Oil Palm Phenolics suppresses Oxidative Stress and Inflammation

by

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Abstract

Water-soluble Oil Palm Phenolics (OPP), derived from Elaeis guineensis, contains a unique blend of plant phenolics. Recent cell and animal studies have demonstrated positive health benefits in a number of different organ systems, with almost no noticeable side effects. Its efficacy and non-toxicity may be in large part due to several active polyphenolic ingredients, rather than a single one. In this work, some of the coordinated mechanisms through which OPP functions to protect glial cells and chemical systems from oxidative stress and inflammation were studied.

The antioxidant effects of OPP were analyzed based on its ability to act as a direct antioxidant against Reactive Oxygen Species (ROS), the selective expression of several antioxidant enzymes when added to U-87 astroglial cells, activation of the inherent antioxidants of the body such as glutathione and change in protein expression of activated enzymes such as Heme Oxygenase 1.

An image analysis assay was developed to study the effects of OPP on cell size, morphology and phagocytosis of Lipopolysaccharides (LPS) induced inflammation in BV-2 microglial cells. This study was followed by experiments to quantify the reduction of nitrite and anti-inflammatory protein expression changes after addition of OPP in LPS-stimulated BV-2 cells.

The insights gained from the study of OPP mixture led to the experiments for probing the synergistic interactions and biological enhancement activity among the individual components of OPP by using chemical and cell-based assays. The results described in this thesis include several advancements towards the goal of manufacturing process development of Oil Palm Phenolics and are highlighted in the relevant sections.

Thesis Supervisor:	Dr. ChoKyun Rha
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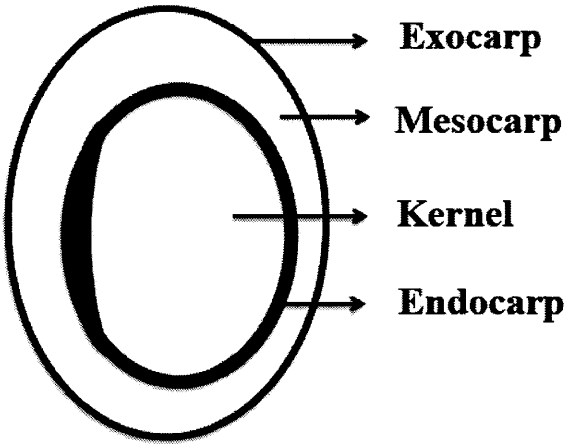
CHAPTER 1: INTRODUCTION

1.1 Oil Palm Phenolics

The Oil Palm (Elaeis Guineensis) is a tropical tree that belongs to the palm family. Indigenous to West Africa, this species has been introduced to Southeast Asia and has flourished in the warm, humid climate. Today, Malaysia accounts for around 50% of the world's palm oil production [1]. Long sought after for the oil that is rich in antioxidants, we now know that the crushed fruit yields a number of valuable compounds in the water-based fraction as well [2].

The palm fruit is the economically vital part of the oil palm plant. The plant bears its fruit in bunches varying in weight from 10 to 40 kg. The individual fruits are made up of an outer skin (the exocarp), the pulp (mesocarp) containing the palm oil in a fibrous matrix, a central nut consisting of a shell (endocarp), and the kernel as shown in figure 1. The oil palm fruit (edible portion) contains 58.4%of fat, 12.5% of carbohydrates, 1.9% of protein, 26% of water and 3.2% of crude fiber [1].

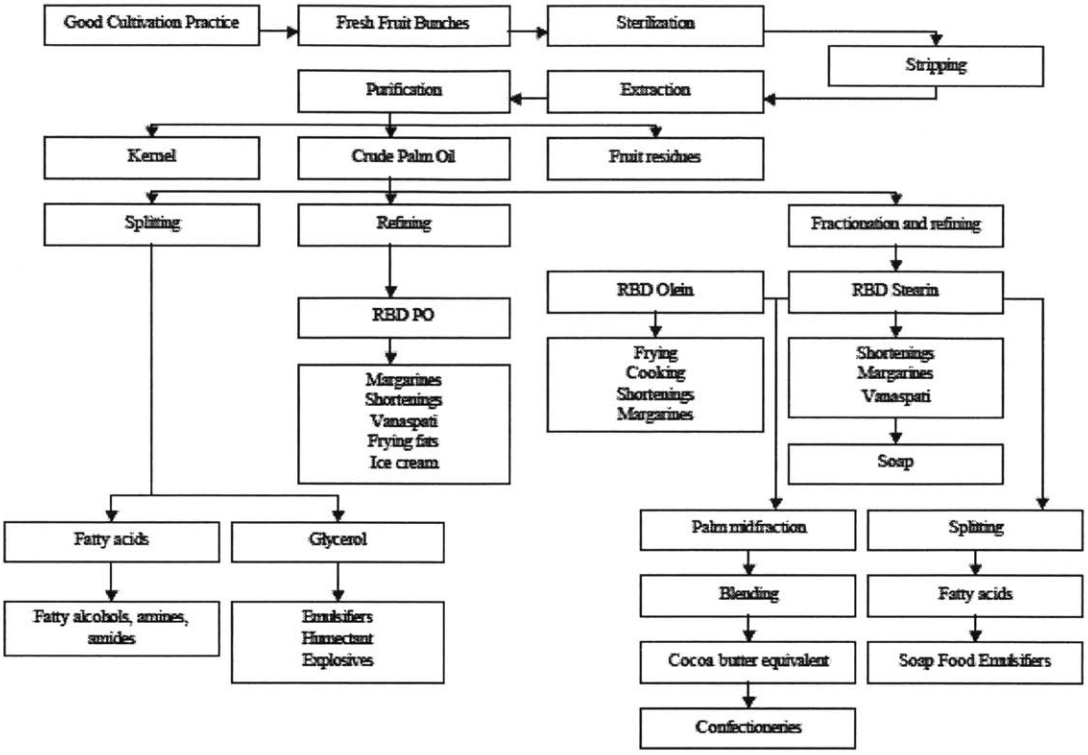
Figure 1: Structure of the palm fruit



Palm Oil is versatile oil that has numerous scientific and economic advantages over other oils. It is a rich source of free fatty acids, vitamins, tocopherols, sterols, proteins and water-soluble antioxidants [1]. These aqueous components could be useful in the manufacture of value added products for the pharmaceutical, consumer goods and specialty chemical industries.

The integrated process in the palm oil production chain consists of several parts starting from good cultivation practice to obtain higher oil extraction yield from the mesocarp to the refining process for facilitating separation and maximizing use of each fractional by-product. The current utilization chart of palm oil is shown in figure 2.

Figure 2: Palm oil utilization chart [1] (RBD: Refined, Bleached and Deodorized)



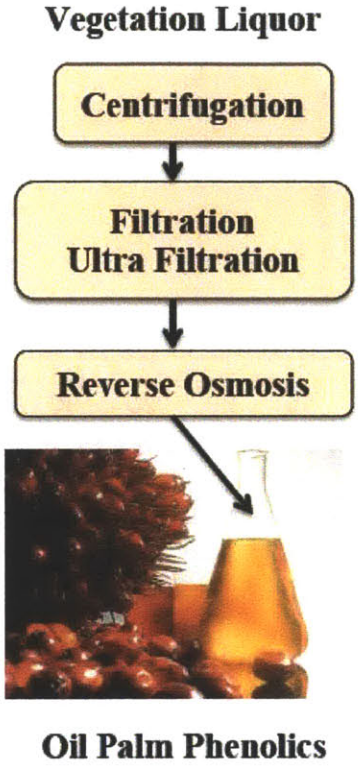
The solid wastes from the palm oil milling process can also be converted into useful industrial products. Empty fruit bunches (EFB) make up 20-28%, palm press fiber (PPF) 11-12% and palm kernel cake (PKC) 5-8% of the expected amount of solid wastes. The palm mill solid wastes can be used as boiler fuel and a potent source of carbon for biogas production [1].

Oil Palm Phenolics (OPP) is derived from the water-soluble phase of pulp of the oil palm plant. The crude oil is refined to remove unwanted odor, flavor and impurities such as the decanter cake after desanding operation. The beneficial components such as antioxidants and vitamins are retained in the aqueous vegetation liquor that results from the removal of crude oil from the crude vegetation liquor. Residual oil, large molecules and excess water are removed from the vegetation liquor to obtain Oil Palm Phenolics (OPP).

For every ton of crude palm oil production, 5 to 7.5 tons of water is required, and more than half of the water will be part of the vegetation liquor [3]. As 85 million tons of palm vegetation liquor is available every year from the Malaysian palm oil milling process, a large amount of Oil Palm Phenolics can be manufactured from the vegetation liquor and converted into useful products [4].

The simplified processing scheme of the manufacture of Oil Palm Phenolics from the vegetation liquor is shown in figure 3.

Figure 3: Production of Oil Palm Phenolics (OPP) from Vegetation Liquor

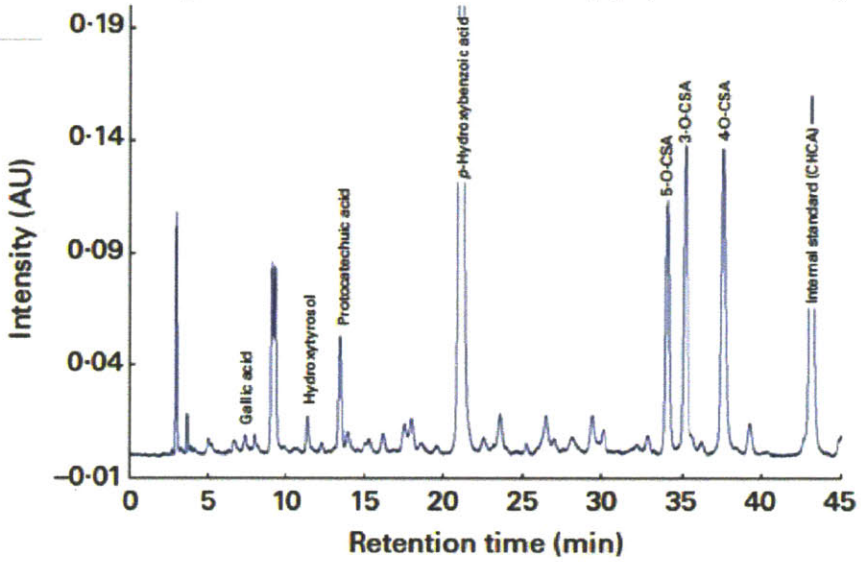


1.2 Polyphenolic compounds in OPP

Polyphenols are a structural class of compounds, many of which occur in natural sources. They are chemically defined as 6-carbon membered benzene (phenyl) rings with two or more hydroxyl groups as substituents on the ring. There is a large amount of in vitro, in vivo and epidemiological studies about the consumption of polyphenolic compounds present in natural sources such as blueberry, red wine, green tea, etc [3]. Polyphenolic compounds can exert neuroprotective actions by modulating intracellular signaling cascades that result in a biological response [5].

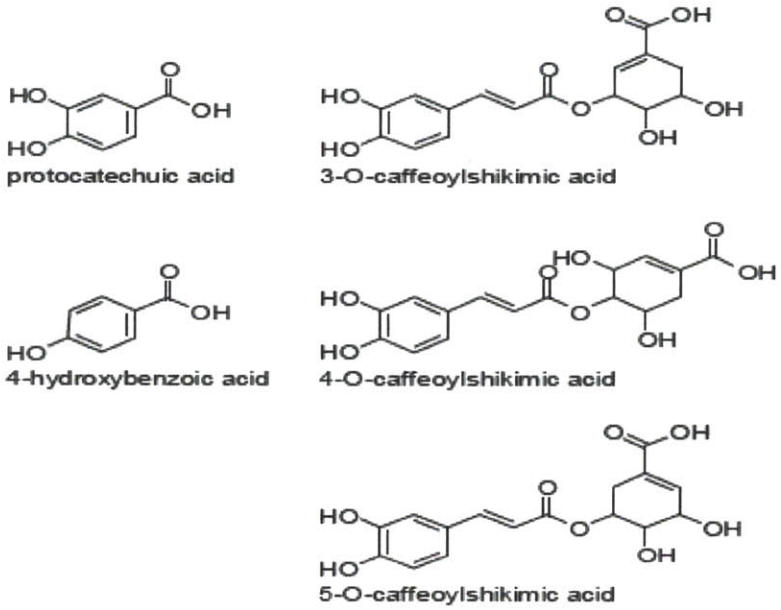
Fingerprinting and characterization of the polyphenolic compounds in Oil Palm Phenolics, obtained from the process shown in figure 3, is achieved by using in vitro biological studies and analytical instrumentation techniques such as reverse phase HPLC (High Pressure Liquid Chromatography) for separation, MS (Mass Spectroscopy) and NMR (Nuclear Magnetic Resonance) spectroscopy for characterization. The HPLC profile of Oil Palm Phenolics (OPP) is shown in figure 4.

Figure 4: HPLC profile of Oil Palm Phenolics [4]. (AU: Arbitrary units)



The HPLC profile indicates the presence of five major phenolic compounds including three isomers of caffeoylshikimic acid (CSA), protocatechuic acid and p-hydroxybenzoic acid as shown in figure 4. The internal standard used in the HPLC analysis of OPP is α-cyano-hydroxycinnamic acid (CHCA). The chemical structures of the five major compounds are shown in figure 5. The antioxidant activities of the polyphenols have been found to increase with the number of hydroxyl groups in them [6]. As protocatechuic acid has 2 hydroxyl groups, 4-hydroxybenzoic acid has 1 hydroxyl group and CSA has 4 hydroxyl groups in its chemical structure, a high antioxidant activity of OPP can be expected.

Figure 5: Chemical structure of the 5 major compounds of Oil Palm Phenolics [4]

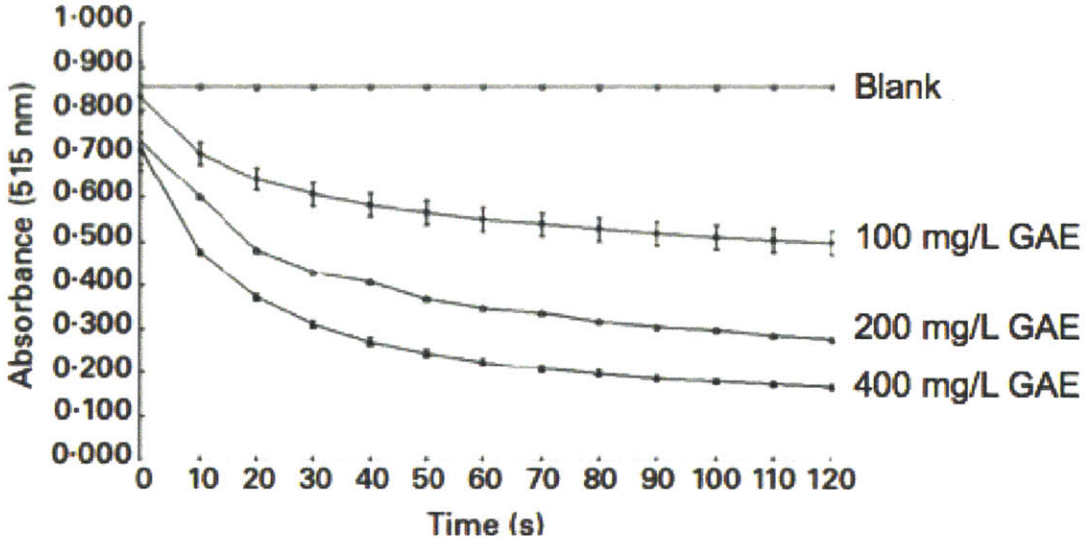


1.3 Free radical scavenging activity of OPP

Reactive oxygen species are highly reactive and cause oxidative damage to the proteins, lipids and nucleic acids of the body [7]. There are 6 major species: peroxyl radicals ($ROO^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), superoxide anion (O_2^{-}), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^{-}$) [8]. Biological cells convert ROS into harmless substances through enzymatic reactions involving the inherent antioxidants of the body. However, peroxyl radicals ($ROO^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), superoxide anion (O_2^{-}) and peroxynitrite ($ONOO^{-}$) cannot be tackled by enzymatic reactions and require the consumption of direct antioxidants to scavenge them. Moreover, this work also elucidates how the phenolic compounds in OPP can activate the inherent antioxidants of the body apart from directly quenching some of the ROS described above.

Previous studies with OPP aimed at determining the antioxidant activity of OPP by measuring the free radical scavenging activity of Oil Palm Phenolics using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent [4]. The results are shown in figure 6.

Figure 6: Free radical scavenging activity of Oil Palm Phenolics [4]*



*GAE: Gallic Acid Equivalents of OPP; the different OPP concentrations led to significantly different antioxidant values ($P<0.01$)

Although several polyphenolic compounds have been shown to exhibit free radical scavenging activity in chemical assays such as the DPPH assay, there is a need to perform both in vitro assays using biological cells and in vivo studies in animal models to confirm the effects of OPP against oxidation and inflammation with biological significance [9]. This thesis aims specifically at probing the in vitro biological effects of OPP and comparing them to the results from chemical assays. A brief summary of the in vivo effects of OPP is provided in the following section.

1.4 In vivo effects of OPP in experimental animal models

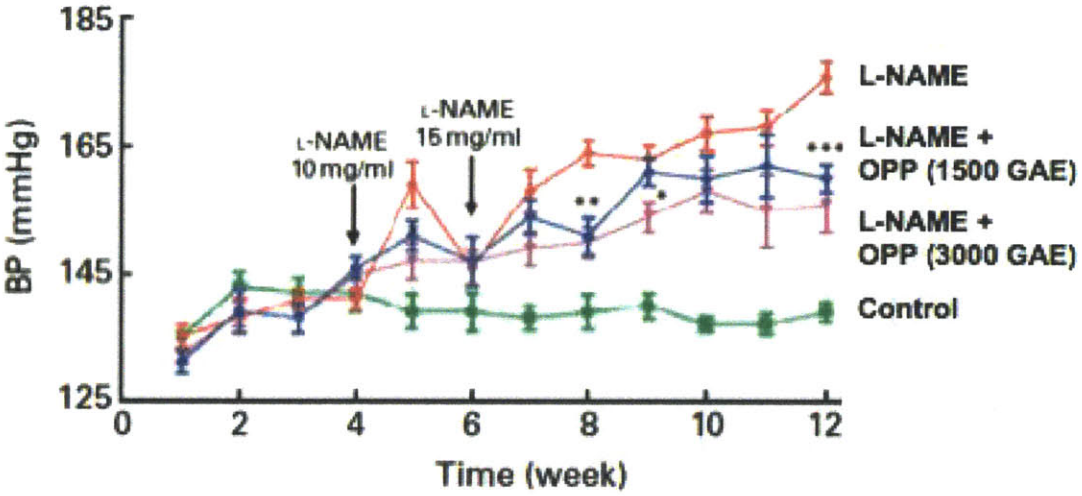
In vivo studies have been performed to study the effects of OPP on cardiovascular, ophthalmic, neurologic, cancer and metabolic diseases.

1.4.1 Cardiovascular effects of OPP

Blood pressure

A NO-deficient rat model that serves as a model of hypertension was used for the studies by treating the mice with L-NAME (N^{ω}-nitro-L-arginine methyl ester), an inhibitor of nitric oxide synthetase [10]. 12 week Sprague-Drawley rats were fed with OPP of different concentrations. OPP reduced the blood pressure in this rat model. These results were consistent with the effects of OPP on isolated vascular preparations to increase the levels of nitric oxide synthetase, which in turn lead to the dilatation of the arterial and arteriolar vessels [4]. The reduction in blood pressure by OPP is shown in figure 7.

Figure 7: Blood pressure reduction by Oil Palm Phenolics [10]



* Values are significantly different from L-NAME group (P<0.05)
** Values are significantly different from L-NAME group (P<0.01)
*** Values are significantly different from L-NAME group (P<0.001)

Cardiac arrhythmia

A rat model of cardiac arrhythmia was used to induce sudden death by occluding the coronary artery [10]. 1500 mg/L GAE of OPP protected against ischemia-induced cardiac arrhythmia in rats after a 4-month feeding period, such as a statistically significant reduction in the incidence of ventricular fibrillation (VF) and ventricular tachycardia (VT). Moreover, OPP also reduced the mean duration of VF and VT, in cases where they occurred. Overall mortality was also reduced by OPP.

Atherosclerosis

Rabbits were fed a high-lipid content diet and the progression of atherosclerosis was monitored [10]. OPP reduced plaque formation and the presence of fatty streaks in the arterial walls. OPP also increased the number of arteries that were free of lesions. As the morphology and development of the lesions were similar to human atherosclerotic lesions, these results of OPP may be an indirect evidence of its bioavailability in humans [11].

1.4.2 Multi-system effects of OPP: Diabetes mellitus

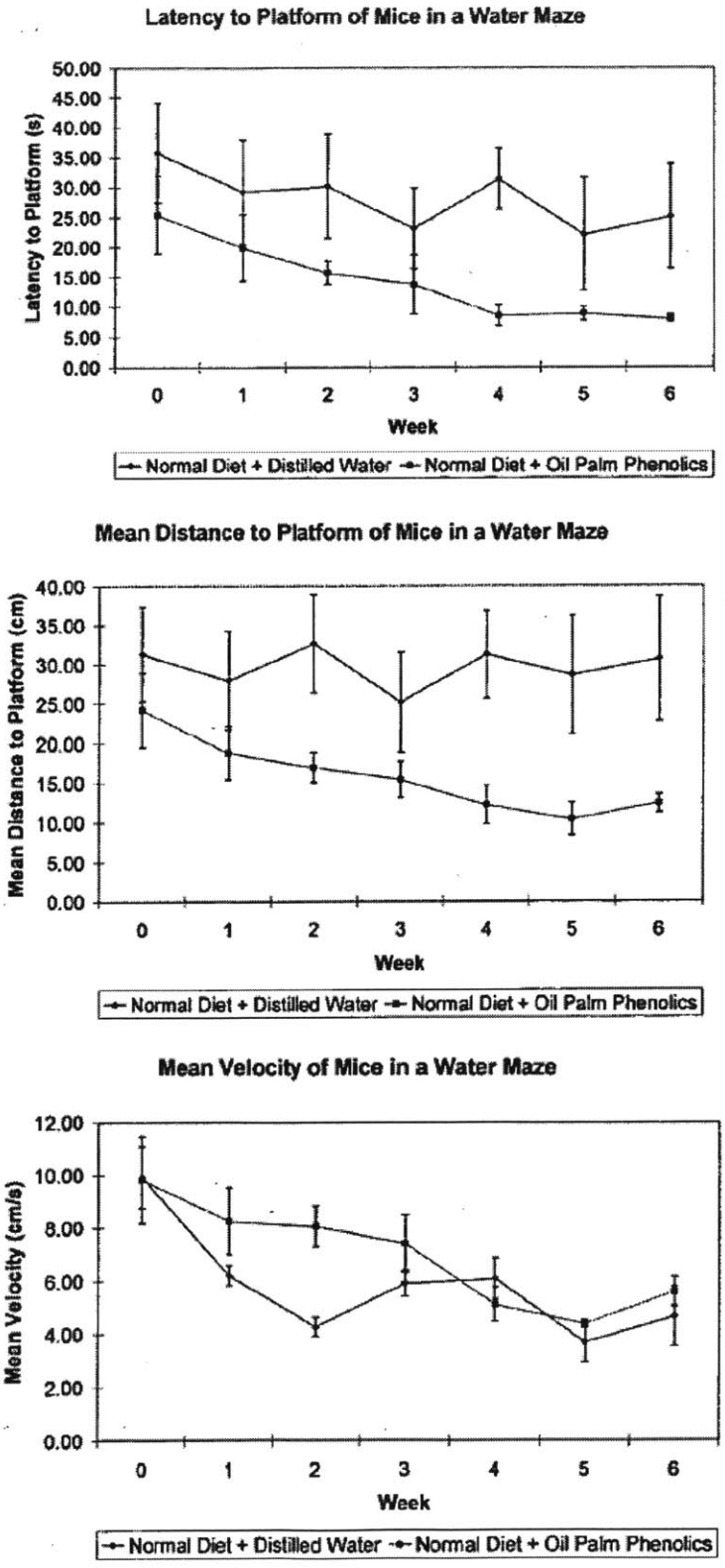
Diabetes is a prevalent disease leading to other complications such as blindness, renal failure and coronary heart disease [12]. A model of the metabolic syndrome and type 2 diabetes was generated in Nile grass rats (*Arvicanthis niloticus*). OPP protected against multiple aspects of the development and progression of the disease. A significant reduction in the hyperglycemia (glucose elevation), hyperlipemia (triglyceride elevation) and hypercholesterolemia (cholesterol elevation) was observed [10].

1.4.3 Neurologic effects of OPP

The neurologic effects of Oil Palm Phenolics are of particular interest as this work uses human/rodent glial cells of the brain as an in vitro model for studying the effects of OPP and its individual components against oxidation and inflammation. The experimental results obtained in BALB/c mice are a strong evidence for the ability of OPP to cross the blood-brain barrier and exhibit bioavailability.

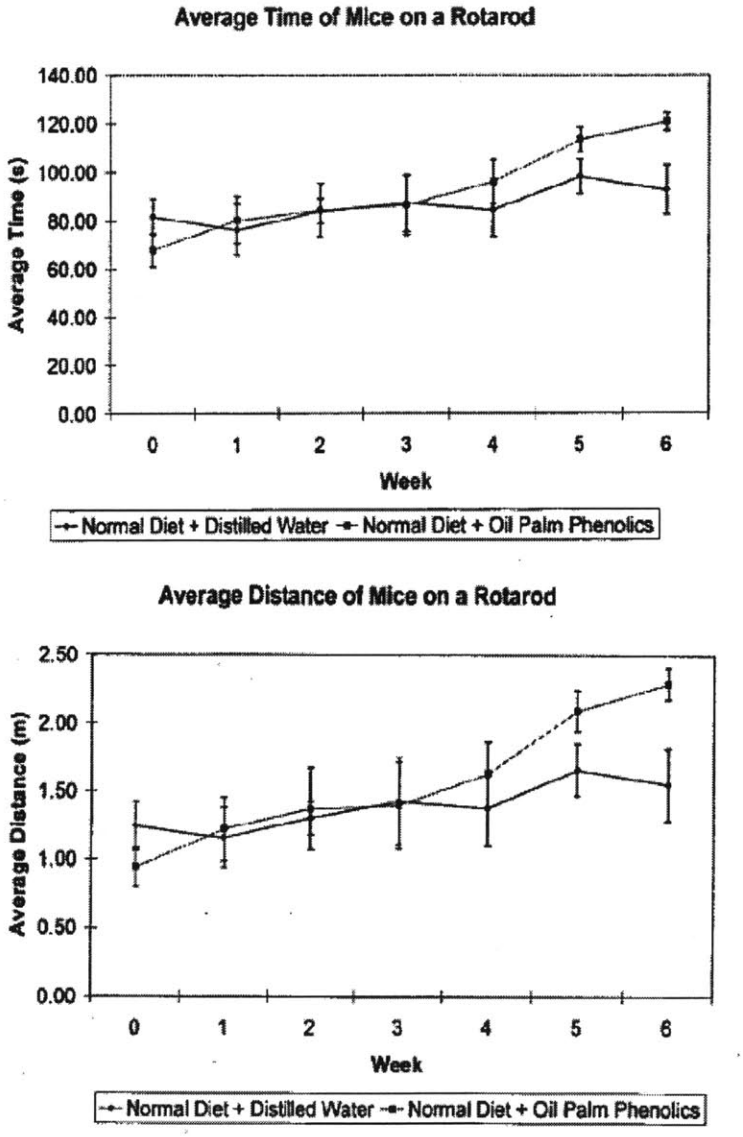
BALB/c mice were fed with OPP for 6 weeks and the control mice were given distilled water [13]. During the feeding period, the animals were tested on a rotarod and a morris water maze every week. The water maze experiment consists of allowing the mice to swim a certain distance to reach the platform. It is based on the premise that over the weeks of testing, mice with better memory will find an optimal path of shortest distance to reach the platform. Video tracking and software is used to measure the latency to platform, mean distance to reach the platform and velocity of travel. The latency and mean distance to reach the platform was significantly reduced in mice that were fed with OPP. As the velocity did not change significantly between the two groups of mice, it indicates an improvement in memory and not swimming speed by OPP. The results are shown in figure 8.

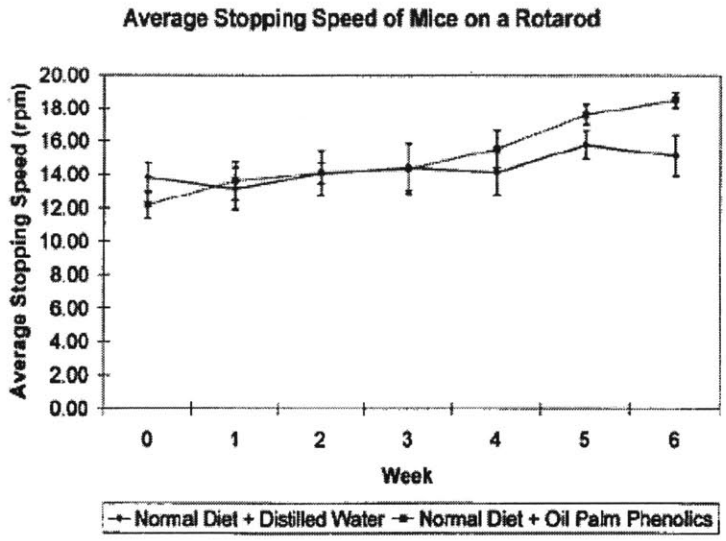
Figure 8: Effects of OPP on the Morris water maze performance of BALB/c mice [14]



The rotarod test consists of placing mice on a rotating rod that runs at two speeds (rpm) by an external motor. Average time of stay, average distance travelled on the rod and average stopping speed before falling off from the rod was higher for the OPP-fed mice. This indicates better balance and motor coordination with the passage of time by the OPP-fed mice. The results are shown in figure 9.

Figure 9: Effects of OPP on rotarod performance of BALB/c mice [14]





Microarray studies and RT-PCR (Real time-Polymerase Chain Reaction) were also performed on the cells of sacrificed mice to study the changes in gene expression due to OPP. An up-regulation in the genes that is important for neurotransmission and formation of synapses, such as a 3-fold change of Fos gene, could also be observed [14].

1.4.4 Opthalmic effects of OPP

Age-related macular degeneration is the most common cause of visual loss in the elderly population. A rodent ocular angiogenesis model was developed for evaluating the anti-angiogenesis properties of OPP [15]. Reduction in loss of retinal neuroepithelium and choroidal vascularization were also elicited by OPP.

1.4.5 Anti-oncologic effects of OPP

7-week old BALB-C mice were inoculated with tumorigenic mouse myeloma cells [10]. The animals fed with OPP *ad libitum* as the only liquid source exhibited significantly reduced tumor volume and tumor weight, although both test and OPP groups had the same levels of tumor incidence. The anti-tumor molecular mechanisms of OPP action were also studied by using microarray studies.

A brief summary of the different animal models and the results are provided in table 1.

Table 1: Experimental animal models in different organs

Organ / System	Health Benefit	Animal Model
Cardiovascular	Anti-arrhythmic effect on Ventricular Tachycardia and Ventricular Fibrillation	Wistar Kyoto rats
	Anti-Hypertensive – lowers blood pressure	NO-deficient rats
	Anti-atherogenic - reduces plaques, streak formation and number of lesions	New Zealand white rabbits
Brain	Improved cognitive function, balance and motor coordination	BALB/c mice
Metabolic (Multi-system)	Prevents development of Diabetes Mellitus	Nile rats
Neoplasia (Multi-system)	Inhibits growth of myeloma tumors	BALB/c mice
Ocular	Inhibits acute macular degeneration	BALB/c mice

1.5 Motivation

Free radical damage and reactive oxygen species have been implied in aging disorders and chronic diseases [7]. The potent antioxidant activity is believed to underlie the multiple biological effects of OPP including: reduction of blood pressure, reduced atherosclerotic arterial degeneration, reduction of glucose levels, etc. While the in vivo models explained above show the efficacy and effectiveness of OPP in different organs of the animals used, it is vital to perform cell-based and chemical-based analysis of Oil Palm Phenolics against oxidation and inflammation in order to understand the biomarkers and mechanisms of action involved in exhibiting the anti-oxidant and anti-inflammatory effects. Some of these methods are also useful to understand the interactions between the individual components of OPP, which in turn facilitate the formulation and manufacturing process development of OPP.

1.6 Thesis Organization

Chapter 2 discusses the anti-oxidative effects of OPP. The antioxidant effects of OPP were analyzed based on its free radical scavenging activity against Reactive Oxygen Species (ROS) in chemical systems, the coordinated expression of several antioxidant genes when added to U-87 astroglial cells, activation of the inherent antioxidants of the body such as glutathione, change in protein expression of activated enzymes such as Heme Oxygenase 1 and the ability to enhance cellular resistance to oxidative stress by activating the Keap1-Nrf2-ARE pathway.

Chapter 3 discusses the anti-inflammatory effects of OPP. The anti-inflammatory effects of OPP were analyzed based on its nitrite scavenging activity, reduction in protein levels of inducible nitric oxide synthase, cyclooxygenase-2 and prostaglandin-2 and the ability to reduce nuclear transcription factor NF-κB subunit p65 in a dose-responsive manner after LPS induced inflammation in BV-2 microglial cells.

