Anti-Inflammatory and Anti-Oxidative enhancement by shikimic acid and dehydroshikimic acid

Dr. T.G. Sambandan
Abaya Sundaresan
Dr. Ravigadevi Sambanthamurthi,
Dr YewAi Tan, Dr Kalyana Sundram
Prof. ChoKyun Rha & Prof. Anthony J. Sinskey
Biomaterials Science and Engineering Lab
Massachusetts Institute of Technology
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Outline

1. Introduction: Characterization of Palm Fruit Bioactives (PFB) / OPP

1. Shikimic acid shows no antioxidant activity in chemical methods (ORAC/TEAC)

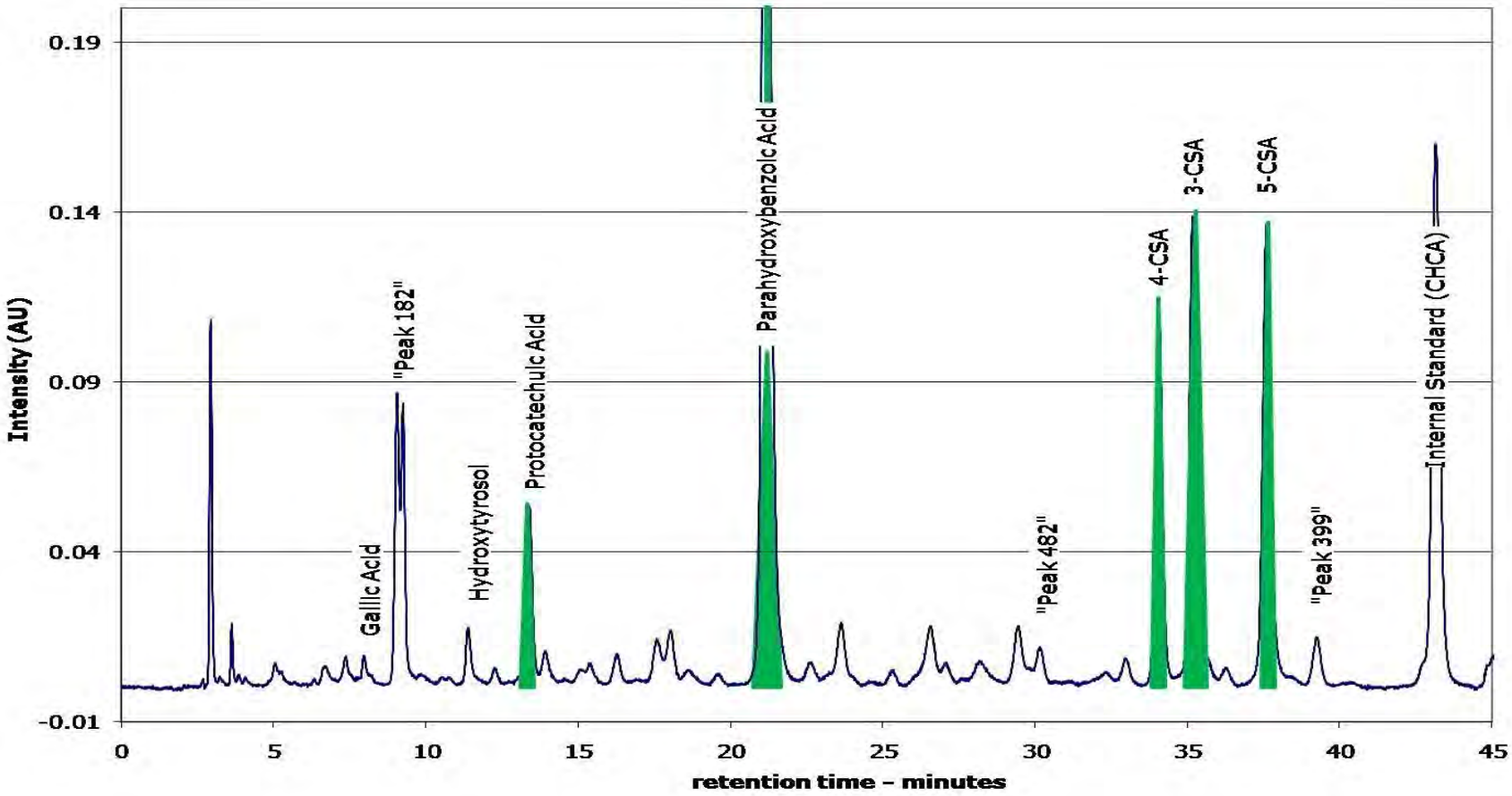
1. Determination of Antioxidant Chemically in chemical environment and in Biological systems

1. Synergy, concerted or other mechanism

1. Biological activity enhancement

1. Conclusions

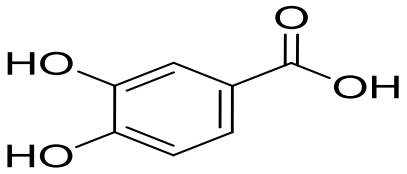
Polyphenol profile in OPP



Column SGE C18
Mobile phase gradient (a) 0.2%% phosphoric acid 10mm Sodium sulfate
(b) 70 /30 V/V methanol and acetonitrile
Detection at 280nM
Flow rate 08 ml /min.

