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PHENOLIC RICH EXTRACTS OBTAINED FROM SMALL RED BEANS IN PREVENTING MACROPHAGE MEDIATED CHRONIC INFLAMMATION

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by

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Small red beans, commonly called Mexican beans, are a part of the legume family, the genus *Phaseolus vulgaris*, i.e., dry edible beans or the common bean. In addition to being a rich source of nutrients, small red beans also contain phenolic compounds, such as flavonoids, tannins, phenolic acids, and anthocyanins that have shown a plethora of health benefits against such conditions as obesity, diabetes, heart disease and cancer. In particular, the phenolic compounds common to the red beans have been reported to protect against chronic inflammation that if left unchecked can lead to various other chronic degenerative diseases. These benefits may be attributed to the phenolic compounds acting in combination as either synergists or additives. Optimal parameters are therefore needed to characterize the type and amount of these diverse phenolic compounds in any food system or matrix, and then to correlate the results to the condition of interest, which in this study is inflammation. However, such studies are non-existent for small red beans despite the presence of chemically diverse phenols at relatively high levels, (depending on the extraction parameters).

Therefore, the objective of this research was to apply response surface methods (RSM) to obtain phenolic rich extracts from two lines of small red beans (NE36 and

NE40). The study was completed using three factor face centered cube design (FCCD) to investigate the effect of three independent variables, solid:solvent ratio, solvent polarity and mix time on response of total phenols (TP), total flavonoids (TF) and anti-oxidative capacity (AC). The most effective factors that resulted in overall maximum TP yields were acetone: solvent (water) composition of 50%, a solid:solvent ratio of 10% and a mix time of 60 min. For optimal TF extractions, an acetone:water composition of 75%, solid:solvent ratio of 10% and mix time of 180 min were required. Maximum AC values were achieved with an ethanol:water composition of 75%, solid:solvent ratio of 10% and a mix time of 180 min. In most cases, a second order polynomial model was developed to optimize the extractions with the exception of TP for ethanol extractions and TF for acetone extractions for NE36. Preliminary data obtained in our lab indicated that higher order models (cubic) better explained the complex interactions. The extractions that produced the highest yields of TP, TF and AC were then tested for the ability to remediate inflammation using lipopolysaccharide (LPS) activated RAW 264.7 macrophages. As nitric oxide is an indicator of inflammation, this test was applied to extract treated cells to determine their ability to remediate inflammation. Only the extracts with high TF show significant anti-inflammatory activities using this vitro model, with the NE36 line showing the most efficacious results. In summary, this study has shown that that optimum phenolic yields (TP and TF) and potent AC and anti-inflammatory extracts are dependent upon the extraction methods and solvents used, and also vary with different lines of red beans. This research is therefore significant as it has shown the potential of small red beans as a health impacting food system, with an emphasis on remediating inflammation.

ACKNOWLEDGEMENT:

I want to start with a note to thank my mother who taught me the value of hard work and education.

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A) LITERATURE REVIEW:

A.1 *Background of Dry Edible Beans*

Dry edible beans (*Phaseolus Vulgaris L.*), (or the common bean), such as pinto, great northern, navy, kidney, pink, red and black beans, are part of the legume family. A legume plant produces seeds in a pod, whereas dry beans are the mature seeds within these pods (Robinson, 2013). Dry edible beans are the world's second most important legume class after soybean and are among the top ten super foods as they play a particularly important role in traditional diets in Africa, India, and Latin America (Xu and Chang, 2009). Common beans are inexpensive in terms of costs, but are rich sources of proteins, carbohydrates, dietary fiber, minerals and vitamins to millions of people in developed and developing countries (Rehman et al, 2001).

Dry beans are grown all over the world with Brazil being the world's leading producer followed by India and China (FAOSTAT, 2011). With 6 percent of the world output, the United States is the sixth-leading producer of dry beans (USDA website). Dry bean production is scattered across 19 states with North Dakota, Michigan, Nebraska, Minnesota and Idaho being the top producers in terms of total yields (yield (bu/acre) or total productions?. According to the Continuing Survey of Food Intakes by Individuals (CSFII, 2013; Lucier et al, 2000), nearly 14 percent of Americans consume at least one food containing cooked dry beans on any given day. The different market classes of red beans are dark red kidney, light red kidney, pink and small red beans. Small red beans are commonly called Mexican beans.

A.2 ***Dry Bean Composition***

Dry edible beans are among the best sources of plant protein and are low in both saturated and total fat. Similar to all plant foods, they are cholesterol-free. One-half cup of raw beans provides approximately 8 grams of protein— about the same amount present in a cup of milk— and between 100 and 130 calories. Comparable to other dry beans, red beans are also a rich source of protein, essential vitamins, minerals, fiber and complex carbohydrates (Table 1) with slight differences in the micro and macronutrients. Dry beans are nutrient dense in that the levels provided per calorie are particularly high. In addition, dry beans contain eight of the nine essential amino acids, i.e., the exception is methionine, in relatively high quantities (Bressani et al, 1963; FAO, 1957). Due to these high protein levels, dry beans hold a position in the protein group of the USDA "my plate guide" (Sath et al, 1984; Deshpande and Damodaran, 1989).

According to the 2005 Dietary Guidelines, nutrients of concern for many Americans include fiber, magnesium, potassium and calcium, all of which are contained in beans. Beans are among the richest sources of dietary fiber, including prebiotic fibers, such as resistant starch, fructoligosaccharides (e.g. stachyose and raffinose) (Reyes-Moreno et al, 1993; USDA, 2012), and insoluble polysaccharides. Prebiotics are fermented in the gut to produce short chain fatty acids (SCFAs), such as propionate and butyrate. These SCFA have been shown to protect against colon cancer, metabolic syndrome, obesity and higher total and LDL cholesterol levels, i.e., the risk factor for cardiovascular disease, among other chronic diseases (Anderson et al, 2009

Table 1. Basic composition of raw red bean (adopted from USDA)

Nutrient	Unit	Value Per 100 g (Raw)
Main Components		
Water	g	11.75
Energy	kcal	333
Protein	g	23.58
Total lipid (fat)	g	0.83
Carbohydrate	g	60.01
Fiber, total dietary	g	24.9
Sugars, total	g	2.23
Minerals		
Calcium, Ca	mg	143
Iron, Fe	mg	8.20
Magnesium, Mg	mg	140
Phosphorus, P	mg	407
Potassium, K	mg	1406
Sodium, Na	mg	24
Zinc, Zn	mg	2.79
Vitamins		
Vitamin C, total ascorbic acid	mg	4.5
Thiamin	mg	0.529
Riboflavin	mg	0.219
Niacin	mg	2.060
Vitamin B-6	mg	0.397
Folate, DFE	micrg	395
Vitamin E (alpha-tocopherol)	mg	0.22
Vitamin K (phylloquinone)	mg	19.0
Lipids		
Fatty acids, total saturated	g	0120
Fatty acids, total monounsaturated	g	0.064
Fatty acids, total polyunsaturated	g	0.457

Bourdan et al, 2001; Brown et al, 1999). A combination of fructoligosaccharides and resistant starch present in dry beans has also shown a synergistic prebiotic effect in rats by increasing the bifidobacteria and lactobacilli in the intestine, which have been linked to multiple health benefits (M.E. Rodríguez-Cabezas et al, 2010; Messina et al, 1999).

Dry beans provide a number of essential nutrients, including the B vitamin folate, vitamin E isomers, (α, γ, δ tocopherols) (Augustin et al, 1981) and minerals, such as iron, zinc, magnesium, copper, potassium and calcium, which are difficult to obtain from other food systems.

The lipid content in red beans is approximately 2.2 to 2.5% with the fatty acids being highly unsaturated, and n3 fatty acids present at 0.6 gm per 100 gm of raw edible portion. The main fatty acids are linoleic acid (18:2n-6) followed by alpha linolenic acid (18:3n-3) comprising approximately 80% of the fatty acid profile (Yoshida et al, 2005). These fatty acids have been shown to exert hypolipidemic, antithrombotic and anti-inflammatory properties in addition to and reducing the risk of cardiovascular heart disease (CHD) (Galli et al, 2006).

In addition to these micro and macronutrients, beans contain phytochemicals that include phenolic compounds, saponins, alpha amylase inhibitors, plant sterols, lignins, lectins and trypsin inhibitors, which have been reported to have numerous health benefits (Vega et al, 2010; Wu et al, 2004; Xu et al, 2007). It must be noted, however, that the nutrient content and bioavailability of these components are dramatically influenced by bean market class, line, cropping environment, storage conditions, processing and final product preparation (Uebersax et al, 2002). As such, beans that are grown in different

regions or the same regions but are different lines could exert different health promoting properties. The level of micronutruents is influenced by both genetic and environmental factors (Elizabeth et al, 2007). Furthermore, the variability in the color of the seed coat is due to diversification and variability in the composition of procyanidins, flavonol glycosides and anthocyanidins (Feenstra, 1960). And, in terms of phenols and other micronutrients, red beans can be quite different than other dry beans.