Chapter 4 discusses the anti-oxidative and anti-inflammatory effects of the components of Oil Palm Phenolics for the purpose of probing the existence of synergistic interactions among the individual components of OPP and/or their derivatives. These studies were also conducted to characterize the difference between the effects of components in chemical and biological systems. The free radical scavenging activity was evaluated by using three different chemical assays: ORAC (Oxygen Radical Absorbance Capacity), TEAC (Trolox Eqivalent Antioxidant Activity) and NO generating sodium nitroprusside (SNP) system to probe $ROO^{\bullet}$, e^{-} and NO_2^{-} respectively. Biological assays to probe H_2O_2 quenching by using CAP-e (Cellular antioxidant protection in erythrocytes), PMN (Polymorphonuclear cells) and NO_2^{-} quenching by using Griess Assay in BV2 microglial cells and RAW 264.7 macrophage cells were also carried out.

Chapter 5 summarizes the work, provides conclusions and suggests future work. References are listed at the end of each chapter.

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CHAPTER 2: ANTI-OXIDATIVE ACTIVITY OF OIL PALM PHENOLICS

2.1 Introduction

A number of studies in recent years have demonstrated OPP to be a strong antioxidant, comparable with many known reducing molecules in a number of assays [1,2]. This protection from oxygen radicals may account for the documented bioactivities of OPP. Given the demonstrated reducing potential of OPP and numerous reports of antioxidant activity by related polyphenolic compounds, I set out to characterize OPP's ability to protect cultured cells from oxidative stress.

It has been suggested that the ingestion of food polyphenols may constitute a strategy for protection from neurodegenerative disorders [3,4]. It is known that in brains of those suffering from most such diseases, there is a chronic and growing condition of oxidative stress, which may amplify DNA damage and protein aggregation, leading to progression of the disease. It is thought that reducing the amount of oxidative radicals in the brain might act as a preventative and/or slow the progression of an already present disease [5]. Astrocytes, being the major cell type in the brain, and containing the highest levels of glutathione and other antioxidant molecules, provide the best model for study in this case.

2.2 Primary Antioxidant Effect: Free Radical Scavenging Activity

OPP has an intrinsic antioxidant activity due to its ability to directly scavenge free radical species. I have evaluated the free radical scavenging activity of OPP by using two different assays: TEAC (Trolox Eqivalent Antioxidant Activity) and ORAC (Oxygen Radical Absorbance Capacity).

2.2.1 TEAC (Trolox Equivalent Antioxidant Activity)

The TEAC Assay is an electron transfer (ET) based assay in which the probe (oxidant) and an electron from the antioxidant react to form a reduced probe and oxidized antioxidant. The probe used in the TEAC assay is ABTS•+ that has an absorbance of 0.7±0.02 at 734 nm and the reduced probe is the colorless $ABTS^{2-}$. The assay is based on the inhibition by antioxidants of the absorbance on the radical cation of ABTS [6]. Hence, the assay utilizes only the inhibition percentage obtained in a short span of time to estimate the antioxidant activity.

Materials and Methods

Stock Solution

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) is dissolved in deionised water to 7 mM concentration. Potassium persulfate is dissolved in deionised water to 2.45 mM concentration. Fresh stocks of ABTS•+ solution are prepared every five days due to self-degradation of the radical.

The Working Solution

The two stock solutions are mixed in equal quantities and allowed to react for 12~16 h at room temperature in the dark. For the evaluation of antioxidant activity, the ABTS•+ working solution is diluted with water to obtain the absorbance of 0.7±0.02 at 734 nm.

Sample preparation

OPP samples from six batches with phenolic solid contents varying from 1.5% to 9% are utilized. Trolox standard with concentration of 0 to 4 mM are used. The dilution factor for each of the OPP samples is chosen such that they lead to radical scavenging activity of 10 to 80% in the 15 minutes.

Then, the TEAC value is calculated by using the formula:

$$TEAC\ value\ \left(\mu.\frac{mol}{g}\right) = \frac{Slope\ of\ OPP\ sample}{Slope\ of\ Trolox}$$

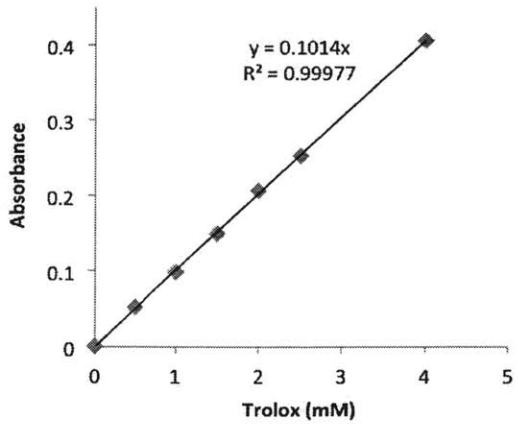
Detection

After vortex mixing 198μL ABTS•+ and 2μL sample solution for exactly 30 s the absorbance reading at 765 nm is taken exactly 1 min after initiation of mixing and thereafter at time intervals of 1, 2, 5, 10, and 15 min.

Results

The standard curve generated between the Trolox concentration and Absorbance value is shown in figure 1. A linear relationship with high R^2 value of 0.9998 could be observed. This justifies the use of Trolox as a standard in this experiment.

Figure 1: Standard curve between the Trolox concentration and Absorbance value



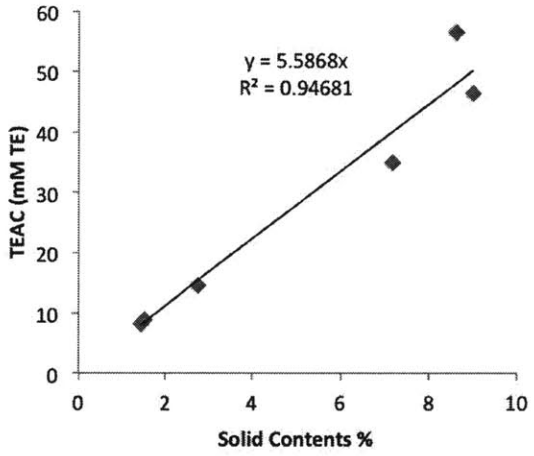
As shown in table 1, increasing solid content of OPP leads to a concomitant rise of its ability to directly scavenge electrons, which is reflected in the Trolox Equivalent (TE) value.

Table 1: Electron Transfer of OPP (TEAC Assay)

OPP Batch	1	2	3	4	5	6
Solid content	1.45%	1.5%	2.76%	7.18%	8.61%	8.98%
Dilution times	6	6	12	32	32	32
TE (mM)	8.13	8.83	14.58	34.87	56.57	46.42

Figure 2 shows the correlation of the concentration of phenolic compounds in OPP and the TEAC value (mM Trolox Equivalent) that represents the free radical scavenging activity. Some differences occur with various batches, as the final sources of antioxidants in OPP are determined by several factors such as fruit bunches, seasonal changes, etc.

Figure 2: Correlation between the solid contents % and TEAC value (mM Trolox Equivalent)



By using the formula, the TEAC value is found to be 559 μmol TE/g OPP.

Conclusion

As there is a clear linear relationship with R^2 value of 0.95 between the phenolic compounds in OPP and TEAC value, the dissolved solids of OPP exhibit electron-scavenging activity.

2.2.2 ORAC (Oxygen Radical Absorbance Capacity)

The ORAC assay is a hydrogen atom transfer (HAT) based assay in which the antioxidant and probe compete for the thermally generated peroxyl radicals ROO$^{\bullet}$ by the decomposition of azo compounds [7]. Antioxidants inhibit the oxidation of fluorescein by hydrogen atom transfer. The method combines free radical inhibition percentage and the inhibition time of antioxidants by using the area under curve (AUC) equation for each antioxidant.

$$AUC = 1 + \frac{f_1}{f_0} + \frac{f_2}{f_0} + \frac{f_3}{f_0} + \frac{f_4}{f_0} + \cdots + \frac{f_n}{f_0}$$

where f_i is the fluorescence at time 'i' and the experiment is carried out upto 'n' minutes.

As the ORAC measurement combine both inhibition time and inhibition percentage of the free radical damage by the antioxidant into a single quantity, it is considered as a robust assay for measuring the antioxidant capacity. In the study described below, the assay is used to measure the antioxidant capacity of OPP liquid solution. The ORAC values of OPP solutions formed by dissolving OPP spray dried powder and OPP freeze dried powder are also determined.

Materials and Methods

Working Solutions

A fluorescein solution is prepared daily in sodium phosphate buffer (75 mM concentration, pH 7.4) by diluting the stock fluorescein to a final concentration of 0.08 μM. 0.4 g of (2,2'-azobis(2-amidino-propane) dihydrochloride) AAPH is dissolved in 10 mL of 75 mM phosphate buffer to a final concentration of 150 mM and kept in the dark in an ice bath. Any extra AAPH is discarded after 8 h.

Detection

100 μL of 0.08 μM fluorescein is added to each well of a black microplate. 45 μL of phosphate buffer is also added to the wells. Finally, 25 μl phosphate buffer (blank), trolox standard (6.25–50 μM) or sample is added to each well. The microplate is preheated at 37 °C for 15 min. The 96-well multi-detection plate reader is set to run fluorescence kinetic read with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. 30 μl of AAPH solution is added to each well and the kinetic read is started immediately. The assay temperature is 37°C with a run duration of 1 h. The experiment was repeated thrice.

Calculation

The final ORAC values are calculated by obtaining a standard curve between the Trolox concentration and the net area under the fluorescein decay curve so that the samples

could be expressed as Trolox equivalents of micromole per liter or per gram. The area under curve (AUC) was calculated as:

$$AUC = 1 + \frac{f_1}{f_0} + \frac{f_2}{f_0} + \frac{f_3}{f_0} + \frac{f_4}{f_0} + \cdots + \frac{f_{35}}{f_0}$$

where f_0 is the fluorescence at time '0', f_i is the fluorescence at time 'i'. The experiment values upto '35' minutes are used in the equation as the fluorescence values for all samples and Trolox standards reach the blank value by 35 minutes.

Net AUC is obtained by subtracting the blank AUC from the AUC of the antioxidant.

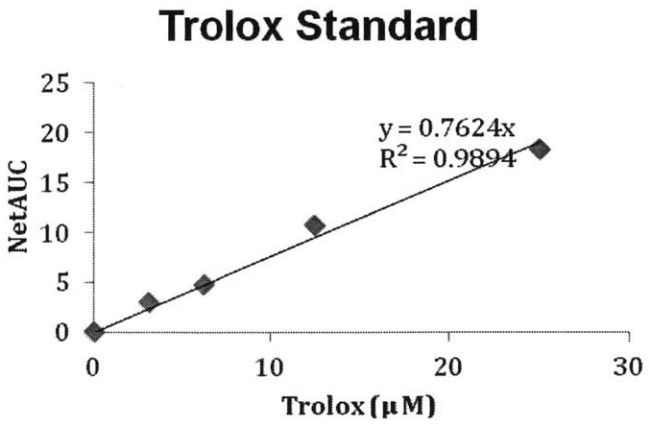
$$\boldsymbol{Net\ AUC = AUC_{antioxidant} - AUC_{blank}}$$

Results

The standard curve generated between the Trolox concentration and Net AUC is shown in figure 3. A perfectly linear relationship could be seen between the AUC and Trolox. This justifies the use of Trolox as a standard in this experiment.

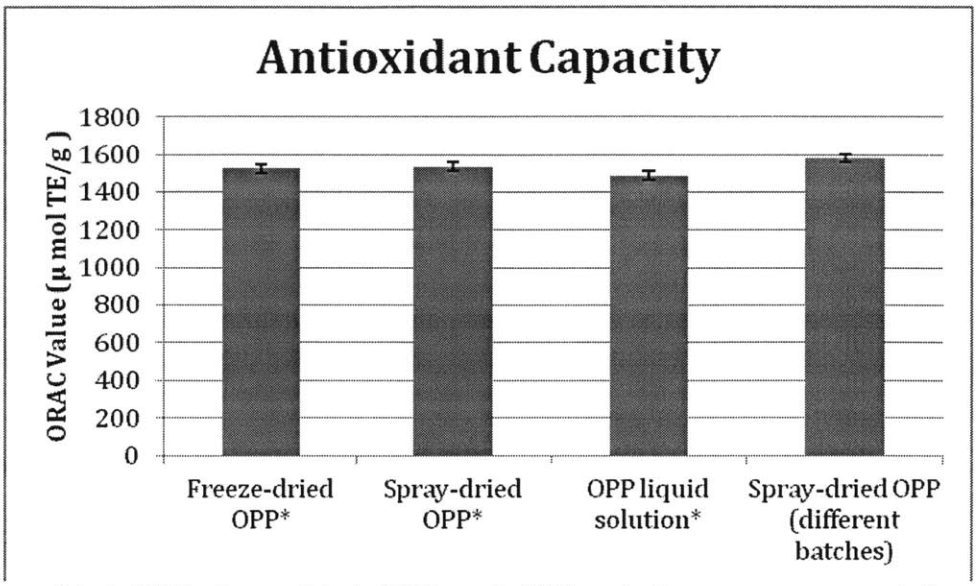
As the concentration of the antioxidant compound increases, oxidation of fluorescein is prevented and this leads to higher fluorescence value and higher AUC. Fluorescence is lost with increasing time, as the excess azo compounds oxidize fluorescein after the antioxidants have all donated the hydrogen atoms to fluorescein. The AUC measure combines both time and concentration of treatment.

Figure 3: Standard curve between the Trolox concentration and Area Under the Curve (AUC)



By using the standard equation above, the ORAC values of OPP solution, spray dried OPP and freeze dried OPP was obtained. The results are shown in figure 4.

Figure 4: Antioxidant capacity of different OPP forms (Mean ± SE) ‡



* Freeze-dried OPP, Spray-dried OPP, and OPP solution were prepared from same batches of OPP. In order to validate the experimental results across batches, the average value from spray-dried OPP of different batches of OPP were also included.
‡ No significant differences were observed using student's t-test ($p < 0.05$)

Conclusion:

The method of drying has economic implications and can be a crucial determinant of quality. Freeze-drying process involves sublimation of the water from the solids by the use of refrigerated vacuum. It is a costly and time-consuming process that usually leads to high quality drying, as heat is not involved. A grinding process usually follows the freeze-drying step.

In the spray drying process, a liquid feedstock is atomized into a spray of fine droplets and then brought into contact with the hot drying gas at sufficient temperature for the moisture evaporation to take place. As the moisture evaporates from the droplets, the solid product is formed, and the powder is readily recovered from the drying gas. The advantages of the spray drying process are the high speed, low cost and easy scale-up.

Some polyphenols are unstable with increased susceptibility to heat and light. The combination of heat and oxygen during the spray drying process is attributed as the cause for the degradation of the polyphenolic compounds, such as the anthocyanins in blueberry [8]. The degradation of the phenolic compounds in turn leads to the reduction of ORAC values.

However, all OPP samples have similar ORAC value with no significant difference based on a student's t-test. This result indicates that the spray-drying process does not have an effect on the antioxidant capacity of OPP. Hence the phenolic compounds present in OPP are heat-stable.

2.3 Secondary Antioxidant effect: Antioxidant genes and enzymes

One of the potential protective effects of astrocytes is the induction of a family of genes encoding phase II antioxidant enzymes, including hemeoxygenase-1 (HO-1), Glutathione S-transferase (GST), γ-glutamyl cysteine ligase (γ-GCL), and NADPH quinone oxidoreductase [9]. These enzymes are also recognized as antioxidant enzymes due to their role in maintaining redox balance, thiol homeostasis, and excretion of reactive metabolites, such as peroxide, epoxides, aldehydes, and quinones. The purpose of this study is to perform an oxidative stress 96-array qPCR to further elucidate the secondary effect of OPP at the gene level to confirm microarray experiment results and check the possibility of other genes that are regulated by OPP.

Apart from the qPCR experiment, the microarray results of genes involved in the glutathione system were exclusively analyzed to understand the effect of OPP in this system.

Materials and Methods

Cell culture

U-87 MG astroglia were purchased from ATCC, aliquoted and frozen in liquid nitrogen. Cells were grown in Eagle's Minimum Essential Medium (Lonza, Walkerville, MD USA) with 10% heat-inactivated fetal bovine serum (Sigma), 1 mM sodium pyruvate (Lonza) and 100U penicillin/streptomycin (Lonza).

Standard incubator conditions were used (37C, 5% CO2). Cells were detached from growth flasks using trypsin/EDTA (Lonza), spun down, resuspended in EMEM and seeded into tissue culture-treated assay plates for all experiments.

Total RNA extraction

Total RNA was purified from U-87 cells directly or after treatment with 800 μg/mL OPP for 12h using the RNeasy Mini Plus Kit (QIAGEN), which includes a step to reduce genomic DNA contamination of the samples. The resulting total RNA extracts were run on the Agilent 2100 bioanalyzer to determine concentration and ensure RNA integrity. This total RNA was used for the qPCR experiments.

Quantitative PCR

Total RNA from 3 biological replicates were subject to reverse transcription using iScript cDNA Synthesis Kit (Bio-Rad) and amplification using the HotStart-IT SYBR Green qPCR Master Mix 2x (Affymetrix). Diluted cDNA mixed with the Master mix was added to the wells of Human Stress Response 96 StellArray qPCR Array (Lonza). Each primer pair in this qPCR array had been validated to ensure specificity and sensitivity. Detection of the SYBR Green dye was achieved using a DNA Engine Opticon Real-Time System (MJ Research). Melting curves were performed post-

amplification to verify single cDNA products and to check for the presence of primer dimers. 18s rRNA control in the qPCR array was used as the normalizer and degree of change was calculated for each gene using the delta-delta C_t method.

Microarray studies and analysis

Gene expression analysis was achieved using Human U133 2.0A DNA chips (Affymetrix). Total RNA samples were processed to reduce rRNA according to the manufacturer's protocols, labeled, hybridized and scanned using an Affymetrix 7G scanner.

The scanned CEL files were processed and RNA normalized with the Expression Console 1.0 software (Affymetrix), and the resultant CHP files were imported into ArrayStar 3.0 software (DNAStar) for heatmap, clustering analysis. The exported results were further examined using Microsoft Excel and the GenMAPP 2.1 software package.

Results

Among the 96 genes tested using qPCR, 30 were up-regulated and 2 were down-regulated. Peroxiredoxin 2 with a 2.8 fold change was the most up-regulated gene and Eosinophil Peroxidase with a -2.2 fold change was the most down-regulated gene (table 2, 3). All the up-regulated genes belonged to a family of antioxidant enzymes, DNA repair or essentially had some other protective role.

Quantitative PCR of several genes of interest corresponded with the microarray data, although the degree of change tended to be generally underestimated by the microarray relative to the qPCR results. 10 stress response genes were up-regulated at 12h in U-87 cells after treatment with 800 μg/mL OPP by a minimum 1.4x fold change in qPCR and a minimum 1.2x fold change correspondingly in microarray (table 4). No gene was correspondingly down regulated in both microarray and qPCR.

The microarray results of antioxidant enzymes show small but synchronized increase of antioxidant-related molecules and enzymes by 12h to increase capacity of the cell to deal with reactive oxygen species. Time-dependent response turns on somewhere between 6h and 12h of treatment. The breakdown product biliverdin is converted into bilirubin, which is a strong antioxidant. This is also evident from the expression levels of biliverdin reductase, which is the enzyme responsible for the conversion of biliverdin to bilirubin [10]. Biliverdin reductase B (cytoplasmic) is upregulated 1.3 fold at 12 h and Biliverdin reductase A (liver) is downregulated 1.3 fold at 12h (table 5).

The microarray results of glutathione-related genes showed a mild up-regulation of glutathione synthase (GSS) and glutamate-cysteine ligase, catalytic subunit (GCLC). Moreover, the increase in glutathione peroxidase (GPX) without a concomitant increase in glutathione reductase (GSR) proves that the up-regulation of GPX is not a result of generation of oxidative stress by OPP, since the induction of GR to recycle GSH from GSSG would be expected in such a case [11]. These results are tabulated in table 6.

Table 2: Most up-regulated genes at 12h in U-87 cells after treatment with 800 µg/mL OPP with minimum 1.4x qPCR fold change*

Gene ID	qPCR
PRDX2	2.8
DNAJA4	2.5
FOXO1	2.5
HSPB2	2.4
HMOX1	2.2
CTNNA1	2.1
HSPA1L	2.1
HSF2	2.0
MBD4	2.0
CDH1	1.9
HSP90AA1	1.9
TP53	1.8
GPX3	1.8
CYR61	1.7
RAC1	1.7

Gene ID	qPCR
XPA	1.7
GPX1	1.7
MSH6	1.6
CAT	1.6
DNAJB4	1.6
DNAJB9	1.6
MSH2	1.5
PRDX1	1.5
CTNNB1	1.5
MAPK8	1.5
HSPB1	1.5
PRDX6	1.5
SOD1	1.5
GSTM4	1.4
HIF1A	1.4

Table 3: Most down-regulated genes at 12h in U-87 cells after treatment with 800 µg/mL OPP with minimum -1.4x qPCR fold change*

Gene ID	qPCR
APP	-1.6
EPX	-2.2

Table 4: Most up-regulated stress response genes at 12 h in U-87 cells after treatment with 800 µg/mL OPP (minimum 1.4x fold-change qPCR and minimum 1.2x fold-change microarray)*

Gene ID	qPCR	Microarray
PRDX2	2.8	1.4
HMOX1	2.2	2.3
XPA	1.7	1.7
GPX1	1.7	1.3
HSPB1	1.5	2.1
MAPK8	1.5	1.4
SOD1	1.5	1.3
PRDX1	1.5	1.2
GSTM4	1.4	1.9
HIF1A	1.4	1.4

Table 5: Microarray results of antioxidant genes at 6h and 12h in U-87 cells after treatment with 800 µg/mL OPP*

Gene ID	6h	12h
BLVRA (liver)	0.739	0.762
BLVRB (cytoplasmic)	0.939	1.321
CAT	0.827	1.148
SOD1 (cytosolic)	0.924	1.257
SOD2 (mitochondrial)	0.942	1.426
SOD3 (extracellular)	0.941	1.008
TXNRD1 (cytoplasmic)	1.127	1.480
TXNRD2 (mitochondrial)	0.971	1.031
TXNRD3 (multiple)	0.842	0.716

Table 6: Microarray results of genes involved in glutathione synthesis and regulation at 12h in U-87 cells after treatment with 800 µg/mL OPP*

Gene ID	Microarray
GSS	1.4
GSR	0.9
GSTA4	1.5
GSTK1	2.3
GSTP1	2.3
GSTM4	1.9
GSTM1	1.6
GPX4	1.6
GCLC	1.2
GCLM	1.2
G6PD	2.3
SLC25A11	2.3

* (Table 2, 3, 4, 5, 6) All values are significantly different from the control ($p < 0.01$ using Tukey-Kramer's Honestly Significant Difference)

Conclusion

In this work, I have demonstrated that OPP also acts as a secondary antioxidant in that it induces the selective expression of several antioxidant enzymes, such as heme oxygenase 1 (HO-1). These enzymes act upon electrophilic, xenobiotic material, conjugating glutathione or other moieties to the molecules in order to mark it for export from the cell. The phytochemical compounds in OPP, though harmless, surely elicit this kind of response. Perhaps it is this heightened state of protection which prepares the cells very well for conditions of oxidative stress.

2.4 Tertiary Antioxidant effect: Glutathione System

In gene expression research of U-87 MG astrocytes, I have observed a secondary antioxidant effect arising from the increased expression of key enzymes involved in glutathione (GSH) synthesis and regulation. GSH has important functions as an antioxidant for cellular defense against ROS. GSH reacts directly with radicals in non-enzymatic reactions and is the electron donor in the reduction of peroxides. Previous studies have shown that an adequate GSH content is essential for the astrocyte-mediated neuro-protection because of the continuous glial delivery of GSH and/or GSH precursors to neurons for GSH synthesis [9].

Healthy cells maintain ~90% reduced form GSH and the rest is the inactive form GSSG [12]. Therefore, the effects of OPP on GSH and GSSG levels in U-87 astrocytes were evaluated.

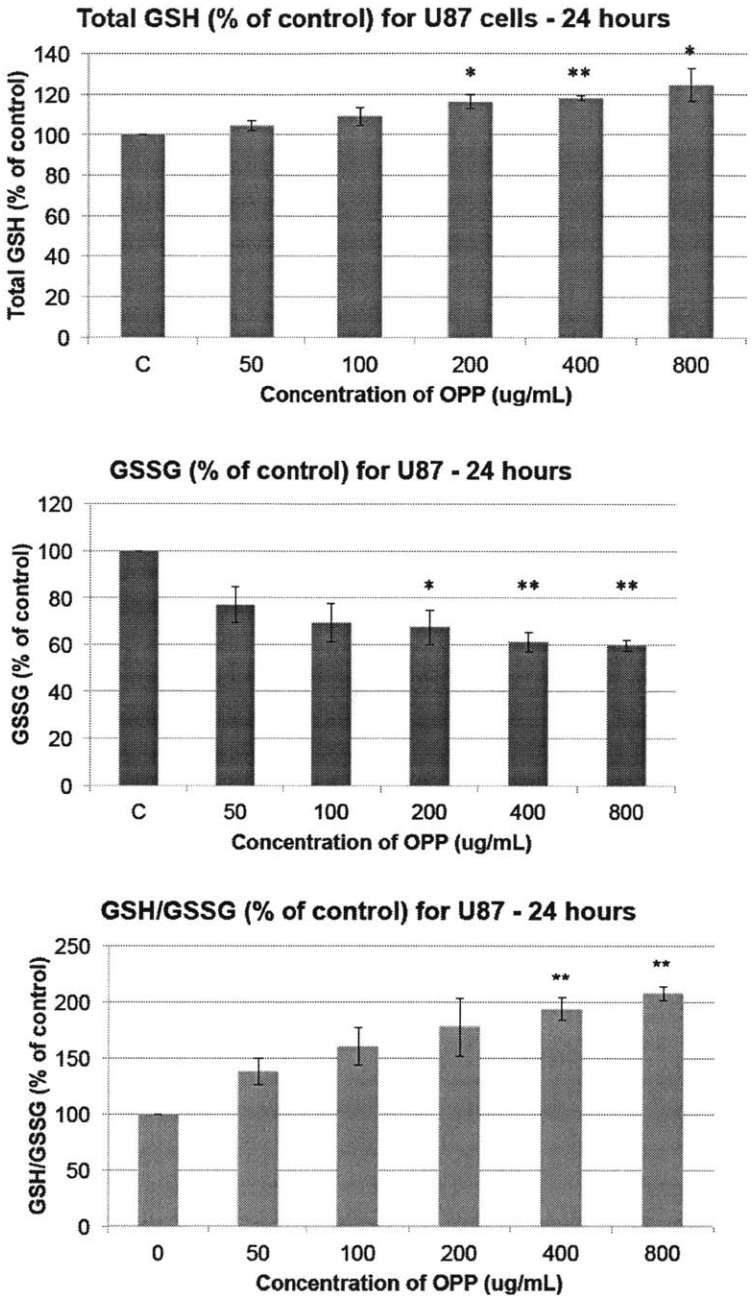
Materials and Methods

Glutathione is determined by a Cayman's GSH assay kit (Cayman Chemical) using an enzymatic recycling method. U-87 cells were cultured as described before and treated with OPP of different concentration for 24 hours. The cells were then concentrated and de-proteinated. GSH was then measured by the reaction of sulfhydryl group of GSH with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) to produce 5-thio-2-nitrobenzoic acid (TNB). Glutathione reductase was added, which in turn reduced GSTNB that is produced along with TNB, to produce more TNB. Concentration of GSH in the sample is a measure of the rate of production of TNB, which was measured as the absorbance at 405 nm. As glutathione reductase was used in the kit, total glutathione is the measure of both GSH and GSSG. However, exclusive quantification of GSSG was also achieved by incubating sample with 2-vinylpyridine (Sigma) and by carrying out the protocol for total GSH.

Results

To determine the effect of OPP on the cell glutathione metabolism, GSH and GSSG levels were examined at 24h after treatment of U-87 MG cells with various concentrations of OPP. As shown in figure 5, OPP treatment induced a concentration-depended increase in the total GSH level of U-87 MG cells. High concentration OPP (800 μg/ml) increased the total GSH level by 30% relative to untreated cells. Even in the present of low concentration OPP (50 μg/ml), a 5% increase of total GSH level was observed in U-87 MG cells (figure 5). Interestingly, OPP also caused a clear decrease in the GSSG levels of U-87 MG cells with the increase of OPP concentration (figure 5). Compared to the untreated cells, only 60% GSSG remained in the cells treated with 800 μg/ml OPP. As a result, with the increase of OPP concentration (50 ~ 800 μg/ml), the GSH/GSSG ratio of astrocytes increased. After 24 h of 800 μg/ml OPP treatment, the ratio is two times higher than that from the untreated cells (figure 5). Therefore, OPP has the ability to regulate astrocyte glutathione metabolism and consequently affects the redox status of the cell.

Figure 5: Effect of OPP on GSH-GSSG levels in U-87 MG astrocytes (Mean ± SE)



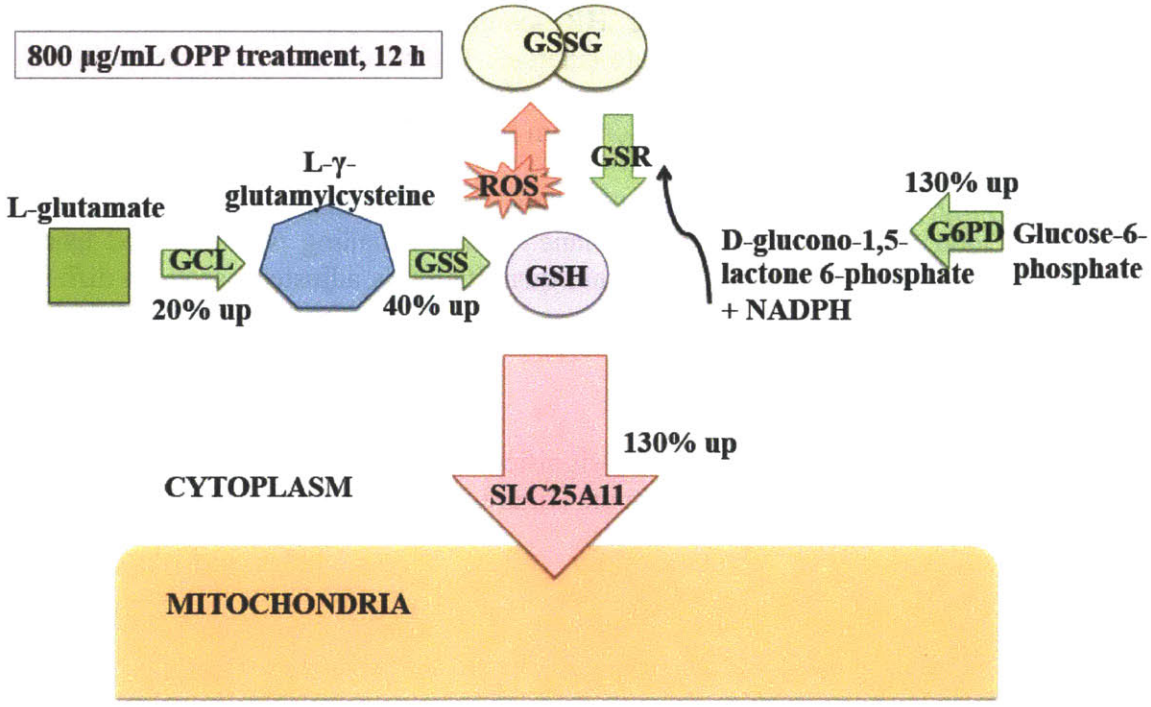
* Values are significantly different from control (P<0.05; Tukey-Kramer's HSD test)
** Values are significantly different from control (P<0.01; Tukey-Kramer's HSD test)

Conclusion

Addition of OPP in the cell culture media induced a modest increase in the expression of three key genes involved in glutathione biosynthesis in U-87 MG astrocytes, glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit

(GCLM), and glutathione synthetase (GSS). Besides, recycling the inactive dimer form (GSSG) back into active GSH requires one mole of NAPDH in order to move forward. The primary source of NAPDH is the pentose phosphate pathway, specifically the first committed reaction, in which glucose-6-phosphate dehydrogenase (G6PD) oxidizes glucose-6-phosphate and produces one mole of NADPH. Our gene expression results show that G6PD is up-regulated significantly in response to OPP, which should result in more available NADPH and drive the GSR reaction forward, generating more active GSH. The increased expression of key enzymes involved in glutathione (GSH) synthesis and regulation leads to a 30% increase in total glutathione (GSH) level, and a decrease in inactive glutathione (GSSG) level in U-87 MG cell line after 24h OPP treatment. As a result, cellular GSH/GSSG ratio, an index directly related to oxidative stress, increases about 2 times in U-87 MG cell line. Consequently, OPP might possess potential protection effects on neurons by inducing an increase in intracellular available glutathione (GSH) levels. A glutathione regulation model by OPP is shown in figure 6.