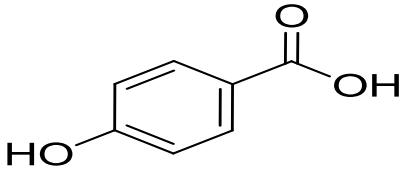
Marker components in OPP

1



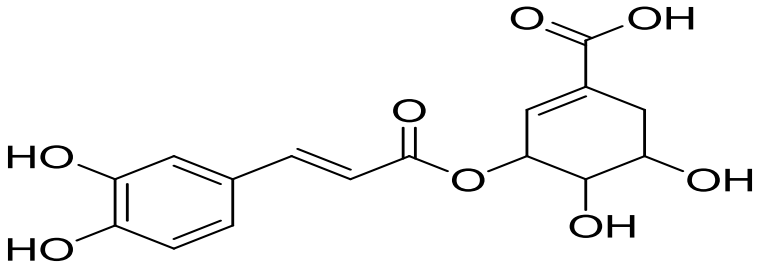
protocatechuic acid

2



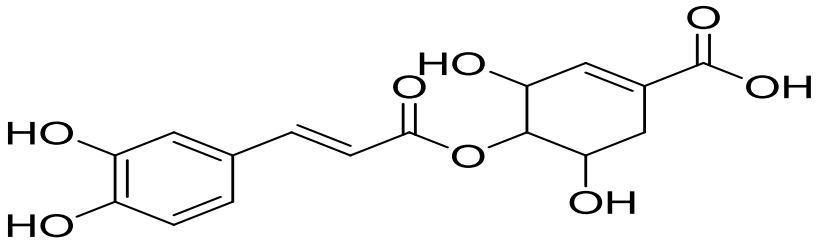
4-hydroxybenzoic acid

3



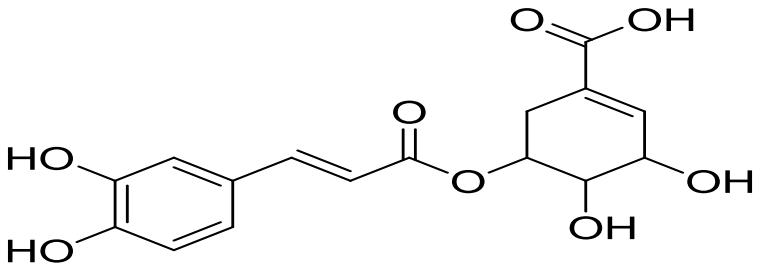
3-O-caffeoylshikimic acid

4



4-O-caffeoylshikimic acid

5



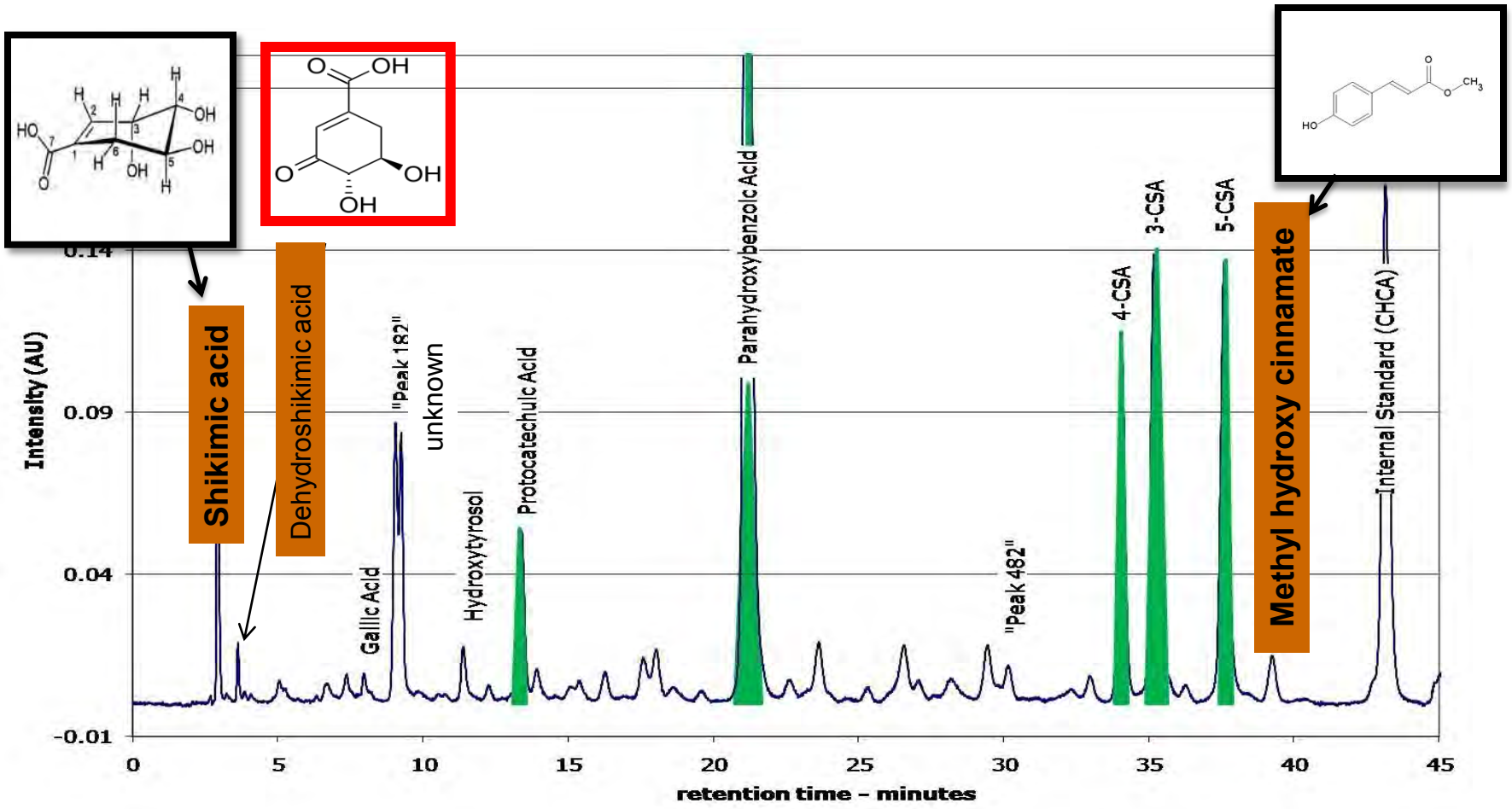
5-O-caffeoylshikimic acid

Marker Components in OPP

OPP Component	Concentration ppm g/g × 10^6	Concentration mmoles/G OPP (Freeze dried)	100 ug /ml OPP = X umole/L
Protocatchuic acid	630	4	4×10^{-1}
p-Hydroxybenzoic acid	7000	50	5
3-O-Caffeoylshikimic acid	3000	9	1
4-O-Caffeoylshikimic acid	3400	10	1
5-O-Caffeoylshikimic acid	4300	13	1
Total Phenolics	18500	88	9

OPP MPOB

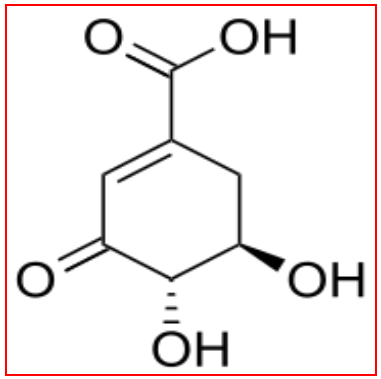
HPLC Chromatogram of Polyphenols in OPP



Column SGE C18
Mobile phase gradient (a) 0.2%% phosphoric acid 10mm Sodium sulfate
(b) 70 /30 V/V methanol and acetonitrile
Detection at 280nM
Flow rate 08 ml /min.

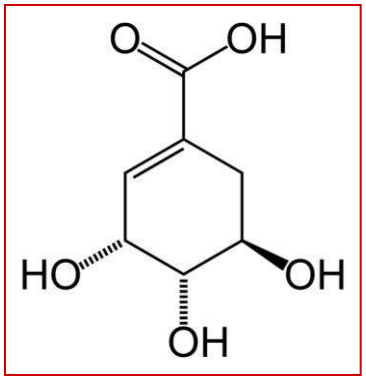
What roles to shikimic acid play in animal cells?

- Other compounds (non phenolic antioxidants) have been identified and may be used for fingerprinting



Dehydroshikimic acid

Molecular weight 173.151



Shikimic acid

Solvent	Solubility (g/100mls)
Water	18.0
Methanol	2.62
Ethanol	2.25
Isopropanol	0.976
Acetonitrile	0.03

Ref: TGS 2012

Phenolic Compounds / Shikimic acids

Phenolic compounds

- Act as cell signaling molecules
- Antioxidants
- Toxins to invading pests

What about "Shikimic acids"

- Spectator compounds?
- Andioxidant?

Immunomodulation by Shikimic acid

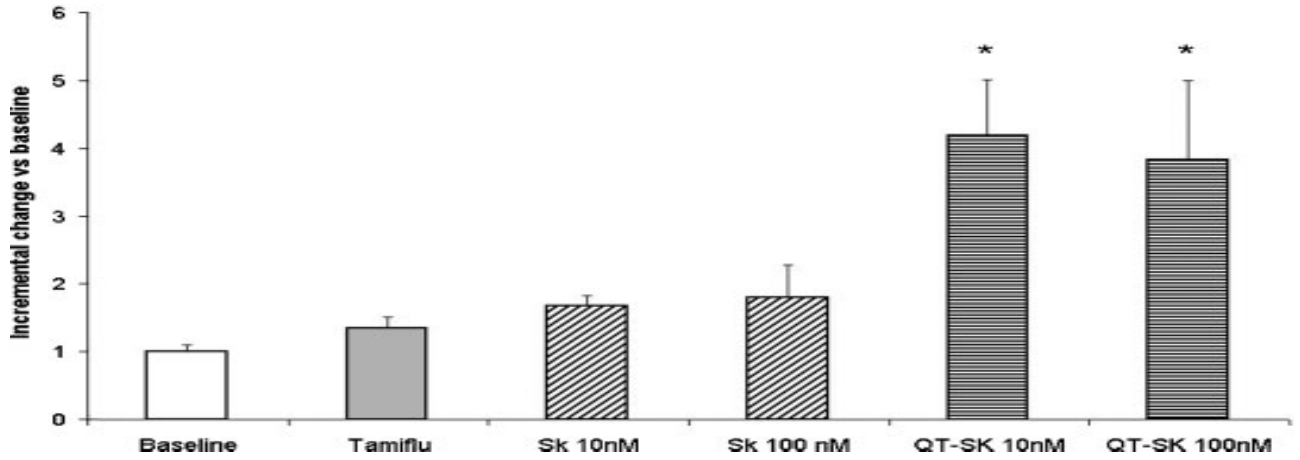


Fig. 1. Change in IL-8 levels in peripheral blood mononuclear cells incubated with Tamiflu, Shikimicacid (SK) and SKþ quercitin (QT). *P<0.05 vs. baseline, Tamiflu, SK 10 nM and SK 100 nM.

