are rich phytochemicals

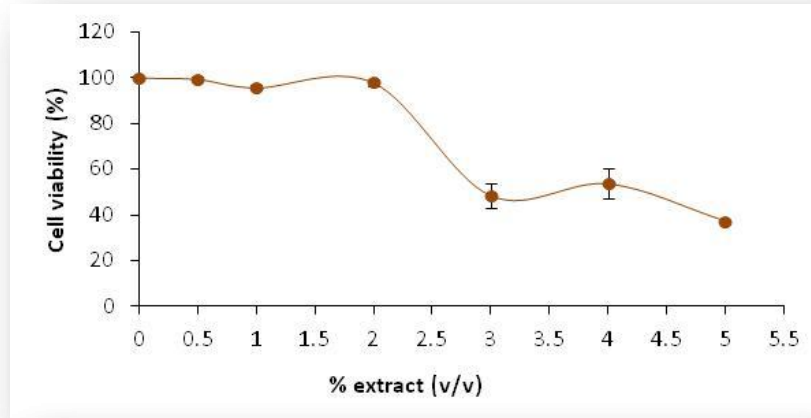
- Cork E1- polyphenols and triterpenes
- Cork E2 – polyphenols (phenolic acids and ellagitanins)

CORK - BASED NATURAL EXTRACTS

- Antiproliferative effect

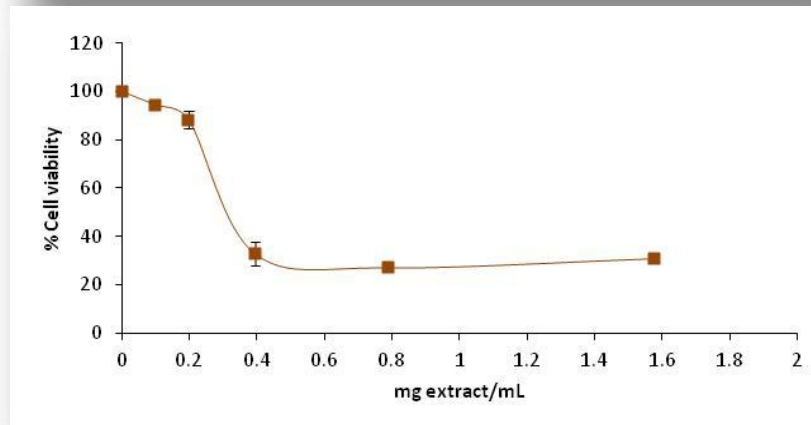


CORK E1



ED50 = 3 mL extract/mL

CORK E2



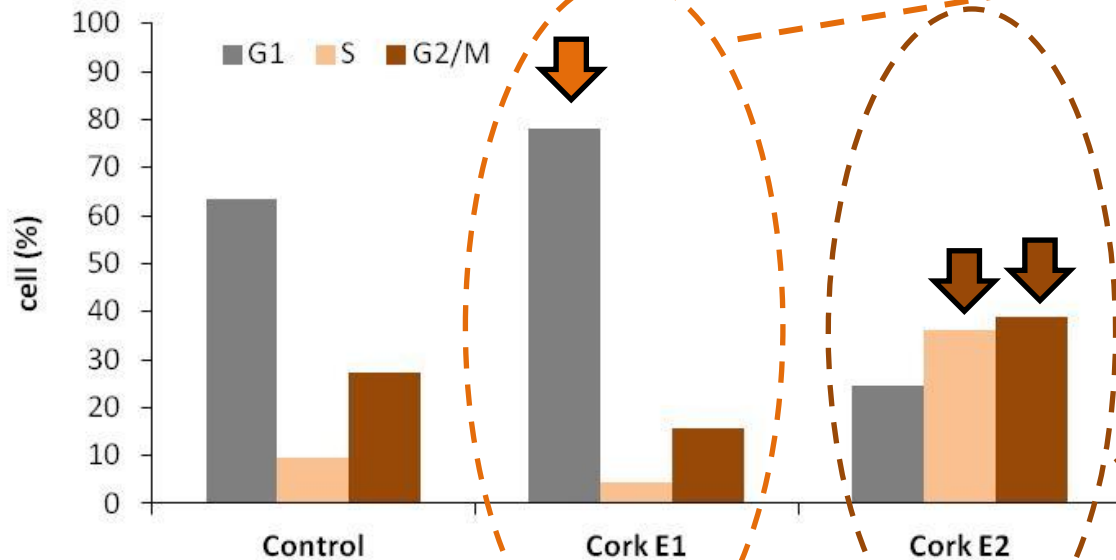
ED50 = 0.25 mg extract/mL

Note: Incubation time- 24 hours

✓ Both cork extracts inhibited cancer cell growth in a dose dependent manner

CORK - BASED NATURAL EXTRACTS

• Cell cycle arrest



Effect of triterpenes on cell cycle arrest

- Betulinic acid – G1 phase
- Ursolic acid- G1 phase

Preliminary experiments in HT29

Effect of phenolics on cell cycle arrest

- Ellagic acid– S phase (HT29 cells)
- Gallic acid- S phase (HT29 cells)
- Ellagitanins- G0/G1 and G2/M phases (HT29 cells)

Kasimsetty et al.(2010)

Khan SA (2009)