Figure 6: Model of glutathione regulation by OPP



2.5 Quarternary Antioxidant effect: Protein expression of antioxidant enzymes and key transcription factors

In the previous sections, the antioxidant effects of OPP at the radical, gene and molecule levels were explained. The gene expression changes may be controlled by master transcription factors. Gene regulation could also lead to or accompany protein expression changes of antioxidant enzymes. The purpose of the following section is to analyze the protein level changes in U-87 cells.

2.5.1 Protein expression of Heme Oxygenase-1 (HO-1)

Heme oxygenase-1 (HO-1) is an important antioxidant enzyme that is expressed in response to oxidative stress and plays an important protective role in the brain [13]. Microarray data show that HO-1 is one of the most up-regulated genes in OPP treated U-87 cells. The result was further confirmed by RT-PCR. In order to verify that up-regulated mRNA level results in higher protein levels, we examined the HO-1 protein level of U-87 cells treated by 100 μg/ml OPP using western blot.

Materials and Methods

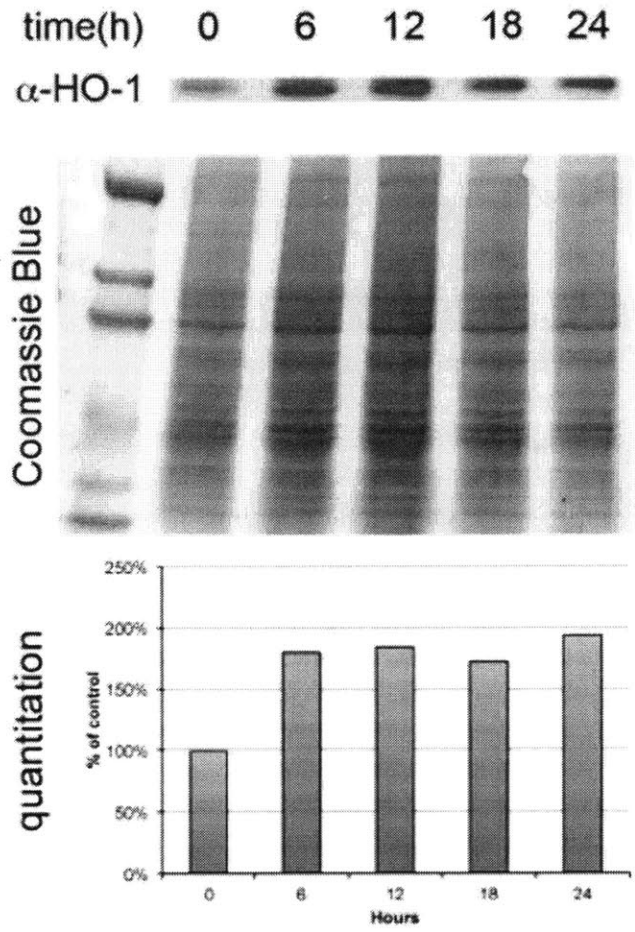
U-87 MG cells are grown using EMEM that was supplemented with various concentrations of OPP based on total density (μg/mL). These cells are washed with PBS and then lysed using RIPA buffer (Upstate) containing mammalian protease inhibitors (Amresco) to give whole cell lysate (WCL). These samples are then separated out using standard SDS-PAGE Ready Gels (Bio-Rad), the bands transferred to PVDF membrane (Millipore), and then blotted using HRP-conjugated antibodies for detection with SuperSignal West Pico ECL Substrate (Pierce). The results are documented in a light-sealed cassette using X-ray film and developed using a Kodak XOMAT. Bands are then quantitated using the ImageJ application from NIH.

After blotting, the membranes are stained using a modified Coomassie Blue dye to visualize total protein loading in each lane. Relative loading is determined using the ImageJ application, and each band's final quantitation is adjusted based on differences in relative loading.

Results

We found that this protein showed a significant expression in response to OPP at a concentration of 100 μg/ml upto 24 hours (figure 7). The protein level is high by 6 hours and this effect is exhibited upto 24 hours.

Figure 7: Levels of heme oxygenase 1 protein (HO-1) are increased in U-87 cells that are treated with 100 μg/mL OPP in a time-dependent manner, as shown by western blotting



Conclusion

Among the highly up-regulated antioxidant enzymes, heme oxygenase 1 (HO-1) has attracted special attention because of its potential protective effect against neurodegenerative diseases. HO-1 is a redox-sensitive inducible stress protein catalyzing the first and rate-limiting step in heme degradation [14]. HO-1 has been reported to produce several antioxidative compounds, including biliverdin and bilirubin for protection of neuronal cells against oxidative stress [10]. In addition, the biological significance of HO-1 as a cellular antioxidant has been confirmed by using gene-knockout and transgenic mice [15]. Thus, the induction of HO-1 in brain could represent a potential defensive mechanism for neurodegenerative disorders and pathologic brain aging. A variety of compounds could act as neuroprotective compounds by transcriptionally up-regulating the expression of HO-1 including curcumin, carnosol and rosolic acid [16, 17]. In this study, U-87 astrocytes treated by OPP exhibited a 2.2-fold increase in HO-1 mRNA (table 2), as well as a 2-fold increase in protein expression (figure 7). These results suggest OPP is capable of inducing HO-1 expression, therefore increasing a cellular resistance to oxidative injury.

2.5.2 Induction of nuclear factor erythroid 2-related factor 2 (Nrf2)

It is well documented that many of these antioxidant-related genes are under control of a master transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2) [18]. When activated by conditions of oxidative stress, this protein translocates from the cytoplasm to the nucleus and binds to antioxidant response elements (AREs) in the promoter DNA of phase II antioxidant enzymes, up-regulating their expression. Considering the coordinated expression of these genes in response to OPP, it is likely that Keap1-Nrf2-ARE pathway is activated by the active components of OPP, resulting in increased protection against oxidative stress. Since a great number of the genes regulated by OPP are indeed under the control of the nuclear factor erythroid 2-related factor (Nrf2) master transcription factor, we assayed nuclear extract fractions of OPP-treated cells.

Materials and Methods

Nuclear extract preparation

After treatment with 100 μg/mL OPP and/or hydrogen peroxide, nuclear extract (NE) fraction and S100 cytosolic fraction are prepared from samples of approximately 10^6 isolated U-87 MG cells using a Kontes dounce homogenizer and ultracentrifugation as previously described in [19]. Due to the small number of cells, the original protocol is modified to use microcentrifugal filters (Millipore, Billerica, MA) for solvent exchange rather than dialysis.

Western blotting

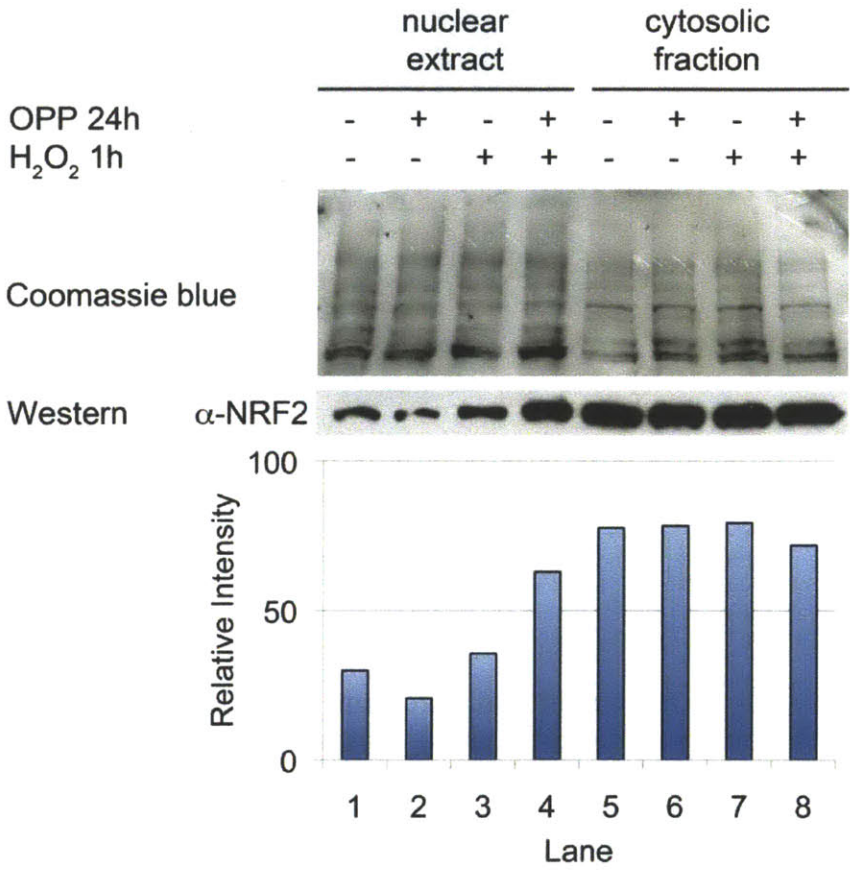
18 μg of treated and untreated NE and S100 cellular fractions are run on a 4-15% Tris-Glycine gel using SDS-PAGE and the protein bands are transferred to PVDF membrane using a lateral transfer tank and apparatus. Membranes are blocked using 0.5% casein in TBS/0.1% Tween 20 (TBST) and washed three times with TBST between steps. Polyclonal anti-NRF2 (C-20) antibody from rabbit (sc-722, Santa Cruz Biotechnology, Santa Cruz, CA) is used as the primary antibody at a 1:1000 dilution, and horseradish peroxidase (HRP)-conjugated polyclonal anti-rabbit IgG from goat (sc-2030, Santa Cruz) was used as the secondary antibody at a 1:10000 dilution. Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used to detect the antibody using X-ray film. After processing of the Western blot, the membrane was stained with Coomassie blue R-250, destained and scanned to demonstrate equal protein loading in each lane.

Results

Treatment of U-87 MG glioblastoma cells with OPP showed activation of the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) which could consequently increase induction of a number of antioxidant response and phase II antioxidant genes (figure 8). Pretreatment of cells with OPP resulted in an enhanced

activation of NRF2 in response to oxidative stress, which suggests a preconditioning effect of OPP.

Figure 8: Pretreatment of U-87 cells with 100 µg/mL OPP results in a significantly greater activation of NRF2 in response to oxidative stress

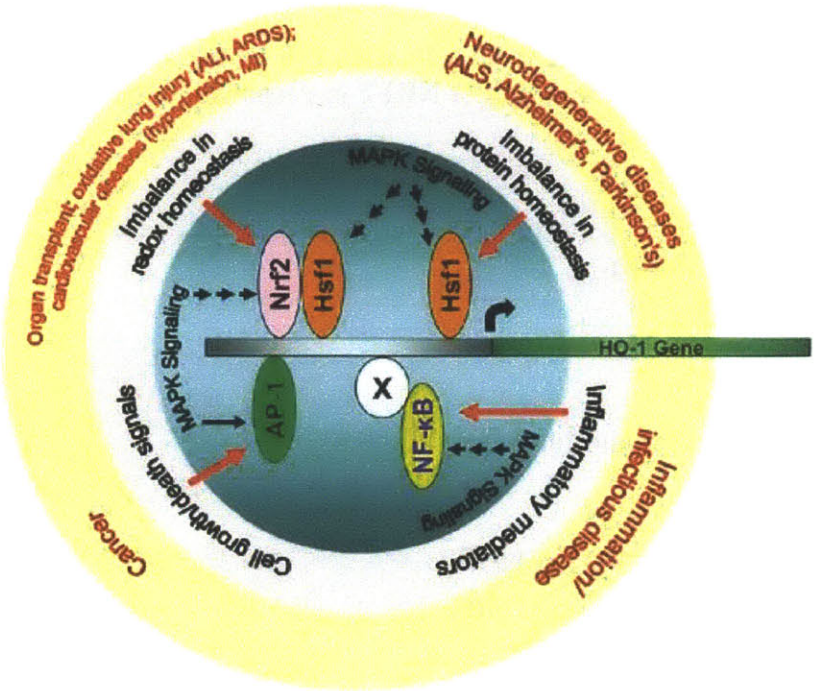


2.6 Discussion

Curcumin strongly enhances Nrf2-mediated expression of HO-1 and other Phase II detoxification proteins in protected PC 12 cells and rat prefrontal cortical neurons from Aβ-induced apoptosis [20,21]. Moreover, other plant-derived phenolic compounds such as carnosol [16], and rosolic acid [17], have been reported to have neuroprotective effects by strongly activating the expression of HO-1. OPP treatment enhanced the expression of a series of phase II antioxidant enzymes in U-87 cells, which are all regulated by Nrf2 signal pathway. Hence it is speculated that OPP has the ability to induce the translocation of Nrf2 from cytoplasm to nucleus and activates the expression of corresponding phase II antioxidant enzymes.

The activation of the HO-1 gene by the various signaling pathways is shown in figure 9. MAPK signaling can lead to activation of AP-1, Nrf2, Hsf1 or NF-κB, which in turn lead to the upregulation of the HO-1 gene.

Figure 9: Regulation of HO-1 gene by the various signaling pathways [22]



Cite: Alam, J., & Cook, J. L./2007/How many transcription factors does it take to turn on the heme oxygenase-1 gene?/*American Journal of Respiratory Cell and Molecular Biology*/36(2)/166-174.
Official journal of the American Thoracic Society.

Polyphenols such as resveratrol and curcumin are great antioxidant molecules, but generally suffer from poor solubility and/or bioavailability [23-25], since the body treats them as xenobiotic materials and works quickly to detoxify them. If their value as antioxidants rests in their ability to act as radical scavengers, they are a hopeless class of compounds. However, I have shown that the presence of OPP can induce genetic and protein-level changes within a few hours, even at low concentrations, which should contribute to protection of the cell from oxidative stresses. If polyphenols have any physiological benefit, it is likely that that benefit comes from the compounds working on multiple levels simultaneously; not only as primary antioxidants, but leaving longer-lasting effects as secondary and tertiary antioxidants as well [26].

In summary, my study demonstrated that the antioxidant capacity of OPP is mediated by three mechanisms: (1) directly scavenging free radicals; (2) up-regulating phase II antioxidant enzymes especially Heme oxygenase-1, which also leads to the induction of protein expression of Heme Oxygenase-1; (3) increasing total cellular glutathione level and decreasing inactive glutathione level. Further, this study suggests that OPP could possess the ability to activate the Keap1-Nrf2-ARE pathway, subsequently enhancing cellular resistance to oxidative stress and leading to protection from stress-related deterioration and disease development.

2.7 References

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CHAPTER 3: ANTI-INFLAMMATORY ACTIVITY OF OIL PALM PHENOLICS

3.1 Introduction

Inflammation in the central nervous system (CNS) increases both during normal aging [1,2] and in age-related neurodegenerative diseases. Neuroinflammation is caused by the activation of glial cells, the astrocytes and microglia, which produce cytokines and other inflammatory mediators [3,4].

The classical signs of redness, heat, swelling, pain, and loss of function characterize inflammation, a complex series of responses to tissue injury and infection. A normal inflammatory response is an acute process that resolves after removal of the inciting stimulus [5]. However, the normal inflammatory response may progress to chronic inflammation due to a long-term failure of corrective response, leading to severe tissue damage. In the CNS, microglia and astrocytes are the main participants in the tissue-damaging chronic inflammatory response [5].

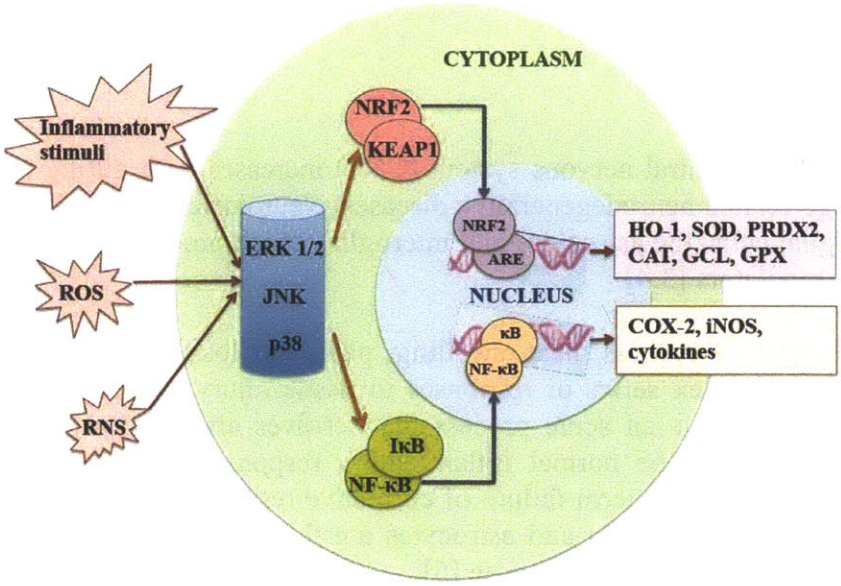
Neuroinflammation is largely mediated by activated microglial cells, which constitute up to 20% of the cell population in the brain [6,7]. The activation of microglia has been attributed to enhanced signal transduction, leading to the induction of the expression of inflammatory enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), and transcription factors such as nuclear factor-kB (NF-kB) [8,9].

Activated microglia generate high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [10, 11]. The inability to balance the production and elimination of ROS results in oxidative stress [12]. NO belongs to a class of inter- and intra-cellular messenger molecules that are small, highly diffusible, gaseous, and highly reactive. The cytotoxic effects of NO are due to a combination of elevated concentration (>10 mM) and the formation of the highly reactive compound peroxynitrite [13].

Oxidative stress-mediated inflammation has been linked to neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD) [11, 14]. Activated microglia overexpressing IL-1 are readily detected in the brains of AD patients [15]. Furthermore, highly activated microglia, though not astrocytes have been found in the substantia nigra of PD brains [16].

Polyphenols are known to be anti-inflammatory in nature. Similar to the anti-oxidant activities of polyphenols, the anti-inflammatory activities are also exhibited through gene targets (inducible nitric oxide synthase), molecular targets (cyclooxygenase, lipoygenase) and cellular targets (macrophages, lymphocytes, endothelial and epithelial cells) [17]. Polyphenols are able to downregulate the pro-inflammatory cytokines such as iNOS and COX-2 by the reduction of NF-κB and MAPK levels [18,19,20]. The signaling pathways in response to oxidative stress and inflammatory signals are shown in figure 1.

Figure 1: Signaling pathways of oxidative stress and inflammatory stimuli



The BV-2 cell line is frequently used as a model microglial system [21]. LPS can activate BV-2 cells, which signals through its Toll-like receptor (TLR-4) [22]. Signal transduction via TLR-4 elicits a cascade of intracellular events, including the transcription of inflammatory genes [23]. The model of LPS-activated microglia has been widely used as an in vitro system for the study of mechanisms underlying neuron damage by various mediators released from activated microglia [24]. Therefore, the present in vitro study investigates the effects of OPP on the inflammatory mediators such as NO and the production of iNOS and COX-2 by LPS-activated BV-2 microglial cells.

LPS activates the transcription factor NF-κB in microglia [25]. NF-κB is crucial in controlling the expression of many pro-inflammatory genes and has been recognized as an important drug target for the treatment of inflammatory diseases [26]. Recently, considerable attention has been focused on dietary and medicinal phytochemicals, such as sources of NF-κB-inhibiting components [27]. Therefore the effects of OPP on the protein levels of the transcription factor NF-κB are also investigated after treating BV-2 cells with LPS.

3.2 Effect of OPP on the morphology, size and phagocytosis in microglia

One phenotype of BV-2 microglial cells is that they can engulf beads via phagocytosis [28,29,30]. ROS-depended pathways govern phagocytosis and the process is dependent upon nitric oxide (NO) and requires activation of p38 MAPK as can be seen in figure 1. Antioxidants such as resveratrol have been shown to affect phagocytosis in LPS-stimualted cells [28].

In order to study if there are any differences in cell size, cell count and morphology after treatment with OPP, an image analysis assay was developed.

Materials and Methods

Cell culture

BV-2 mouse microglia were kindly provided by Dr. Jae-Shyong Hong (NIEHS, NC, U.S.A). They are aliquoted and frozen at -80C. Cells are grown in Dulbecco's Minimum Essential Medium (Sigma) with 10% heat inactivated fetal bovine serum (Sigma) and 100U penicillin/streptomycin (Lonza).

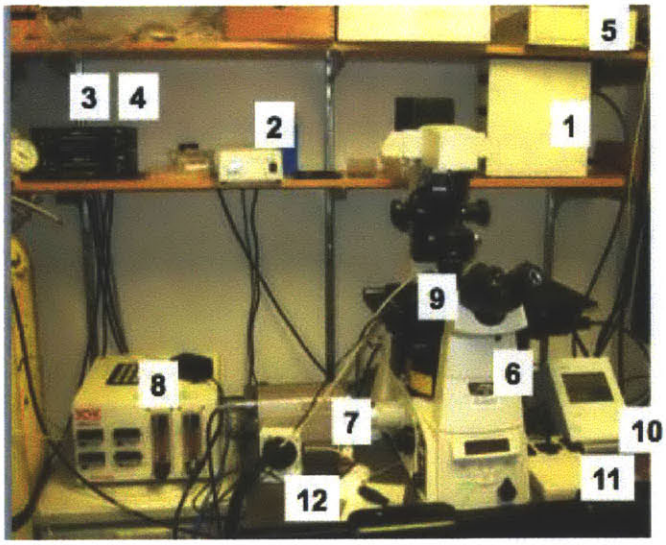
Standard incubator conditions are used (37C, 5% CO_2). Cells are detached from growth flasks using trypsin/EDTA (Lonza), spun down, resuspended in DMEM and seeded into 6 well plates.

Bright field and Texas Red λ microscopy

OPP of different concentrations (50 to 800 μg/mL) is added to the cells in the presence and absence of lipopolysaccharide (LPS; 1 μg/mL). Images of the cells are taken on Nikon Eclipse TI at 24 hours and the experiment is repeated twice.

The Nikon Eclipse TI equipment has a perfect focus system and is compatible with 6 to 96 well microplates. The image of the system is shown below in figure 2.

Figure 2: Nikon Eclipse Ti System



1. **Fluorescence source**
2. **Bright field source**
3. **Shutter**
4. **Shutter**
5. **Stage**
6. **Scope**
7. **Camera**
8. **Humidity, CO2, temp controller**
9. **Environmental chamber**
10. **Scope hand pad**
11. **Joystick**
12. **Perfect focus dial**

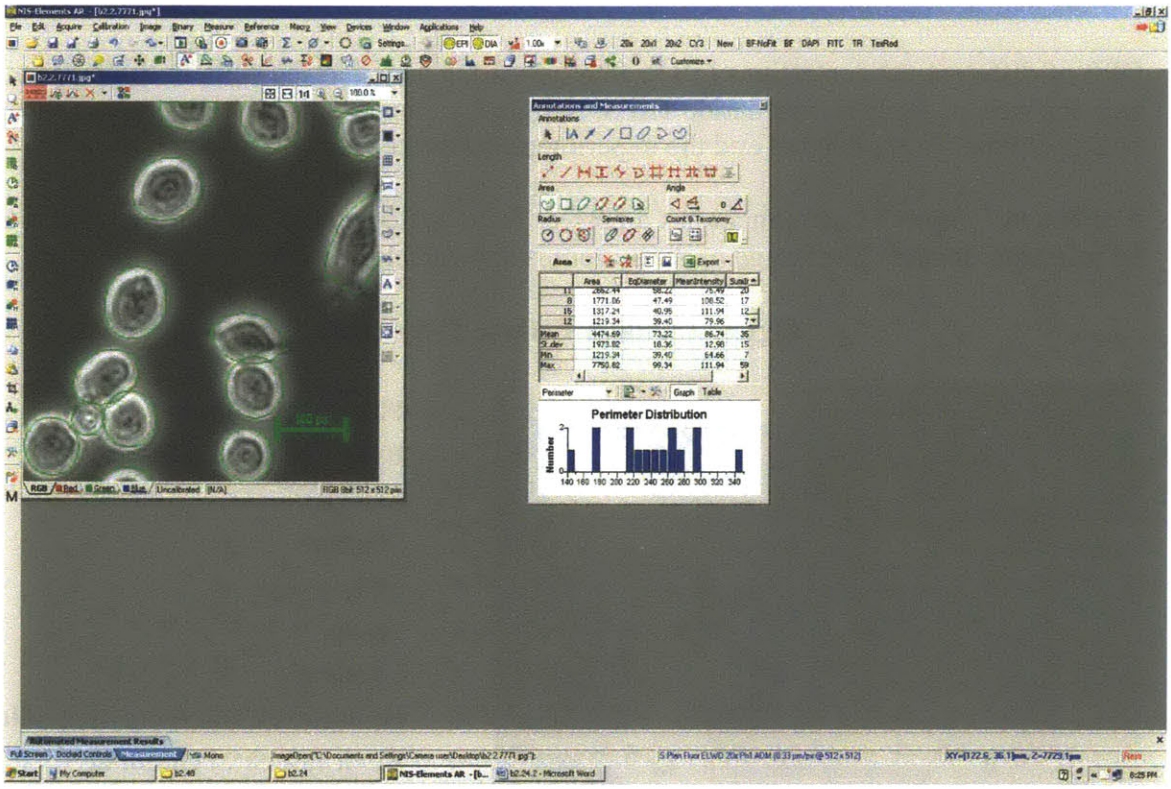
For the phagocytosis assay, fluorescent beads are added after 24 hours treatment with OPP in the presence or absence of LPS. The cells are incubated overnight. Just before the images are taken, excess beads are removed and the cells are fixed in formaldehyde.

Apart from taking the images, it is also possible to control the temperature at 37°C and maintain humidity and CO_2 at the required level by using the Nikon Eclipse TI. This ensures that the cell effects are not due to change of environmental conditions.

Image Analysis

After the images are obtained using the microscope, they are analyzed by using the NIS Elements software. Cell area and percentage of area occupied by cells is measured. To obtain the percentage of area occupied by cells, all the cell areas are added and divided by the total area of the image window (pixels^2). A typical image on NIS Elements is shown in figure 3.

Figure 3: A typical image (OPP 200 µg/ml - 24 hours) on NIS Elements



Results

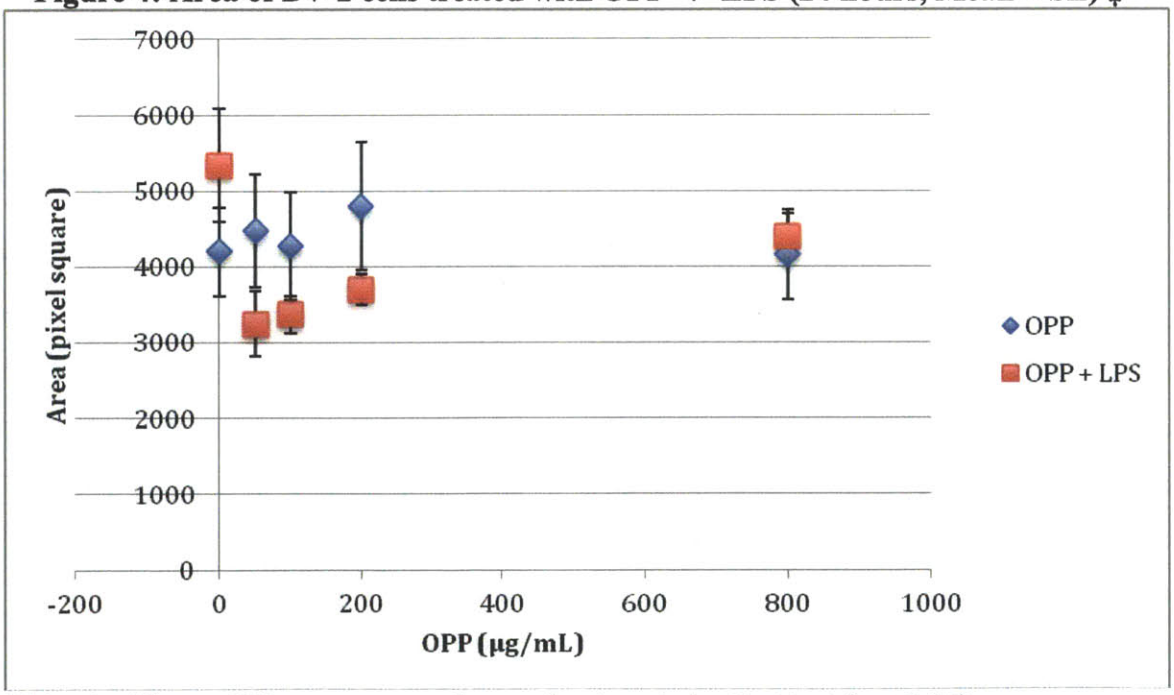
The results of average of three experiments are shown in table 1. OPP of different concentrations did not lead to an increase/decrease in average area of the cells when compared to the control cells that did not have OPP or LPS. Addition of LPS lead to an increase in average cells area that was nullified by the addition of OPP along with LPS. However, there was no significant difference between the two groups. These results are shown in figure 4.

Table 1: Effects of OPP and LPS on cell area and percentage of cells in a given area

Condition	Average area +/- SD (pixel square)	Average number of cells*	Average % area occupied*
C	4201.7 +/-1638.55	8	0.15891
OPP 50	4474.69 +/-1973.82	7	0.15798
OPP 100	4273.9 +/- 2134.78	9	0.15566
OPP 200	4793.72 +/-2067.99	6	0.14964
OPP 800	4154.73 +/-1784.71	9	0.16658
LPS	5334.93 +/-1819.75	6	0.14849
OPP 50 & LPS	3253.87 +/- 1121.38	7	0.14078
OPP 100 & LPS	3373.97 +/- 930.72	14	0.15366
OPP 200 & LPS	3698.85 +/- 606.47	9	0.16008
OPP 800 & LPS	4400.5 +/- 887.75	9	0.16692

* No significant differences were observed using student's t-test ($p < 0.05$)

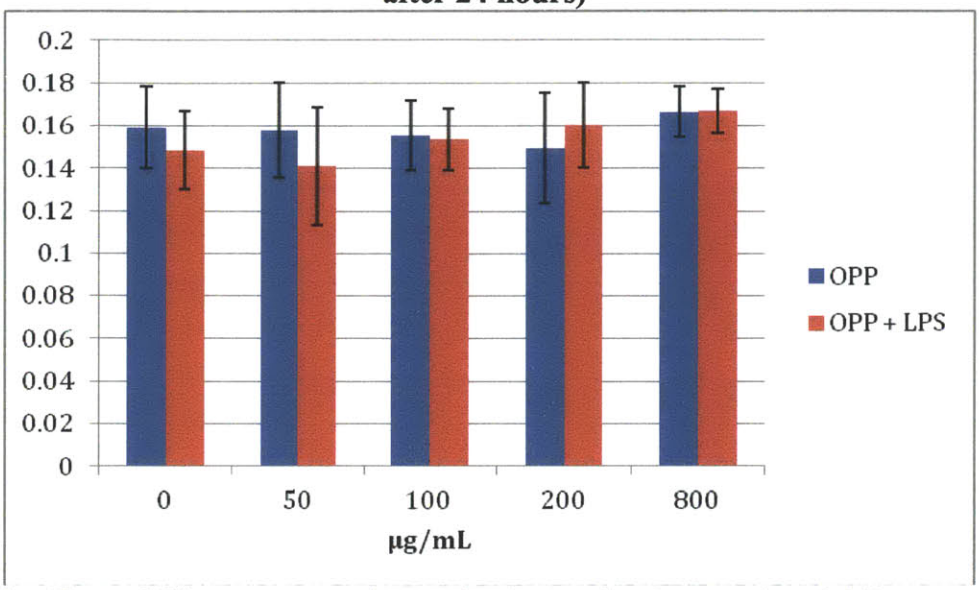
Figure 4: Area of BV-2 cells treated with OPP +/- LPS (24 hours, Mean ± SE) ‡



‡ No significant differences were observed using student's t-test ($p < 0.05$)

It could be seen that there was no significant change in percentage of area occupied by cells or number of cells in an image window (figure 5). Hence, OPP or LPS does not lead to proliferation of cells.