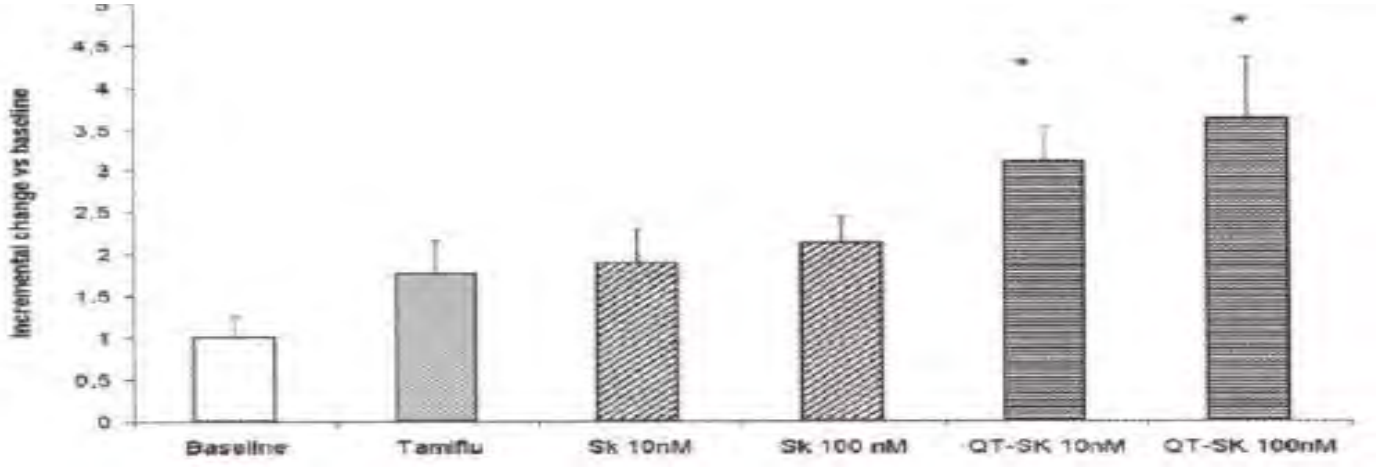


Fig. 2. Change in IL-6 levels in peripheral blood mononuclear cells incubated with Tamiflu, Shikimicacid (SK) and SKþ quercitin (QT). *P<0.05 vs. baseline, Tamiflu, SK 10 nM and SK 100 nM

A.A.E. Bertelli, et al .Journal of Medical Virology 80:741–745 (2008)

Is Shikimic acid (SA) an antioxidant ?

- Chemical analysis, ORAC & TEAC assay show **low value**
- **Bio. assays (cell based):** Is SA an anti-oxidant ?

Bioassay based on the bactericidal action of ROO*¥ Hiroshi Maeda*et al 1999

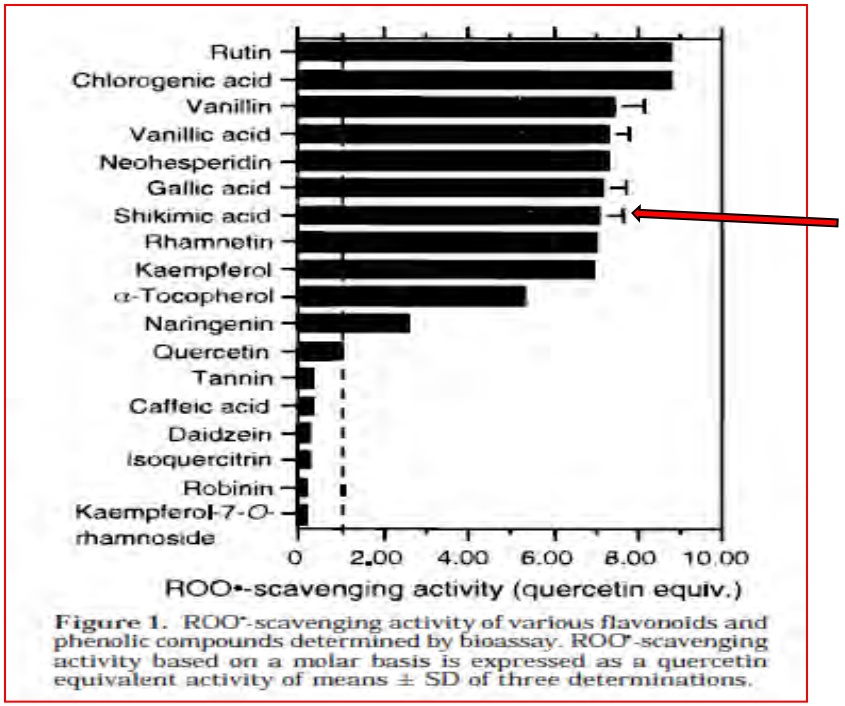
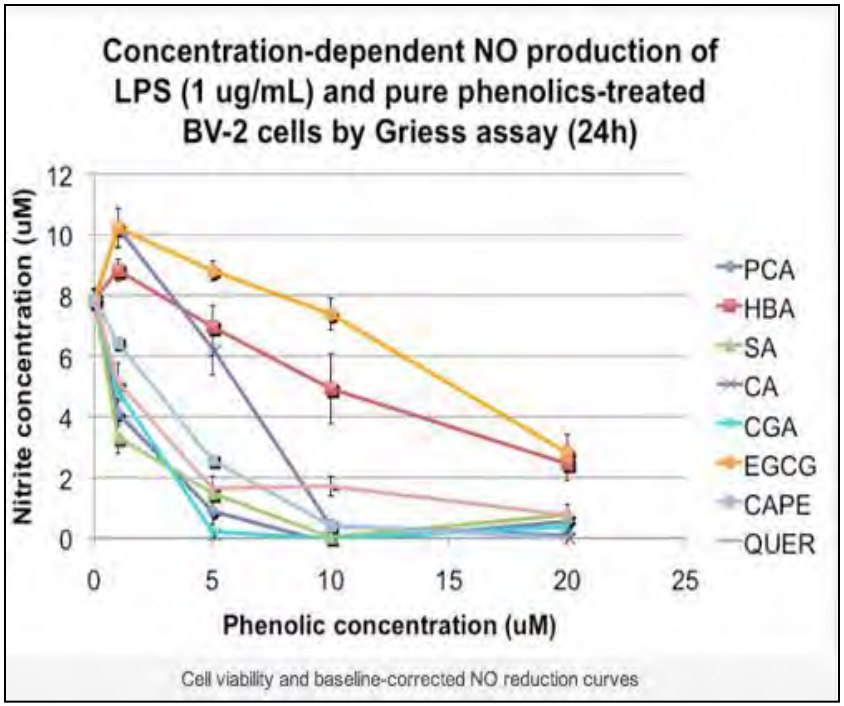


Figure 1. ROO•-scavenging activity of various flavonoids and phenolic compounds determined by bioassay. ROO•-scavenging activity based on a molar basis is expressed as a quercetin equivalent activity of means ± SD of three determinations.

NO determination in BV2-cells



ABTS+ ABTS

Antioxidants

- Chemical property – Plays with electron acceptor mechanisms on Proxidants.
- Antioxidant property of a substance – Electron grabber – proxidant in different chemical Environment
- Antioxidants are not interchangeable
- Each one has unique chemical behaviors and biological properties –elaborate network systems
- No single substance can do the work of the entire organism / single cell completely

Reactive oxygen and nitrogen species

Table 1. Reactive oxygen species (ROS)
Free radicals
Hydroxyl OH.
Superoxide O.−2
Nitric oxide NO.
Thiyl RS.
Peroxyl RO.2

Table 2. Non-radical reactive oxygen species
Non-radicals
Peroxynitrite ONOO−
Hypochlorous acid HOCl
Hydrogen peroxide H2O2
Singlet oxygen 1g (−1O2)
Ozone O3
Lipid peroxide LOOH

Table 3. Reactive nitrogen species (RNS)
Nitrous oxide N2O Nitrosyl cation NO+
Peroxynitrite OONO− Nitrogen dioxide NO.2
Peroxynitrous acid ONOOH Dinitrogen trioxide N2O3
Nitroxyl anion NO− Nitrous acid HNO2
Nitryl chloride NO2Cl

OPP MPOB

Determination Antioxidant Activity

A variety of in-vitro chemical methods are being used to determine the antioxidant activity of products and ingredients

1. **ORAC* (oxygen radical absorbance capacity)**
2. **TEAC (Trolox equivalent antioxidant capacity method)**
3. **DPPH (reduction of 2,2 diphenylpieryhydrazyl)**
4. **FRAP (ferric reducing /antioxidant power)**
5. **Folin Ciocalteau Total Phenolic Assay (metal oxides are reduced by phenolic antioxidants)**

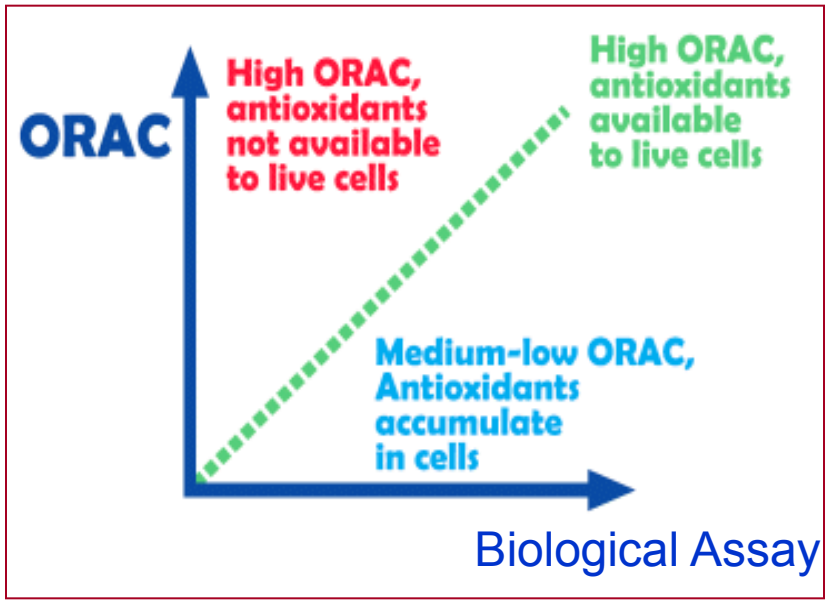
*** ORAC** assay is a robust analytical method that determines the antioxidant potential of foods botanical and biological samples. It is an vitro test that can measure to what degree a substance of interest can scavenge various free radicals. It also most cited & developed by NIH and USD.A

Hydroxyl radical peroxylradical peroxynitrite singlet oxygen and superoxide anion

OPP MPOB

Determination Antioxidant Activity

‘Whether the results have any bearing on the effectiveness in the human body’?