A.3. *Phenolic Compounds and Red Beans*

Red beans have been long recognized for their protein content (Messina 1999), but recently their other chemically diverse nutrients have become a topic of interest, including phenolic compounds, saponins, alpha amylase inhibitors, plant sterols, lignins, lectins and trypsin inhibitors. (Vega et al, 2010). In particular, polyphenolic compounds are a group of secondary metabolites that are ubiquitous in fruits, vegetables, and other plants. These compounds perform various endogenous functions, but primarily protect the plant from environmental stressors, such as pathogens and insect pressure, through their potent anti-oxidative properties (Wildman, 2006).

There are approximately 8,000 structural variants of phenolic compounds that are categorized by the presence of an aromatic ring(s) bearing one or more hydroxyl moieties (Bravo et al, 1998). Specific subgroups are further subdivided into different classes based upon the number of phenolic rings and other functional groups that link these rings. As an outcome, different phenolic classes have been formed, as shown in Figure 1. However, it must be emphasized that all have one or more phenol groups in their structural backbone (Figure 2).

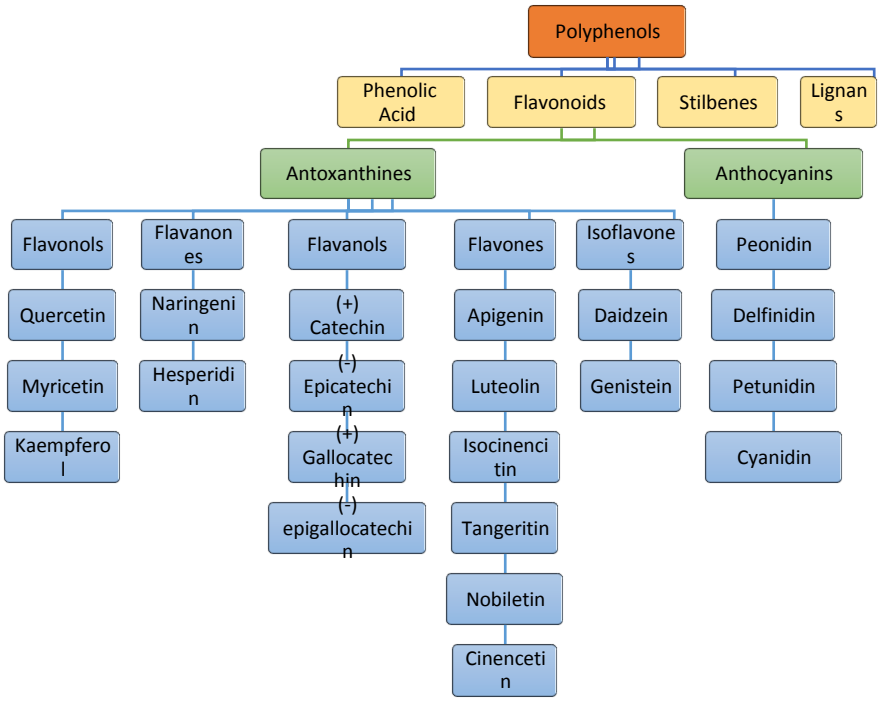


Figure 1. Classification of the main polyphenols. (Robards, 1999; Morton et al, 2000; Aherne and O'Brien, 2002; Tsao, 2010).

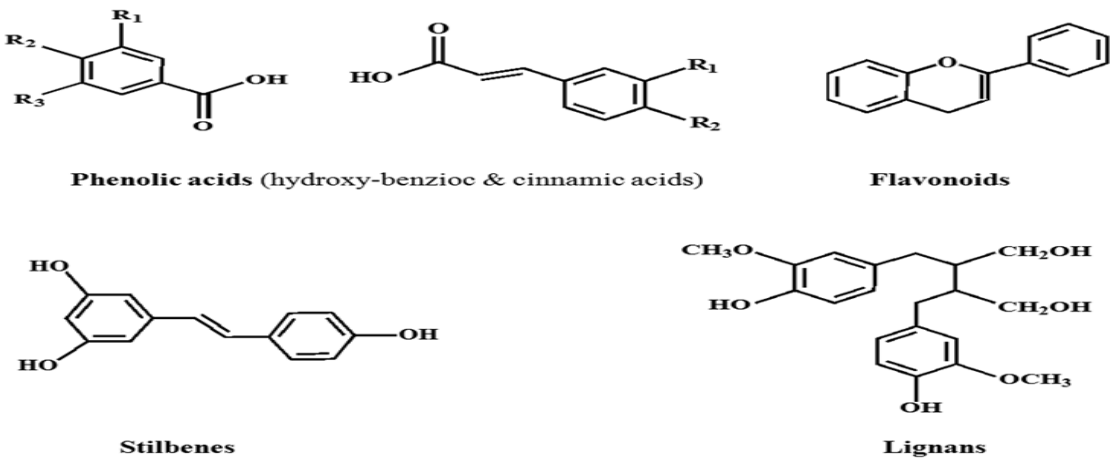


Figure 2. Chemical structures of the different classes of polyphenols. (Adapted from Pandey et al, 2009).

For example, the phenolic acids are subclasses derived from hydroxybenzoic acids, such as gallic acid and from hydroxycinnamic acid, and also include, but are not limited to, caffeic, ferulic, and coumaric acid (Han et al, 2007). These classes of phenolic acids contain one aromatic ring, a carboxylic acid group and one or more hydroxyl groups. Alternatively, the flavonoids are unique phenols that are composed of three heterocyclic rings in their backbone and are further separated into different classes based upon the position of the rings relative to one another, their degree of conjugation or the presence / position of their hydroxyl groups (Figure 2) (Shahidi and Naczk, 1995). Different classes include flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols. Of the phenols, the flavonoids are considered to be particularly potent antioxidants, most specifically the anthocyanins and tannins (Beecher, 2003). Anthocyanins are known for their red, blue or purple color depending on the pH, whereas condensed tannins are basically polymers of anthocyanins. Flavonoids have been reported to possess antiinflammatory activities by inhibiting various pathways, such as cyclooxygenase, lipooxygenase and inducible nitric oxide (NO) synthase (iNOS) pathways (Yoon et al 2005). The other classes of polyphenols are the stilbenes, the lignans and the polymeric lignins (Han et al, 2007).

Red beans contain phenols at levels higher than most other types of legumes, or at comparable or higher amounts than other types of bean market classes, depending on the extraction methods used (Tables 2-4) (Wu et al, 2004; Luthria and Pastor-Corrales, 2006). The phenolic content of common beans ranges between 34-280 mg/100 of grams of dry matter (Bravo 1998) while red beans contain 35.9 ± 8.2 mg/gm by dry weight (Vinson et al, 1998) with phenolic acid content of 28.6 mg/100 gm (Luthria et al, 2006), which is

Table 2. TPC, TFC, CTC and ORAC for different market classes of beans

Market Classes	TPC-Total Phenol	TFC-Total Flavonoid	CTC Condensed Tannin	ORAC
Small Red	5.76 ± 0.38	4.24±0.10	5.16 ± 0.11	70.58±3.24
Black	3.37± 0.15	2.51±0.12	4.09±0.10	48.91±2.04
Pinto	3.76 ± 0.06	2.99±0.12	3.23 ± 0.11	51.13±3.64
Navy	0.57 ± 0.05	0.92±0.02	0.47 ± 0.01	13.30±0.55

Values reported for TPC, TFC and CTC are in mg/g and ORAC in µmol Trolox Eq/g. Results are shown as the mean +/- standard deviation (n=3) on dry weight basis. Different market classes of Common beans (Phaseolus vulgaris L) were used .The bean flour (0.5 g each) were extracted with mixture of acetone/water/acetic acid (70:29.5:0.5, v/v/v) .The mixture were extracted for 3 hrs under horizontal shake for 300 rpm follow by another 12 hrs of overnight incubation in dark. The residues were reextracted with 5 ml of respective extraction solvents and the extracts were combined and stored at 4 C. Total phenol, flavonoid, and condensed tannins were determined using a colorimetric method while ORAC was done by fluorescein decay method . (Xu et al, 2007)

Table 3. Phenolic acid content for different market classes dry beans

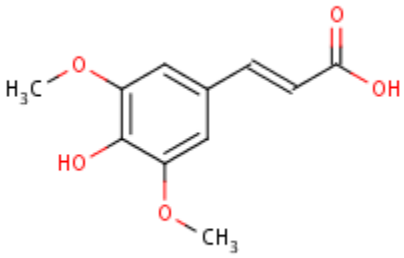
Bean Market Class	Phenolic acid concentration (mg/100 g) n=3				Total phenolic acid content (mg/100 g)
	Caffeic acid	*P*coumaric acid	Ferulic acid	Sinapic acid	
Small Red	ND*	5.8	17.4	5.4	28.6
Pinto	ND	4.5	16.0	9.0	29.5
Great Northern	ND	4.0	17.0	9.4	30.4
Navy	ND	12.4	26.6	9.2	48.2
Black	1.1	9.42	20.62	7.2	37.25
Dark Red Kidney	ND	1.8	15.3	3.8	20.9
Pink	ND	6.8	19.4	8.2	34.4

Not Detected (ND) * Ground beans were extracted with MeOH containing 0.2% TBH (2, 3-tertbutyl- 4-hydroxy anisole) and 10% acetic acid (85:15). The mixture was sonicated for 30 min and the volume of the extract was adjusted to 10 mL with distill water. Individual phenolic acids were quantitated by HPLC Diode array detection (Luthria et al, 2006).