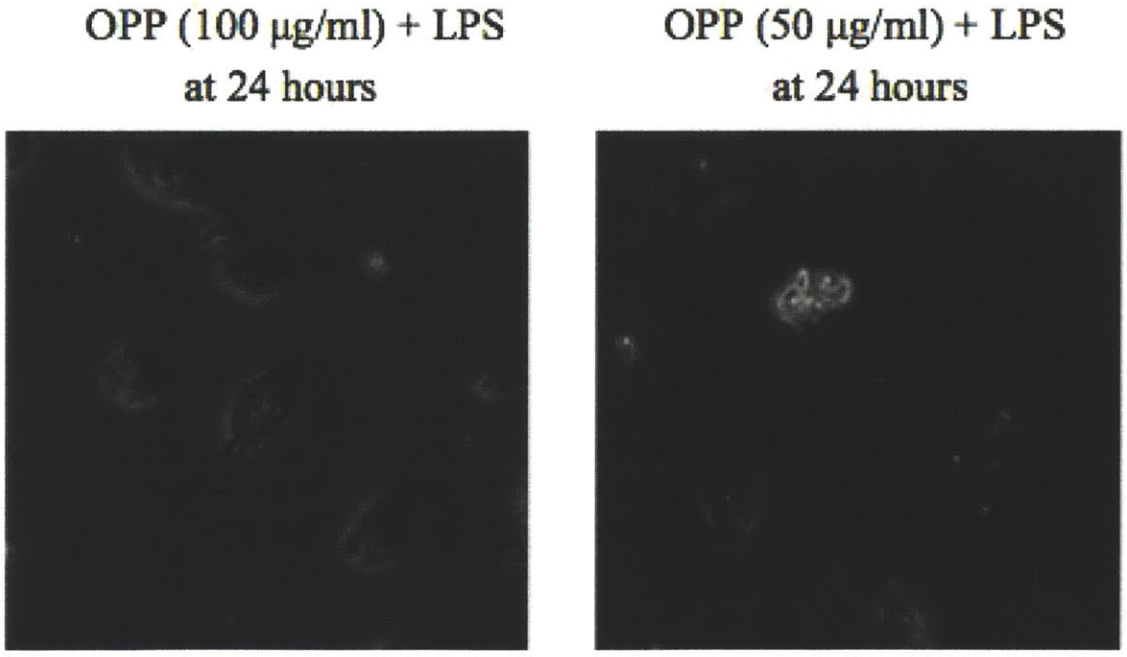
Figure 5: % Area occupied by BV-2 cells treated with OPP +/- LPS (Mean ± SE after 24 hours)*



* No significant differences were observed using student's t-test ($p < 0.05$)

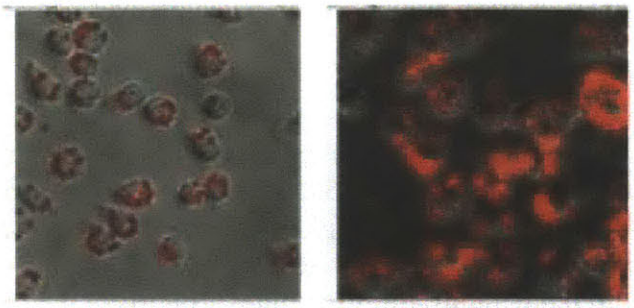
Another interesting observation is the existence of phagosome-like structures when the cells were treated with OPP and LPS. Two images are shown in figure 6.

Figure 6: Phagosome-like structures in images of cells treated with OPP and LPS

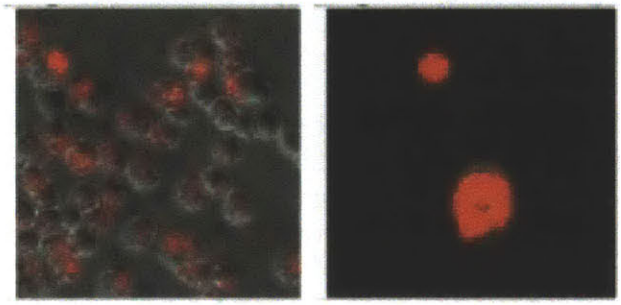


When latex beads are added overnight, there was more phagocytosis in cells that had LPS when compared to the cells without LPS. These images are shown in figure 7.

Figure 7: Phagocytosis after overnight incubation with latex beads



a) Control b) LPS



c) 800 μg/mL OPP d) LPS + 800 μg/mL OPP

Conclusion

There were no significant changes in the area and number of BV-2 cells after adding LPS and OPP. Phagocytosis was present in the cells and the fluorescent intensity was highest in the cells with LPS and OPP treatment.

3.3 Nitrite Quantification

Nitric oxide is an important messenger molecule in influencing inflammatory responses [13]. The Griess colorimetric assay is a method to measure the levels of nitrite (NO_2^-) in aqueous solutions. In our study, the assay is used to measure the nitric oxide production in LPS-stimulated cells, as NO_2^- is one of the two primary stable and nonvolatile breakdown products of nitric oxide (NO).

Materials and Methods

Working solutions

1x Griess Reagent: 250 mL water is added to the modified Griess reagent (Sigma-Aldrich). The contents are mixed by inversion of the bottle for 5 mins. The reconstituted solution can be stored at room temperature for 3 months.

Sodium nitrite (100 mL, 0.1 M): 0.68995 g of $NaNO_2$ is added to a volumetric flask, dissolved in some water (after vortexing) and water is added till volume reaches 100 mL. The solution is stored at 4° C

Sodium nitrite (10 mL, 100 uM): To 10 μL of 0.1M $NaNO_2$ solutions, 10 mL of buffer used for samples is added. A fresh solution is prepared on every day of testing.

Cell viability

The CellTiter Glo Luminescent Cell Viability assay (Promega) is used to test the cell viability. The optimum number of cells (50000/well) was plated overnight. 90 μL of media with/without 1 μg/mL LPS and 10 μL of sample/media were added to the cells after aspirating the existing media. After 24 hours of incubation, the contents of the plate are equilibrated at room temperature for 30 minutes, 100 μL of the CellTiter Glo reagent are added and the luminescence of each well is recorded.

Griess Assay Sample preparation

BV-2 cells are seeded and grown in 24-well plates using the same conditions as above. Each well received 750 μL of media with addition of OPP over a range of concentration and LPS (1 μg/mL) to make a total volume of 1 mL per well. After 24 hours of incubation, 50 μL of medium is removed from each well thrice to 3 wells of a 96 well plate.

Standard curve for the Griess Assay

In order to generate a standard curve, sodium nitrite solution from concentration 0 to 100 μM is used as the standard. Absorbance at 540 nm is measured and a plot of absorbance versus concentration is generated. The line equation of this plot is used to determine the nitrite concentration of samples.

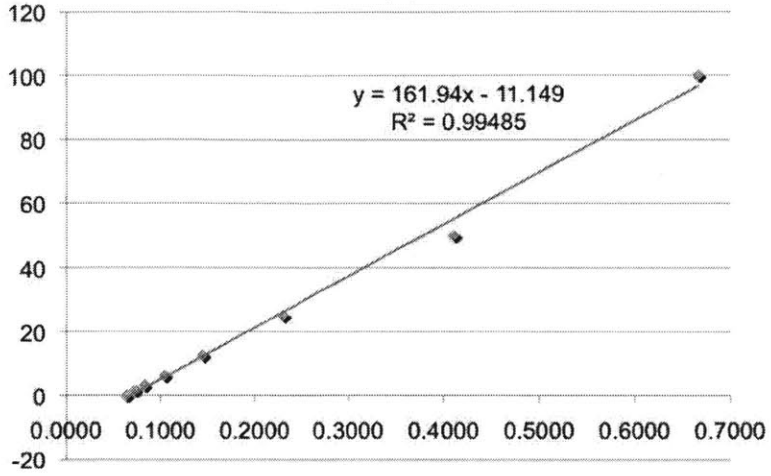
Nitrite (or) nitric oxide measurement using Griess Assay

The spontaneous oxidation of nitric oxide leads to the formation of nitrite (NO_2^-) in biological systems. To measure the nitrite concentration, 50 μL of the 1x Griess solution is added to each of the 96 wells to which 50 μL of medium is also added. The plate is incubated at room temperature for 15 minutes and absorbance at 540 nm is measured immediately after incubation.

Results

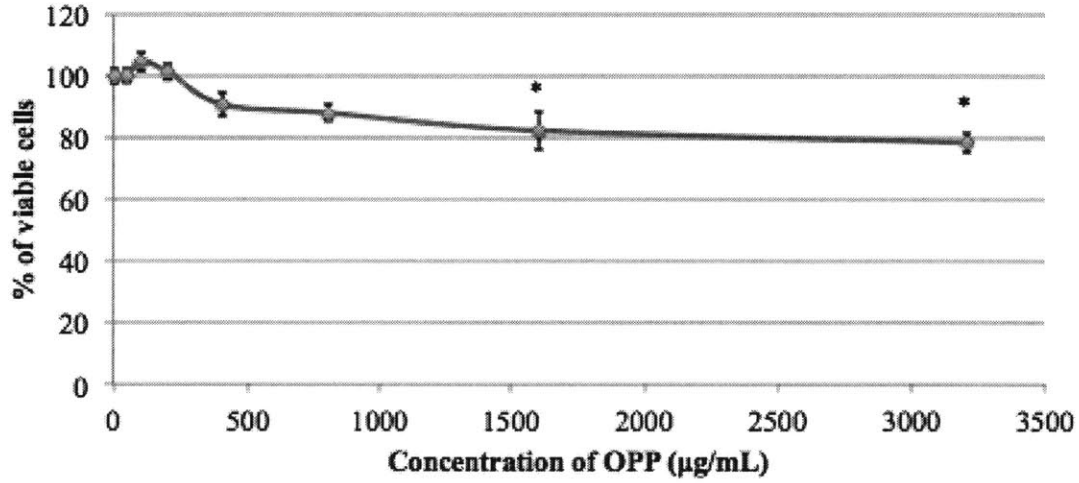
The standard curve generated between the nitrite concentration and absorbance is shown in figure 8. A linear relationship could be seen between the absorbance and nitrite. This justifies the use of sodium nitrite as a standard in this experiment.

Figure 8: Standard curve between sodium nitrite concentration (Y), absorbance (X)



The cell viability results are shown in figure 9.

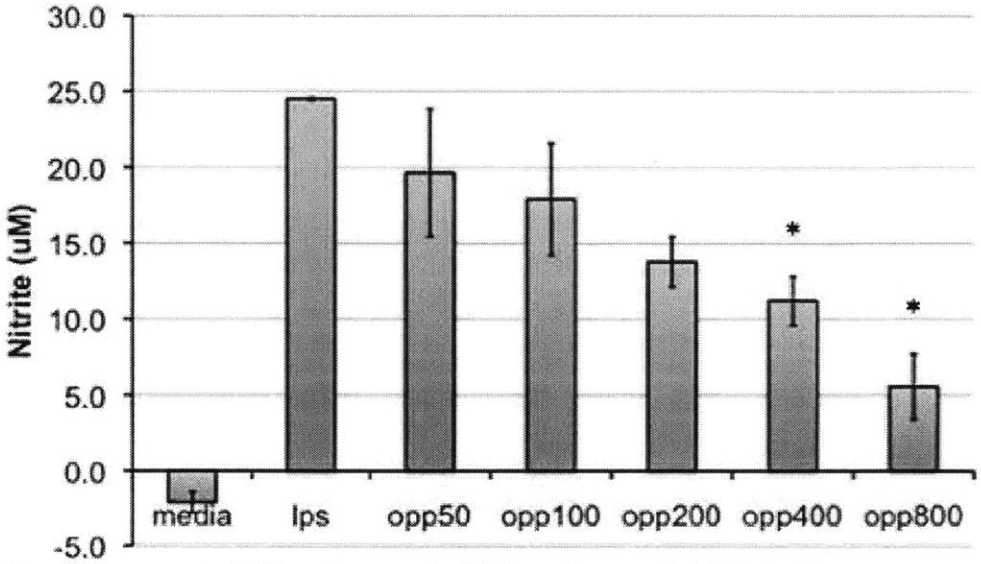
Figure 9: Viability of BV-2 cells treated with OPP (0 to 3200 µg/mL) and LPS (1 µg/mL) for 24 h (Mean ± SE)



* $p < 0.05$ with respect to LPS-only sample (Tukey-Kramer's HSD test)

More cells die with an increase in OPP concentration when compared to the LPS-only treatment. This means that the total NO production that is dependent on the total number of cells actively secreting NO will change. Hence, the cell viability must be taken into account in order to properly compare NO reduction over a range of OPP concentration. Each NO value should be divided by the fraction of cells left alive with LPS + OPP compared to LPS alone to obtain a corrected value for each data point. The cells + media only is used as the zero value so that the contribution of LPS to elevate NO production over unstressed cells can be measured. The cell viability adjusted nitrite levels are shown in figure 10. A dose-dependent decrease of nitrite is seen with the amount of OPP added to the BV-2 cells.

Figure 10: BV-2 cells treated with OPP (μg/mL) + 1 μg/mL LPS (Griess assay, Mean ± SE, 24 h)*



* $p < 0.05$ with respect to LPS-only sample (Tukey-Kramer's HSD test)

Conclusion

A previous lab study indicated that multi-component phenolics from Oil Palm Phenolics (OPP) are effective in preventing oxidant-induced hydro peroxide response deficits in U-87 cells. The present study shows that inflammation-induced activation of microglia is prevented when OPP is added. Results indicate that treatment with OPP reduces the production of the inflammatory mediator nitric oxide (NO) in cell-conditioned media from lipopolysaccharide (LPS)-activated BV-2 microglia.

3.4 Protein Expression by Immunoblot Analysis

Methods and Materials

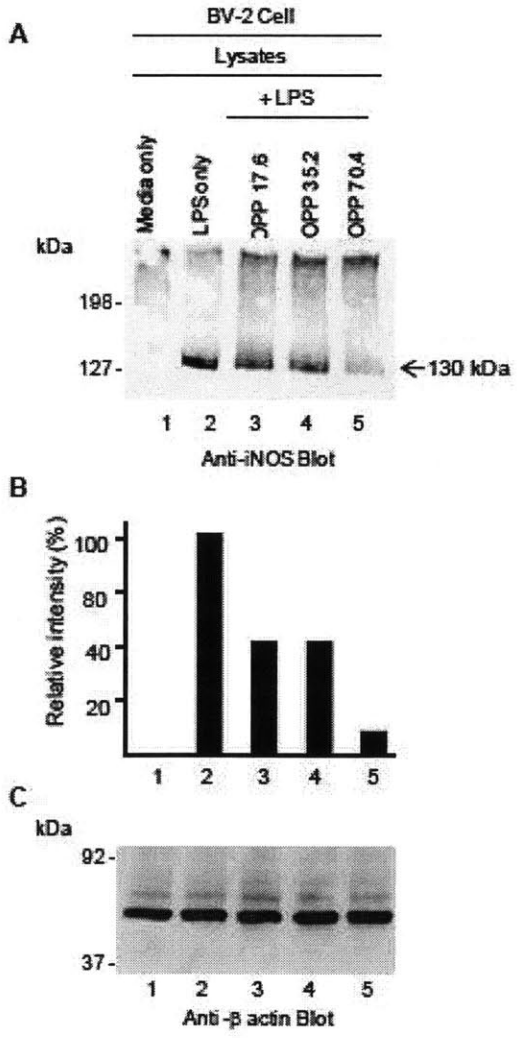
Cell culture conditions and treatments are the same as those used in nitrite assays using griess reagent. After cell-conditioned media is collected for nitrite measurements, cells are washed twice with PBS and 100 μl of CelLytic-M mammalian cell lysis-extraction reagent (Sigma-Aldrich) is added to each well. Cells are lysed at 4°C with rocking for 1 hr and centrifuged at 12,000g for 15 min at 4 °C. Cell lysates are collected, protein concentration is determined by the DC protein assay (Bio-Rad). Equal amounts of denatured protein samples (10 μg per lane) are separated by 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The reagents for immunoblotting assay, except for the primary antibodies, are from enhanced chemiluminescence. Primary antibody for COX-2 (Santa Cruz Biotechnology), iNOS (Upstate Cell Signaling), Prostaglandin-2 (Cayman Chemical) and NF- κB (Santa Cruz Biotechnology) are purchased. The results are documented in a light-sealed cassette using X-ray film and developed using a Kodak X-OMAT. Bands are then quantitated using the ImageJ application from NIH.

Results

3.4.1 Inducible Nitric Oxide Synthase

OPP suppressed the protein expression of iNOS and COX-2. OPP dose responsively inhibited the LPS-induced increase in iNOS protein levels (figure 11). The protein levels of iNOS are undetectable in untreated control cells. Upon LPS stimulation, a dramatic increase in the protein expression of iNOS is observed in the BV-2 cells. At the lowest concentration (100 µg/ml), OPP treatment is not able to reduce the LPS-stimulated iNOS protein expression. However, at higher concentration (800 µg/ml), OPP treatment significantly reduces the protein levels of iNOS in LPS-activated cells (figure 11).

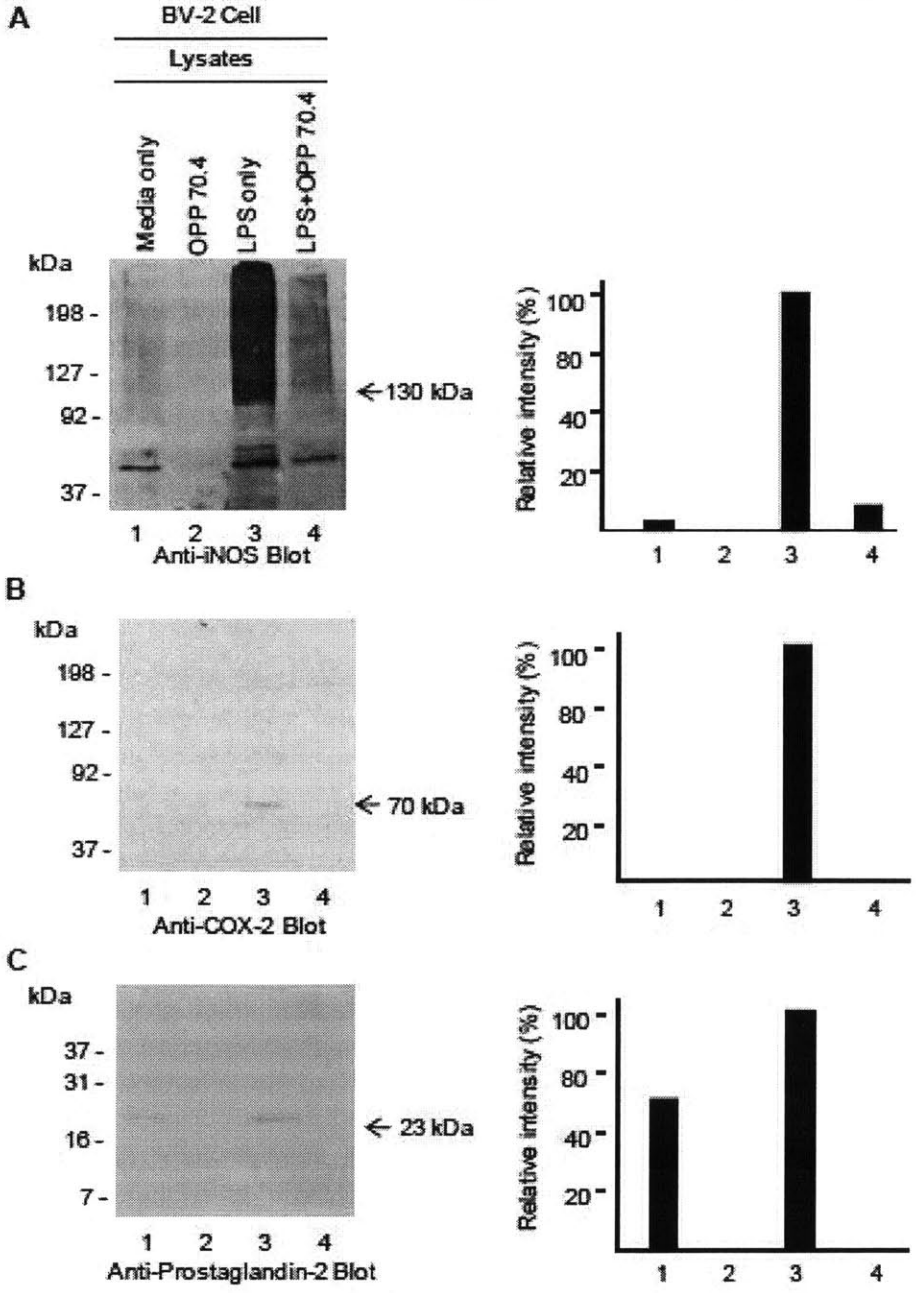
Figure 11: Effects of LPS on inducible nitric oxide synthase (iNOS) protein level A. Blots. B. Densitometric analysis 1. Media alone, 2. LPS alone, 3. LPS + OPP 200 µg/ml, 4. LPS + OPP 400 µg/ml, 5. LPS + OPP 800 µg/ml. C. Blotting against β-actin was employed as a loading control. OPP 200, 400 and 800 µg/ml contains 17.6 µM, 35.2 µM and 70.4 µM total phenolics, respectively



3.4.2 Cyclooxygenase-2 and Prostaglandin-2

In contrast, OPP treatment dose dependently suppressed the protein expression of COX-2 and Prostaglandin-2 at high concentration (800 µg/ml of OPP). OPP treatment was more effective in suppressing COX-2 and Prostaglandin-2 than iNOS protein expression (figure 12).

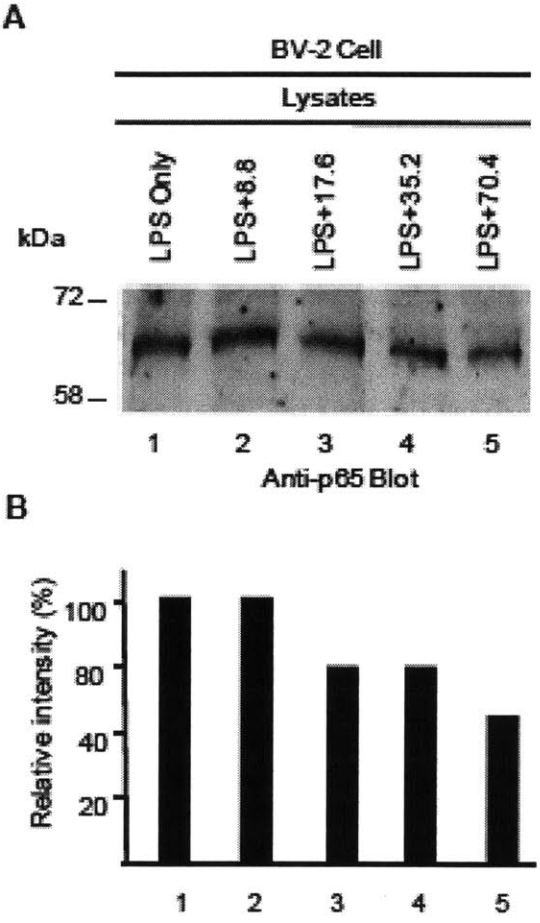
Figure 12: Effect of LPS on A. inducible nitric oxide synthase (iNOS) protein level B. Cyclooxygenase-2 and C. Prostaglandin-2 in LPS-stimulated BV-2 cells are significantly reduced by treatment with OPP. 1. Media alone, 2. OPP alone, 3. LPS alone, 4. LPS + OPP 800 µg/ml. OPP 800 µg/ml contains 70.4 µM total phenolics



3.4.3 Nuclear transcription factor NF-κB

As many anti-inflammatory mediators act by modifying NF-kB activity, the effects of OPP on NF-kB activity are measured by immunoblot analysis. OPP treatment significantly reduced the protein levels of NF-kB in LPS-activated cells (figure 13).

Figure 13: The effect of OPP on NF-kB activity in LPS-mediated BV-2 cells. 1. LPS alone, 2. LPS + OPP 100 µg/ml, 3. LPS + OPP 200 µg/ml, 4. LPS + OPP 400 µg/ml, 5. LPS + OPP 800 µg/ml. OPP 100, 200, 400 and 800 LPS + OPP 200 µg/ml contains 8.8 µM, 17.6 µM, 35.2 µM and 70.4 µM total phenolics, respectively



Conclusion

Protein levels of inducible nitric oxide synthase, cyclooxygenase-2 and prostaglandin-2 in LPS-stimulated BV-2 cells were significantly reduced by treatments with OPP. Nuclear transcription factor NF-κB subunit p65 was inhibited in a dose-responsive manner by OPP. The results suggest that OPP attenuates the inflammatory responses of brain microglia and could be potentially useful in modulating inflammatory conditions in the central nervous system.

3.5 Discussion

OPP down-regulates the production of nitric oxide (NO) by LPS stimulated microglia. Microglia, the resident immune cells in the brain, play a pivotal role in immune surveillance, host defense, and tissue repair in the central nervous system. Microglia affects NO production. Although NO is required for normal maintenance and function of the cell, NO is also a potent neurotoxin. The hyperactivation of microglia has been implicated in neurological disorders [31]. Microglia is also a key source of the proinflammatory factors TNF-α, IL-6, and inducible NO synthase (iNOS), which are over-expressed in many neurologic pathologies [31].

NO plays several roles, depending not only on the cell or tissue which produces it but also on the particular component of the inflammatory process with which it interacts. In many cell types in vitro, inflammatory cytokines either enhance or suppress iNOS expression and NO production. NO is secreted by activated macrophages, one of the most important classes of effector cells involved in the inflammatory response [32]. The LPS-induced production of NO in macrophages is inhibited by flavonoids [33].

Our results indicate that NO production is more strongly inhibited in LPS-stimulated inflammatory BV-2 microglial cells than in non-activated microglial cells. Further, the cell size and number of cells were unaffected by OPP or LPS. Higher concentrations of OPP did not diminish the viability in LPS-stimulated BV-2 cells. Protein levels of inducible nitric oxide synthase, cyclooxygenase-2, prostaglandin-2 and NF-κB in LPS-stimulated BV-2 cells were also significantly reduced by treatment with OPP. Hence, OPP may have therapeutic applications for inhibiting uncontrolled or elevated inflammatory response in the brain.

3.6 References

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CHAPTER 4: ANTI-INFLAMMATORY AND ANTI-OXIDATIVE ACTIVITY OF THE COMPONENTS OF OIL PALM PHENOLICS

4.1 Introduction

Oil Palm Phenolics is composed of 5 major components: protocatechuic acid, p-hydroxybenzoic acid, 4-caffeoylshikimic acid, 3-caffeoylshikimic acid and 5-caffeoylshikimic acid. Moreover, the presence of a sixth component, shikimic acid was also recently identified in Oil Palm Phenolics. The structures of the six compounds are shown in figure 1. The HPLC profile is shown in figure 2.

Figure 1: Structure of six major compounds of Oil Palm Phenolics [1]

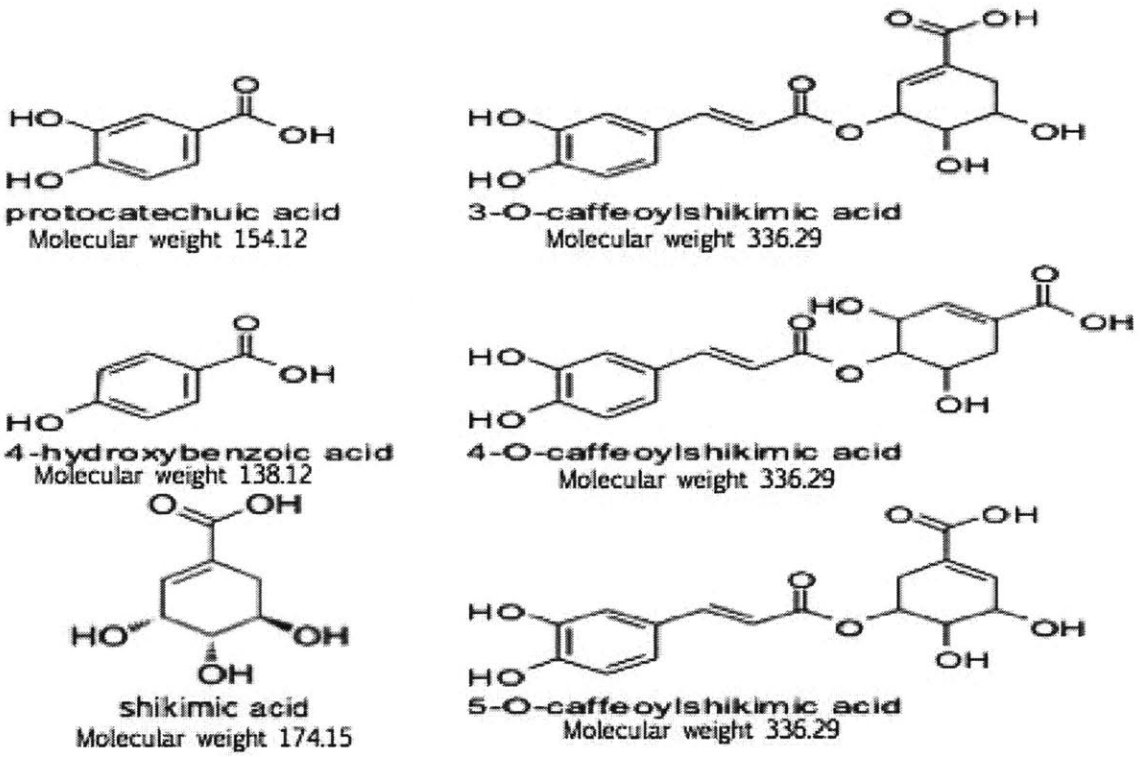
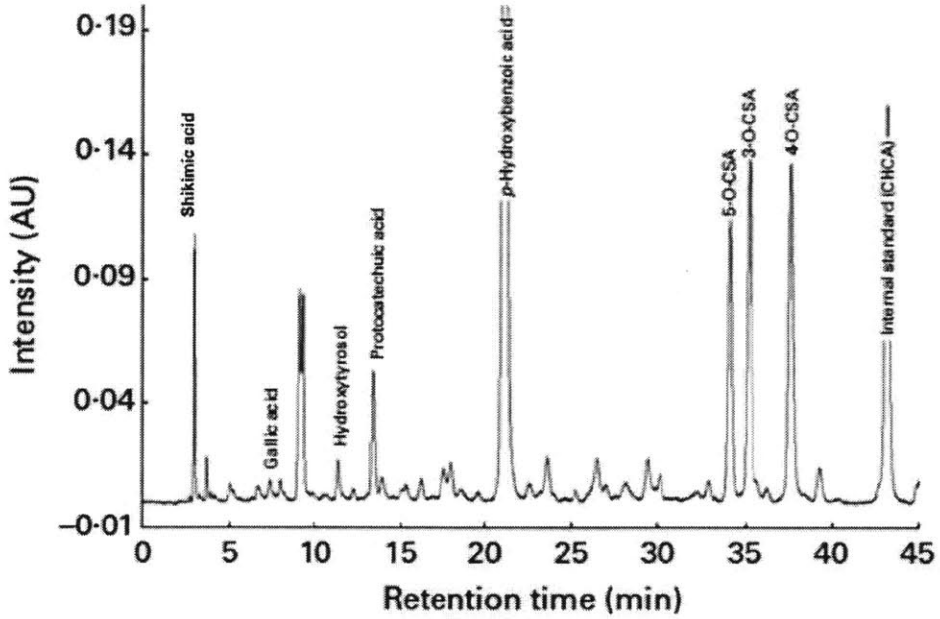


Figure 2: HPLC profile of Oil Palm Phenolics [1]. (AU: Arbitrary units)



The concentration of the 5 major compounds obtained from ten samples of OPP in different mills and locations is shown in table 1. Mean values are represented on a dry weight basis (mg of each major phenolic component per kg of freeze dried OPP) [1].

It can be seen in table 1 that caffeoylshikimic acid is the major phenolic acid in OPP, accounting for more than 1% of total solids. It can be hydrolyzed into shikimic acid and caffeic acid, when subject to appropriate conditions. Moreover, there is also the presence of 1% endogenous shikimic acid in Oil Palm Phenolics [2].

Shikimic acid has exhibited several therapeutic effects in the areas of antagonization of focal cerebral ischemic injury [3], analgesic activities and inhibition of platelet aggregration [4]. It is used as a specialty chemical in the pharmaceutical industry for the production of *Tamiflu*™, an anti-viral drug for the treatment and prevention of flu [5]. It is worthwhile noticing that a short supply of shikimic acid was cited as the major bottle-neck in the production of *Tamiflu*™ [6], as only a few natural sources have a high enough concentration of the acid to be profitable for industrial scale extraction [7].

In the previous two chapters, I studied the anti-oxidative and anti-inflammatory effects of OPP composition, as a whole, in mammalian brain cells and chemical systems at the gene, protein and molecular levels. The reduction in oxidative radicals and inflammatory species can be facilitated by antioxidants, such as Oil Palm Phenolics (OPP) through a combination of effects and interactions among its individual components. The final effect against oxidation and inflammation can be measured by using chemical systems or biological cell-based methods that differ from each other in terms of the substrate, probes or radical measured, conditions of reaction and/or method of quantification [8].

Table 1: Composition of individual components in OPP‡

Sample	Protocatechuic acid	p-Hydroxybenzoic acid	3-O-Caffeoylshikimic acid	5-O-Caffeoylshikimic acid	4-O-Caffeoylshikimic acid
A	660(+/-180)	6,400(+/-220)	3,200(+/-400)	3,600(+/-390)	4,700(+/-350)
B	610(+/-100)	7,300(+/-500)	2,500(+/-350)	2,800(+/-70)	4,100(+/-80)
C	640(+/-120)	6,900(+/-220)	2,900(+/-160)	3,200(+/-90)	3,800(+/-310)
D	620(+/-50)	7,600(+/-110)	3,100(+/-550)	3,700(+/-120)	4,500(+/-520)
E	850(+/-140)	5,300(+/-260)	2,300(+/-110)	2,700(+/-210)	2,700(+/-960)
F	560(+/-100)	8,600(+/-750)	4,100(+/-270)	4,800(+/-410)	6,200(+/-290)
G	510(+/-90)	6,700(+/-210)	2,400(+/-20)	2,500(+/-160)	3,700(+/-340)
H	550(+/-90)	6,600(+/-330)	4,400(+/-1,700)	4,300(+/-570)	5,200(+/-340)
I	440(+/-70)	6,300(+/-780)	3,400(+/-60)	3,900(+/-170)	4,700(+/-240)
J	830(+/-30)	8,100(+/-380)	1,900(+/-260)	2,800(+/-70)	3,300(+/-210)
Average (mg/kg)	**630**	**7,000**	**3,000**	**3,400**	**4,300**
% (dried OPP)	**0.063%**	**0.7%**	**0.3%**	**0.34%**	**0.43%**
Standard Deviation	**110**	**440**	**600**	**280**	**430**

‡ All data are represented as Mean ± SD

This chapter explains some of the experiments for studying the chemical versus biological characterization of the individual 5 major components of OPP, shikimic acid and its derivatives in six different chemical or biological systems. It also contains some studies for exploring the possibility of OPP's efficacy through synergistic interactions between the individual components. The experimental setup is shown in table 2.