Antioxidant compounds absorbed upon consumption
Local effects in gut tissue
Entry into blood circulation
Entry into living cells
Ability to protect cells from oxidative damage

ORAC cannot be used predict what effect or benefit an antioxidant substance will have within the human body in attenuating free radical production or coping with oxidative stress. As an in vitro test ORAC cannot predict such in vitro effects nor can any other chemical assay

Determining Antioxidant Activity

Berries are high in antioxidant activity

Can we assume that something high on list in vitro tests will be high in the body?

Berries with **anthocyanins** as their primary component –blue berries , black berries are generally high in anti oxidants but the **anthocyanins don't appear to be stable in the body**. As a result the amount that needs to consumed is higher than other foods that have lower anti oxidants levels

Additional research is needed on factors affecting their ***absorption and metabolism***

Ronald L prior Research chemist / Nutritionist USDA/ ARS Arkanas chidren Nutrition Ctr.

Oxygen Radical Absorbance Capacity (ORAC assay Trolox equivalent TE per gram)

- Chemical based antioxidant measurement

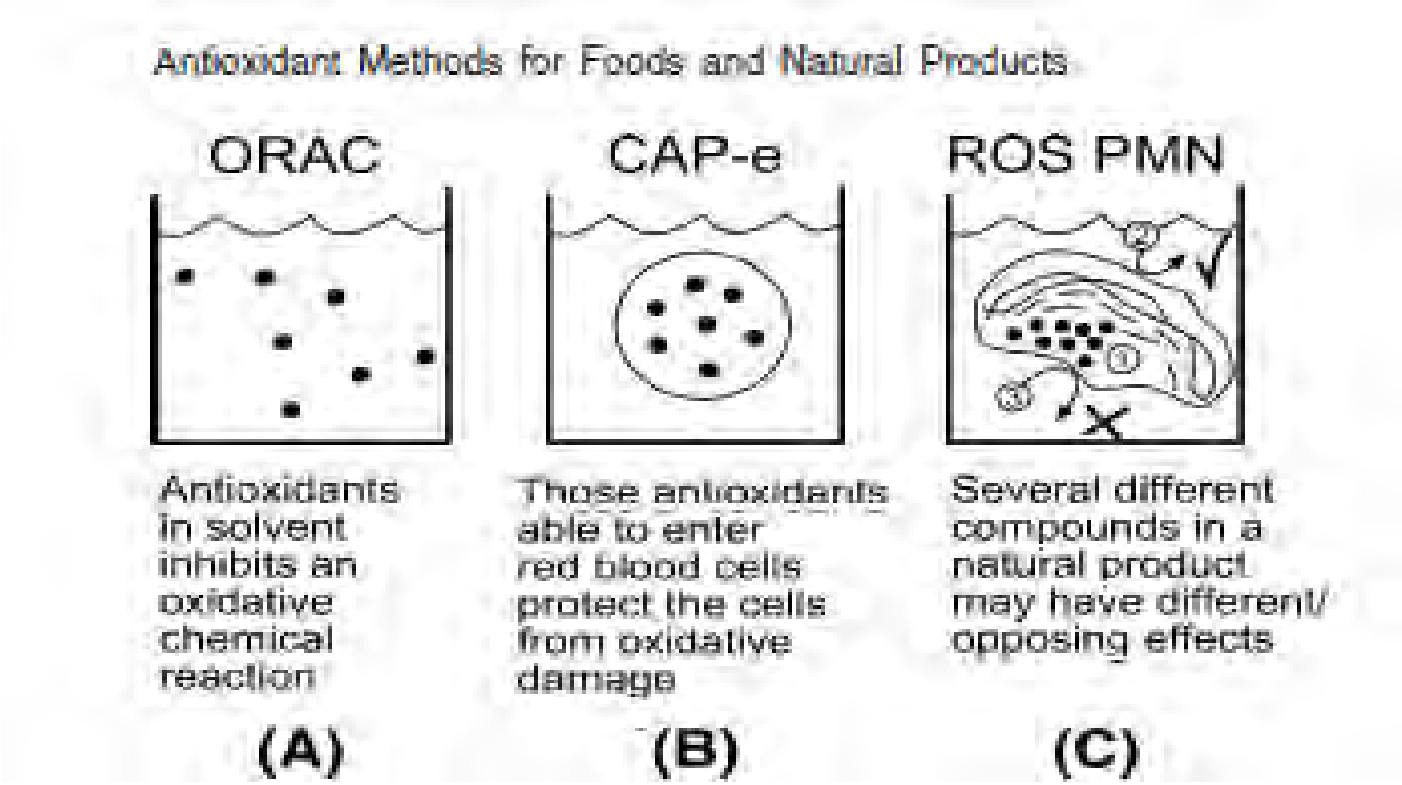
  Cannot predict or correlate to in vivo biological (USDA dilemma)

ORAC values of some super foods*:

Super foods	ORAC (TE /gm)	Biological activity
Immunel (bovine colostrum)	18	high biological activity
EpiCor	614	high biological activity

ref: Jensen et al. J. Agric. Food Chem. 2008, 56, 8319-8325

Developing multifacted data for PFB by Parallel testing Using Erythrocytes and polymorphononuclear cells



Dana Honzel, Steve G. Carter, Kimberlee A. Redman, Alexander G. Schauss, John R. Endres and Gitte S. Jensen

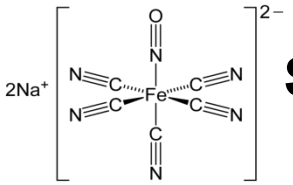
J. Agric. Food Chem., 2008, 56 (18), pp 8319–8325

Measurments methods

Methods	Substrate	Probes	Time of reaction	Method of quantification
TEAC	Chemical	Electron	15 min	Absorbance
ORAC	Chemical	ROO*	120 min	Fluorescence
SNP	Chemical	NO_2^-	6 hours	Absorbance (plate reader)
CAP-E	RBC cells	H_2O_2	90 minutes	Fluorescence (flow cytometer)
PMN	PMN cells	H_2O_2	90 minutes	Fluorescence (flow cytometer)
Cell-based Griess	BV2 cells (or) RAW macrophages	NO_2^-	24 hours	Absorbance (plate reader)

The Principles for ORAC and TEAC

ORAC	**Antioxidant reaction with peroxyl radicals, induced by AAPH (2,2'-azobis-2-amidino-propane)**	**Loss of fluorescence of fluorescein**
TEAC	**Antioxidant reaction with an organic cation radical ABTS (22'-azio-bis(3-3ethylbenzothiazoline-6-sulphonic acid)**	**Colorimetry**



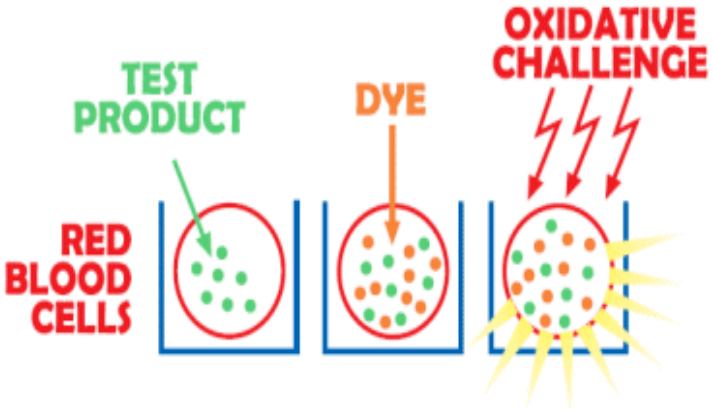
SNP

Sodium nitro prusside

In phosphate buffer (7.4) release Nitric acid . NO reacts with Oxygen to form (NO2-)