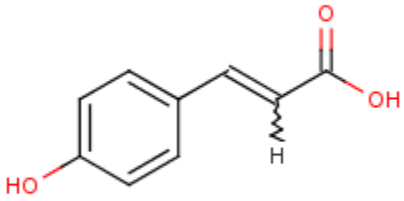
Table 4. Anthocyanin content in common bean market classes

Bean Market Class	Anthocyanin Content (mg/g)
Small Red	0.32
Pinto	0.05
Black	0.40
Navy	0.15

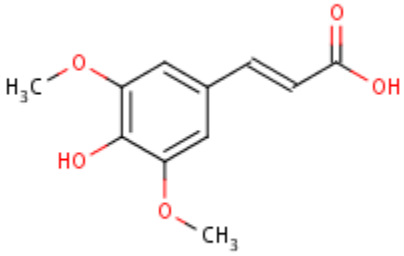
Results are shown as the mean +/- standard deviation (n=3) on dry weight basis Concentrations of anthocyanin are expressed as mg cyaniding-3-glucoside equivalents per gm of bean sample.400 mg of ground bean sample was extracted with 300µl of methanol and 1% HCl overnight in a refrigerator.200ml milliQ of water and 500 µl of chloroform were then added and then centrifuged at highest rpm for 2-5 min. The supernatant was taken and the volume made up with methanol 1% HCl and water and absorbance measured at 530 nm and 657 nm. (Peters et al, 2001).



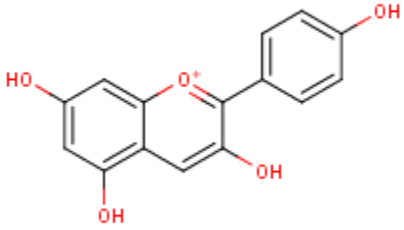
Sinapic Acid



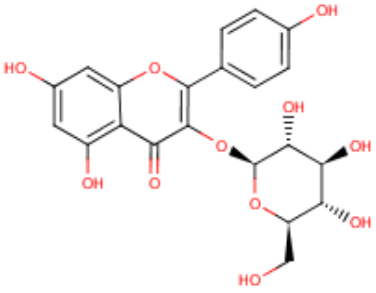
P coumaric acid



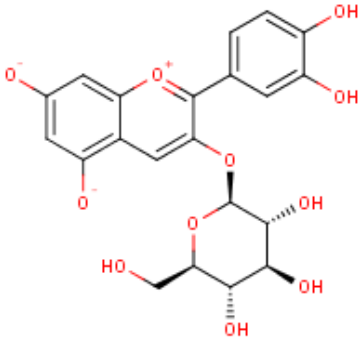
Ferulic Acid



Pelargonidin



kaempferol 3-O-glucoside



Cyanadin 3 O 4 glucoside

Figure 3. Major Polyphenolic Compounds present in Red Beans (CHEBI database)

comparable to other market classes of beans. The three major phenolic acids identified in red beans are p-coumaric, ferulic, and sinapic acids, whereas the flavonoids consist of kaempferol 3-O-glucoside, pelargonidin and cyanidin 3-O 4 glucoside (Lin et al, 2008) (Figure 3).

A.4. Macrophage *Mediated Chronic Inflammation and Phenols*

Inflammation is an essential response to repair tissue injury caused by noxious physical, chemical or microbiological stimulus (Sarkar et al, 2005). Macrophages are a major component of the mononuclear phagocyte system that consists of closely related cells of bone marrow origin, including blood monocytes, and tissue macrophages (Fujiwara et al, 2005). From the blood, monocytes migrate into various tissues and transform into macrophages. In inflammation, macrophages have three major functions; antigen presentation, phagocytosis and immunomodulation (Haschemi, 2012). Macrophages are activated to perform these functions through various signaling agents that include indigenous cytokines (e.g. interferon γ, granulocyte-monocyte colony stimulating factor, and tumor necrosis factor α), bacterial lipopolysaccharide (LPS), extracellular matrix proteins, and other chemical mediators (such as nitric oxide NO). The pathological consequences can lead to tissue edema and abnormal histological change (Wang et al, 2006). Inhibition of inflammation occurs when the above cited mediators are deactivated or removed, and inflammatory effector cells are permitted to repair damaged tissues (Fujiwara et al, 2005). However, an imbalance between the pro-inflammatory activating agents and the anti-inflammatory signals leads to macrophage mediated chronic inflammation. This can develop into a self-perpetuating cellular stress

that if left unchecked, can lead to atherosclerosis, diabetes, neurodegenerative diseases and even cancer (Lafuente et al, 2009; Tracey 2002). Figure 4 shows the various diseases caused by chronic inflammation in the body. In order to stop this cycle, pro-inflammatory macrophages (M1) must be deactivated or converted to the anti-inflammatory phenotype (M2). Additionally, the deactivated macrophage (M0) must remain so even in the midst of pro-inflammatory activating signals or proceed directly to the anti-inflammatory tissue repair state (M2).

Epidemiological studies have indicated that populations who consume foods rich in specific phenols have lower incidences of chronic inflammatory diseases (Yoon et al, 2005). Many studies have shown that five different flavonoids, such asgenistin, quercitin and luteolin, are able to modulate the arachidonic acid metabolizing enzymes (phospholipase A2 (PLA2), cyclooxygenase (COX), lipoxygenase (LOX) and nitric oxide radical (NO) by impacting the inducible nitric oxide synthase (iNOS) pathway in some way (Nijveldt et al, 2001). Such in vivo flavonoid anti-inflammatory actions include antioxidant control, inhibition of eicosanoid generating enzymes or the down-regulation of pro-inflammatory molecules. The inhibition of these enzymes reduces the production of arachidonic acid (aa), prostaglandins, leukotrienes, and nitric oxide (NO), which are crucial mediators of inflammation (A.Garcia-Lafuente et al, 2009). Apart from these enzymes, several other cytokines, such as TNF alpha, IL-6 and IL-1Beta, are associated with chronic inflammation.

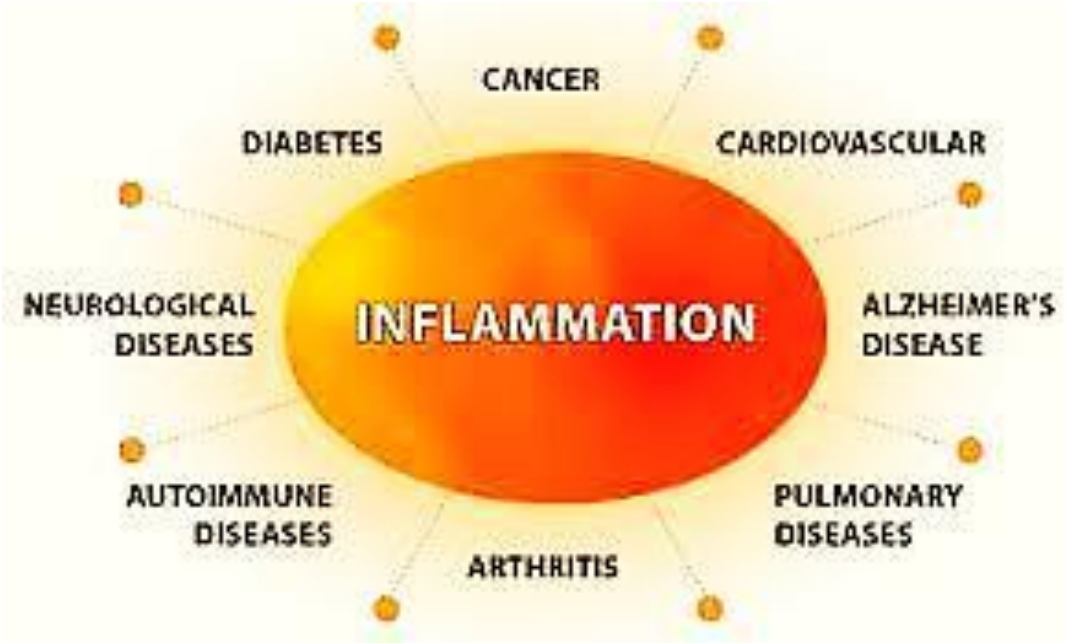


Figure 4.Various diseases caused by chronic inflammation

Several studies have shown that flavonoids inhibit these cytokines (Middleton et al, 2000; Cook et al, 1996). For example, studies with the soy isoflavones, genistein, daidzein and glycitein, revealed that all are able to suppress NO production in LPS-activated murine macrophages in a dose dependent manner. The following three mechanisms have been proposed that include scavenging of NO radicals, inhibition of INOS enzyme activity and inhibition of iNOS gene expression (A.Garcia-Lafuente et al, 2009). Several mechanisms explaining the anti-inflammatory activity of flavonoids present in red beans are described in the Figure 5. Cyclooxygenase (COX) produces prostaglandins (PG) and thromboxanes from AA and some flavonoids, such as luteolin, galangin or morin, inhibit COX and thus these inflammatory mediators (Bauman et al, 1980). Moreover, phenolic acids such as p-coumaric, caffeic, ferulic and syringic acid isolated from *S. frutescens* have shown anti-inflammatory properties by facilitating leukocyte migration to inflamed sites and acting as free radical scavengers (Fernandez et al, 1998). These polyphenolic compounds are also present in small red beans and this forms the basis of our selection of these beans for our study.