Note: Incubation time- 24 hours; C Cork E1=2.4%; C Cork E2= 0.22mg/mL


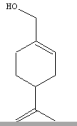



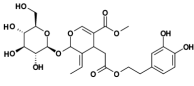
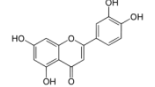

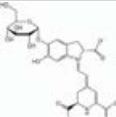

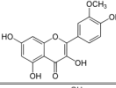
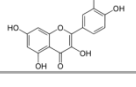

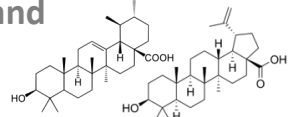
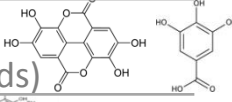


✓ Cork extracts exhibited different responses on cell cycle modulation, which could be related with the distinct composition of samples:

- Cork E1 extract induced cell cycle arrest into G1
- Cork E2 extract induced cell cycle arrest in S and G2/M phases

- ✓ This study demonstrates that cherry, cactus pear, olive and cork-based natural extracts inhibit the growth of HT29 through cell cycle modulation.



Conclusions

Natural extracts	Induction of cell cycle arrest	Induction of cell cycle arrest	Compounds
 CHERRIES POH-RICH EXTRACT	G1	G1	Perillyl alcohol (monoterpene) 
 OLIVE HT-RICH EXTRACT	G1	G1	Hydroxytyrosol (phenolic acid) 
 OLIVE LT-OL-RICH EXTRACT	S and G2/M	S	Oleuropein (tyrosol esters) 
		G2/M	Luteolin (flavonoid) 
 OPUNTIA RUBUSTA EXTRACT	G1	G1 ?	Betanin (betaleins) 
 OPUNTIA FICUS EXTRACT	S	S and G2/M ?	Isorhamnetin (flavonoid) 
		S ?	Quercetin (flavonoid) 
		?
 CORK EXTRACT 1	G1	G1	Betulinic acid and Ursolic acid (triterpenes) 
		S	Ellagic and gallic acids (phenolic acids) 
 CORK EXTRACT 2	S and G2/M	G0/G1 and G2/M	Ellagitannins 

- ✓ This study demonstrates that cherry, cactus pear, olive and cork-based natural extracts inhibit the growth of HT29 through cell cycle modulation.
- ✓ The natural extracts used in this study have the potential to improve the research and development of new chemotherapeutic agents for colon cancer.

Future work

- Evaluation of other natural extracts and ingredients
- Evaluation of more anticancer effects (induction of apoptosis, 3D cell-based assays, ...)



Nutraceuticals and Controlled Delivery Lab IBET/ITQB-UNL

Group Leader: Dr. Catarina Duarte

Joana Poejo, Inês Silva

Dr. Ana Matias

Analytical Chemistry Lab ITQB/IBET

Dr. Rosário Bronze



CORTICEIRA AMORIM, S.G.P.S., S.A. (Portugal)

Dr. Susana P. Silva, Patrícia Correia

Polymer Processing and Supercritical Technology Lab FCT-UC

Dr. Herminio de Sousa

Dr. Mara Braga, Dr. Inês Seabra

Financial Support:

- FEDEP/COMPETE -QREN project “NUTRACork” (ref. 21597/2011)
- Fundação para a Ciência e Tecnologia (FCT)
 - PTDC/AGR-AAM/099645/2008 project
 - PEst-OE/EQB/LA0004/2011 grant
 - REDE/1518/REM/2005

IBET – Instituto de Biologia Experimental e Tecnológica



Oeiras, Portugal

iBET is a private not for profit research organization in the area of biotechnology and life sciences.

IBET – Instituto de Biologia Experimental e Tecnológica

- The largest Biotechnology Research Organisation in Portugal
- Brings together as partners and collaborators, public institutions and private companies

Main Activities

- Health – Pharma
- Agro – Industry
 - Plants – Forestry
- Environment

Mission

- Foster the competitiveness of its customers and partners
- Create wealth out of knowledge of Chemistry, Biochemistry and Biology

IBET – Instituto de Biología Experimental e Tecnológica

Agro – Industry

- Nutraceuticals and Controlled Delivery Lab
- Analytical Chemistry Lab

Identification and quantification of several classes of compounds

- Nutraceuticals and Controlled Delivery Lab

Development of adequate and efficient systems to :

- Increase Shelf life and stability of the bioactive
- Increase solubility
- Increase bioavailability
-

Processing

Development of alternative green technologies for the isolation of natural of bioactive compounds with high added-value and application in food, cosmetic and pharmaceutical industries

- Nutraceuticals and Controlled Delivery Lab
- Membrane Processes Lab

Chemical characterization

Biological characterization

Development and application of cell-based assays

- Nutraceuticals and Controlled Delivery Lab
- Animal Cell Technology Lab

Formulation

Thank you for your attention

12TH INTERNATIONAL CONFERENCE

**FUNCTIONAL FOOD INGREDIENTS AND
NUTRACEUTICALS IN CHRONIC
DISEASE: SCIENCE AND PRACTICE**

**NOVEMBER 29-DECEMBER 2
DALLAS, TX, USA**



<http://www.itqb.unl.pt/labs/nutraceuticals-and-delivery>

e-mail: tserra@ibet.pt