Colorimetry Griess reagent

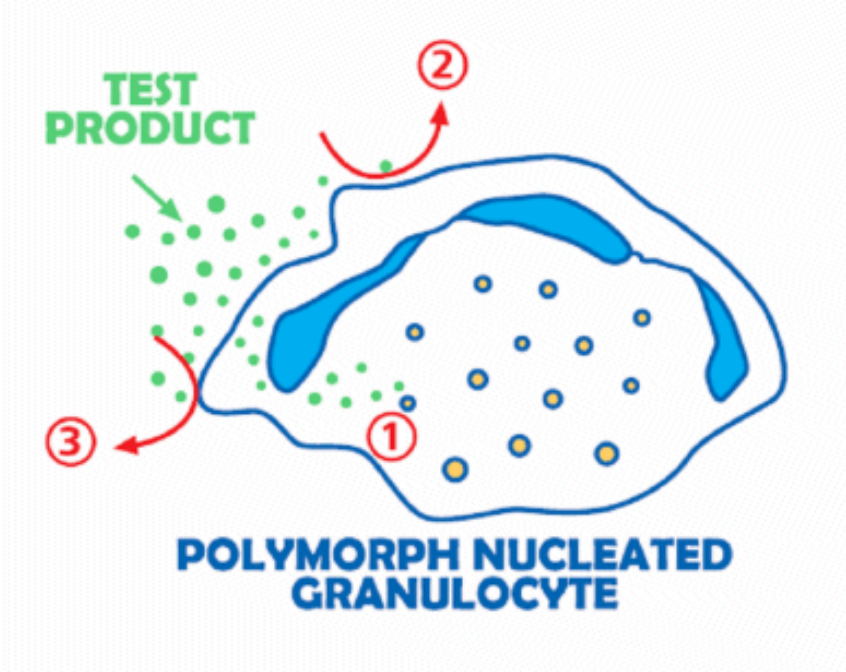
Principle for the CAP-e assay.



- Erythrocytes (red blood cells) are exposed to a natural product to allow antioxidants to enter into the cells.
- Unabsorbed antioxidants are removed, and the cells are loaded with a precursor dye.
- Upon an oxidative challenge, the precursor dye emits fluorescent light in proportion to the amount of oxidative damage.
- A reduction of fluorescence is proportional to antioxidant protection.

ref: Jensen et al. J. Agric. Food Chem. 2008, 56, 8319-8325

Principle for the PMN assay



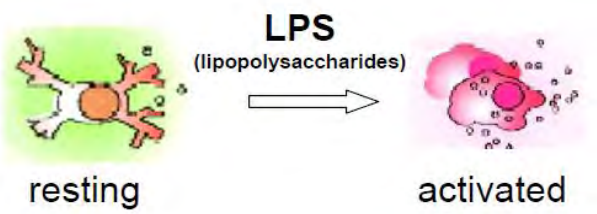
The essential methodology is similar to the CAP-e, except that the cell type used is the inflammatory PMN cell. This cell type can respond to natural products in the following three ways:

1) Antioxidants enter the intracellular space and neutralizes ROS;

2) Compounds within the natural product may bind to receptors on the cell surface and mediate a signal without entering the cell, leaving the cell less able to secrete ROS;

3) Compounds within the product may bind to other types of cell surface receptors, and trigger signaling towards a more pro-inflammatory behavior, leading to increased ROS formation.

Measurement of NO Production



Murin BV-2 cells were cultured in 24-well plate

Treatment with LPS in the presence and absence of OPP

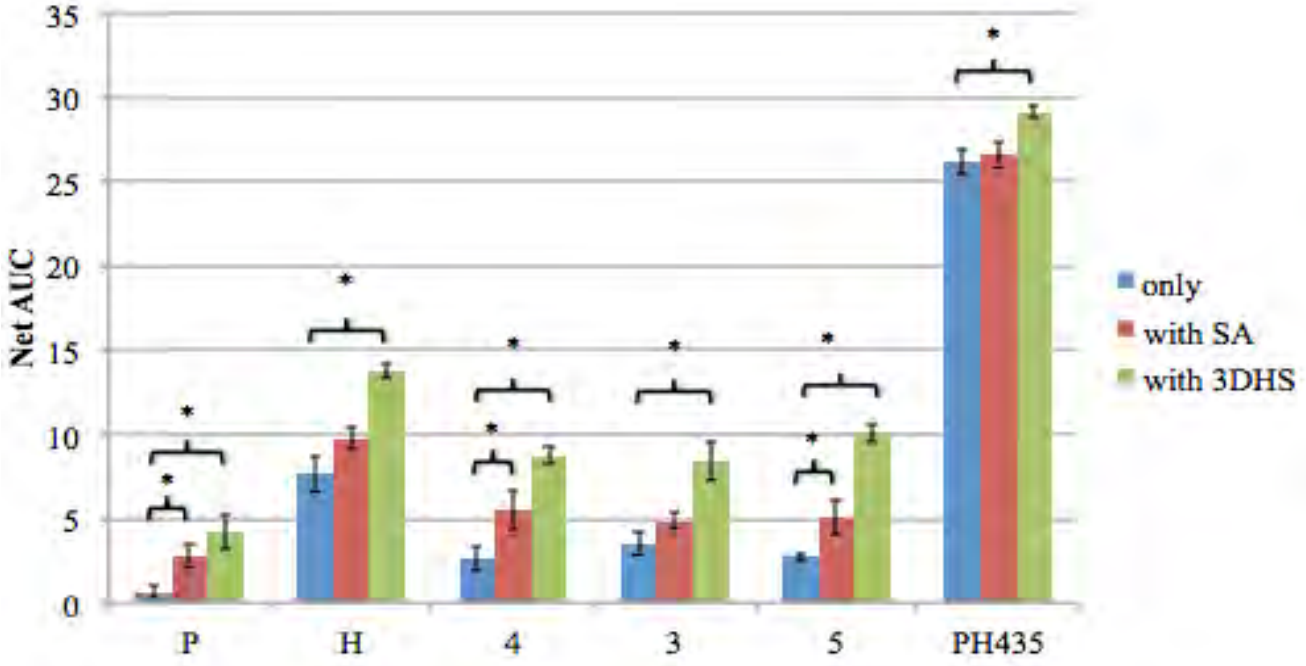
NO Determination

Measurement

Measure absorbance in Plate reader with a filter 540 nm
Nitrite concentrations were calculated from a sodium nitrite standard curve

Mitchell et al., 1992 , Mol Pharmacology, 41, 1163-68

The enhancement activity of SA and DSA with other phenolic compounds in quenching of NO radicals (SNP)



Chemical based ROO• scavenging activity assay (ORAC assay)*

Sample	Amount added (μM)#	Net Area Under the Curve (AUC)	Mean free radical scavenging activity w.r.t OPP (%) (± S.E.)
Protocatechuic acid (P)	0.14	0.6	2.1 (± 1.2)
p-hydoxybenzoic acid (H)	1.8	7.7	25.6 (± 3.6)
4-caffeoylshikimic acid (4)	0.31	2.6	8.8 (± 2.6)
3-caffeoylshikimic acid (3)	0.35	3.5	11.8 (± 2.3)
5-caffeoylshikimic acid (5)	0.45	2.7	9.1 (± 0.8)
Shikimic acid (S)	2.0	-Not detected-	-Not detected-
3-dehydroshikimic acid (D)	2.0	-Not detected-	-Not detected-
Mathematical sum (P+H+4+3+5) of Net AUC values		17.2	57.4 (± 5.2)
Mathematical sum (P+H+4+3+5+S) of Net AUC values		17.2	57.4 (± 5.6)
Mathematical sum (P+H+4+3+5+D) of Net AUC values		17.2	57.4 (± 5.3)
Chemical combination (PH435) by adding the 5 components at above-mentioned amounts together		26.2	87.5 (± 2.4)
Chemical combination (PH435S) by adding the 6 components at above-mentioned amounts together		26.6	88.9 (± 2.5)
Chemical combination (PH435D) by adding the 6 components at above-mentioned amounts together		29.2	97.5 (± 1.2) ‡
Oil Palm Phenolics	35ug/mL	29.9	100 (± 1.7)