A.5. ***Role of Nitric Oxide (NO) in Inflammation***

Nitric oxide is an important intra and intercellular regulatory molecule. It is enzymatically synthesized via the oxidation of the terminal guanidine nitrogen atom of L-arginine by nitric oxide synthase (NOS), which are either constitutive (cNOS) or inducible (iNOS) (Moncada et al, 2002). Figure 6 shows the iNOS pathway for production of NO. Inducible nitric oxide synthase is not detectable in healthy tissues but is expressed after an immunological challenge or injury to cells that include smooth

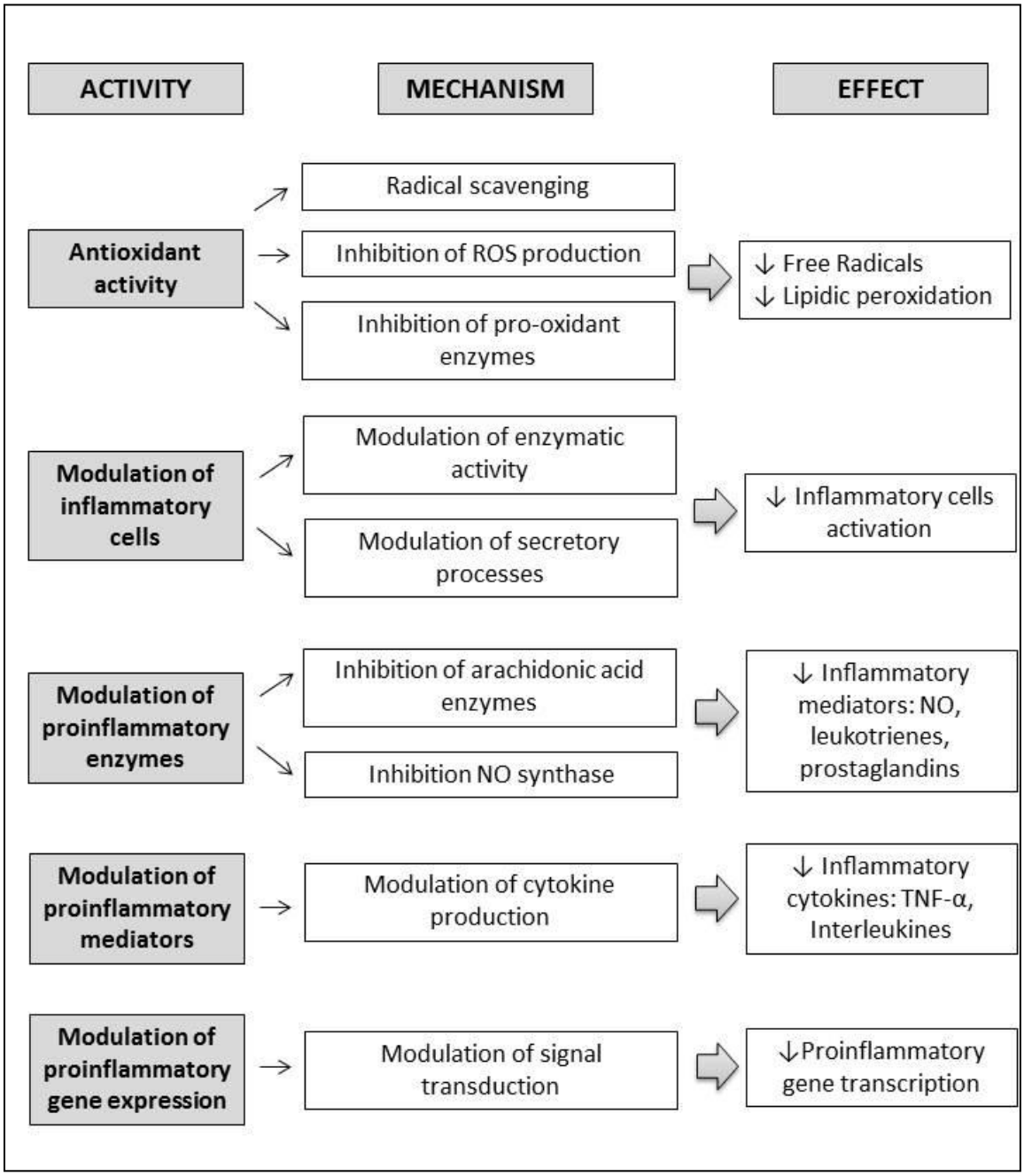


Figure 5. The mechanism of action of flavonoids in inflammation (A. Garcia Lafuente et al, 2009)

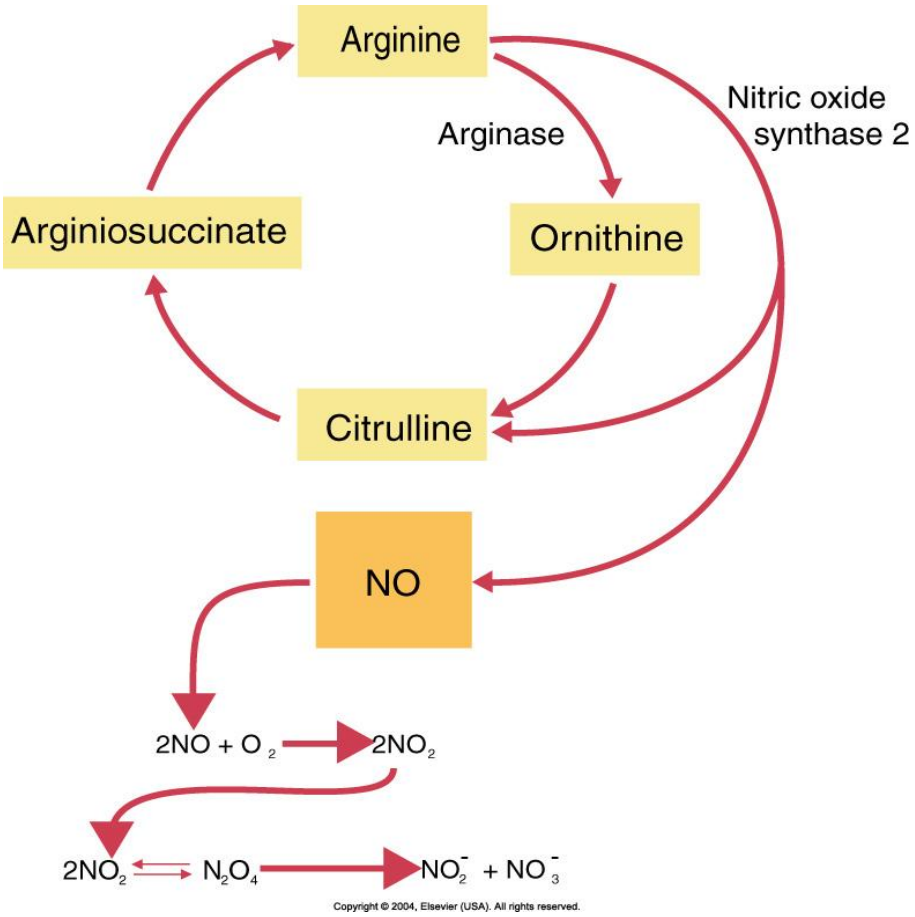


Figure 6 Inducible nitric oxide synthase (iNOS) pathway

muscle cells, macrophages and hepatocytes after exposure to specific stimulants such as cytokines (Busse et al, 1990; Zhang et al, 1993).

Nitric Oxide synthesized by the enzyme inducible nitric oxide synthase (iNOS) has been reported as a mediator of inflammation and is involved in both acute inflammation, chronic inflammation, and pathophysiology of a variety of diseases (Heras et al, 2001, Zamora et al, 2000). The damage from NO during the inflammation might be decreased by NO scavengers and iNOS enzyme inhibitors. A number of studies show that phytochemicals, such as quercetin, tocopherol, and catechins inhibit the damage caused by NO (Arroyo et al 1992, Chan et al 1997, Kawada et al, 1998). Phagocytic cells, especially macrophages, have been implicated in immunopathological disorders related to oxidative stress, including inflammation and diseases (Fujiwara et al, 2005). Macrophages are sensitive to changes in the oxidant-antioxidant balance because ROS and RNS production is part of their normal function. Therefore, macrophages offer an excellent model system to study the antioxidant and NO inhibitory activities of natural materials (Saha et al, 2004).

Moreover, the RAW 264.7 mouse macrophage cell line is widely used for studies of inflammation due to its reproducible response to lipopolysaccharide (LPS), which is mediated by toll-like receptor 4 (TLR4) (Cao et al., 2006). Many stimuli, such as lipopolysaccharides (LPS), can activate the transcription factor nuclear factor kappa β (NF- κβ), which in turn regulates iNOS expression leading to NO production (Marks et al, 1998). Thus, NO is a marker for inflammation and can be used to assess the effect of phytochemicals as anti- inflammatory agents. Various studies have shown the inhibitory

effects on NO production by polyphenolic compounds found in common vegetables (Jung et al, 2006). Malaysian medicinal plants (Saha et al, 2004; Lee et al, 2011) and flavonoids (Kim et al, 1999).