4.2 Chemical Systems

OPP and its individual components have an intrinsic antioxidant activity due to their ability to directly scavenge free radical species in chemical systems. The three chemical systems used in my study are ORAC (Oxygen Radical Absorbance Capacity), TEAC (Trolox Eqivalent Antioxidant Activity) and NO generating sodium nitroprusside (SNP) system.

Table 2: Experiments set-up

Name of assay	Substrate	Probes	Time of reaction	Method of quantification	Purpose
ORAC	Chemical	$ROO^{\bullet}$	60 to 120 minutes	Fluorescence (plate reader)	Chemical characterization, synergy study
TEAC	Chemical	e^{-}	15 minutes	Absorbance (plate reader)	Chemical characterization
SNP	Chemical	NO_2^{-}	6 hours	Absorbance (plate reader)	Chemical characterization
CAP-E	RBC cells	all ROS	90 minutes	Fluorescence (flow cytometer)	Biological characterization
PMN	PMN cells	all ROS	90 minutes	Fluorescence (flow cytometer)	Biological characterization
Cell-based Griess	BV2 cells (or) RAW macrophages	NO_2^{-}	24 hours	Absorbance (plate reader)	Biological characterization, synergy study

4.2.1 Electron transfer measurement (Trolox equivalence antioxidant capacity – TEAC Assay)

The TEAC Assay is an electron transfer (ET) based assay in which the probe (oxidant) and an electron from the antioxidant react to form a reduced probe and oxidized antioxidant [8]. The probe used in the TEAC assay is ABTS•+. The assay utilizes only the inhibition percentage obtained in a short span of time to estimate the antioxidant activity. The method is the same as described in section 2.2.1.

Detection

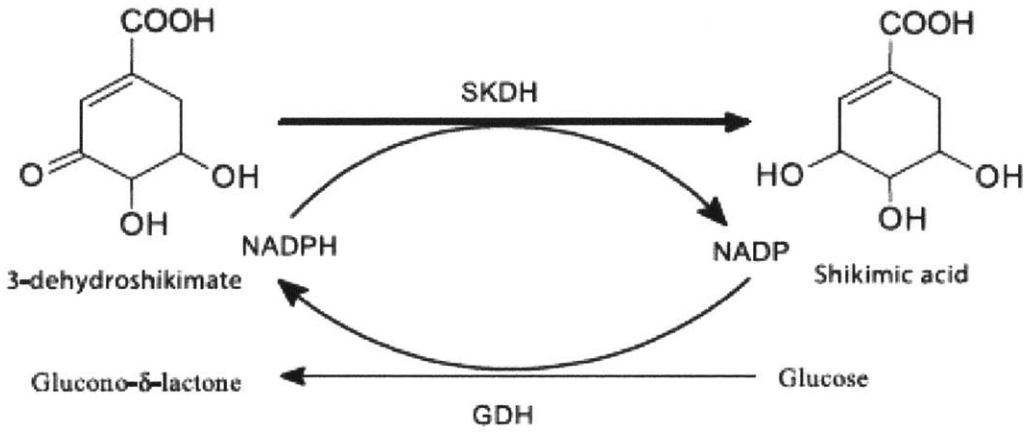
After vortex mixing 198μL ABTS•+ and 2μL sample solution for exactly 30 s, the absorbance reading at 765 nm (wavelength of maximum absorbance) is taken exactly 1 min after initiation of mixing and thereafter at time intervals of 1, 2, 5, 10, and 15 min. All experiments were conducted thrice.

Materials

When shikimic acid was identified as the sixth major component of OPP, the TEAC experiment was conducted to see if it had any antioxidant capacity, and if it could enhance the activity of the five major compounds (PCA - protocatechuic Acid; HBA - p-hydroxybenzoic acid; CSA4 - 4-caffeoylshikimic acid; CSA3 - 3-caffeoylshikimic acid; and CSA5 - 5-caffeoylshikimic acid) to the level of OPP. Chemical combination of the major compounds could be formed by adding them according to the amounts present in a given μg/mL of OPP.

3-dehydroshikimic acid is a chemical compound that can be reduced to shikimic acid by the enzyme shikimate dehydrogenase by using a molecule of NADPH as a biological cofactor. Shikimic acid can also be converted back to 3-dehydroshikimate in the presence of glucose and the enzyme, glucose dehydrogenase. The reaction is shown below [9].

Figure 3: Reaction between shikimic acid and 3-dehydroshikimic acid [9]



3-dehydroshikimic acid has been included in the set of experiments that have shikimic acid, as it is a compound which exhibits weak to strong antioxidant activity in liposomes, emulsions and bulk oil [10]. Hence, comparing the activity of 3-dehydroshikimic acid to shikimic acid may help us probe further into the mechanism of action.

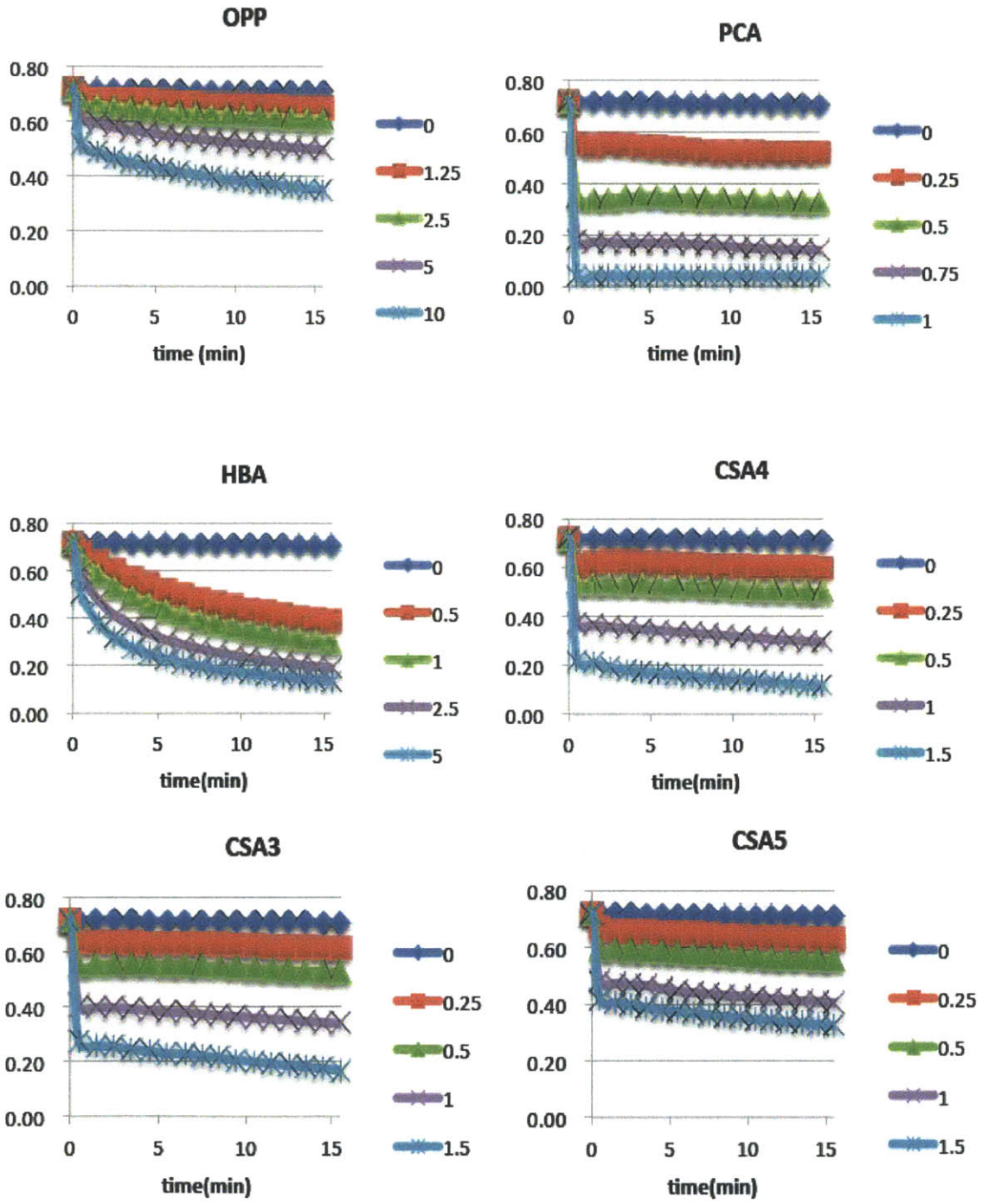
Results

As shown in figure 4, all five phenolic acids in OPP show the ability to directly scavenge free radical species. With the concentration of each compound increased, more ABTS•+ is reduced. Shikimic acid and 3-dehydroshikimic acid do not show any radical scavenging activity in this assay as individual compounds.

Figure 5 shows the correlation of the concentration of phenolic compound (mg/mL) and the % inhibition that represents the free radical scavenging activity. Within the measured concentration range, PCA and three CSA isomers show linear relationship, except HBA. Consequently, concentration of each compound leading to 50% free radical scavenging activity is calculated and shown in table 3.

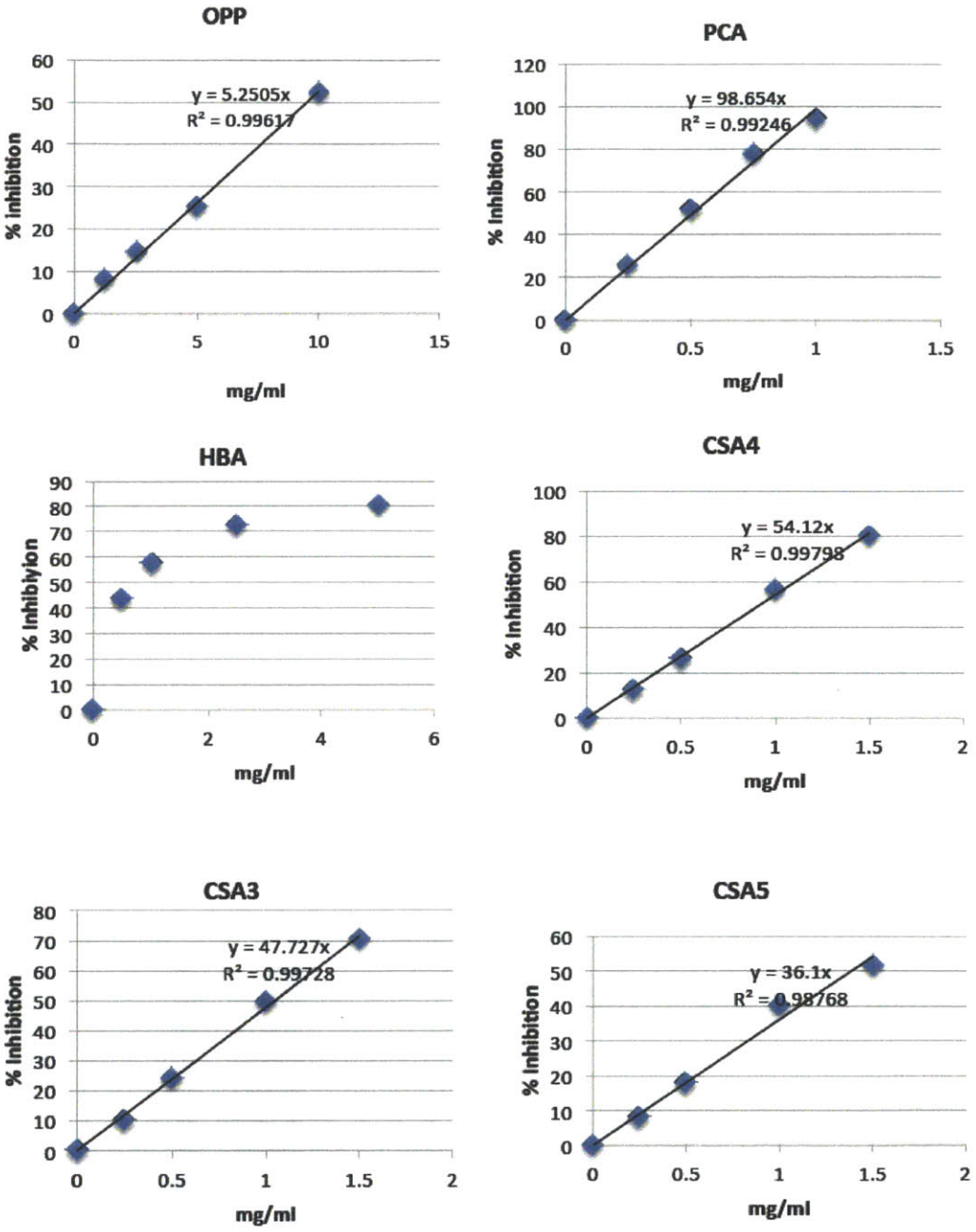
Among the five major phenolic acids in OPP, protocatechuic acid (PCA) has the highest free-radical scavenging activity, as a low concentration of 0.5 mg/ml can lead to 50% radical scavenging activity.

Figure 4: TEAC of each individual phenolic compound in OPP‡



‡Data from one representative experiment are presented

Figure 5: % Radical scavenging activity = [1- A_{sample}/A_0] × 100‡



‡Data from one representative experiment at 15 minutes are presented

Table 3: Chemical-based free radical scavenging activity (TEAC assay)*

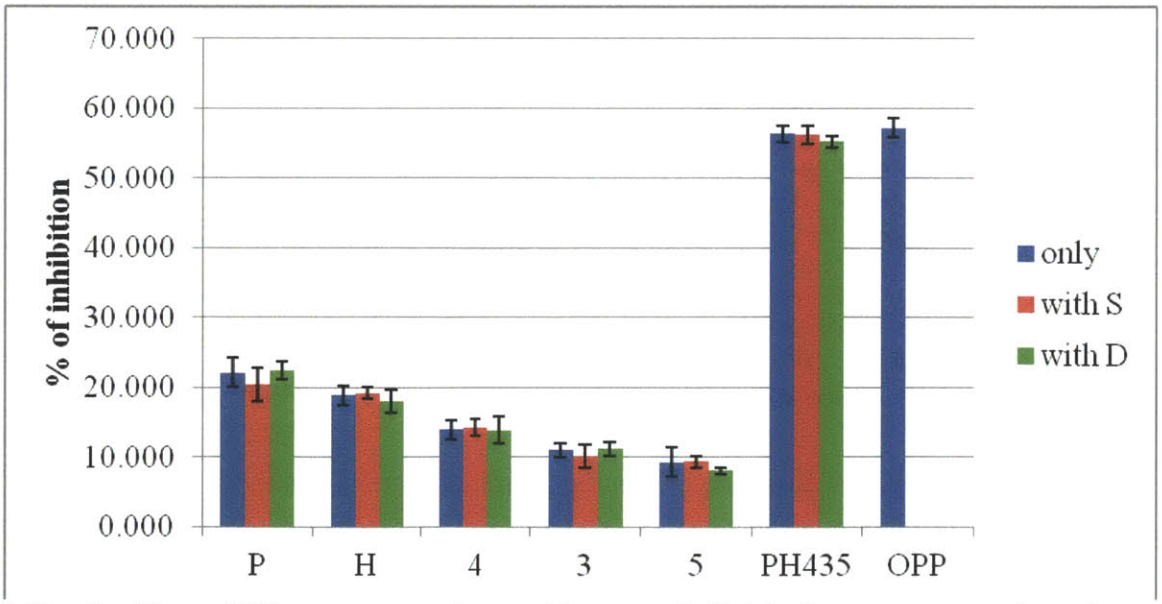
Description‡	Concentration leading to 50% inhibition of absorbance of ABTS•+ (mg/mL)	S.E.
Protocatechuic acid (P)	0.5	0.04
p-hydoxybenzoic acid (H)	0.91	0.1
4-caffeoylshikimic acid (4)	0.93	0.08
3-caffeoylshikimic acid (3)	1.05	0.06
5-caffeoylshikimic acid (5)	1.39	0.09
Shikimic acid (S)	-Not detected-	-NA-
3-dehydroshikimic acid (D)	-Not detected-	-NA-
Oil Palm Phenolics	9.5 (i.e. 0.19 mg/mL of phenolic compounds#)	1.2

*The free-radical scavenging activity of all components was measured in a range of concentration (0.5 to 10 mg/mL). The experiment was conducted thrice and the mean data along with standard error after 15 minutes is shown.

‡Among the five major phenolic acids in OPP, protocatechuic acid (PCA) has the highest free-radical scavenging activity

#9.5 mg/mL of OPP will have 0.19 mg of phenolic compounds (i.e. 2%) in it

Figure 6: Percentage of inhibition with/without shikimic acid or 3-dehydroshikimic acid (Mean ± SE)* ‡



* No significant differences were observed between individual components only and individuals with S, D using Tukey-Kramer's Honestly Significant Difference ($p < 0.05$);
‡ Pure compounds and OPP of 0.25 mg/mL were used. PH435 with/without S, D was prepared according to the composition of individual components in 0.25 mg/mL OPP

From figure 6, it can be seen that shikimic acid and 3-dehydroshikimic acid do not show any chemical enhancement of the percentage of inhibitions of the individual components and that of the combination of all five compounds.

Moreover, there is no significant difference between the combination of five major compounds, combination of six compounds (i.e PH435 with shikimic acid (or) 3-dehydroshikimic acid) and OPP. This result may be because the assay does not have very high sensitivity.

4.2.2 Peroxyl radical measurement (Oxygen Radical Absorbance capacity - ORAC)

The working principle, materials and methods of the ORAC assay have already been described in section 2.2.2. Briefly, antioxidants and fluorescein substrate compete for quenching the peroxyl radicals ($ROO^{\bullet}$) that are generated through the decomposition of azo compounds. Hence, better antioxidants lead to extended fluorescence of fluorescein. The method combines free radical inhibition percentage and the inhibition time of antioxidants by using the area under curve (AUC) equation for each antioxidant.

$$AUC = 1 + \frac{f_1}{f_0} + \frac{f_2}{f_0} + \frac{f_3}{f_0} + \frac{f_4}{f_0} + \cdots + \frac{f_n}{f_0}$$

where f_i is the fluorescence at time 'i' and the experiment is carried out upto 'n' minutes.

Net AUC is obtained by subtracting the blank AUC from the AUC of the antioxidant.

$$Net\ AUC = AUC_{antioxidant} - AUC_{blank}$$

The ORAC measurement is considered as a robust assay for measuring the antioxidant capacity as it combines both inhibition percentage and inhibition time. This assay is a convenient method to perform synergy studies as it is relatively simple and can be performed for many different combinations. All experiments were repeated thrice with duplicate samples in every one of them.

4.2.2.1 Synergistic interactions among the five major compounds of OPP

Phenolic compounds are known to have antioxidant properties [11]. The interactive antioxidant capacity of phenolic compounds has not yet been well explored. Interactions of the five individual phenolic compounds at the concentrations found in OPP were analyzed by using the ORAC assay. Mixtures of two, three, four and all five phenolic compounds were prepared. The samples were prepared according to the composition present in 35 µg/mL of OPP (table 1 and table 4).

Table 4: Sample preparation for synergy study among the five major components

Single Compound

No.	PCA	HBA	CSA4	CSA3	CSA5
S 1	√				
S 2		√			
S 3			√		
S 4				√	
S 5					√

Quadruple Mixture

No.	PCA	HBA	CSA4	CSA3	CSA5
Q 1	√	√	√	√	
Q 2	√	√	√		√
Q 3	√	√		√	√
Q 4	√		√	√	√
Q 5		√	√	√	√

Double Mixture

No.	PCA	HBA	CSA4	CSA3	CSA5
D 1	√	√			
D 2	√		√		
D 3	√			√	
D 4	√				√
D 5		√	√		
D 6		√		√	
D 7		√			√
D 8			√	√	
D 9			√		√
D 10				√	√

Triple Mixture

No.	PCA	HBA	CSA4	CSA3	CSA5
T 1	√	√	√		
T 2	√	√		√	
T 3	√	√			√
T 4	√		√	√	
T 5	√		√		√
T 6	√			√	√
T 7			√	√	√
T 8		√	√	√	
T 9		√	√		√
T 10		√		√	√

Quintuple Mixture

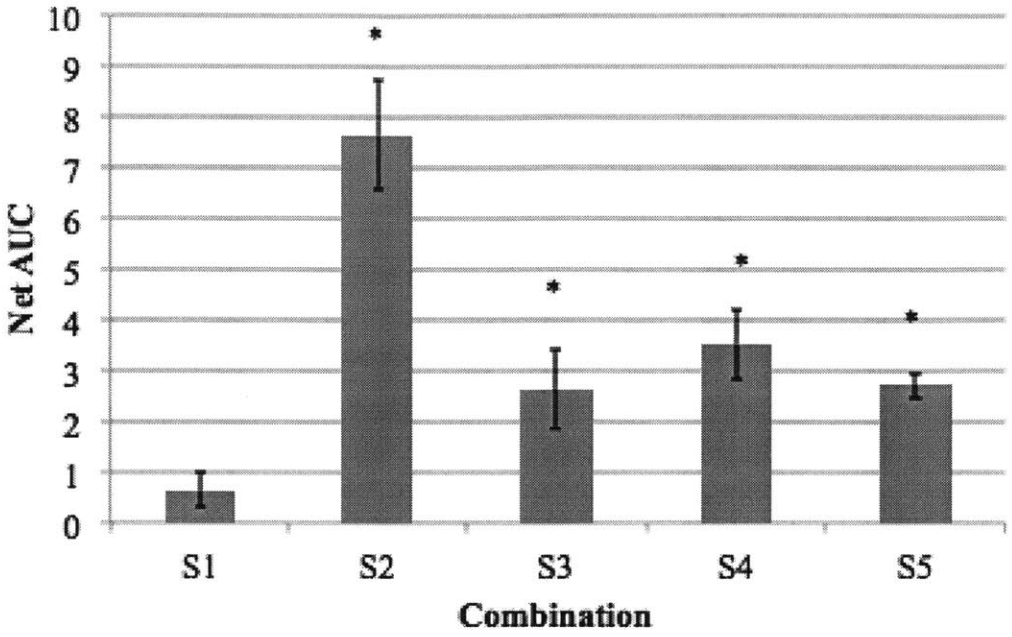
No.	PCA	HBA	CSA 4	CSA 3	CSA 5
F 1	√	√	√	√	√

Results and Statistics

Figure 7, 8, 9 and 10 show the AUC values for the individual phenolic compounds, double mixture, triple mixture, quadruple, quintuple mixtures and OPP respectively.

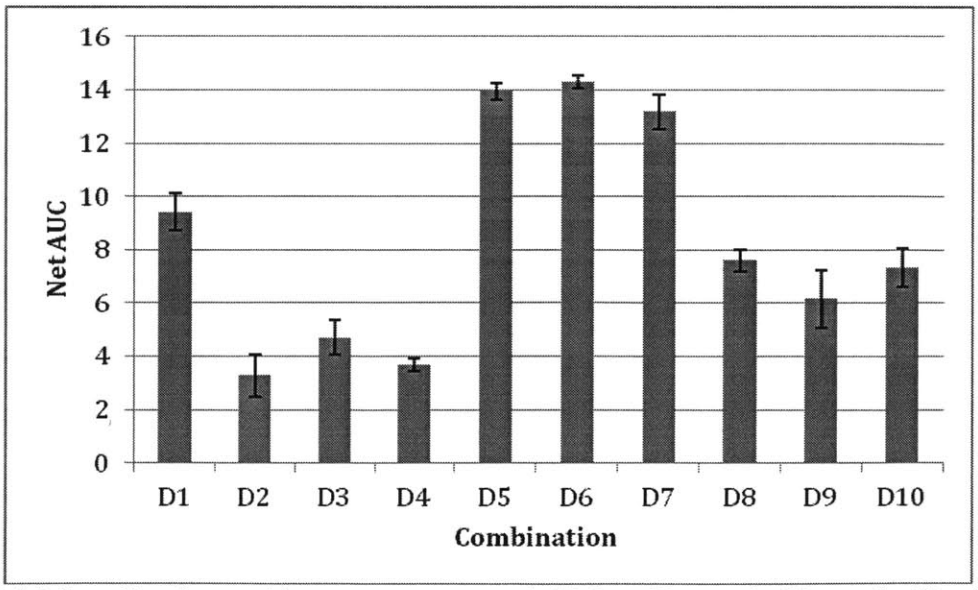
JMP Pro 10 (SAS Institute) was used to determine significance of combinations when compared to blank values, which takes into account error terms when data are combined. The net AUC values were compared through an ANOVA of the individuals/combinations and blank/sugar results, and using the differences for post hoc tests to determine the effect of the individual compounds and/or their combinations.

Figure 7: Net AUC values of individual compounds (Mean ± SE)



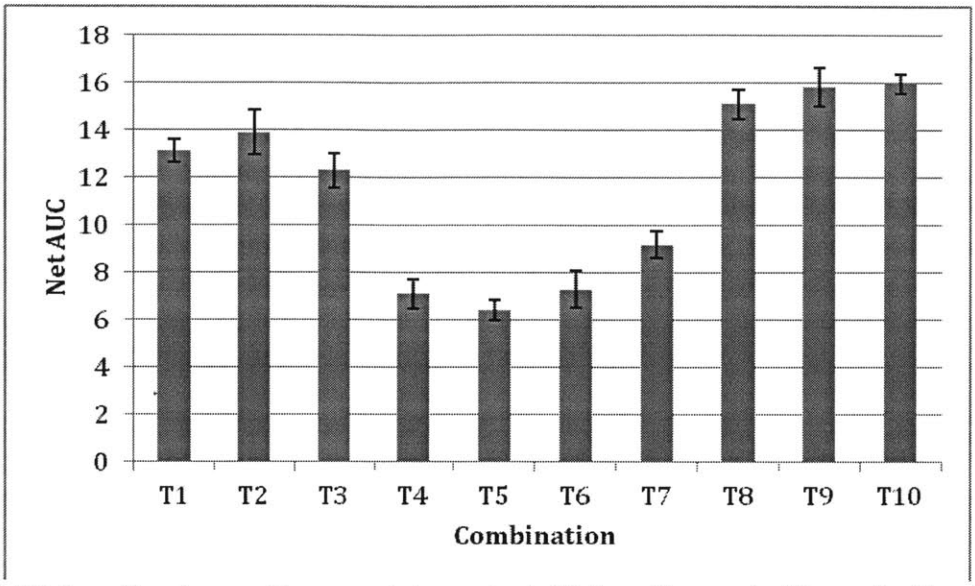
* $p < 0.05$ with respect to control (Tukey-Kramer's Honestly Significant Difference (HSD))

Figure 8: Net AUC values of double mixtures (Mean ± SE)*



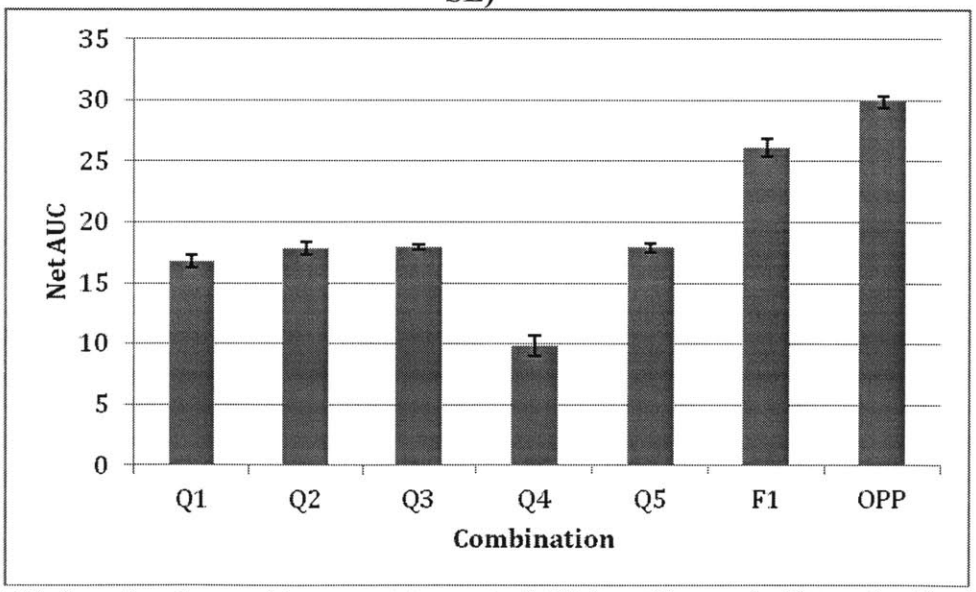
* $p < 0.05$ for all values with respect to control (Tukey-Kramer's Honestly Significant Difference (HSD))

Figure 9: Net AUC values of triple mixtures (Mean ± SE)*



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

Figure 10: Net AUC value of quadruple, quintuple mixtures and OPP (Mean ± SE)*



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

The positive values of all net AUC denote the presence of antioxidant activity in all 5 major compounds and their combinations. For double combinations, a difference was calculated by subtracting the sum of the average ORAC values for the individual compounds from the resulting average ORAC value of the combination of both compounds.

$$\text{Mean AUC difference} = (\text{combination ab}) - (\text{individual a} + \text{individual b})$$

Similarly, for combinations of 3, 4 and 5, the difference was calculated by subtracting the sum of the averages of the individual 3, 4 or 5 compounds from their combination.

$$\text{Mean AUC difference} = (\text{combination abc}) - (\text{a} + \text{b} + \text{c})$$

$$\text{Mean AUC difference} = (\text{combination abcd}) - (\text{a} + \text{b} + \text{c} + \text{d})$$

$$\text{Mean AUC difference} = (\text{combination abcde}) - (\text{a} + \text{b} + \text{c} + \text{d} + \text{e})$$

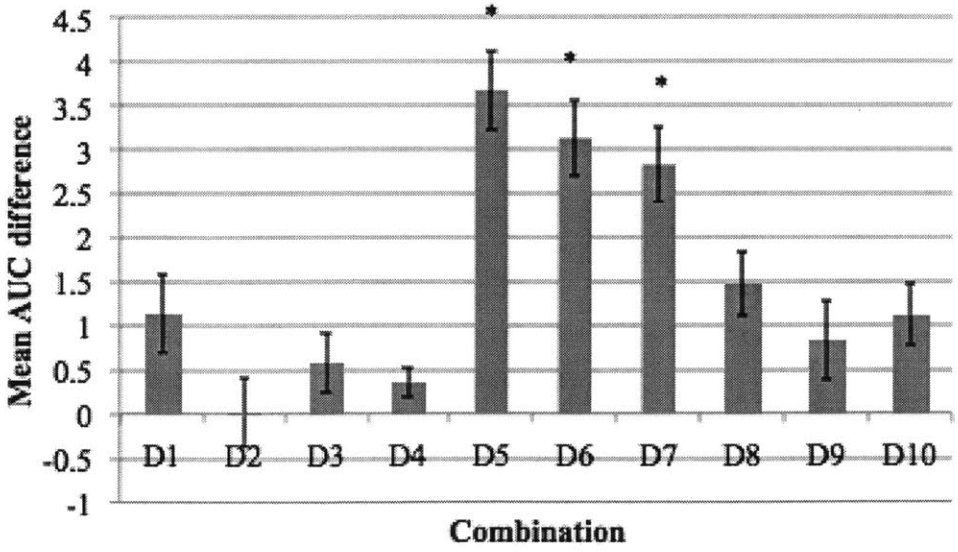
A difference was also calculated between the net AUC value of OPP and the quintuple combination in order to identify the amount of antioxidant capacity of OPP that was explained by the five major compounds.

$$\text{Mean AUC difference} = \text{OPP} - (\text{combination abcde})$$

JMP Pro 10 (SAS Institute) was used to determine significance of combinations using estimate statistics, which take into account error terms when data are combined. The mean AUC differences were compared through an ANOVA of the sum of individuals and combination results of the net AUC values, and forming the differences as post hoc tests to determine the effect of combining the individual compounds.