TEAC

Sample	Amount added (mg/mL) #	Mean percentage of inhibition of ABTS•+	Mean inhibition % w.r.t OPP (± S.E.)
Protocatechuic acid (P)	0.25	22.1	38.7 (± 3.7)
p-hydoxybenzoic acid (H)	0.25	18.9	33.1 (± 2.4)
4-caffeoylshikimic acid (4)	0.25	14.0	24.4 (± 2.4)
3-caffeoylshikimic acid (3)	0.25	11.0	19.3 (± 1.7)
5-caffeoylshikimic acid (5)	0.25	9.3	16.3 (± 3.7)
Shikimic acid (S)	0.25	-Not detected-	-Not detected-
3-dehydroshikimic acid (D)	0.25	-Not detected-	-Not detected-
Chemical combination (PH435)		56.3	98.5 (± 2.1)
Chemical combination (PH435S)		56.2	98.3 (± 2.3)
Chemical combination (PH435D)		55.3	96.6 (± 1.4)
Oil Palm Phenolics (OPP)	0.25	57.2	100 (± 2.3)

OPP MPOB

Chemical based NO scavenging activity by using SNP assay *

Description	Amount added (µM)#	Mean percentage of inhibition of nitrite	Mean nitrite scavenging activity w.r.t OPP (%) (± S.E.)
p-hydoxybenzoic acid (H)	81.1	20.6	31.7 (± 2)
Shikimic acid (S)	91.9	9.1	14 (± 4.1)
3-dehydroshikimic acid (D)	93.0	9.7	14.9 (± 1.7)
Chemical combination (HS)		14.2	21.8 (± 12.1)
Chemical combination (HD)		12.7	19.4 (± 9.1)
Chemical combination (PH435)		66.1	101.6 (± 3.4)
Chemical combination (PH435S)		65.0	99.9 (± 6.6)
Chemical combination (PH435D)		66.2	101.7 (± 6.4)
Oil Palm Phenolics (OPP)	1.6 mg/mL	65.1	100 (± 0.8)

Cell-based ROS scavenging activity (CAP-e assay) *

Description	Amount added (mM) #	Actual % inhibition of ROS	% inhibition of ROS in comparison to OPP (± S.E)
p-hydoxybenzoic acid (H)	0.51	22.5	30.6 (± 0.2)
Caffeoylshikimic acid (C)	0.3	25.9	35.2 (± 0.1)
Shikimic acid (S)	0.57	-Not detected-	-Not detected-
3-dehydroshikimic acid (D)	0.58	9.9	13.4 (± 0.05)
Mathematical sum (H+C+S) of % inhibition of ROS		48.4	65.7 (± 0.2)
Mathematical sum (H+C+D) of % inhibition of ROS		58.3	79.2 (± 0.2)
Chemical combination (HCS) by adding the 3 components at above-mentioned amounts together		57.4	77.9 (± 0.6)
Chemical combination (HCD) by adding the 3 components at above-mentioned amounts together		68.5	93 (± 0.8)
OPP	10 mg/mL	73.7	100 (± 0.8)

OPP MPOB

Cell-based ROS scavenging activity (PMN assay)

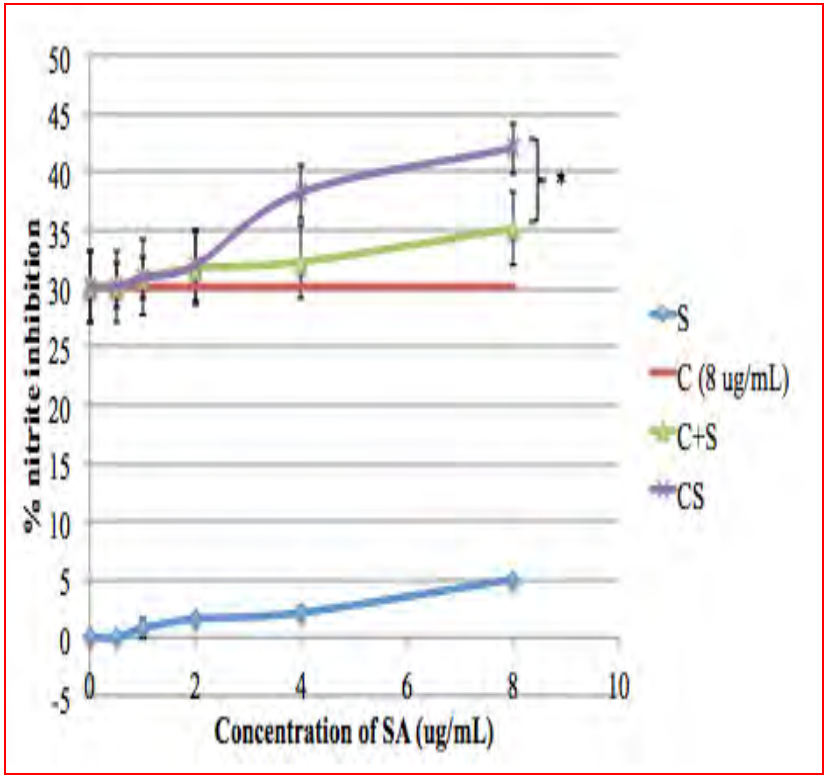
Description	Amount added (μM)#	Actual % inhibition of ROS	% inhibition of ROS in comparison to OPP (± S.E)
p-hydoxybenzoic acid (H)	81.1	13	18.2 (± 0.1)
caffeoylshikimic acid (C)	47.6	23.3	32.5 (± 0.1)
shikimic acid (S)	91.9	23.8	33.2 (± 0.2)
3-dehydroshikimic acid (D)	92.9	22	30.7 (± 0.2)
Mathematical sum (H+C+S) of % inhibition of ROS		60.1	83.9 (± 0.3)
Mathematical sum (H+C+D) of % inhibition of ROS		58.3	81.4 (± 0.3)
Chemical combination (HCS) by adding the 3 components at above-mentioned amounts together		67.9	94.8 (± 2.2) *
Chemical combination (HCD) by adding the 3 components at above-mentioned amounts together		66.6	93 (± 1.8)
OPP	1.6 mg/mL	71.7	100 (± 2.3)

Synergy study in cell-based NO_2^- scavenging activity (Griess assay, BV2 cells)

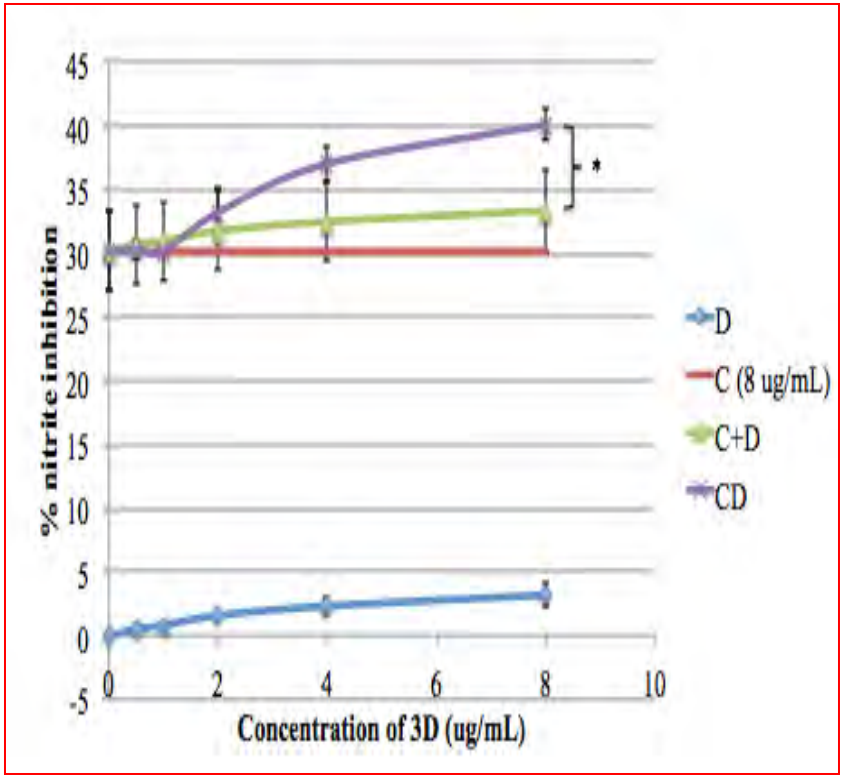
Description	Amount added (μM) ‡	Actual % inhibition of NO_2^-	% inhibition of NO_2^- in comparison to OPP (± S.E)
Protocatechuic acid (P)	3.3	8.6	13 (± 0.7)
p-hydoxybenzoic acid (H)	40.5	11.4	17.3 (± 2.8)
4-caffeoylshikimic acid (4)	7.1	8.4	12.7 (± 2.5)
3-caffeoylshikimic acid (3)	8.1	12.3	18.6 (± 2.3)
5-caffeoylshikimic acid (5)	10.2	8.8	13.4 (± 3.7)
Shikimic acid (S)	45.9	3.4	5.2 (± 0.7)
Mathematical sum (P+H+4+3+5) of % inhibition of NO_2^-		49.4	75 (± 5.8)
Mathematical sum (P+H+4+3+5+S) of % inhibition of NO_2^-		52.9	80.2 (± 5.8)
Chemical combination (PH435) by adding the 5 components at above-mentioned amounts together		56.3	85.4 (± 1.7)
Chemical combination (PH435S) by adding the 6 components at above-mentioned amounts together		62	94.1 (± 0.8) *
OPP	800 μg/mL	65.9	100 (± 3.2)