A.6. *Response Surface methods*

Nonetheless, these beneficial properties of phenols in any natural system may result from different interactions or combinations of the chemically diverse phenols that impart greater protective properties on one biological response relative to another. As such, different types and ratios of the phenols may be responsible for a given health promoting propertiy, and which together may act as synergists, additives or potentiates. The optimal parameters to isolate these compounds (quantities, types, and ratios) relative to a given natural system and their overall oxidative protective benefits as a whole food are not known. This lack of knowledge impedes our ability to produce consistently safe and efficacious red beans targeted at specific cellular stressor diseases.

Additionally, the efficiency of extraction of phenols from whole foods is significantly influenced by multiple factors, such as solvent composition, extraction time, extraction temperature, solvent to solid ratio and extraction pressure (Shahidi et al, 2005; Wettasinghe et al, 1999; Cacace et al, 2003a). Classical optimization studies use a one factor at a time approach while other factors are kept constant. As a result, the potential interaction between several variables is not studied by this timely approach. Considering the chemical diversity of phenolic compounds, however, an interactive influence among the variables is expected. Thus, to obtain extracts that are either chemically diverse or exert a potent biological response, (and mostly likely both), and to ultimately understand

the phenolic composition of the product as a whole, a more comprehensive extraction approach must be applied. The statistical approach must also account for the interactive influence of various variables used for optimization.

Response surface methodology (RSM), originally described by Box and Wilson (1951), enables the evaluation of several process variables and their interaction on response variables. Thus RSM is a collection of statistical and mathematical techniques that has been successfully used for developing, improving and optimizing processes (Myers and Montgomery, 2002). A response surface method has many advantages, such as providing information to characterize interactions between multiple processes, determining kinetic constants and investigating enzyme stability /kinetics (Cheynier et al, 1983). The response can be represented graphically by three dimensional space or contour plots to easily visualize the output from the RSM. With respect to extracting phenols from natural systems, RSM has been applied to wheat (Chandrika and Shahidia, 2005), peanut skins (Ballard et al, 2009), Inga edulis leaves (Silva et al, 2007), and fruits of Euterpe oleracea (Pompeu et al, 2009). This method has thus been successfully used to model and optimize biochemical and biotechnological processes related to food systems (Cacace et al, 2003b; Parajo et al, 1995; Senanayake et al 1999; Senanayake et al, 2002; Telez-Luis et al, 2003; Vasquez et al, 1998).

In the context of this work, RSM was applied to red beans to characterize phenolic compounds in terms of total phenols, flavonoids, antioxidant capacity and total tannins. This information was needed to ultimately understand the phenolic composition of the bean as affected by environmental/genetic effectors. Based on the RSM

experiments, select extracts were screened to determine the anti-inflammatory effects in further cell experiments to determine the biological / health effect.

B. <u>OBJECTIVE AND SPECIFIC AIMS</u>:

The *objective of this research project* was to determine the ability of phenolic rich extracts recovered from two lines of small red beans to prevent macrophage mediated chronic inflammation. These studies are needed as research on the anti-inflammatory properties of red beans does not exist. As different extraction methods will recover extracts with different levels and composition profiles of phenols, a response surface method (RSM) was applied to each set of beans to obtain samples containing the three highest levels of phenols and flavonoids or exhibiting the highest anti-oxidative capacity. The objective of this project was completed by performing the following two specific aims (SPA).

SPA 1: ***<u>To apply a response surface design to the extraction procedures as a means to obtain phenolic rich extracts from two lines of small red beans (NE36 and NE40)</u>.*** This specific aim was accomplished with three extraction solvents (methanol, ethanol and acetone) using RSM that incorporates 3 factors (solvent polarity, mixing time, and solid / solvent ratio) and three levels for each factor. Each extract was then analyzed for total phenols, flavonoids and antioxidant capacity. The latter assay was completed to minimally access chemical diversity of an extract relative to the total phenolic content.

SPA 2: ***<u>To determine the ability of select extracts of small red beans identified from SPA 1 to prevent macrophage mediated chronic inflammation</u>.*** This specific aim was completed by preparing extracts based on their degree of phenolic diversity and total

amounts, as determined in SPA 1. The selected extracts were screened by exposing RAW 264.7 mouse macrophages to an inflammation activating agent, lipopolysaccharide (LPS) for 24 hrs followed by nontoxic dosages of the extracts for another 24 hrs. The anti-inflammatory effect was then determined by monitoring nitric oxide levels which was normalized against the Bradford assay.

C. MATERIALS AND METHODS:

C.1 *Chemicals, Reagents, and Beans for all SPA*:

Extraction solvents, methanol, ethanol, and acetone were purchased from Fisher Scientific Co. (Fair Lawn, New Jersey). Other reagents used for the study that were procured from Fisher Scientific included sodium carbonate, sodium nitrite, hydrochloric acid and potassium phosphate. Other reagents were purchased from various vendors, including Folin-Ciocalteu (MP Biomedical Inc.; Solon, OH), aluminum chloride (Acros Organics Inc.; Fair Lawn, NJ), sodium hydroxide (BD, West Chester, PA), Fluorescein (J.T. Baker: Center Valley, PA), 2-2′-azobis (2-amino-propane) dihydrochloride (AAPH), Dimethylsulfoxide (DMSO) and lipopolysaccharide (LPS) from *Salmonella enterica typhimurium* (Sigma-Aldrich., ST. Louis, MO), Fetal bovine serum (FBS) (Atlanta Biologicals ,GA), penicillin/streptomycin stock mixture (10,000 I U/ml and 10,000 µg/ml, respectively) (Mediatech, Inc. Herndon, VA), sodium bicarbonate (Sigma, St. Louis, MO), Bradford reagent (Biorad labs, Hercules, CA) and Griess assay kit (Enzo life sciences ,Farmingdale, NY). The standards used for the phenolic (gallic acid), flavonoid (catechins),tannin (catechins) and oxygen radical absorbance capacity (Trolox) assays and, Dulbelcco's Modified Eagle Medium (DMEM) were obtained from Sigma-Aldrich, ST. Louis, MO. The yellow tetrazolium 3-(4, 5-dimethylthiazolyl-2)-2, 5-

diphenyltetrazolium bromide MTT reagent kit and RAW 264.7 mouse macrophages was obtained from ATCC .The Small Red beans (two lines NE36 and NE40) were provided by Dr. Carlos Urrea (University of Nebraska Panhandle Research and Extension Center, Scotsbluff). The beans were maintained at -20 ^{o}C until preparated for further analysis.

C2. *Specific Aim 1*: *Surface Response and Extract Testing*

C.2.1 *Extraction Procedures:* The extract selection process for SPA 2 was based on RSM (three factors), i.e., a three-factor-three-level face-centered cube design (Table 5 and 6) in order to achieve the highest phenols, flavonoids and anti-oxidative capacity levels. This design was accomplished by initially homogenizing the beans into a fine powder with an electric grinder. The effects of three different solvents (methanol, ethanol, and acetone) on the three responses of the cited compounds and the anti-oxidative capacity were monitored by adjusting the water to solvent polarity, solid to solvent ratio (10-30%), and mix time. (The actual levels used are shown in Table 5). For this study, the solid levels were adjusted accordingly to maintain a 3-5 ml final extraction volume. The suspension was mixed horizontally under steady rocking for the designated time period as per the experimental design at room temperature. The samples were then centrifuged at 25 ºC for at least 10 minutes. The supernatant was collected and analyzed for total phenols, flavonoids and, antioxidant capacity, as described below. Each extraction was performed as cited in Table 6, in triplicate replications and tests were performed in triplicate.

Table 5: Levels of independent variables for extraction process based on central composite face centered design.

Independent Variable	Units	Factor	Coded Levels		
			-1	0	+ 1
Organic Solvent *: Water	(v/v)	X_1	25:75	50:50	75:25
Solid : Volume	(%)	X_2	10%	20%	30%
Time	(min)	X_3	60	120	180

* Methanol, Ethanol, or Acetone to Water Ratio

Table 6: Three factors, three- level face-centered cube design was used for RSM.

Standard Order	Factor X_1	Factor X_2	Factor X_3	Solvent Ratio	Solid:Vol (%)	Time (min)
1	1	0	0	75:25	20	120
2	0	0	1	50:50	20	180
3	1	-1	-1	75:25	10	60
4	0	0	-1	50:50	20	60
5	0	1	0	50:50	30	120
6	0	0	0	50:50	20	120
7	0	-1	0	50:50	10	120
8	1	1	1	75:25	30	180
9	-1	-1	1	25:75	10	180
10	1	-1	1	75:25	10	180
11	0	0	0	50:50	20	120
12	-1	1	1	25:75	30	180
13	1	1	-1	75:25	30	60
14	-1	-1	-1	25:75	10	60
15	-1	1	-1	25:75	30	60
16	0	0	0	50:50	20	120
17	-1	0	0	25:75	20	120

Table 5 shows the coded and uncoded levels.