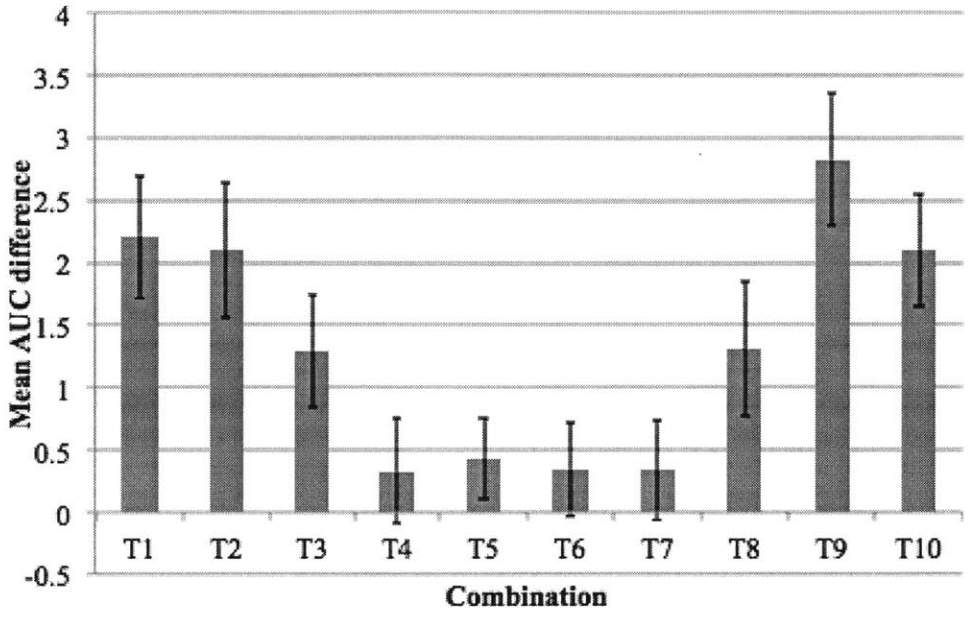
The Mean AUC difference for double mixture, triple mixture, quadruple and quintuple mixtures are shown in figure 11, 12 and 13 respectively.

Figure 11: Mean AUC difference of double mixtures (Mean ± SE)



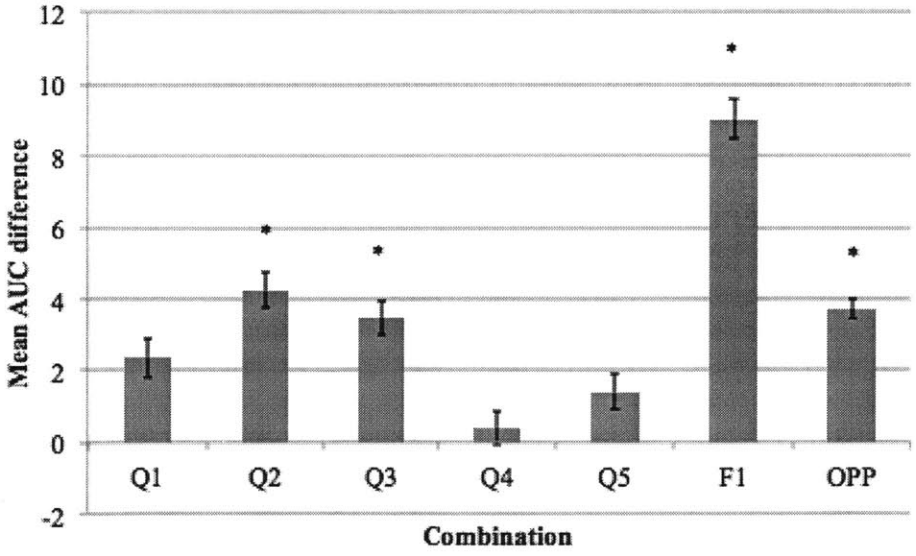
* Significant mean AUC differences ($p < 0.05$) using Tukey-Kramer's Honestly Significant Difference (HSD)

Figure 12: Mean AUC difference of triple mixtures (Mean ± SE)*



*The mean difference for triple combinations were insignificant at $p < 0.05$

Figure 13: Mean AUC difference of quadruple, quintuple and OPP mixtures (Mean ± SE)



* Significant mean AUC differences ($p < 0.05$) using Tukey-Kramer's Honestly Significant Difference (HSD)

From figures 11, 12 and 13, it can be seen that all five individual phenolic compounds show a mean synergistic interaction in all combinations except D2 (PCA, CSA4) and Q4 (PCA, CSA4, CSA3 and CSA5). Some combinations do not show a significant mean AUC difference. However, these combinations definitely show at least an additive effect.

Combinations containing HBA show significantly higher synergistic effect on antioxidant capacity. This may be due to higher synergistic effect of HBA or higher HBA concentration in OPP than other individual phenolic compounds.

There is a significant mean difference between OPP and the quintuple combination of five major compounds as shown in the OPP column of figure 13. This result suggests that there may be other active compounds contributing to the antioxidant capacity besides the five major compounds in OPP.

4.2.2.2 Synergistic enhancement by Shikimic acid and its derivative, 3-dehydroshikimic acid

As shikimic acid was identified as the sixth major component of OPP, the ORAC experiment was conducted to see if shikimic acid or its derivative, 3-dehydroshikimic acid, had any antioxidant capacity, and if it could enhance the activity of the five major compounds to the level of OPP. A table of components used is shown below.

Table 5: Components used in the study of synergistic enhancement

Abbreviation	Compound
P	Protocatechuic acid
H	4-hydroxybenzoic acid
4	4-caffeoylshikimic acid
3	4-caffeoylshikimic acid
5	4-caffeoylshikimic acid
S	Shikimic acid
D	3-dehydroshikimic acid
Sugar	Sugar solution

Results

The Net AUC values of the six major compounds added in the concentration that they are present in 35 μg/mL of OPP is shown in figure 14. The sample preparation table is shown in table 6.

Table 6: Sample preparation of chemical combinations

Single Compound

Name	PCA	HBA	CSA4	CSA3	CSA5	SA	3DHS
P	√						
H		√					
4			√				
3				√			
5					√		
S						√	
D							√

Double mixture

Name	PCA	HBA	CSA4	CSA3	CSA5	SA	3DHS
PS	√					√	
HS		√				√	
4S			√			√	
3S				√		√	
5S					√	√	
PD	√						√
HD		√					√
4D			√				√
3D				√			√
5D					√		√

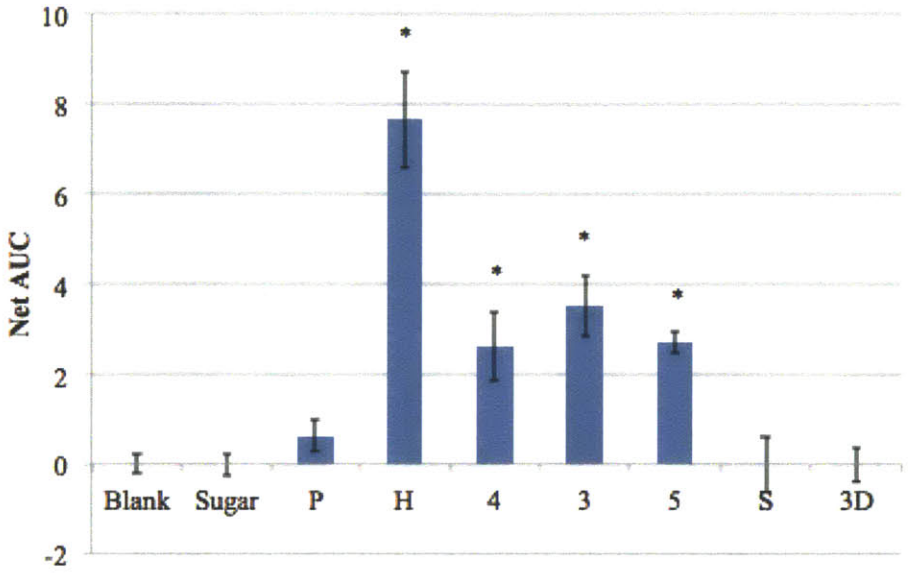
Quintuple and Sextuple mixtures

Name	PCA	HBA	CSA4	CSA3	CSA5	SA	3DHS
PH435	√	√	√	√	√		
PH435S	√	√	√	√	√	√	
PH435D	√	√	√	√	√		√

On their own, shikimic acid and 3-DHS do not show any antioxidant activity (figure 14). Even when their range of concentration was increased to include 0 to 4.5 mM of these two compounds in their pure form, both compounds still showed no activity in this assay.

They were then added to the other 5 compounds of OPP to form double combinations. Sextuple combinations of five major compounds with shikimic acid and 3DHS were also analyzed.

Figure 14: Net AUC values of individual components (Mean ± SE)



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

For double combinations, a difference was calculated by subtracting the sum of the average ORAC values for the individual compounds from the resulting average ORAC value of the combination of both.

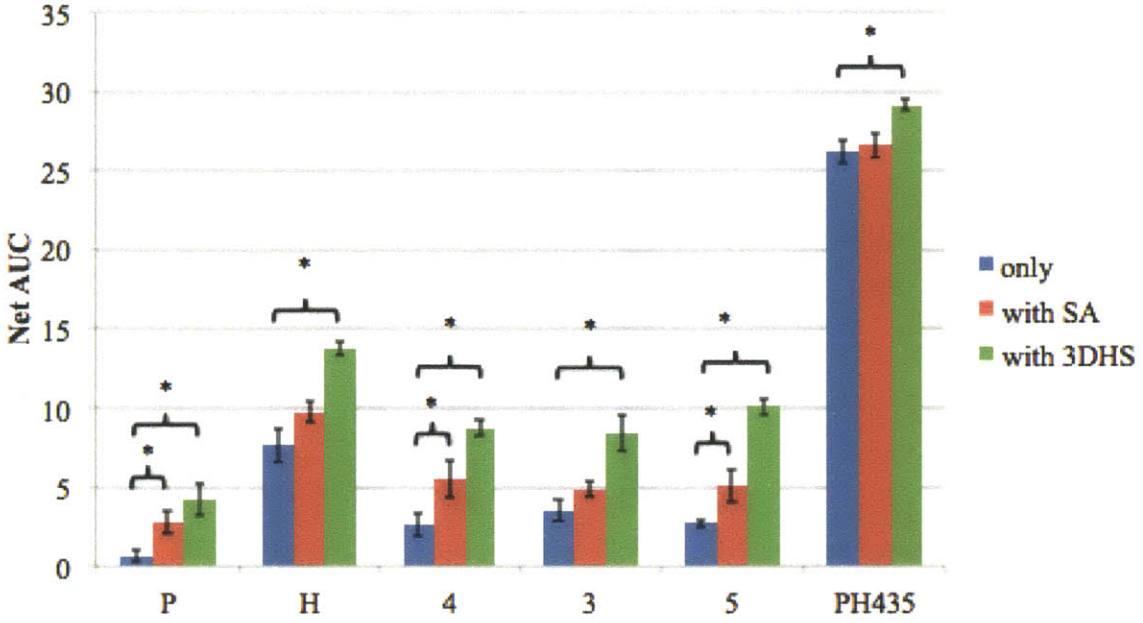
Mean AUC difference = (combination ab) − (individual a + individual b)

For sextuple combinations, a difference was calculated by subtracting the sum of the average ORAC values for the individual compound and 5 major compound combination from the resulting average ORAC value of the combination of both.

Mean AUC difference = (combination abcdef) − (combination abcde + individual f)

In figure 15, it can be seen that although shikimic acid and 3-dehydroshikimic acid do not show any antioxidant activity in the ORAC assay, they are able to enhance the AUC value of the individual components and that of the combination of all five compounds. 3DHS has a higher enhancement than shikimic acid in this assay. In order to understand how these values compare to OPP, the antioxidant activity is represented in terms of free radical scavenging activity % in comparison to OPP (table 7).

Figure 15: Net AUC values of double and sextuple combinations (Mean ± SE)



* Significant mean AUC differences ($p < 0.05$) using Tukey-Kramer's Honestly Significant Difference (HSD)

OPP has a significantly higher inhibition percentage than the mathematical sum of the inhibition percentages of the five major components. The chemical combination of the five major components, without shikimic acid, is able to explain only 87.5% of the free radical scavenging activity of OPP. When shikimic acid is added to the five major compounds, the percentage improves slightly to 88.9%. The five major compounds along with 3DHS are able to account for 97.5% of the antioxidant activity of OPP.

Table 7: Chemical based ROO• scavenging activity assay (ORAC assay)*

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-
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	35 μg/mL	29.9	100 (± 1.7)

The components were added according to the amount present in 35 μg/mL of OPP
* All compounds/combinations except ‡ have $p < 0.05$ with respect to OPP using Tukey-Kramer's Honestly Significant Difference (HSD)

4.2.3 Nitrite measurement (Sodium nitroprusside – SNP assay)

Nitric oxide (NO) is a short-lived free radical generated endogenously. It influences a number of physiological functions such as neurotransmission. However, excess production can lead to pathology. It is known to be responsible for the vasodilation and hypotension observed in septic shock and inflammation [12]. Inhibitors of NO may be useful candidates for the treatment of inflammatory diseases that are accompanies by overproduction of NO.

Materials and Methods

Sodium nitroprusside (Sigma-Aldrich) in aqueous solution and light at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce a nitrite ion that can be estimated by using the modified griess reagent (Sigma-Aldrich). The absorbance of the chromophore can be read at 540 nm (wavelength of maximum absorbance) in a plate reader (Tecan). The nitrite concentration and percentage of inhibition can be calculated by referring to the absorbance of standard solutions of sodium nitrite (Sigma-Aldrich). The experiments were repeated thrice and the data are indicated as mean ± standard error.

Results

A standard curve was generated between the absorbance and sodium nitrite standard. Every 0.1 units of absorbance were found to correspond to 20 µM of nitrite (figure 16).

The incubation time for nitrite production from SNP was decided as 360 mins based on the saturation point of a plot of concentration of nitrite (absorbance) versus incubation time (figure 17).

Figure 16: Calibration curve between absorbance and nitrite concentration

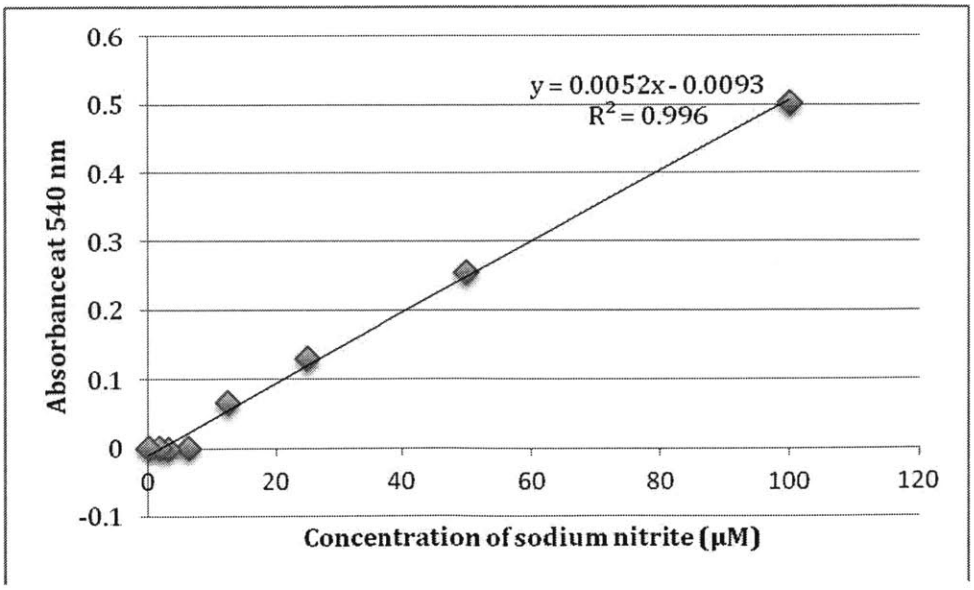
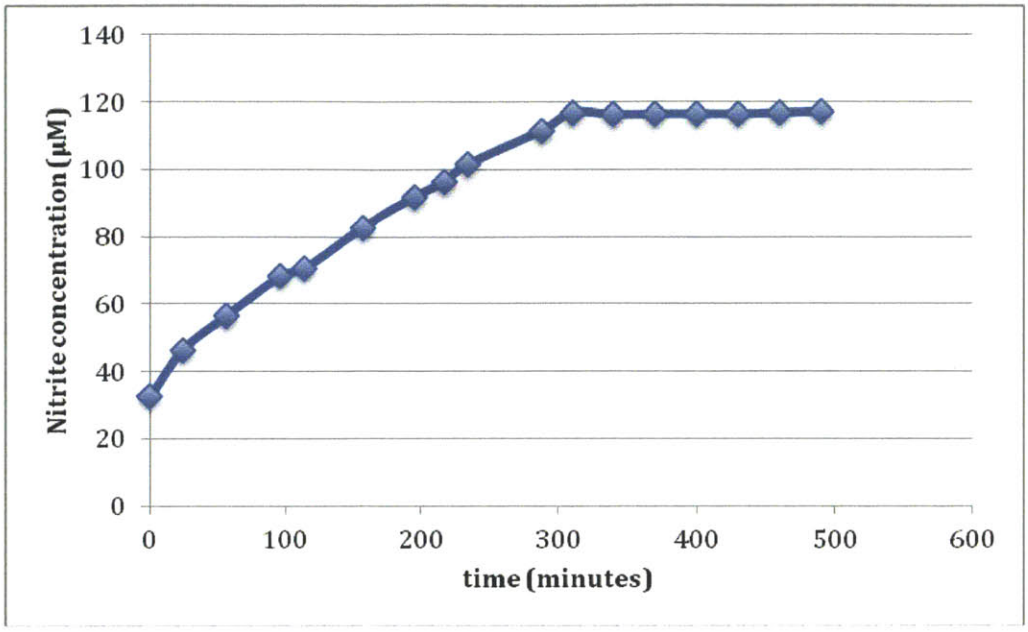


Figure 17: Plot of concentration of nitrite versus incubation time



4.2.3.1 Chemical characterization of pure compounds

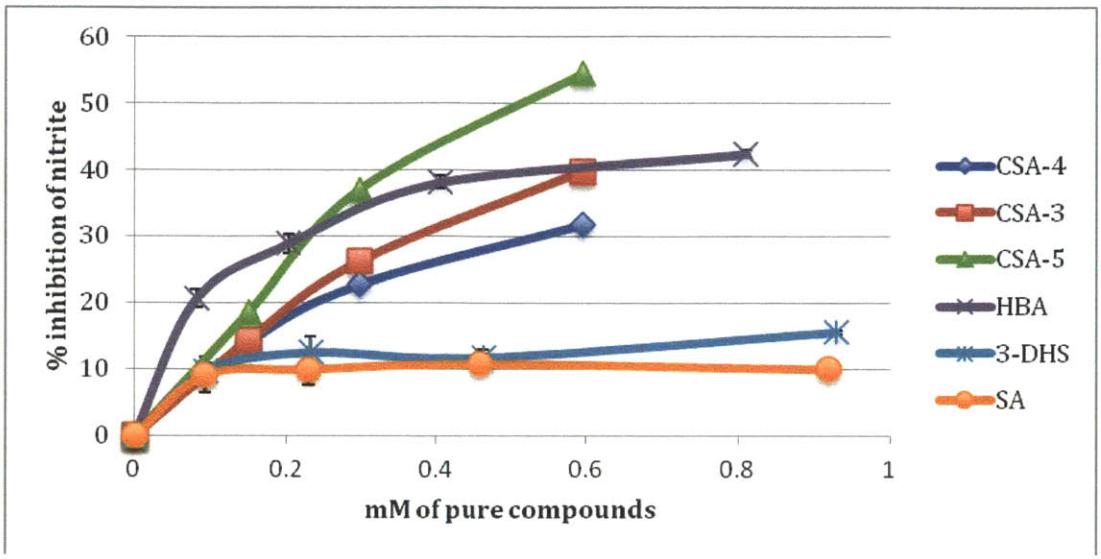
The derivatives of shikimic acid used in this study are: 1) caffeoylshikimic acid (CSA4, CSA3, CSA5), the primary marker compound present in OPP and 2) 3-dehydroshikimic acid (3DHS), a compound that can be reduced to form shikimic acid by the enzyme shikimate dehydrogenase. Apart from these two compounds, 3) Oil Palm Phenolics (OPP), and 4) p-hydroxybenzoic acid (HBA), the compound which is present in large quantity (0.7%) in OPP are also used for the sake of comparison.

Various concentrations (0 to 1 mM) of these test compounds were incubated with SNP for 360 minutes. A control was also run without any test compounds (an equivalent amount of vehicle was used). The nitrite concentration and percentage of inhibition can be calculated by referring to the absorbance of standard solutions of sodium nitrite.

Results

From figure 18, it can be seen that HBA and CSA's exhibit an increase in percentage of nitrite inhibition with an increase in their concentration. However, SA and 3DHS seem to saturate at 10% inhibition at a very low concentration of around 0.1 mM each.

Figure 18: Chemical based NO scavenging activity by using SNP assay (Mean ± SE)



4.2.3.2 Comparison of chemical enhancement by shikimic acid to OPP

By recalling the results from ORAC assay, it is known that shikimic acid does not show any activity as individual compounds but can enhance the activity of other compounds. Hence, SA and 3DHS were added along with the chemical combination of five major compounds (PH435) and HBA, the major compound in OPP. As this study involves combinations, the amounts were added according to the ratios in which they are present in OPP. The combinations are given in table 8. The results are shown in figure 19.

Table 8: Sample preparation of chemical combinations for probing enhancement

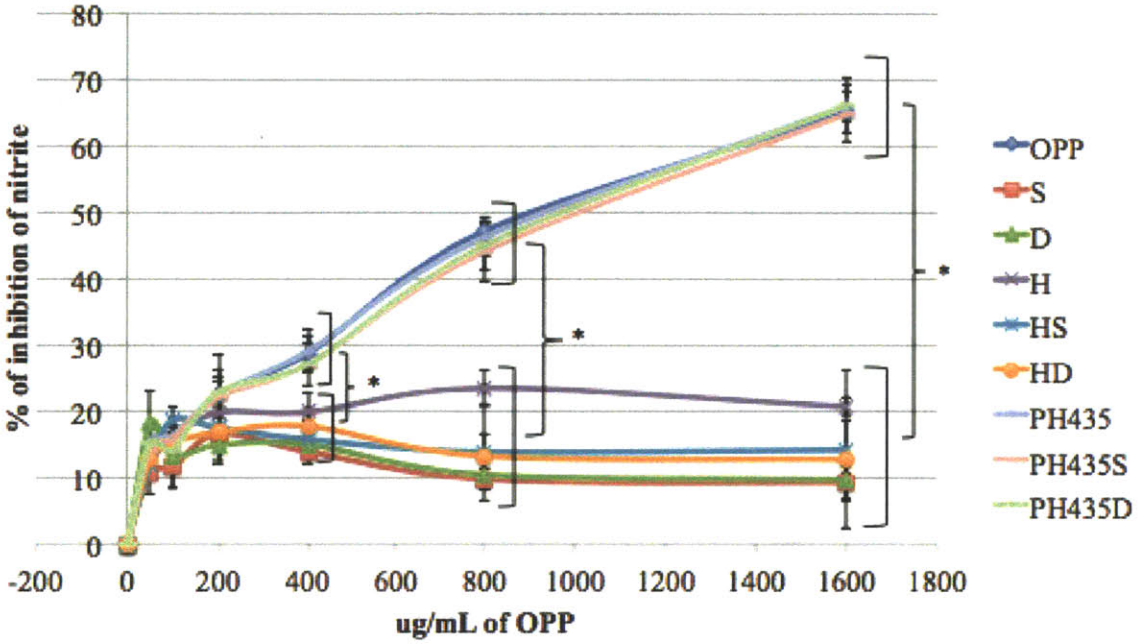
Name	PCA	HBA	CSA4	CSA3	CSA5	SA	3DHS
S						√	
D							√
H		√					
HS		√				√	
HD		√					√
PH435	√	√	√	√	√		
PH435S	√	√	√	√	√	√	
PH435D	√	√	√	√	√		√

Statistics

JMP Pro 10 (SAS Institute) was used to determine significance of combinations, which take into account error terms when data are combined. The inhibition percentages of nitrite were compared through an ANOVA of the individuals/combinations at each concentration equivalents of OPP. For all the concentration levels for which the

ANOVA F-test rejected the null hypothesis, the Tukey-Kramer HSD test was used to compare the mean of all pairs.

Figure 19: Study to probe the existence of chemical enhancement by shikimic acid (Mean ± SE) ‡



‡ All components were added according to the amounts present in the denoted μg/mL of OPP
* $p < 0.05$ using Tukey-Kramer's Honestly Significant Difference (HSD); ANOVA F-test fails at 50, 100 and 200 μg/mL OPP levels.

Results

SA and 3DHS do not show any clear enhancement in this assay, when added along with HBA. This result may be because this assay measures only the chemical activity.

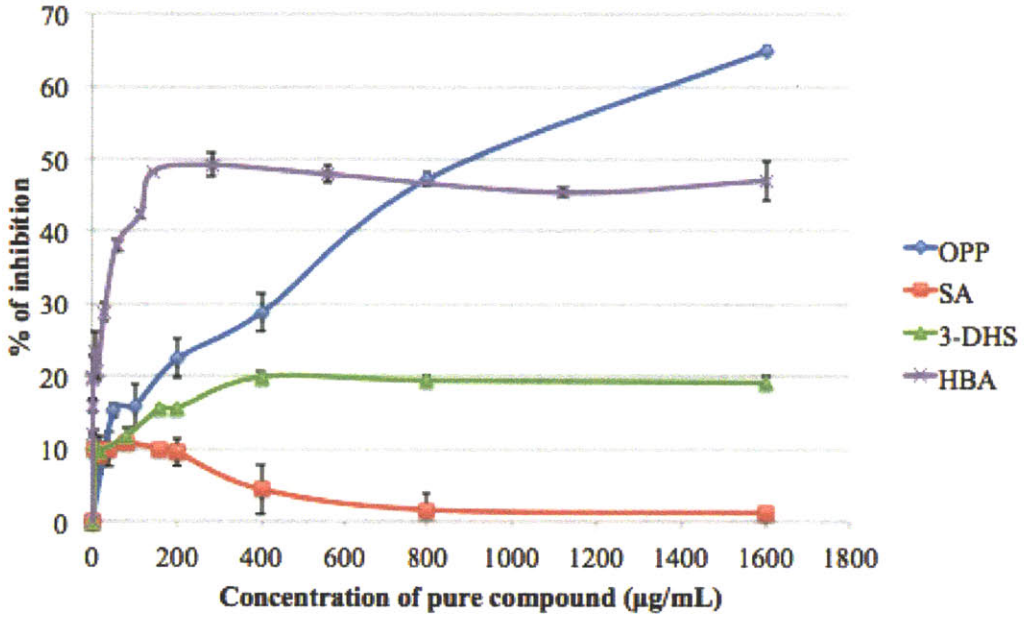
The 4 combinations of OPP, PH435, PH435S, PH435D seem to exhibit very similar percentages of nitrite inhibition, with any differences among them being beyond the detection limits of the assay.

4.2.3.3 Characterization of Shikimic acid in an extended range

As it remains uncertain whether the saturation in percentage of inhibition of nitrite by SA and 3DHS as individual compounds (figure 18, 19) is a result of assay threshold value or indicates the presence of mild activity by shikimic acid, the assay was repeated for an extended range of concentration (approximately 0 to 10 mM of each pure compound).

From figure 20, it can be seen that HBA saturates at around 100 µg/mL and OPP continues to exhibit increasing % of nitrite inhibition upto 1600 µg/mL. However, SA exhibits a unique behavior, whereby the percentage of nitrite inhibition increases steeply till around 100 µg/mL and then it begins to decrease. In the case of 3DHS, the % of nitrite inhibition increases steeply till around 100 µg/mL and then it begins to saturate.

Figure 20: Chemical based NO scavenging activity in an extended range (SNP assay) (Mean ± SE) ‡



‡ All concentrations denote µg/mL of pure compounds (not amount present in OPP).

4.2.3.4 Reverse phase HPLC analytical assay

This unique solution-behavior of shikimic acid, somewhere around 100 µg/mL was probed further by using a reverse phase HPLC (RP-HPLC) procedure [13]. Solutions of various concentrations below and above the possible peak values of SA and 3DHS were made in 2X PBS by serial dilution, passed through an HPLC system at a flow rate of 1 mL/min. The mobile phase was 0.05% H3PO4 in water (A) and acetonitrile (B). A gradient table was developed for separation of compounds. The peak area versus phenolic compound concentration was plotted.

This RP-HPLC method is known to be useful for determining the critical micelle concentration (CMC) whereby monomers and micelles each have distinct retention times [13]. However, in our measurements, we did not observe two distinct peaks above the CMC but only a single peak. We discovered a break in the linearity of the HPLC area curve of the single observed peak. The breaks in the calibration curves of peak area versus concentration represent CMC and are in agreement with the solution-behavior of SA and 3DHS as determined by the SNP assay (figure 21, 22).

Figure 21: HPLC calibration curve of shikimic acid (Break point of 100 µg/mL (or) 0.57 mM)

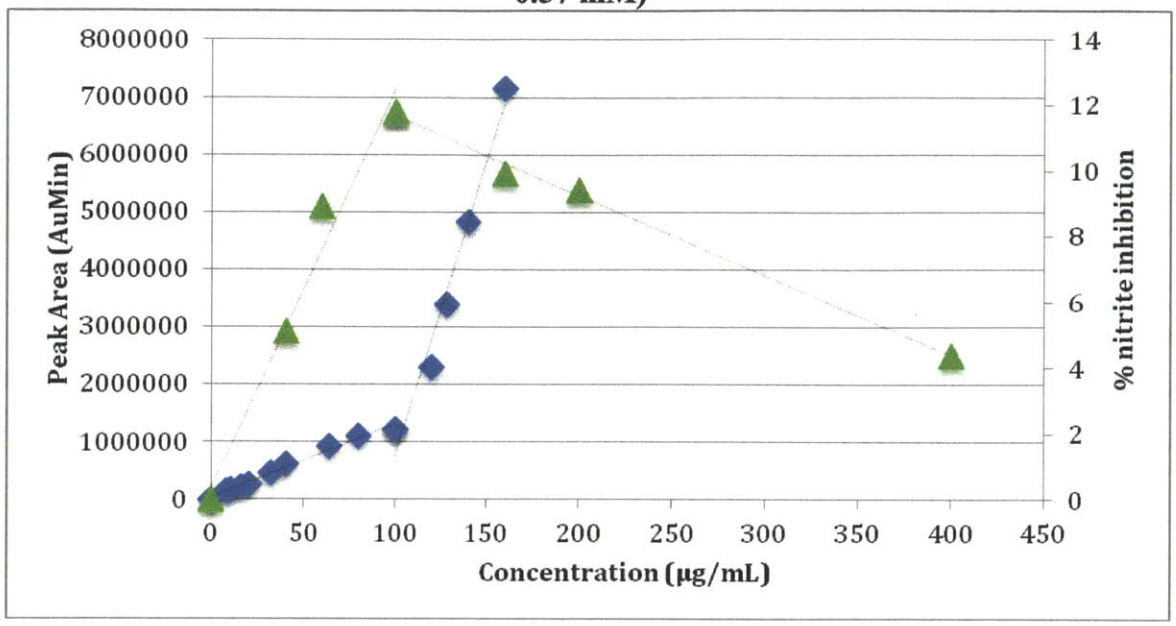
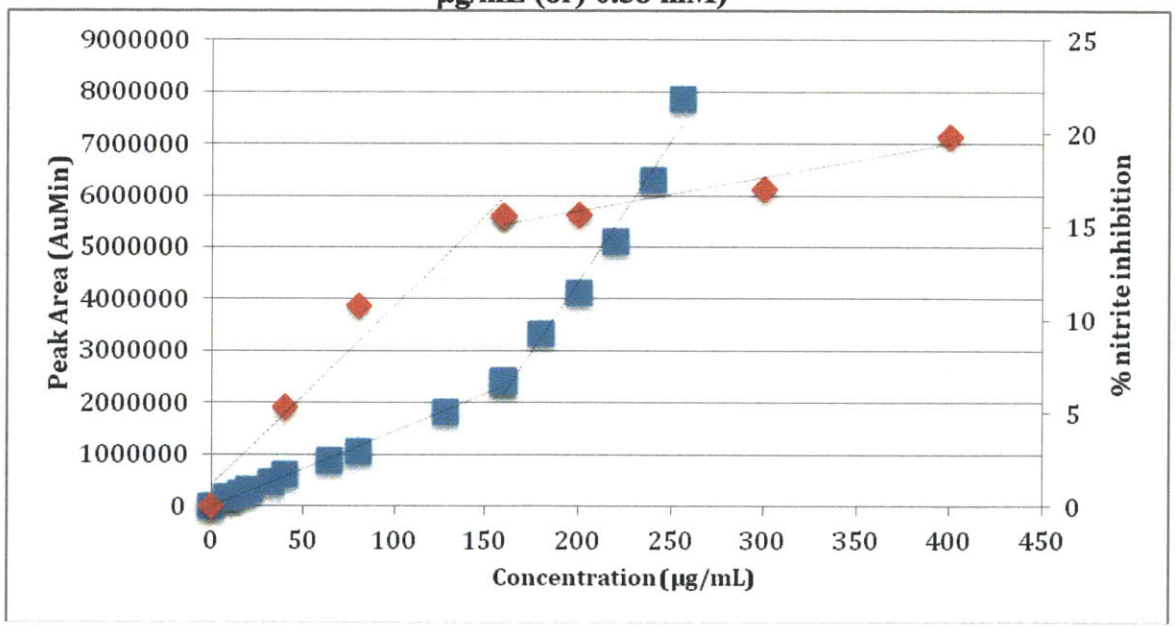


Figure 22: HPLC calibration curve of 3-dehydroshikimic acid (Break point of 160 µg/mL (or) 0.58 mM)



Conclusion

Although this assay does not provide evidence about a significant enhancement of chemical nitrite scavenging activity by shikimic acid and 3-dehydroshikimic acid, it has led to several useful insights about the solution-behavior of shikimic acid and 3-dehydroshikimic acid.