NO_2^- scavenging activity (Griess assay-raw macrophage)

Caffeoylshikimic acid + Shikimic acid



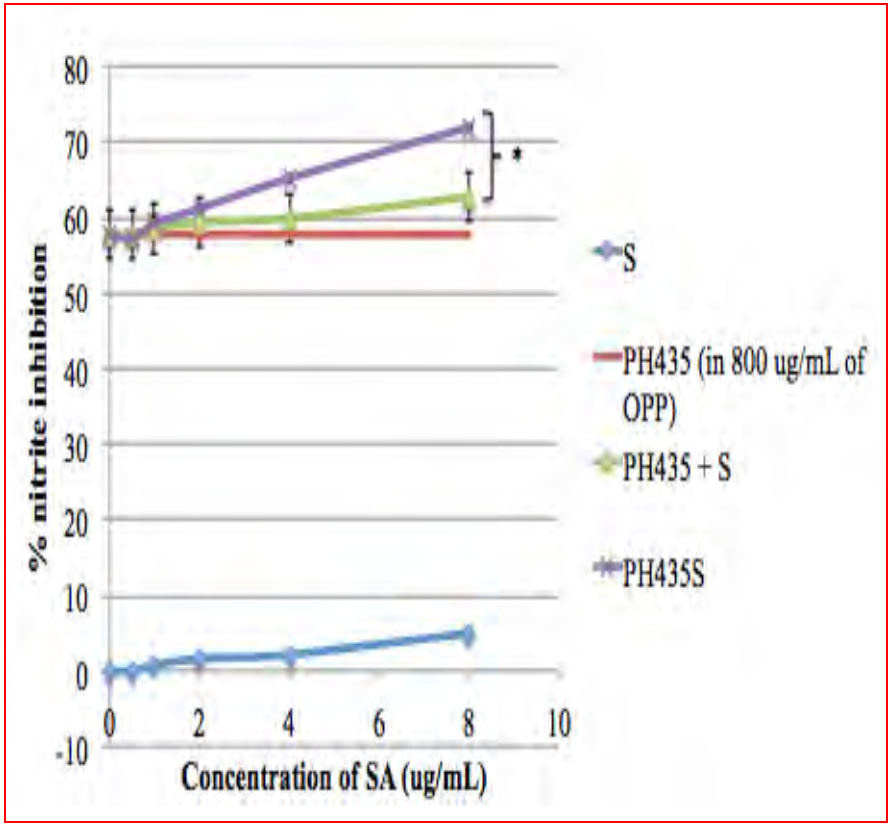
Caffeoylshikimic acid + Dehydro-shikimic acid



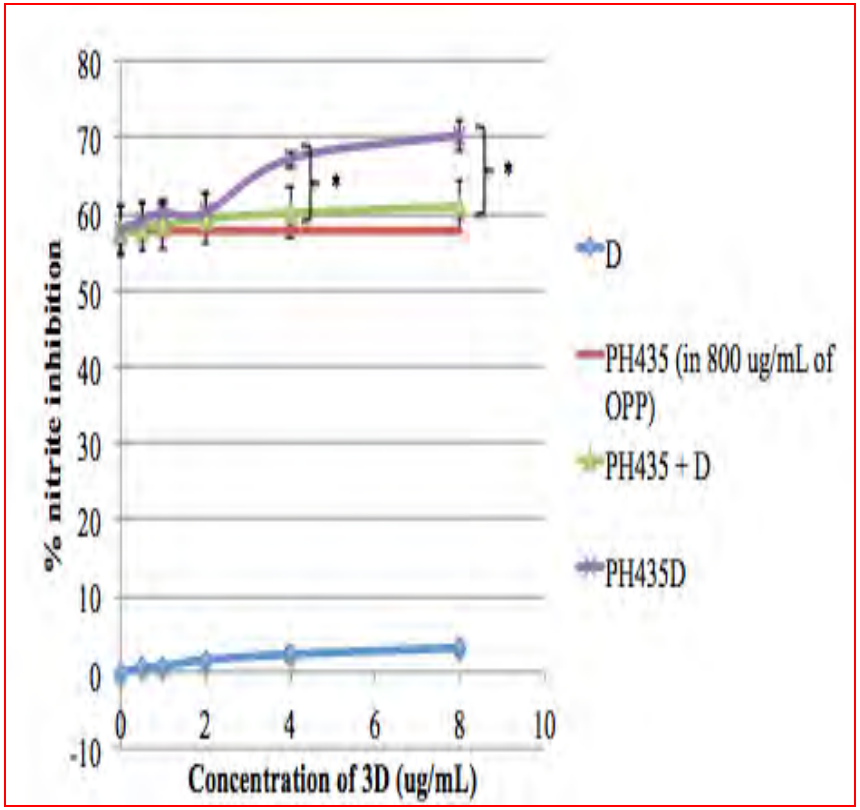
* Combination is significantly higher than sum of components indicated ($p<0.05$, Student's t test)

NO_2^-scavenging activity (Griess assay-raw macrophage)

PH435 + SA

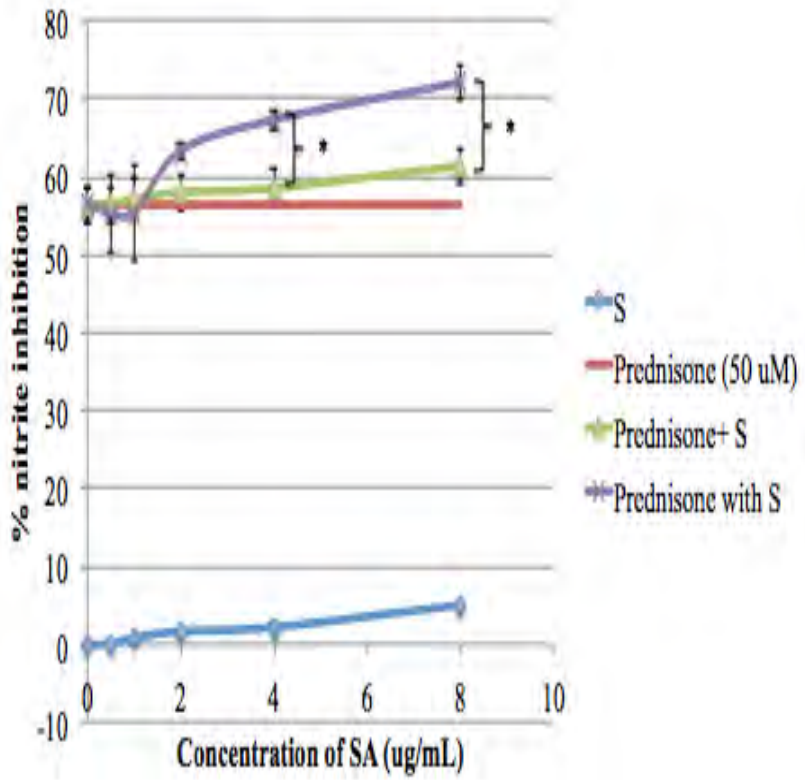


PH345+DSA

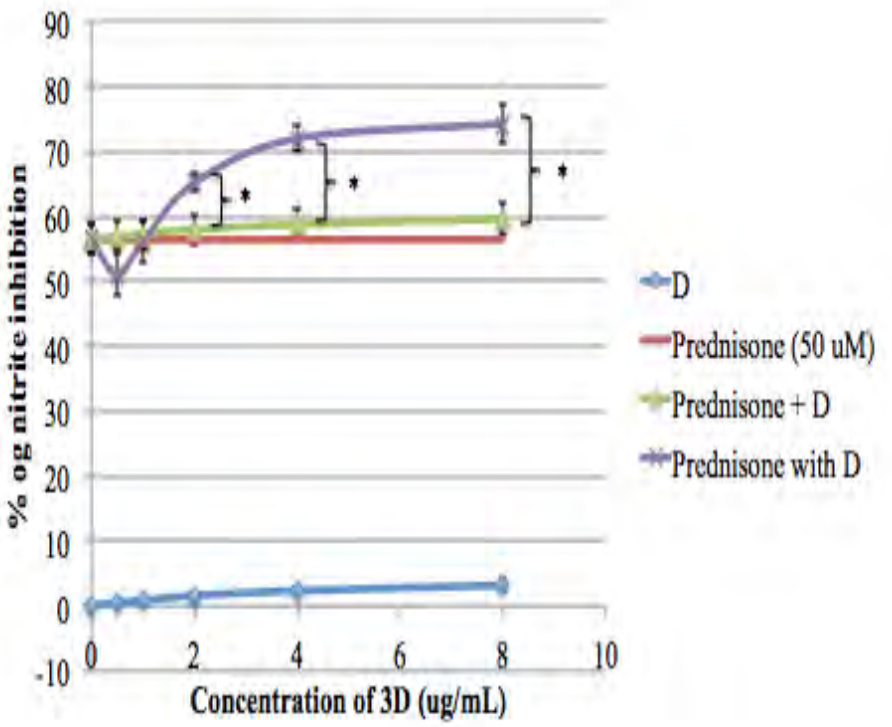


NO_2^-scavenging activity (Griess assay-raw macrophage)