C.2.2 *Total Phenolic Assay:* The Folin-Ciocalteu method was used to determine total phenols as described by Singleton and Rossi, (1965). Briefly, a sample aliquot (100 µL) was combined with 100 µL of Folin-Ciocalteu reagent and 4.5 mL of nanopure water. After 3 minutes of shaking at room temperature, 0.3 mL of 2% (w/v) sodium carbonate was added to the samples followed by a reaction time of 2 hrs at room temperature with intermittent shaking. Detection of the phenols was achieved with a UV-Vis spectrometer (Beckman Coulter, Brea, CA) at a wavelength of 760 nm. A standard calibration curve using gallic acid was plotted to calculate the results. Total phenols were expressed in mg *gallic acid* / g red bean powder as mean +/- standard deviation

C.2.3 *Total Flavonoids Assay*: Quantification of flavonoids was accomplished by combining 125 µL of the sample supernatant obtained from centrifuging the RSM extracts with 37.5 µL of 5% (w/v) sodium nitrite and 0.625 mL of nanopure water according to Adom and Liu, (2002). After allowing the reagent to react with the sample for 4-6 minutes at room temperature, 75 µL of 10% (w/v) aluminum chloride was added to each sample, followed by 0.25 mL of 1.0 M sodium hydroxide. Nanopure water (0.4 mL) was added after allowing the sample to mix for 5-7 minutes. After vortexing the mixture, an aliquot was measured at a wavelength 510 nm. Total flavonoids were expressed as mg *catechin* / g red bean powder as mean +/- standard deviation.

C.2.4 *Oxygen Radical Absorbance Capacity:* Antioxidant capacity was measured with the oxygen radical absorbance capacity (ORAC) as described by Huang et al, (2002). A standard stock solution was prepared by dissolving 0.010 g of Trolox (a water soluble derivative of Vitamin E) in 10 mL of 75 mM potassium phosphate buffer, pH 7.4.

Standard dilution concentrations were prepared that ranged from 0.46–62.50 µg/mL. Fluorescein (8.16 x 10^{-5} mM) was incubated with the diluted standards and test samples for 10 minutes. The reaction was then activated by adding a radical initiator, 153 mM 2, 2'-azobis (2-amidinopropane) hydrochloride, to generate peroxyl radicals. All samples/standards were prepared in 96 well plates and monitored with a fluorescent microplate reader (BMG LABTECH GmbH, Offenburg, Germany). Fluorescence was measured every 1.5 minutes at excitation and emission wavelengths of 485 nm and 520 nm, respectively, until the values plateaued. The area under the curve (AUC) and Net AUC were calculated to plot Net AUC vs. Trolox (µg/mL) calibration curves. The results were expressed as µmol Trolox / g red bean powder in mean +/- standard deviation.

C.2.5 *RSM Analysis and Regression Equations*: The behavior of each the extraction parameters relative to extracting the given components were analyzed by a second degree polynomial equation, as shown below:

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} b_{ji} X_i X_j$$

where Y is the response, b_o is the constant coefficient, b_i are the linear coefficients, b_{ii} are the quadratic coefficients, b_{ij} are the interaction coefficients, and X_i and X_j are the coded values of the independent variables. In the event, the method did not show a good fit, a higher mode, cubic, or lower model, linear, was applied to the results. To perform this operation, Stats Graphic, Centerium, (version 26, Warrenton, VA) was used to develop a regression equation between extraction variables and total phenols, total flavonoids, and anti-oxidative capacity.

C.2.6 *Statistical Analysis and Verification of the Model:* All determinations were completed in triplicate and the experimental results were expressed as the mean +/- SD. The statistical analysis was performed using StatsGraph Centerium (version 26, Warrenton, VA). The RSM experimental data were analyzed by multiple regression analysis through the least squares method. Two different tests, i.e., the sequential sum of the squares and model summary statistics, were applied to the experimental data to determine the adequacy of various models. The model and the regression coefficients involved in the model and their effect were analyzed by Pareto ANOVA charts and were considered significant at $p < 0.05$. The fitness of the regression curve was further evaluated by determining the correlation coefficient for the model R^2 (>75)?, whereas the ability of the model to fit the experimental data was assessed by a lack of fit test ($p>0.05$). Regression equations were formulated based on whether the data obtained from each solvent system complied with the criteria stated in this section.

C3. ***Specific Aim 2***: ***Anti-Inflammation Evaluation of Extractions***

C.3.1 *Cell Culture Preparation*: Raw 264.7 mouse macrophages were maintained in an atmosphere containing 5% CO_2, 90% relative humidity and a temperature of 37 ^oC. The cells were cultured in 75 cm^3 polystyrene flasks (Corning Inc., Corning, NY) with Dulbelcco's Modified Eagle Medium (DMEM)), solubilized in water for injection and supplemented with 57 ml Fetal Bovine Serum (FBS) 5.7 ml of penicillin/streptomycin stock mixture (10,000 I U/ml and 10,000 µg/ml respectively) and 11 ml L-glutamine per 500 ml of DMEM medium. Prior to supplementation, 3.7 g of sodium bicarbonate per L of medium was added. The pH of the medium was adjusted to 7.2 with 0.5 N HCl, which was then filtered through a 0.2 µm filter (Thermo Fischer Scientific Inc) into sterile 500

ml media bottles. Supplements were added using the same filter, and the medium was stored at 4^{o}C until used. The cells were passaged every two to three days by scraping for detachment. For the inflammation trials, 1 x 10^{6} cells per well were plated into 96 well culture plates and were allowed to grow for 24 hrs prior to treatment.

C.3.2 *Preparation of Extracts:* As stated previously, the extracts were selected based on the RSM data that showed high ORAC, total phenols and total flavonoids in the three different solvents (methanol, ethanol and acetone). The selected extracts were prepared for the anti-inflammatory studies by initially removing the organic solvent using a rotary evaporator (rotavap) and/or vacuum evaporation. The concentrated extract was then transferred to a pre-weighed container and any remaining extraction solvent was removed with a final nitrogen purge. The residue was then resuspended in (0.1%) DMSO. The extracts were then stored at -20^{o}C until further analysis was performed.

C.3.3 *Viability Testing* Macrophage viability in response to different doses of the selected extracts were determined by using the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay (or MTT assay) using the MTT assay kit. Cells were plated at a concentration of 2 x10^{4} cells per well in 96 well plates and grown for 24 hrs prior to treatment. The treatments consisted of a control of DMEM medium, DMSO in DMEM and different concentrations of select red bean extracts in DMEM.These concentrations were prepared from a stock solution of 100 mg/ml of the selected extract in DMSO. Treatments were completed for 24 hrs, at which time 15 µl of MTT reagent was added to each of the wells for 2 hrs, followed by lysis with 100 µl of detergent reagent. After 4 hrs, the absorbance was measured at 580 and 620 nm with a FLUOstar Optima microplate reader from BMG Labtech (Durham, NC) equipped with Optima

analysis software. The % viability relative to control that was not incubated with red bean extract was calculated. Concentrations that allowed >80% cell viability that were not toxic to the cells were then used for the remaining studies. (Cell viability was determined by comparing with untreated cells.)

C.3.4 *LPS and extract treatment of macrophages:* Cells were plated at a concentration of 1x 10^6 cells per well in 96 well plates in unsupplemented media and grown for 24 hrs prior to treatment. The cells were washed with 200 µl of media and then treated with 200 µl of 200 ng/ml LPS and incubated at 37 ºC for 24 hrs. The cells were again washed with media and treated with 4 to 5 different concentrations of extracts that did not kill the cells, as determined from the MTT assay. Cells supplemented with DMEM containing DMSO and treatment concentrations but minus the inflammatory inducing agent, LPS, served as served as negative controls, and DMEM media containing cells with LPS served as positive controls. After incubation for 24 hrs at 37 ºC, the nitric oxide assay as described below was performed on all the samples.

C.3.5 *Nitric Oxide Assay:* Nitric oxide was monitored to determine the ability of select red beans to protect against macrophage-mediated inflammation. The NO assay was completed as described by Zhang et al (2011). Briefly, equal volumes of cell aliquots (50 µl) from tests completed as described in C.3.4 were treated with 50 µl of Greiss reagent, incubated at room temperature for 10 minutes, and the absorbance was measured at 550 nm. The results will be expressed as NO production/inhibition (%) relative to the controls. Two biological replications and three technical replicates were performed.

C.3.6 *Bradford Assay:* The cell lysate that was prepared by treating the cells with 100 µl of boiling nanopure water was used for the Bradford Assay in order to normalize the cells to protein levels. The cell lysate (10 µl) was treated with 290 µl of Bradford reagent, incubated at 37 ºC at room temperature for 10 minutes, and the absorbance was measured at 595 nm. The results were used to normalize the NO assay results.

C.3.7 Statistical *Analysis*: Data was analyzed using SAS (Statistical Analysis System) software. Analysis of variance (ANOVA) tests were performed on the cell culture results (NO) to determine whether the treatments differed significantly from the controls at the 90% confidence interval ($p < 0.10$). A randomized complete block design was used and the blocking was done by passage (bio replicates) and there were three technical replicates within each passage.

D. RESULTS AND DISCUSSION:

D.1 *Specific Aim 1: Surface Response and Extract Testing*

D.1.1 *RSM Characterization: Selection of Independent Variables*

The extraction of bioactive components from plant materials is the first step in the characterization of the plant systems (phenolic rich systems). Solvent extractions are the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency and wide applicability (Dai et al, 2010). Solid–liquid extraction uses a solvent to remove a soluble fraction from an insoluble, permeable solid (Cacace et al, 2003). The efficiency of the extraction of any compound is influenced by multiple parameters, such as temperature, extraction time, temperature, solvent polarity, pressure,

sample to solvent ratio / and matrix properties of the system. These effects may be either independent or interactive (Montgomery, 2001; Hernández et al, 2009).