The break-points in RP-HPLC peak area versus concentration calibration curves for SA and 3DHS may provide some guidance for deciding the cytotoxic limits or bioactive limits of SA and 3DHS.

4.3 Cell-based systems

Experimental evidence has shown the presence of six major reactive oxygen/nitrogen species (ROS/RNS) causing oxidative damage in the human body. These are superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($ROO^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$) [8]. The peroxyl radical ($ROO^{\bullet}$) has been the most commonly used ROS in the methods because it is an important radical in autoxidation and it can be conveniently generated from the thermal decomposition of azo compounds [8]. However, in order to comprehensively measure the oxidant-scavenging capacity of a material, several assays that are specifically designed to study all of these ROS/RNS have to be carried out.

The chemical systems that were explained in section 4.2 are useful to identify the compounds that have antioxidant properties and compounds that can enhance the antioxidant properties of other compounds. However, it is important to determine the antioxidant compounds with biological properties, some of which can be biologically active compounds. This section aims to make such distinction.

4.3.1 ROS measurement (Cell-based antioxidant protection in erythrocytes CAP-e assay)

Red blood cells can be used as a cellular model to quantify reactive oxygen species (ROS) [14]. As these cells lose their nuclei at the erythroblast stage, they cannot perform transcription and translation for bringing about genetic and protein level changes. Hence, the ROS measurement signifies the ability of the antioxidant compound to enter the cytosol and quench free radicals.

Materials

Phosphate-buffered saline (PBS), hydrogen peroxide 30% solution (H_2O_2), dimethyl sulfoxide (DMSO), Histopaque 1077 and Histopaque 1119 were obtained from Sigma-Aldrich. Dichlorofluorescein diacetate (DCFDA) was obtained from Santa Cruz Biotech.

Natural compounds

Samples were prepared by dissolving 100 mg in 1 mL of PBS. The products are mixed by vortexing, placed on a shaker in the dark and incubated at RT for an hour. Any remaining solid was removed by centrifuging the products at 2400 rpm for 10 minutes. A 0.22 μm low protein binding cellulose acetate syringe filter was used to filter the supernatant. The list of phenolic compounds used in this study and their notations are given below.

Table 9: List of phenolic compounds

Component	Abbreviation
H	p-hydoxybenzoic acid
C	caffeoylshikimic acid
S	shikimic acid
D	3-dehydroshikimic acid
HCS	Chemical combination by adding the 3 components together in the concentration that they are present in OPP
HCD	Chemical combination by adding the 3 components together in the concentration that they are present in OPP
OPP	Oil Palm Phenolics

Working solutions

0.55045 mL of 30% hydrogen peroxide solution is dissolved in 10 mL of water. 0.18 mL of DMSO is added to 0.05 mg of DCF-DA and vortexed 3 times for 15 s. A working solution is prepared by adding 0.1 mL of stock to 100 mL of PBS.

Purification of red blood cells

Peripheral blood samples were obtained from healthy human volunteers between the age of 20 and 50 years (Lampire Biological Laboratories) and drawn into sodium heparin. 24 mL of blood was layered onto a double gradient of Histopaque 1119 (12 mL) and 1077 (12 mL) and centrifuged at 700g for 30 min at RT. Two distinct opaque layers could be observed after the centrifugation. Plasma is discarded to within 0.5 cm of the upper layer. Cells are transferred from this layer to a tube labeled "mononuclear". The remaining fluid to within 0.5 cm of the lower layer is aspirated and discarded. Cells from this layer are transferred to a tube labeled "granulocytes". Then, Histopaque 1119 is aspirated and discarded. The bottom RBC fraction is washed twice in 10 mL of PBS without calcium or magnesium at 2400 rpm for 25 min by using vials. RBC cell suspension was prepared for the CAP-e assay by adding packed RBC (0.1 ml) to PBS (10 ml). RBC aliquots could also be stored at 4°C until use in the CAP-e assay.

Assay procedure

The procedure described in [14] was used. Briefly, around 1 million red blood cells obtained from whole blood are treated with the different antioxidants present in 1 to 10 mg/mL of OPP for a period of 90 minutes. 1 mL of DCF-DA working solution, a green fluorescent dye, which is responsive to reactive oxygen species, is incubated with the cells for 1 hour. Then, the cells undergo oxidative stress by the addition of 167 mM H_2O_2 solution (i.e. 100 µL of working solution) for a period of 45 minutes. Samples were washed twice in PBS to remove the peroxide, transferred to cold PBS, and stored on ice in preparation for immediate analysis by the FACSCalibur flow cytometer (BD Biosciences).

Data acquisition was by using the CellQuest Pro Software and the cells were analyzed by using FlowJo software. Reduction of MFI in samples that were treated with the different compounds is measured in terms of percentage of inhibition of ROS oxidation. Experiments were repeated thrice.

Synergistic interactions in components of OPP and their derivatives

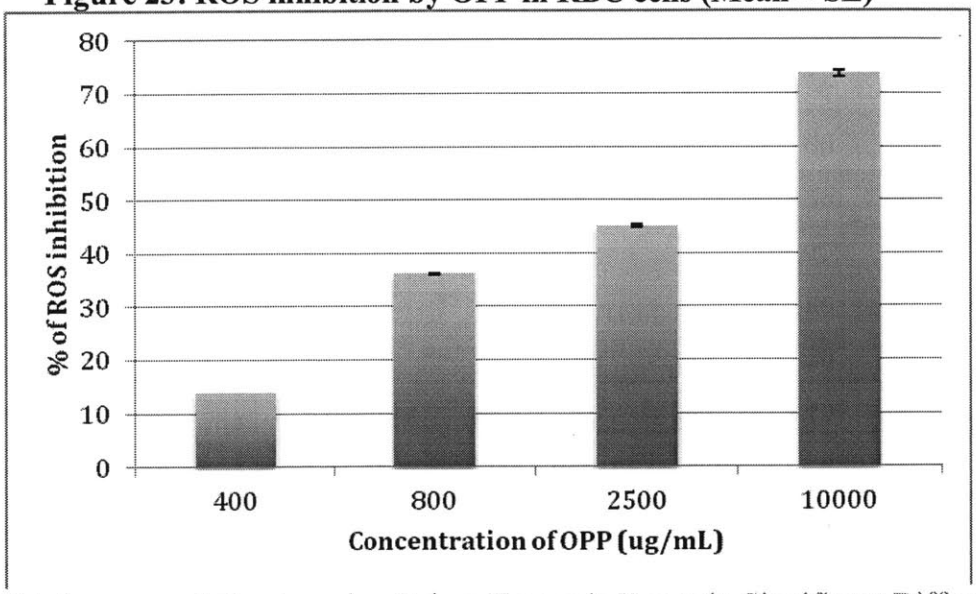
Whenever two components are denoted with a plus sign (Eg: A+B), it refers to the sum of their individual percentages of ROS inhibition.

Whenever two components are denoted together (Eg: AB), it refers to the percentage of ROS inhibition obtained when they are chemically combined together and added to cells.

For a chemical combination to be synergistic,
$AB > A + B$
A chemical combination is said to be antagonistic if:
$AB < A + B$
A chemical combination is additive if:
$AB = A + B$

A concentration of 10000 µg/mL OPP was chosen based on the results of a screening experiment in which OPP showed greater than 70% ROS inhibition for this concentration. A high inhibition percentage by OPP usually means that all of its components can be expected to exhibit ROS scavenging activity that will be within the detectable assay limits. The results of the screening experiment are shown in figure 23.

Figure 23: ROS inhibition by OPP in RBC cells (Mean ± SE)*



* $p < 0.001$ for mean of all pairs using Tukey-Kramer's Honestly Significant Difference

The results of the synergy study are shown in table 10.

Table 10: 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)

‡ Components were added according to the concentration present in 10 mg/mL of OPP
* All values show statistical significance in comparison to OPP ($P < 0.01$, Tukey-Kramer Honestly Significant Differences test)

In this method, shikimic acid does not show any effect on reducing ROS. However, all the other compounds are able to inhibit ROS oxidation. Both HCS and HCD combinations are synergistic as they are greater than H+C+S and H+C+D respectively. An interesting observation is that the chemical combination of HCS, the predominant compounds of OPP, is able to explain only 77.9% of the inhibition of ROS by OPP. However, when 3-dehydroshikimic acid is added along with H and C, it leads to 93% inhibition of ROS when compared to OPP.

4.3.2 ROS measurement (Cell-based antioxidant protection in granulocytes/polymorphonuclear - PMN cells assay)

ROS measurement in polymorphonuclear cells are a combination of effects from the compounds' ability to enter the cytosol and neutralizing free radicals, ability of anti-inflammatory compounds to enter the cytosol and reprogram the PMN cell for a reduced inflammatory behavior, which in turn affects the ROS measurement and pro-inflammatory compounds that can lead to inflammation when subjected with ROS [14].

Materials

RPMI-1640 culture medium, phosphate-buffered saline (PBS), hydrogen peroxide 30% solution (H_2O_2), dimethyl sulfoxide (DMSO), Histopaque 1077 and Histopaque 1119 were obtained from Sigma-Aldrich. Dichlorofluorescein diacetate (DCFDA) was obtained from Santa Cruz Biotech. 20% solution of Pluronic F-127 solution was obtained from Invitrogen.

Working solutions

0.55045 mL of 30% hydrogen peroxide solution is dissolved in 10 mL of water. The precursor dye DCF-DA was prepared by adding 0.18 mL of DMSO and 0.02 mL of a 20% solution of Pluronic F-127 in DMSO to a 50 μg aliquot of DCF-DA and vortexing 3 times for 15 s. A working solution of DCF-DA was then prepared by adding 0.1 mL stock to 100 mL PBS.

Purification of Polymorphonuclear cells (Granulocytes)

Peripheral blood samples were obtained from healthy human volunteers between the age of 20 and 50 years (Lampire Biological Laboratories) and drawn into sodium heparin. 24 mL of blood was layered onto a double gradient of Histopaque 1119 (12 mL) and 1077 (12 mL) and centrifuged at 700g for 30 min at RT. Two distinct opaque layers could be observed after the centrifugation. Plasma is discarded to within 0.5 cm of the upper layer. Cells are transferred from this layer to a tube labeled "mononuclear". The remaining fluid to within 0.5 cm of the lower layer is aspirated and discarded. Cells from this layer are transferred to a tube labeled "granulocytes".

The cells from "granulocytes" tube were washed twice by adding 10 mL PBS without calcium and magnesium and centrifuged at 2400 rpm for 25 minutes. PMN pellet was treated with 4 mL of distilled water for 30s to lyse RBCs not removed by the gradient centrifugation. Immediately, 4 mL of 1.8% saline (2x PBS) was added to restore the physiological isotonic condition. Subsequently, 4 mL of PBS was added, and the PMNs were centrifuged for 10 min at 2400 rpm. All PMN cells obtained are used immediately.

Assay Procedure

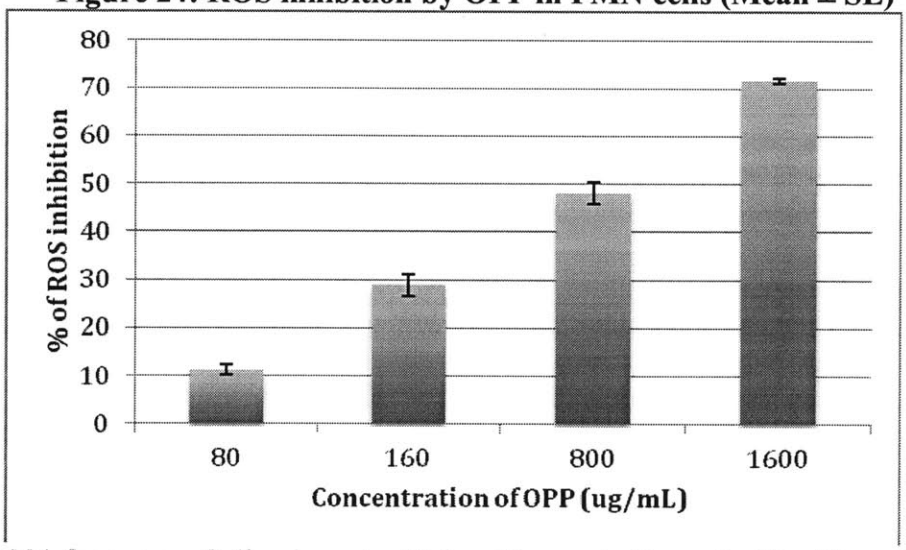
The procedure described in [14] was used. Briefly, around 1 million polymorphonuclear cells obtained from whole blood are treated with the different antioxidants present in 0.08 to 1.6 mg/mL of OPP for a period of 90 minutes. 1 mL of DCF-DA working solution, a green fluorescent dye, which is responsive to reactive oxygen species, is incubated with the cells for 1 hour. Then, the cells undergo oxidative stress by the addition of 167 mM H_2O_2 solution for a period of 45 minutes. Samples were washed twice in PBS to remove the peroxide, transferred to cold RPMI medium, and stored on ice in preparation for immediate analysis by the FACSCalibur flow cytometer (BD Biosciences).

Data acquisition was by using the CellQuest Pro Software and the cells were analyzed by using FlowJo software. Reduction of MFI in samples that were treated with the different compounds is measured in terms of percentage of inhibition of ROS oxidation. Experiments were repeated thrice.

Synergistic interactions in components of OPP and their derivatives

This assay was performed to study the possibility of synergistic interactions in OPP. A concentration of 1600 µg/mL of OPP was chosen based on the results of a screening experiment in which OPP showed greater than 70% ROS inhibition for this concentration. A high inhibition percentage by OPP usually means that all of its components can be expected to exhibit ROS scavenging activity that can be within the detectable assay limits. The results of the screening experiment are shown in figure 24. The results of the synergy study are shown in table 11.

Figure 24: ROS inhibition by OPP in PMN cells (Mean ± SE)*



* $p < 0.001$ for mean of all pairs using Tukey-Kramer's Honestly Significant Difference

In this method, shikimic acid is able to inhibit ROS oxidation even as an individual compound. Both HCS and HCD combinations are synergistic as they are greater than H+C+S and H+C+D respectively. 3-dehydroshikimic acid, a compound that can be reduced to form shikimic acid by the enzyme shikimate dehydrogenase in a cell-based system also exhibits a similar effect. A very interesting observation is that the combination of HCS leads to almost as much percentage inhibition of ROS as OPP.

Table 11: 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)

‡ Components were added according to the concentration present in 1.6 mg/mL of OPP
* All compounds/combinations except HCD show statistical significance ($p < 0.05$) with respect to OPP using Tukey-Kramer's Honestly Significant Difference (HSD)

Conclusion

Hence, shikimic acid can enter the cytosol and reprograms the PMN cells for an anti-inflammatory action, which in turn reduces the ROS oxidation. In RBC cells it shows no activity as it lacks direct antioxidant activity. It may also be possible that shikimic acid cannot get converted into its active form 3-dehydroshikimic acid in RBC cells due to lack of GDH [15], but the conversion can be possible in granulocytes. It could also be a result of the anti-inflammatory signaling actions by shikimic acid in combination with other compounds.

4.3.3 Nitrite measurement (BV2 microglial cells and RAW 264.7 macrophages – Griess assay)

The murine microglial cell line BV2 was stimulated to produce nitric oxide (NO) by adding lipopolysaccharide (LPS) [16, 17]. The different compounds of OPP were added to these cells for a period of 24 hours and the nitrite content was measured by using Griess reagent. The key to the compounds is listed in table 12.

Table 12: Components used in the nitrite quantification study

Abbreviation	Compound	Solvent‡
PCA	Protocatechuic acid	Water
HBA	4-hydroxybenzoic acid	Water
SA	Shikimic acid	Water
CA	Caffeic acid	DMSO
CGA	Chlorogenic acid	Water
EGCG	Epigallocatechin gallate	Water
CAPE	Caffeic acid phenethyl ester	DMSO
QUER	Quercetin	DMSO

‡ Wherever DMSO is specified, the solid was dissolved in DMSO, then 10 µL were added to 4 mL cell culture media. So the final concentration of DMSO is 0.25% in the cell culture medium.

Cell viability

The CellTiter Glo Luminescent Cell Viability assay (Promega) is used to test the cell viability as described in section 3.3.1.2. After 24 hours of incubating the cells with pure compounds and LPS, the contents of the plate are equilibrated at room temperature for 30 minutes, 100 µL of the CellTiter Glo reagent are added and the luminescence of each well is recorded.

Standard curve

In order to generate a standard curve, sodium nitrite solution (Sigma-Aldrich) from concentration 0 to 100 µM is used as the standard. Absorbance at 540 nm (wavelength of maximum absorbance) is measured and a plot of absorbance versus concentration is generated. The line equation of this plot is used to determine the nitrite concentration of samples.

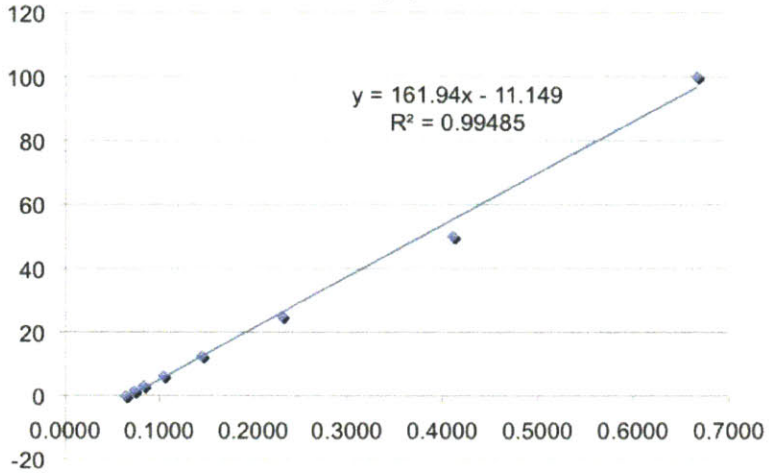
Nitrite measurement

To measure the nitrite concentration, 50 µL of the 1x Griess solution (Sigma-Aldrich) is added to each of the 96 wells to which 50 µL of medium is also added. The plate is incubated at room temperature for 15 minutes and absorbance at 540 nm (wavelength of maximum absorbance) is measured immediately after incubation. Experiments were repeated thrice.

4.3.3.1 Biological characterization of pure compounds

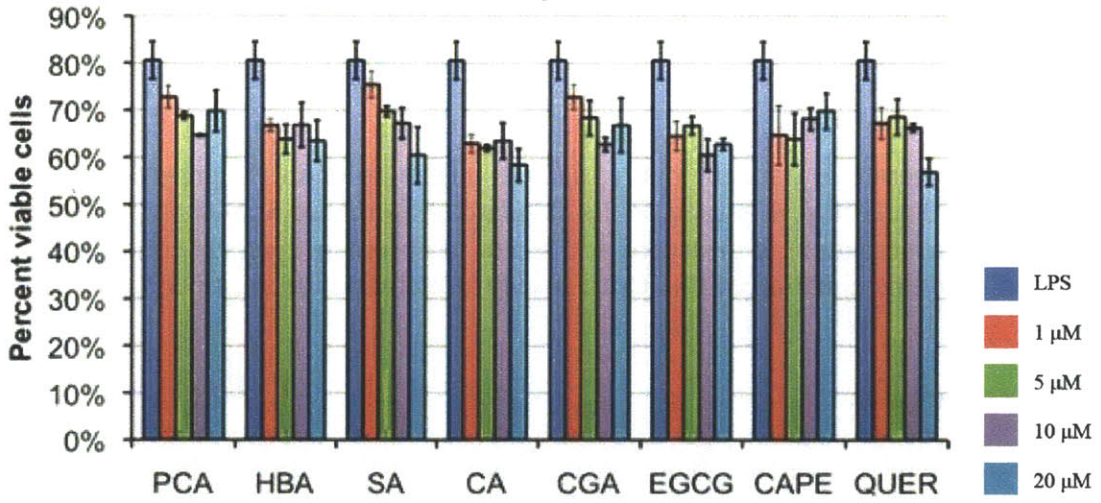
The standard curve generated between the nitrite concentration and absorbance is shown in figure 25. A linear relationship could be seen between the absorbance and nitrite. This justifies the use of sodium nitrite as a standard in this experiment.

Figure 25: Standard curve between sodium nitrite concentration (Y), absorbance (X)



The cell viability results are shown in figure 26.

Figure 26: Cell viability of BV-2 cells treated with LPS (1 µg/mL), pure phenolics and CellTiter Glo reagent (24 h) (Mean ± SE)*

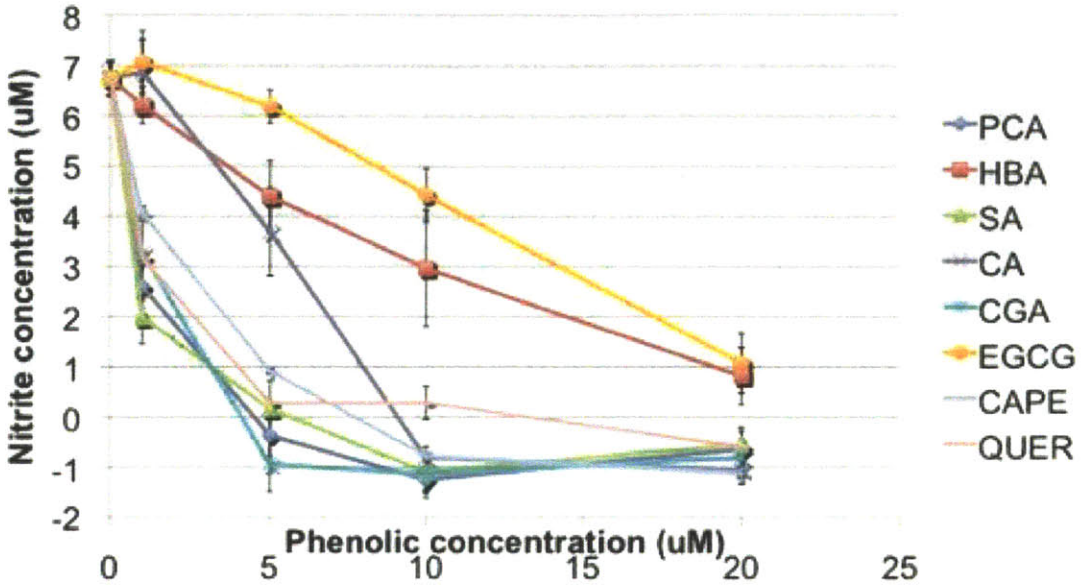


*All values at 5, 10 and 20 µM are significantly lower than LPS-only ($p<0.05$, Tukey-Kramer HSD)

As more cells die with an increase in phenolics concentration than LPS-only treatment, the total NO production that is dependent on the total number of cells actively secreting NO will change.

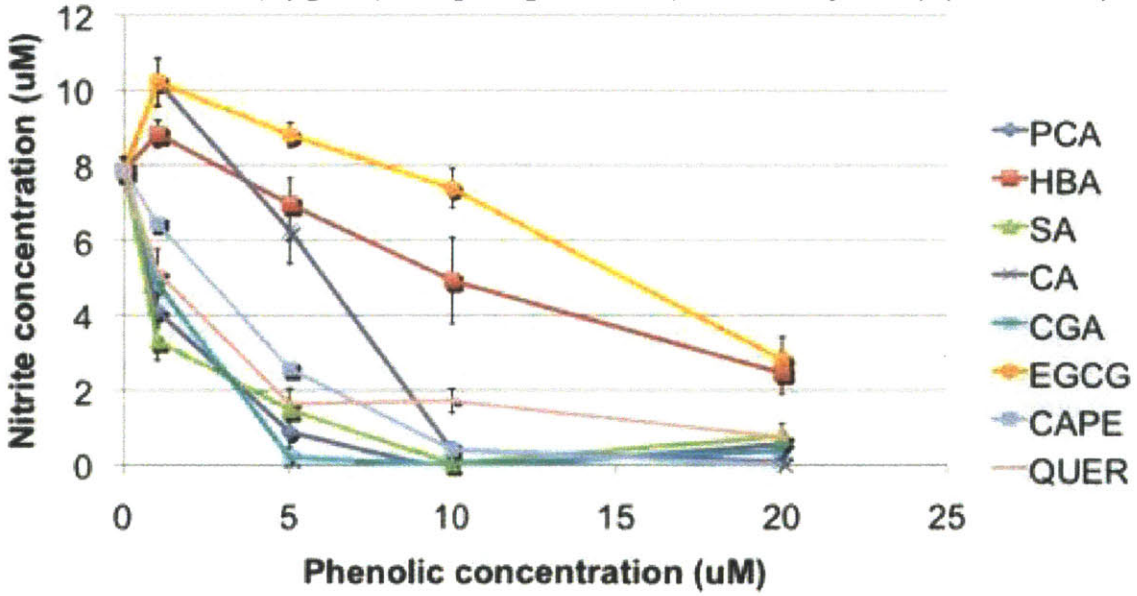
The NO levels of the different compounds are shown in figure 27. The pure compounds showed significant reduction of NO production at higher concentrations. The most effective at NO reduction appear to be protocatechuic acid (PCA), chlorogenic acid (CGA) and surprisingly, shikimic acid (SA). The weakest effect was found in EGCG and 4-hydroxybenzoic acid (HBA)

Figure 27: Concentration-dependent NO production of BV-2 cells treated with LPS (1 µg/mL) and pure phenolics (Griess assay, 24 h) (Mean ± SE)



As noted previously, the cell viability must be taken into account in order to properly compare NO reduction for these curves. Each NO value is divided by the fraction of cells left alive with LPS + phenolics compared to LPS alone to obtain a corrected value for each data point. The cells and media alone are used as the zero value so that the contribution of LPS to elevate NO production over unstressed cells can be measured. The cell viability adjusted NO levels are shown in figure 28.

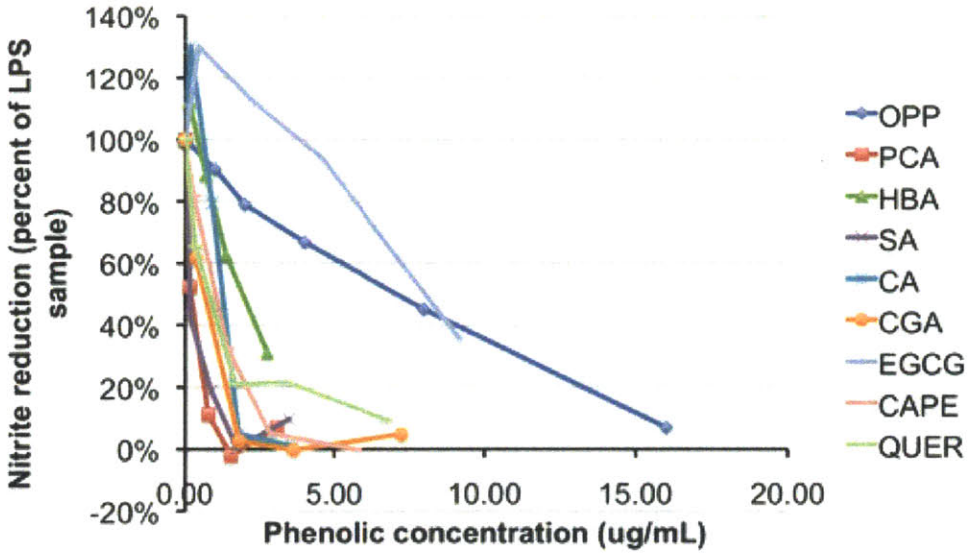
Figure 28: Cell viability and baseline-corrected NO production of BV-2 cells treated with LPS (1 µg/mL) and pure phenolics (Griess assay, 24 h) (Mean ± SE)



It can be seen from figure 28 that EGCG is by far the least effective compound of the group. All the compounds except EGCG, HBA and QUER are able to return NO production to baseline levels by around 10 μM of phenolic concentration.

In order to see where OPP fits into this hierarchy, the molecular weight of each of these pure compounds is used to convert the μM concentration into μg/mL, since the concentration of OPP is measured in units of μg/mL. Then, an approximation is made for the phenolics in OPP. Since roughly 2% of the solid weight of OPP is phenolics, the concentration of OPP is multiplied by 2% to get the approximate concentration of phenolics in each sample. Matching up the percent NO decrease from the griess assay results of OPP alone, the potent anti-inflammatory effect of OPP is compared to pure phenolics in figure 29.

Figure 29: NO reduction on LPS-treated BV-2 cells, OPP vs pure compounds (24 h)



By looking at the phenolic concentration in OPP, it can be seen that it compares very well with pure compounds as an anti-inflammatory agent in the LPS-BV-2 model. In fact, it performs better than EGCG on a gram-per-gram basis. The other phenolic compounds including PCA, HBA, SA and CA are more effective than the phenolics in OPP at reducing NO in this cell line by about one order of magnitude.

The unique solution-behavior concentration of SA was estimated as 100 μg/mL (or 0.57 mM) in section 4.2.3.4. This lies much higher than the highest concentration of 20 μM in this assay. Hence, the unique solution-behavior concentration of SA falls in the cytotoxic range.

To be more specific, this assay has LPS resulting in 8 μM of nitrite formation. It takes 10 μM of pure shikimic acid (or) around 2 μg/mL of pure shikimic acid (or) the shikimic acid present in 200 μg/mL of OPP to bring it to base level.

4.3.3.2 Biological enhancement of the components of OPP by shikimic acid

Whenever two components are denoted with a plus sign (Eg: A+B), it refers to the sum of their individual percentages of nitrite inhibition.

Whenever two components are denoted together (Eg: AB), it refers to the percentage of nitrite inhibition obtained when they are chemically combined together and added to cells.

For a chemical combination to be synergistic,
$AB > A + B$
A chemical combination is said to be antagonistic if:
$AB < A + B$
A chemical combination is additive if:
$AB = A + B$
A concentration of 800 µg/mL OPP was chosen based on the ability of all compounds to exhibit nitrite-scavenging activity in this region. The results are shown in table 13.

Table 13: Synergy study in cell-based NO_2^- scavenging activity (Griess assay)

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)

‡ Components were added according to the concentration present in 800 µg/mL of OPP
* All components except PH435S show statistical significance ($p < 0.05$) with respect to OPP using Tukey-Kramer's Honestly Significant Difference (HSD)

Shikimic acid by itself leads to 5.17% inhibition of nitrite when compared to OPP. More interestingly, the chemical combination of 5 components of OPP is able to explain only 85.36% of the inhibition of nitrite. However, when shikimic acid is added along with the 5 major components, it leads to 94.07% inhibition of nitrite when compared to OPP. This means that these six components are the major bioactive components of OPP in this cell-based assay. It also shows the biological enhancement of the five major compounds by shikimic acid.

4.3.3.3 Biological enhancement of anti-inflammatory compounds by shikimic acid and its derivative

Macrophage cells play an important role in combating inflammation because of their ability to activate proinflammatory cytokines [18]. Modulation of proinflammatory cytokines by different compounds is a proven approach to reduce inflammatory diseases [19].

Prednisone is a commonly prescribed corticosteroid drug that reduces inflammation and initiate gene, protein expression changes in several cell lines [20]. Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) that has shown inhibition of nitrite along with gene and protein expression changes to exhibit its anti-inflammatory action [21]. These two drugs are used in this experiment both as a control and for studying the biological enhancement potential of shikimic acid and 3-dehydroshikimic acid.

In this study, murine RAW 264.7 macrophage cells were subjected to LPS-induced inflammation after incubation with the different compounds for an hour. The nitrite values and the percentage inhibition of nitrite by the different compounds were measured by using the Griess reagent.

Results

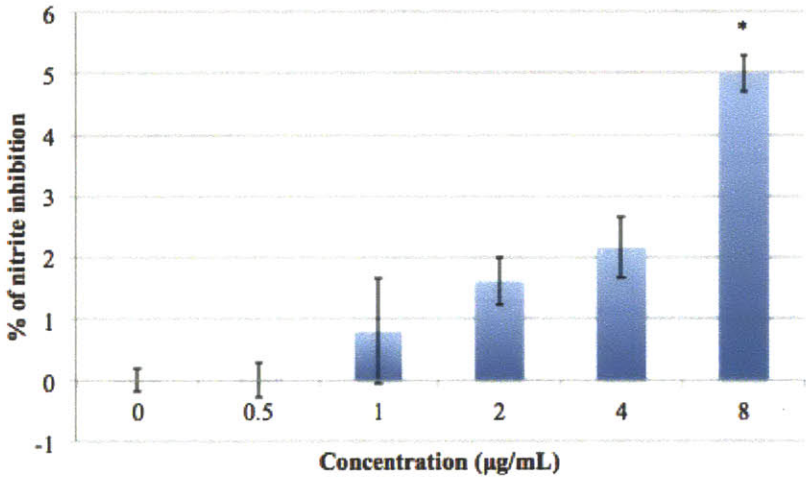
From the previous section, it is known that the concentration of 800 μg/mL OPP leads to around 65% inhibition of nitrite. When the components of OPP such as CSA are added to cells according to the amount present in 800 μg/mL of OPP, they exhibit some inhibition of nitrite. Similar results can be expected in both RAW 264.7 cells and BV-2 cells, as the amount of LPS added (1 μg/mL) remains the same. Previous studies have also established the IC50 value of Ibuprofen and Prednisone for this assay to be around 50 μM [20, 21, 22]. The above information was used to decide the concentration of compounds added in the following set of experiments. The combined amount of caffeoylshikimic acid present in OPP is around 1%. Hence, 5-caffeoylshikimic acid constituting 1% of OPP is used. A table of components and abbreviations are presented below.