Prednisone + Shikimic acid

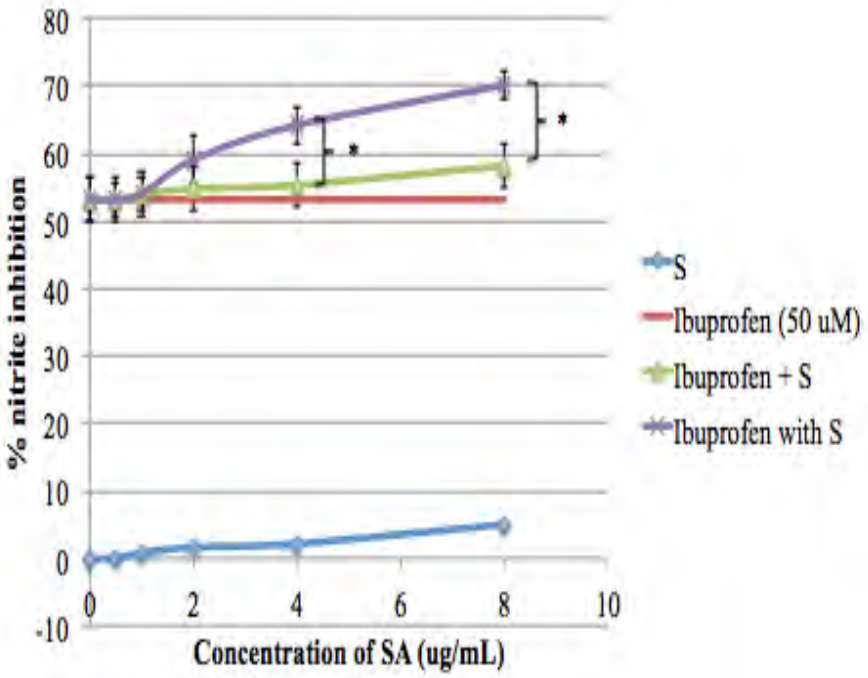


Predniosne + Dehydroshikimic acid

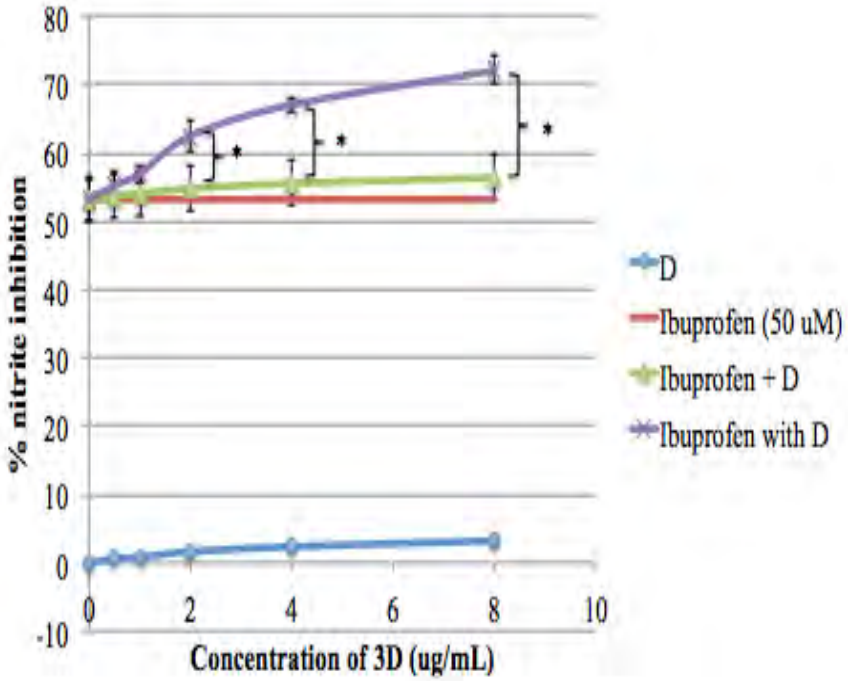


NO_2^-scavenging activity (Griess assay-raw macrophage)

Ibuprofen + shikimic acid



Ibuprofen + Dehydroshikimic acid



‡ 50 µM Ibuprofen was chosen based on IC50 for this assay from previous studies [13]

* Combination is significantly higher than sum of components indicated ($p<0.05$, Student's t test)

Conclusions

- Shikimic acid (SA) / 3-dehydroshikimic (DSA) alone have little or no antioxidant activity as measured by chemical systems
- SA/DSA may promote concerted effect in chemical systems
- SA/DSA in combination of phenolic compounds enhances anti-inflammatory effects in biological systems.

REMEMBER

Unity is Strength

THANK YOU

A collaborative effort between MIT and MPOB

The support of the Director General of MPOB:
Datuk Dr. Choo Yuen May

Correlation between ORAC and CAP-e

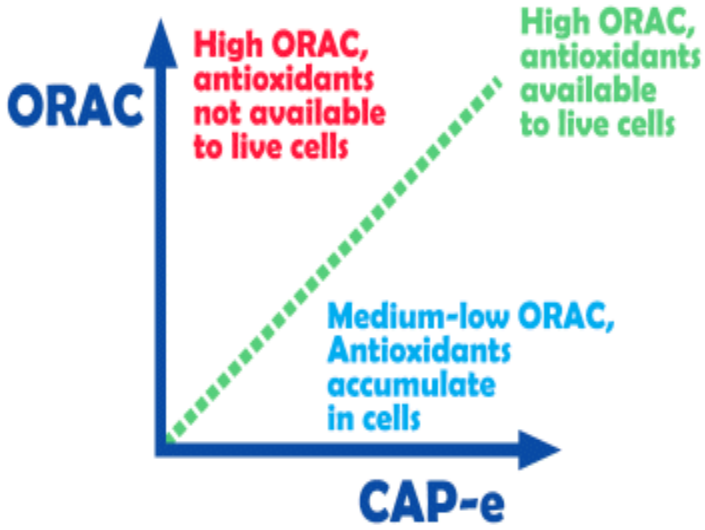


Diagram showing several possible situations pertaining to antioxidant capacity of natural products, when comparing data obtained by ORAC versus CAP-e:

a) A product may have a high ORAC value but be unable to enter and protect cells from oxidative damage;

b) a product may perform well in both assays;

c) a product may show better protection of cells than what would be expected based on the ORAC value alone

ref: Jensen et al. J. Agric. Food Chem. 2008, 56, 8319-8325

TEAC (Trolox Eqivalent Antioxidant Activity)

Description	Amount added (µM)#	Net Area Under the Curve (AUC) ‡	Mean free radical scavenging activity w.r.t OPP (%) (± S.E.)
Protocatechuic acid (P)	0.14	0.6	2.1 (± 1.2)
p-hydoxybenzoic acid (H)	1.77	7.7	25.6 (± 3.6)*
4-caffeoylshikimic acid (4)	0.31	2.6	8.8 (± 2.6)*
3-caffeoylshikimic acid (3)	0.35	3.5	11.8 (± 2.3)*
5-caffeoylshikimic acid (5)	0.45	2.7	9.1 (± 0.8)*
Shikimic acid (S)	2	-Not detected-	-Not detected-
3-dehydroshikimic acid (D)	2	-Not detected-	-Not detected-
Oil Palm Phenolics	35 µg/mL	29.9	100 (± 1.7)*

Appendix A (i)

Net AUC values of individual components

The components were added according to the amount present in 35 µg/mL of OPP

‡ Higher AUC denotes higher antioxidant capacity

* $p < 0.05$ with respect to control (Tukey-Kramer's Honestly Significant Difference)