In particular, phenolic based compounds present in all plant materials vary in classes, from simple monomers (phenolic acids, anthocyanins) to highly polymerized substances (tannins) and in different quantities. Moreover, phenols may complex with other plant components, such as carbohydrates, proteins, organic acids and fats (Dai et al, 2010). Therefore, no universal extraction procedure is suitable for extracting all plant phenolic compounds from a single type of plant let alone from different types of plants. Furthermore, the level of phenolic compounds in plant sources also depends on such factors as cultivation techniques, line, growing conditions, ripening process, as well as processing and storage conditions, among others (Naczk et al, 2006).The recovery of phenolic compounds from plant materials is influenced by the extraction time and temperature, and other parameters (Robards et al, 2003). However, many phenolic compounds are easily hydrolyzed and / or oxidized when using long extraction times and high temperatures, thereby affecting possible bioactivity and amounts (Pathirana et al, 2005; Gan et al, 2011). It is thus critical to select efficient extraction procedures to maintain the stability of phenolic compounds.The influence of extraction variables on the recovery of phenolic compounds from red beans has not yet been reported.

For these studies, extractions were carried out with methanol, ethanol and acetone adjusted with 25 % to 75% water as studies have shown that water promotes the solubility of phenolic compounds (Rostanogo et al, 2004), as well as affects the amount and rate of polyphenols extracted (Xu et al, 2007). The cited solvents were selected based

on the literature review that showed that each were effective in extracting phenols from a number of different natural systems. More specifically, methanol has generally been reported to be more efficient in extraction of lower molecular weight polyphenols while the higher molecular weight flavanols are more readily extracted with aqueous acetone (Metivier et al 1980; Prior et al 2001; Guyot et al, 2001; and Labarbe et al, 1999). Ethanol is another highly suitable solvent for polyphenol extraction and is safe for human consumption due to its generally recognized as safe (GRAS) status (Naczk et al, 2006).

The two fundamental processes that govern the extractions are equilibrium and mass transfer rate (Cacace et al 2003). Along with solvent composition, other factors, such as solid to liquid ratio, mixing time and temperature influence the mass transfer. (Wettasinghe et al, 1999; Azizah et al, 1999; Pinelo et al,2005). Therefore, for this study, solid: liquid ratio (10%-30% w/v), mixing time (60-180 min), and solvent type / composition were evaluated (Table 5). A rocker was used to ensure steady mixing and close contact between the solvent and bean powder. Total phenols, total flavonoids and antioxidant capacity were measured in response to these variables as a means to understand the phenolic composition of red beans and their biological (anti-inflammatory) response, while characterizing the extraction methods used to recover phenols from red beans.

D.1.2 *Total Phenols (TP)*

D.1.2.a *Total phenol results obtained from FCCD-RSM:* The levels of total phenols (TP) in response to each solvent system (methanol, ethanol, and acetone), while adjusting for water levels, solid to solvent ratio and mix time were evaluated using a three

factor, three level faced centered composite design (FCCD). This design involved 17 different extractions with three center points (Table 6). The results obtained for NE36 and NE40 for each solvent are shown in Tables 7 and 9 respectively, and expressed as the mean +/- standard deviation of three replicates. From these results, the range in TP levels was determined for NE36 (Table 8) and NE40 (Table 10). The TP yields were the greatest for both lines with acetone, and the lowest wih methanol for NE36 and ethanol for NE40, with a difference of ~ 1.8 mg/g. This difference may be due to different types of phenolics being extracted by different solvents.

Total phenols extraction efficiencies were the greatest for both lines (3.45 mg/g 36 NE and 3.52 mg/g 40 NE with extraction 7 (Table 6) in acetone using the same factors / independent variables. The lowest overall level of 0.29 mg/g was obtained from the NE40 lines using methanol and extraction 13 parameters (Table 8 and Table 6), while the NE36 low was 0.46 mg/g with methanol and extraction 12 parameters (Table 6).

Table 7. Total phenols response (in mg/g) of red bean extracts under different extraction conditions and solvent systems for line NE36.

Std. Order	Methanol	Ethanol	Acetone
1	0.62 ± 0.04	0.61 ± 0.01	2.36 ± 0.20
2	0.84 ± 0.09	1.06 ± 0.03	1.98 ± 0.10
3	0.84 ± 0.09	0.88 ± 0.03	2.46 ± 0.22
4	0.91 ± 0.01	0.97 ± 0.01	2.60 ± 0.15
5	0.68 ± 0.01	0.70 ± 0.02	2.41 ± 0.13
6	0.87 ± 0.03	0.86 ± 0.02	3.15 ± 0.25
7	1.11 ± 0.04	1.05 ± 0.14	3.45 ± 0.17
8	0.91 ± 0.01	0.57 ± 0.02	2.26 ± 0.07
9	0.54 ± 0.04	1.25 ± 0.03	1.72 ± 0.15
10	1.16 ± 0.06	1.00 ± 0.06	2.51 ± 0.25
11	0.84 ± 0.03	0.85 ± 0.01	2.77 ± 0.22
12	0.46 ± 0.03	0.77 ± 0.01	1.12 ± 0.07
13	0.54 ± 0.04	0.53 ± 0.05	2.24 ± 0.18
14	1.16 ± 0.07	1.64 ± 0.07	1.81 ± 0.23
15	0.47 ± 0.03	0.91 ± 0.08	0.99 ± 0.05
16	0.80 ± 0.02	0.87 ± 0.00	2.64 ± 0.05
17	0.69 ± 0.09	0.78 ± 0.08	1.70 ± 0.12

* Data are shown as the mean ± standard deviation (n=3)

Table 8. Ranges of total phenols for each solvent system (NE36)

Extraction Solvent	Total Phenols (mg/g)	Range (mg/g)
Methanol	0.46 – 1.16	0.70
Ethanol	0.53 –1.64	1.11
Acetone	0.99 – 3.45	2.46

Table 9. Total phenolic response (in mg/g) of red beans extracts under different extraction conditions and solvent systems for line NE40

Std. Order	Methanol	Ethanol	Acetone
1	0.67 ± 0.03	0.64 ± 0.03	2.99 ± 0.26
2	0.89 ± 0.06	1.23 ± 0.07	1.08 ± 0.02
3	0.86 ± 0.07	1.13 ± 0.16	2.95 ± 0.02
4	0.90 ± 0.02	0.91 ± 0.03	2.62 ± 0.09
5	0.74 ± 0.09	0.71 ± 0.09	2.84 ± 0.09
6	0.99 ± 0.04	0.88 ± 0.01	3.17 ± 0.10
7	1.33 ± 0.08	1.15 ± 0.03	3.52 ± 0.09
8	0.59 ± 0.02	0.72 ± 0.03	2.14 ± 0.10
9	1.29 ± 0.10	1.69 ± 0.17	1.19 ± 0.09
10	0.82 ± 0.03	1.28 ± 0.05	1.53 ± 0.06
11	0.97 ± 0.02	0.88 ± 0.01	3.03 ± 0.17
12	0.65 ± 0.10	0.85 ± 0.05	0.88 ± 0.05
13	0.29 ± 0.00	0.58 ± 0.02	2.49 ± 0.07
14	1.50 ± 0.02	1.30 ± 0.10	2.37 ± 0.37
15	0.58 ± 0.02	0.56 ± 0.06	1.16 ± 0.20
16	0.90 ± 0.02	0.86 ± 0.05	3.08 ± 0.23
17	0.68 ± 0.10	0.75 ± 0.06	1.60 ± 0.11

* Data are shown as the mean ± standard deviation (n=3).

Table 10: Ranges of total phenols for each solvent system. (NE40)

Extraction Solvent	Total Phenols (mg/g)	Range (mg/g)
Methanol	0.29 – 1.50	1.21
Ethanol	0.56 – 1.69	1.13
Acetone	0.88 – 3.52	2.64

The results show that acetone was able to extract the highest overall TP yields, suggesting that the majority of the phenols present are non-polar, or lack a polar conjugate. Many studies have used either methanol, ethanol, or acetone to extract the phenolics from vegetables, fruits and cereals (Chavan et al 2001,Shahidi et al 2001, Matilla et al 2000, Labarbe et al 1999, Hertog et al., Sun & Ho, 2005; Xu and Chang, 2007), but have not used RSM to determine an appropriate solvent system for total phenol levels in different food systems.

D.1.2.b *Fitting the TP model*: Multiple regression equations were generated relating response variables to uncoded levels of independent variables. Regression coefficients were determined by applying the least squares technique to the results obtained for each solvent system (Myers and Montgomery 2002) to predict the quadratic polynomial (equations. The analysis of variance (ANOVA) of the quadratic model for the solvents showed a significant p value < 0.05 for all three solvents for line NE40, but only acetone and ethanol for NE36 (Table 11 and 12). However, the R^2 value was high for methanol, ethanol and acetone for both lines indicating that most of the variability could be explained, which supports the adequacy of this model for these solvent based TP extractions from red beans according to Le, Behera and Park (2010) and Chauhan and Gupta (2004). (A high R^2 coefficient provides assurance for low dispersion of the experimental data.) On the other hand, the model fit for the methanol extraction of line NE36 shows R^2 value of 81.9 and $p>0.05$. Conversely, the ANOVA of the model for the methanol for the line NE40, generated p values of <0.05 and R^2 above 80.