0.5, 1, 2, 4 and 8 μg/mL of SA and 3DHS was added to RAW 264.7 cells and then subjected to inflammation by 1 μg/mL of LPS. The percentage of inhibition of nitrite is shown in figure 30 and 31. A dose-dependent increase in % of nitrite inhibition can be observed in both cases.

Table 14: Components and abbreviations

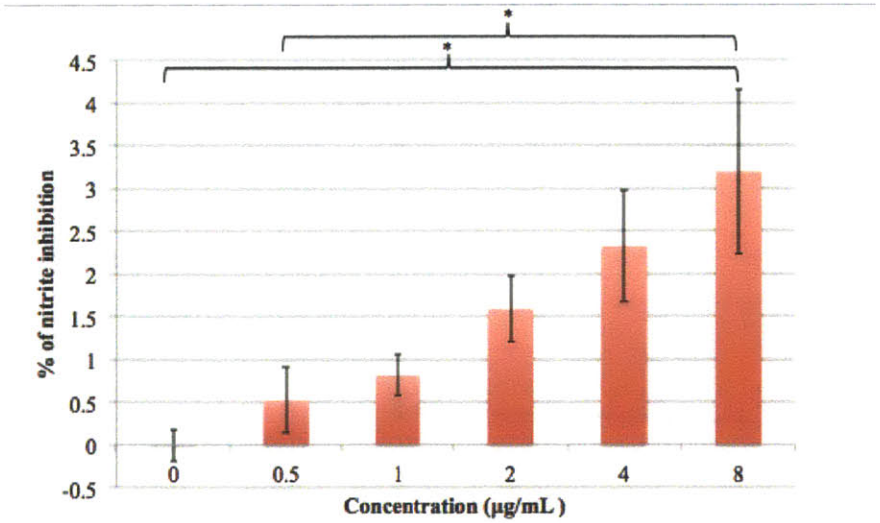
Component	Abbreviation
Shikimic acid	S
3-dehydroshikimic acid	D
Caffeoylshikimic acid	C
Chemical combination of OPP's 5 major compounds	PH435

Figure 30: Concentration-dependent nitrite inhibition of RAW 264.7 cells treated with LPS (1 µg/mL) and Shikimic acid (Griess assay, 24 h) (Mean ± SE)



* Significantly higher than all other values ($p<0.05$, Tukey-Kramer HSD)

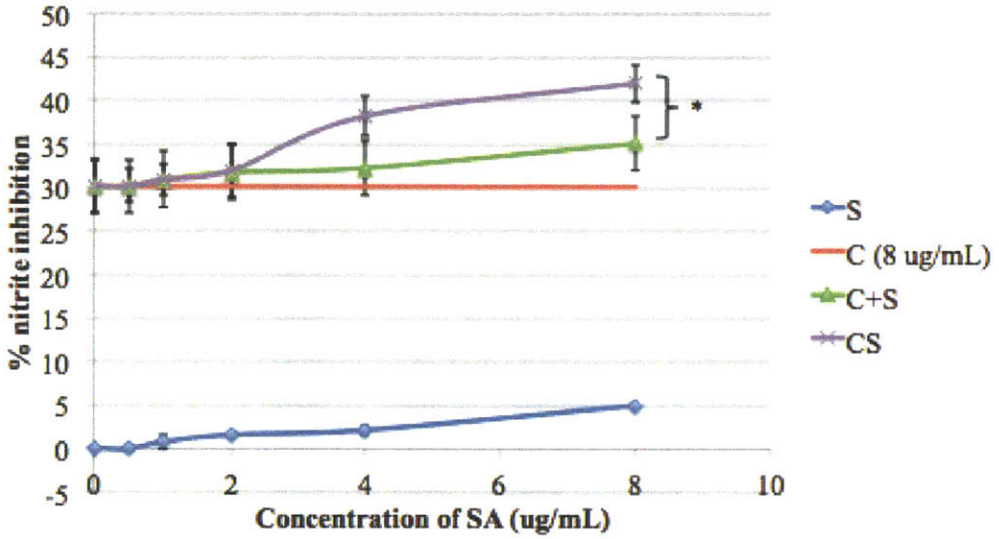
Figure 31: Concentration-dependent nitrite inhibition of RAW 264.7 cells treated with LPS (1 µg/mL) and 3-dehydroshikimic acid (Griess assay, 24 h) (Mean ± SE)



* Significant difference between indicated values ($p<0.05$, Tukey-Kramer HSD)

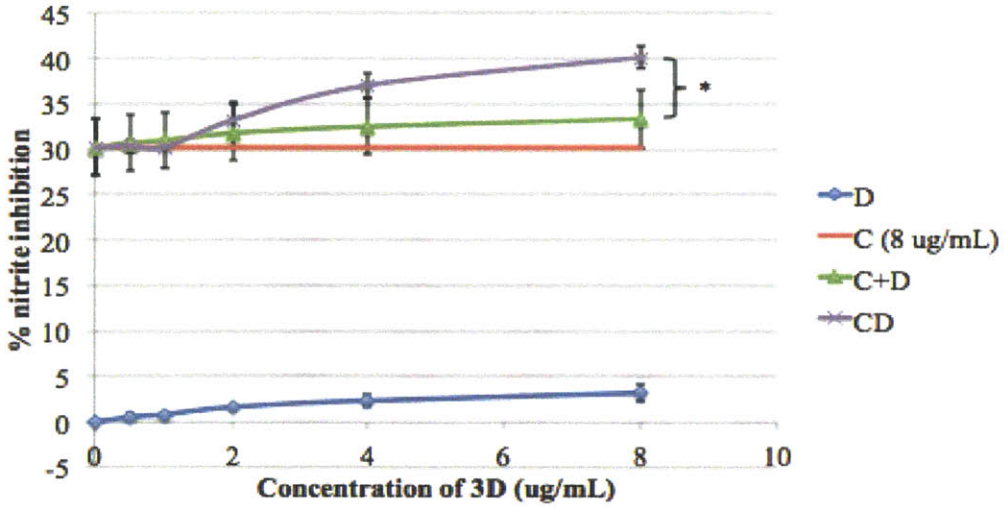
A fixed concentration of 8 µg/mL CSA-5 was added to RAW 264.7 cells, with and without 0.5, 1, 2, 4 and 8 µg/mL of SA and 3DHS, which were then subjected to inflammation by 1 µg/mL of LPS. The percentage of inhibition of nitrite is shown in figure 32 and 33.

Figure 32: Concentration-dependent nitrite inhibition of RAW 264.7 cells treated with LPS (1 µg/mL), 5-caffeoylshikimic acid and shikimic acid (Griess assay, 24 h) (Mean ± SE)



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

Figure 33: Concentration-dependent nitrite inhibition of RAW 264.7 cells treated with LPS (1 µg/mL), 5-caffeoylshikimic acid and 3-dehydroshikimic acid (Griess assay, 24 h) (Mean ± SE)

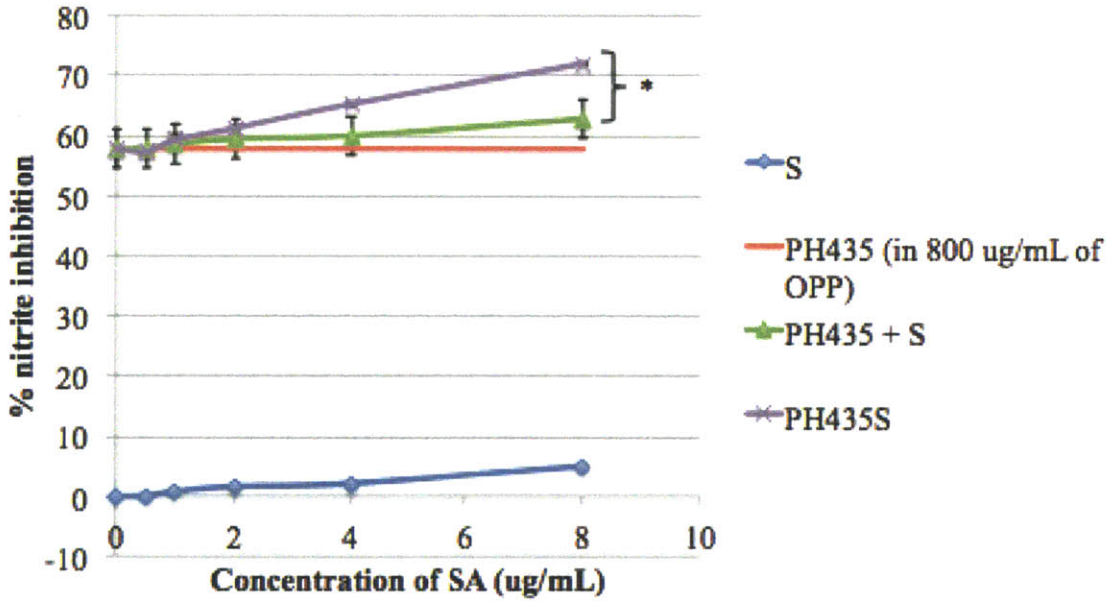


* Combination is significantly higher than sum of components indicated ($p<0.05$, Student's t test)

It can be seen that shikimic acid and 3-dehydroshikimic acid exhibit only a mild increase in the percentage of nitrite inhibition when they are added as individual compounds. However, both compounds can biologically enhance the activity of caffeoylshikimic acid. This effect is particularly visible for the higher concentration of 4 and 8 µg/mL of SA, 3D.

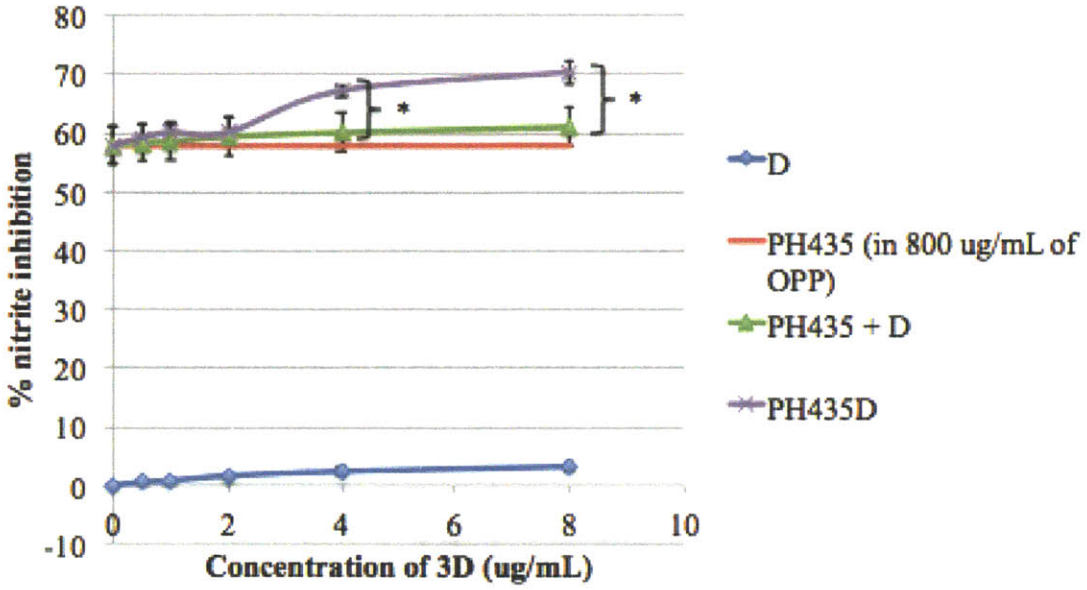
A fixed concentration of the chemical combination of 5 major compounds present in OPP was prepared according to their composition in 800 µg/mL of OPP. This was added to RAW 264.7 cells, with and without 0.5, 1, 2, 4 and 8 µg/mL of SA and 3DHS, which were then subjected to inflammation by 1 µg/mL of LPS. The percentage of inhibition of nitrite is shown in figure 34 and 35.

Figure 34: Concentration-dependent nitrite inhibition of RAW 264.7 cells treated with LPS (1 µg/mL), chemical combination of 5 major compounds in OPP and shikimic acid (Griess assay, 24 h) (Mean ± SE)



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

Figure 35: Concentration-dependent nitrite inhibition of RAW 264.7 cells treated with LPS (1 µg/mL), chemical combination of 5 major compounds in OPP and 3-dehydroshikimic acid (Griess assay, 24 h) (Mean ± SE)



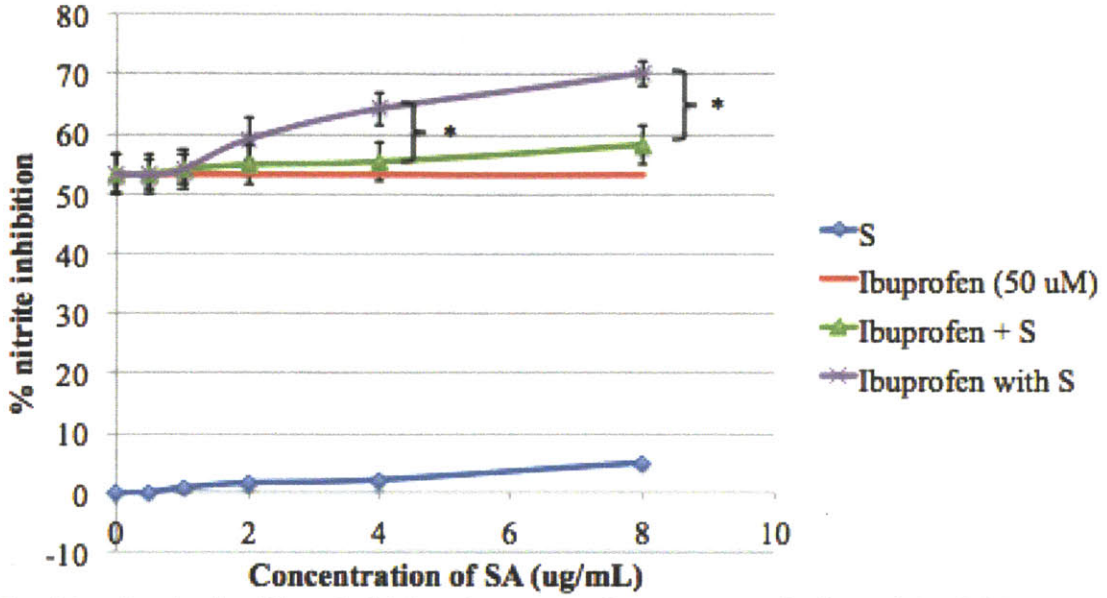
* Combination is significantly higher than sum of components indicated ($p<0.05$, Student's t test)

Shikimic acid and 3-dehydroshikimic acid of higher concentrations (4 and 8 µg/mL) led to biological enhancement of the nitrite quenching activity of PH435.

A fixed concentration of the NSAID Ibuprofen (50 µM) was added to RAW 264.7 cells, with and without 0.5, 1, 2, 4 and 8 µg/mL of SA and 3DHS, which were then subjected to inflammation by 1 µg/mL of LPS. The percentage of inhibition of nitrite is shown in figure 36 and 37.

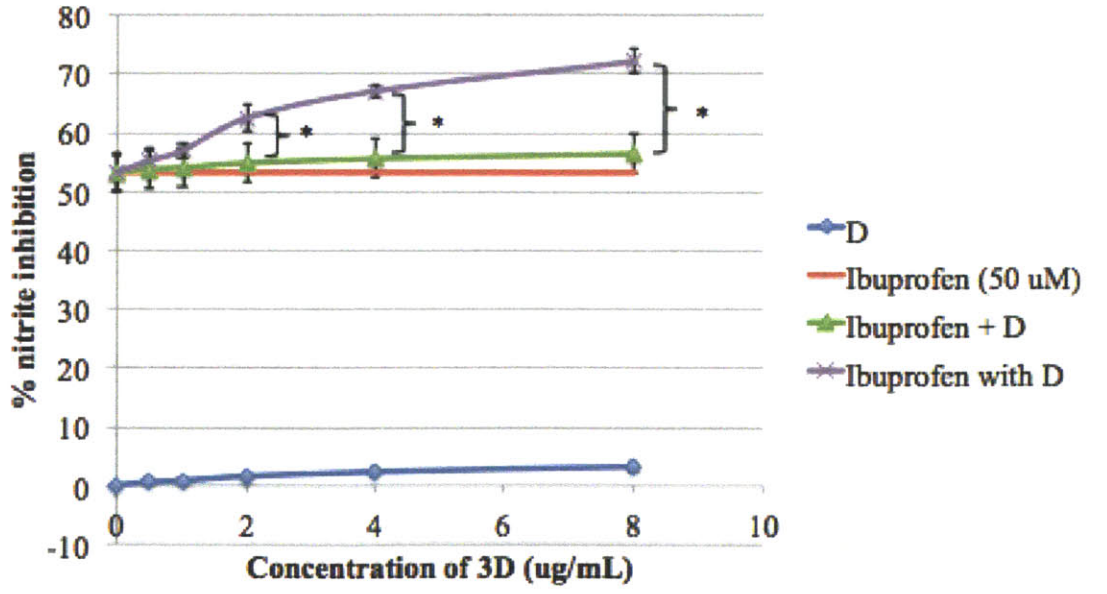
A biological enhancement of the nitrite scavenging activity of Ibuprofen by shikimic acid and 3-dehydroshikimic acid is clearly visible at even a low concentration of 2 µg/mL SA, 3D.

Figure 36: Concentration-dependent nitrite inhibition of RAW 264.7 cells treated with LPS (1 µg/mL), Ibuprofen and shikimic acid (Griess assay, 24 h) (Mean ± SE)



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

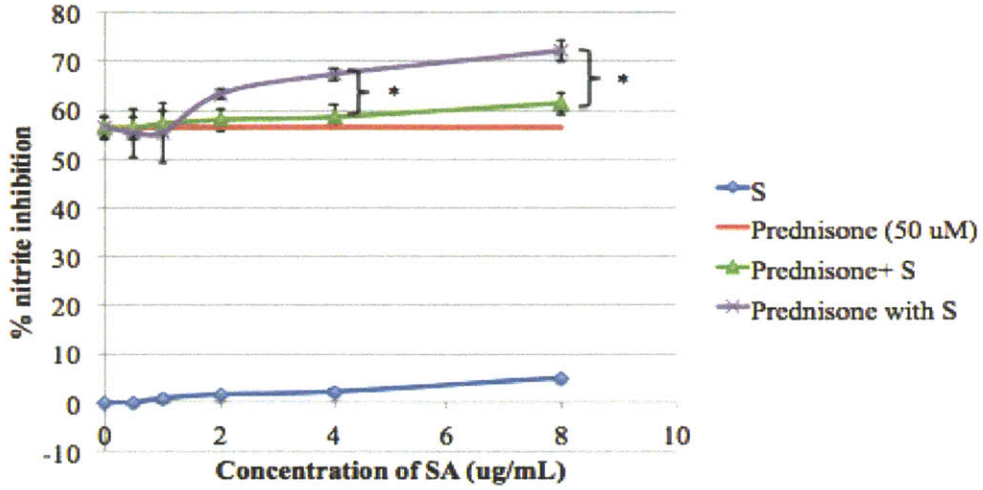
Figure 37: Concentration-dependent nitrite inhibition of RAW 264.7 cells treated with LPS (1 µg/mL), Ibuprofen and 3-dehydroshikimic acid (Griess assay, 24 h) (Mean ± SE)



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

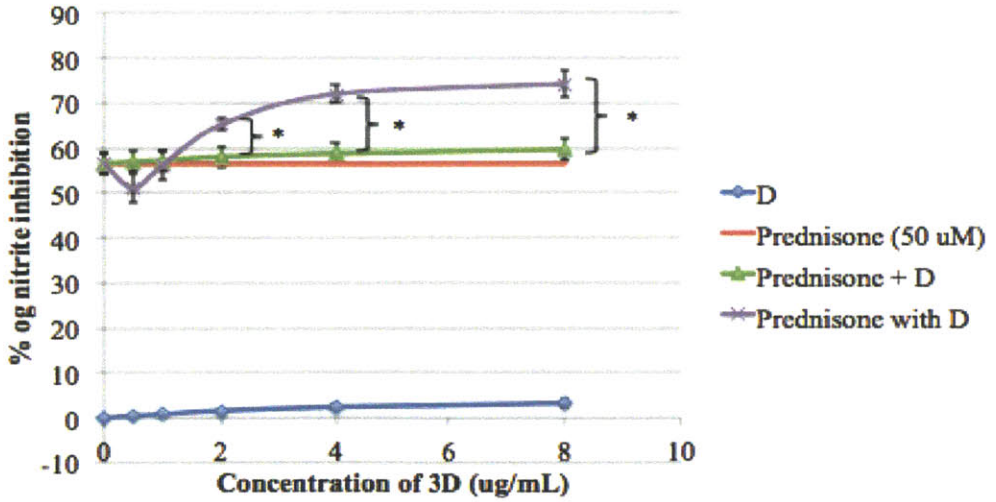
A fixed concentration of the corticosteroid Prednisone (50 µM) was added to RAW 264.7 cells, with and without 0.5, 1, 2, 4 and 8 µg/mL of SA and 3DHS, which were then subjected to inflammation by 1 µg/mL of LPS. The percentage of inhibition of nitrite is shown in figure 38 and 39.

Figure 38: Concentration-dependent nitrite inhibition of RAW 264.7 cells treated with LPS (1 µg/mL), Prednisone and shikimic acid (Griess assay, 24 h) (Mean ± SE)



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

Figure 39: Concentration-dependent nitrite inhibition of RAW 264.7 cells treated with LPS (1 µg/mL), Prednisone and 3-dehydroshikimic acid (Griess assay, 24 h) (Mean ± SE)



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

Similar to Ibuprofen, biological enhancement of the nitrite scavenging activity of prednisone by shikimic acid and 3-dehydroshikimic acid is clearly visible at even a low concentration of 2 or 4 μg/mL SA, 3D.

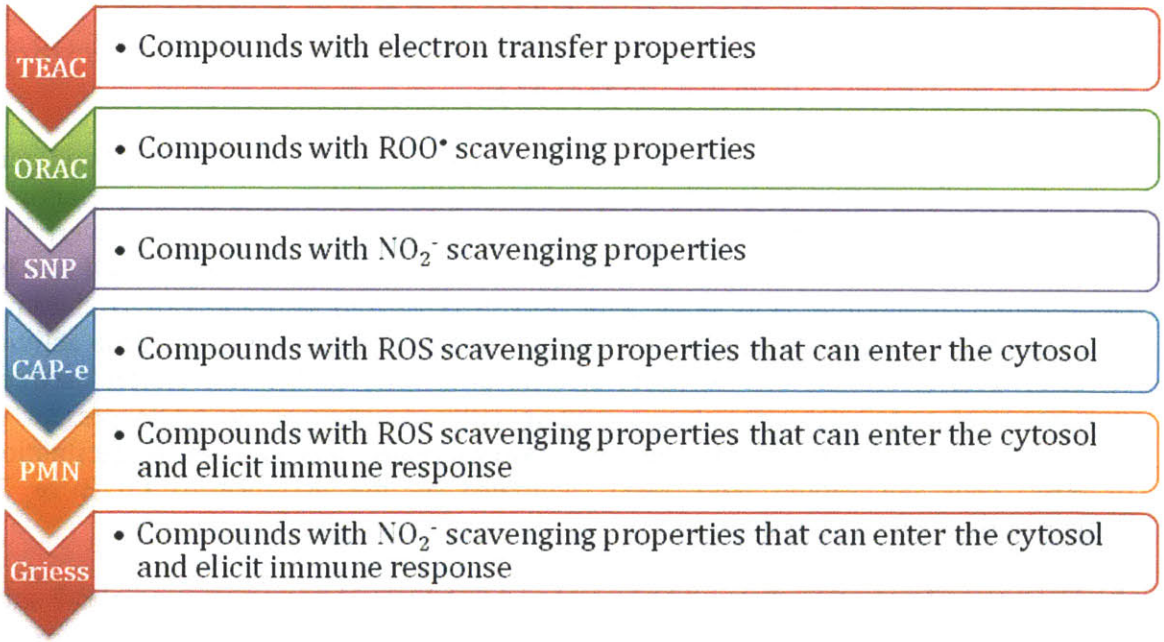
Conclusion

All phenolic compounds analyzed in this study were able to exhibit a decrease in NO with their increasing concentration. The novel finding that shikimic acid and 3-dehydroshikimic acid can biologically enhance the anti-inflammatory activity of other compounds is a useful one.

4.4 Discussion

The differences between various methods of testing the anti-oxidant potential and anti-inflammatory activity of natural compounds have been a topic of debate [14]. Many natural compounds have been shown to exhibit activity in chemical methods but lack such activity in biological cells. However, I have made the converse observation with respect to shikimic acid by taking a systemic approach of testing the individual components of Oil Palm Phenolics in six different systems, each of them evaluating for a different ROS/RNS under various reaction conditions (figure 40).

Figure 40: Chemical and Biological systems encompassing several ROS/RNS



The Trolox Equivalent Antioxidant Capacity Assay (TEAC) results showed antioxidant capacity for all the 6 major compounds, except shikimic acid. However, Oil Palm Phenolics was found to show very high activity. These observations made me realize the possibility of the inability of shikimic acid to quench free radicals by itself and the potential for synergistic interactions among the individual compounds.

Synergistic and antagonistic interactions among the compounds present in navel oranges have been evaluated by using the Oxygen Radical Absorbance capacity Assay (ORAC) [11]. Similar to the TEAC assay results, shikimic acid showed no activity on its own while the other five components of OPP showed antioxidant capacity. This led to the initial speculation that the five major compounds were the only active compounds of OPP. Yet, when they were added together, they did not match up to the high ORAC of OPP. At this point, both shikimic acid and its derivative, 3-dehydroshikimic acid were found to chemically enhance the antioxidant capacity of the individual components or their combinations. When shikimic acid and 3-dehydroshikimic acid were chemically added to the combination of 5 major compounds, they led to a high antioxidant capacity almost as high as that of OPP. This finding is proof to the fact that the five major compounds along with shikimic acid are the chemically active compounds of OPP. As 3-dehydroshikimic acid along with the five major compounds led to a higher value than the five major compounds with shikimic acid, it also leads to the possibility of a reaction balance between shikimic acid and 3-dehydroshikimic acid in OPP.

Shikimic acid and its derivative, 3-dehydroshikimic acid continued to show no clear effect as individual compounds in the Sodium Nitroprusside Assay (SNP) for quantification of nitrite. Their unique solution behavior may also help us decide on the concentration levels for the other assays.

In the cell-based antioxidant protection assay using RBC cells, shikimic acid showed no potential to quench ROS. The observation that 3-dehydroshikimic acid showed ROS quenching activity in this assay but not in ORAC assay may be due to the type of ROS measured in these two assays. While CAP-e assay can help us measure all ROS, ORAC assay can only measure a particular ROS ($ROO^{\bullet}$).

In the CAP-e assay, 3-dehydroshikimic acid added along with p-hydroxybenzoic acid and caffeoyshikimic acid, the major compounds of OPP, exhibits synergistic interactions when compared to the sum of individual ROS quenching abilities. Moroever, this combination led to a radical quenching activity almost as high as that of OPP. The combination of shikimic acid along with p-hydroxybenzoic acid and caffeoyshikimic acid, the major compounds of OPP, exhibited synergism for around 80% of the activity of OPP. This is second line of proof for the possibility of the conversion between shikimic acid and 3-dehydroshikimic acid in OPP. As there is no GDH present in erythrocytes [15], the conversion could not have happened only through the facilitation of RBC cells but should have been facilitated by the presence of GDH or 3-dehydroshikimic acid directly in OPP.

An important observation that shikimic acid and 3-dehydroshikimic acid can show anti-inflammatory activity on their own was made by using the PMN assay. As the only difference between the CAP-e assay and PMN assay is the ability of the PMN cells to elicit genetic and protein level responses, it remains clear that shikimic acid and 3-dehydroshikimic acid can activate some signaling pathways for exhibiting this anti-inflammatory activity. As we have already studied the anti-oxidant and anti-inflammatory activity of OPP in depth in chapters 2 and 3, it may be possible that

shikimic acid and its derivative can also lead to gene, protein expression changes and the modulation of nuclear transcription factor NF-κB and nuclear factor erythroid 2-related factor (Nrf2). There is also a possibility of the ability of these individual compounds to activate the inherent antioxidants of the body, such as glutathione.

The PMN assay also shows synergism between p-hydroxybenzoic acid and caffeoylshikimic acid with both shikimic acid and 3-dehydroshikimic acid. Most interestingly, these combinations almost match up to the ROS quenching activity of OPP.

The Griess Assay in RAW 264.7 and BV2 cells were used as a robust assay system to confirm many observations made in the other assays. In this assay, shikimic acid and 3-dehydroshikimic acid showed the ability to quench nitrite species, thereby confirming the results made in PMN assay. In the synergy study for Oil Palm Phenolics, six major compounds including shikimic acid and five major compounds were found to be synergistic combinations. The combination with six major compounds had greater nitrite quenching activity than the combination with five major compounds. It had nitrite scavenging activity that almost matched to that of OPP. Hence, these six compounds are the major compounds of OPP. As similar results were observed with 3-dehydroshikimic acid, a balance may be existing between shikimic acid and 3-dehydroshikimic acid in OPP. The biological enhancement activity of shikimic acid and 3-dehydroshikimic acid were also confirmed with two pharmaceutical standards, Ibuprofen and Prednisone.

4.5 References

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CHAPTER 5: CONCLUSION AND FUTURE WORK

5.1 Summary of Goals and Achievements

The principal aim of my research was to analyze the anti-oxidative and anti-inflammatory effects of Oil Palm Phenolics. In order to achieve this aim, I accomplished the following goals:

- Characterized the anti-oxidative effects of the composition of Oil Palm Phenolics at the molecular, gene and protein level.
    - The primary antioxidant activity of OPP to directly reduce reactive oxygen species was studied by using the ORAC and TEAC assay.
    - The secondary effects to induce the selective expression of several antioxidant enzymes, such as heme oxygenase 1 were studied by using microarray and qPCR experiments in U87 astroglia.
    - The tertiary effect to activate the inherent antioxidants, such as glutathione, was conducted by using a glutathione colorimetric assay in U87 glial cells.
    - The quarternary effect to analyze the changes in protein expression of activated enzymes such as Heme Oxygenase 1 and induction of nuclear factor erythroid 2-related factor (Nrf2) was conducted by using western blots.

- Characterized the anti-inflammatory effects of the composition of Oil Palm Phenolics at the molecular, gene and protein level.
    - An image analysis assay to study the effect of OPP on the morphology, size and phagocytosis in microglia was developed by using a fluorescent microscope.
    - The ability of OPP to quench nitrite in BV2 cells with LPS inflammation was quantified with the Griess assay.
    - The protein expression changes of OPP to reduce Inducible Nitric Oxide Synthase, Cyclooxygenase-2, Prostaglandin-2 and Nuclear transcription factor NF-κB was measured by using western blots.

- Developed a robust step-by-step six-method approach to perform the characterization and synergy studies for the individual components of Oil Palm Phenolics by using flow cytometry, fluorescent and absorbance assays.
    - Performed chemical and biological characterization of the individual components of Oil Palm Phenolics and their combinations.
    - Performed synergy studies for the individual components of Oil Palm Phenolics and their combinations in chemical and cell-based systems
    - Obtained several key insights about the nature in which the individual components of OPP are interacting in the different chemical and biological studies.

5.2 Opportunities for future work

Process Development

My research has shown the anti-inflammatory and biological enhancement potential of shikimic acid. As the demand for shikimic acid is growing dramatically due to its scarcity and its potential use in many industrial and pharmaceutical applications [1], I believe that the current priority is to develop a bench scale process and scale it to industrial scale extraction.

Human clinical study

A human clinical study can be developed to assess the antioxidant potential of OPP after its oral administration by using some of the experiments that I have detailed in my thesis. The CAP-e and PMN assays are especially promising candidates as it is a common procedure to obtain the whole blood samples and analyze their CAP-e, PMN values by using flow cytometry [2]. The glutathione levels can also be quantified from plasma samples [3].

There is also an opportunity to develop assays to measure the subcellular localization and mechanism of action of these antioxidant compounds.

Computational simulations

The unique solution behavior by shikimic acid and 3-dehydroshikimic acid can be further explored by using molecular dynamic simulations.

Mechanism of Action

The mechanism of action by shikimic acid and 3-dehydroshikimic acid to biologically enhance the activity of other anti-inflammatory compounds can be further studied. The mechanism of action studies for OPP in chapters 2 and 3 of this thesis may serve as good starting points to conduct such research.

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