Table 11. Regression coefficients (coded) predicted by the quadratic polynomial model for phenols when extracted with the cited solvent systems for line NE36

Coefficient	Methanol	Ethanol	Acetone
b_o	0.842	0.807	2.88
Linear			
b_1 (SP)	0.056	-0.180*	0.385
b_2 (S:S)	-0.167**	-0.238*	-0.243
b_3 (MT)	-0.022	-0.002	-0.030
Quadratic			
b_{11} (SP x SP)	-0.087	-0.055	-0.556
b_{22} (S:S x S:S)	0.091	0.718	0.278
b_{33} (MT x MT)	-0.088	0.111	-0.713
Cross product			
b_{12} (SP x S:S)	0.0263	0.053	0.118
b_{13} (SP x MT)	0.1652	0.086	0.003
b_{23} (S:S x MT)	0.0823**	0.023	0.024
R^2	81.9	87.5	86.17
p values			
Model	0.0551	0.0180	0.0247
Lack of Fit	0.1706	0.1107	0.7275

SP – Solvent Polarity, S:S – Solid: Solvent, MT – Mix Time

***** significant at 1%, ** significant at 5%

Table 12. Regression coefficients (coded) predicted by the quadratic polynomial model for phenols when extracted with the cited solvent systems for line NE40

Coefficient	Methanol	Ethanol	Acetone
b_o	0.925	0.859	2.940
Linear			
b_1 (SP)	-0.149**	- 0.081	0.511*
b_2 (S:S)	-0.293*	-0.313	-0.224*
b_3 (MT)	0.010	0.130	-0.497*
Quadratic			
b_{11} (SP x SP)	-0.229**	-0.154	-0.531*
b_{22} (S:S x S:S)	0.130	0.078	0.351**
b_{33} (MT x MT)	-0.008	0.224	-0.976*
Cross product			
b_{12} (SP x S:S)	0.096**	0.057	0.231**
b_{13} (SP x MT)	-0.049	-0.048	-0.013
b_{23} (S:S x MT)	-0.077	-0.015	0.220**
R^2	95.64	98.13	96.09
p values			
Model	0.0006	0.000	0.0004
Lack of Fit	0.1930	0.0322	0.0516

SP – Solvent Polarity, S:S – Solid:Solvent, MT – Mix Time
* significant at 1%, ** significant at 5%

D.1.2.c *Adequacy of the TP models*: The ability of each model to fit the experimental data was then determined to provide assurance of obtaining predictable results. In general, a fitted response surface may produce poor or misleading results unless the model exhibits an adequate fit (Myers & Montgomery, 2002). The model's adequacy was evaluated by comparing the difference between the residuals of the current model with that of observed data (Maren et al. 2013), which indicates a "lack of fit". If the model residuals correspond to that of the experimental, a p value > 0.05 is expected indicating that data fits the model. All three solvents for both lines NE36 and NE40 passed this test with the notable exception for ethanol for line NE40 (Table 11 and 12). A higher more complex model may fit for ethanol.

D.1.2.d *Regression coefficients equations and Pareto charts*: The TP regression equations for the methanol and ethanol extraction of line NE36 and acetone and methanol extractions of NE40 are provided in Table 13 based on the criteria for accepting a model, as described in the Material and Methods section, (Section C.2.5). For NE36 acetone extractions, the model fits but there were no significant interactions so higher models with more complex interactions may be involved. These equations are based on the significance of individual regression coefficients only ($p < 0.10$). Furthermore, Pareto charts are also shown to describe the overall contribution of each coefficient (Figure 7 and 9).

The different solvents show different effects on TP extraction from red beans as shown by comparison of charts. For line NE36, the TP extractions with methanol were mainly affected by solid volume, with higher amounts negatively affecting TP yields for the linear coefficient (Figure 7a). The next parameter that effected TP yields

Table 13: Regression equations fitting the model and passing lack of fit test for TP.

NE36
TP methanol = 0.842 - 0.091*Xss* +*0.1652XspXmt*
TP ethanol =0.807-0.167*Xss*-0.056*Xsp*
For TP acetone, The model fits but no significant interactions were found so higher models can be used for more complex interactions.
NE40
TP acetone = 2.940 +0.511*Xsp* –*0.976 XmtXmt* – *0.497Xmt* –*0.531 XspXsp*-0.224Xss+ 0.231*XspXss*+0.220*XssXm*t +0.351*XssXss*
TP methanol = 0.925-0.293*Xss*-0.149*Xsp*-0.229*XspXsp*+0.096XssXsp

Xsp: solvent polarity ,Xmt: mixing time ; Xss: solid solvent

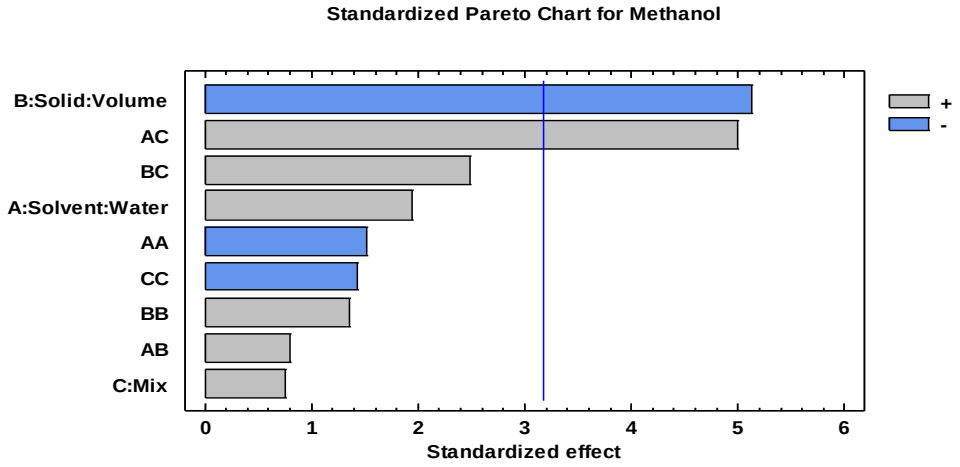


Figure 7a

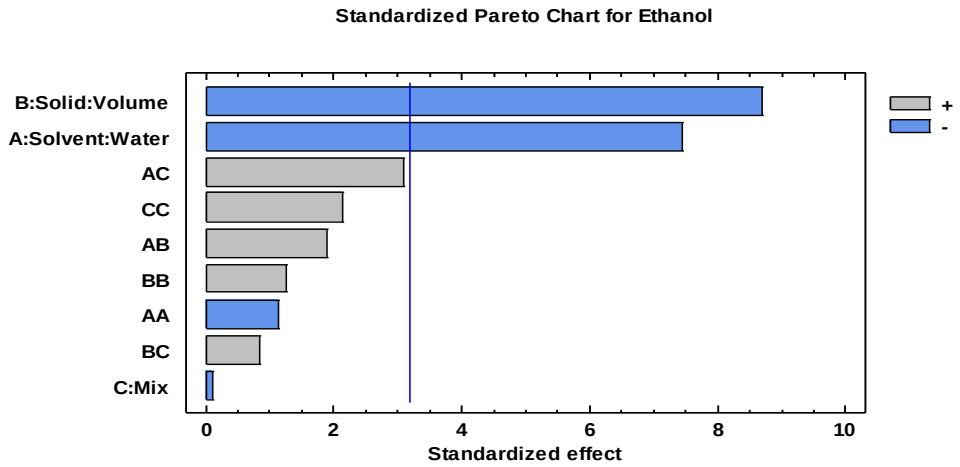


Figure 7b

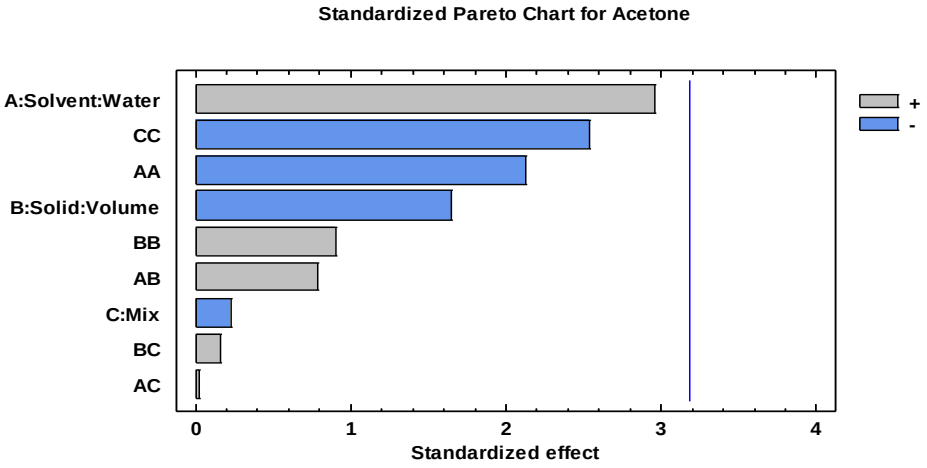


Figure7c

Figure 7: Pareto charts showing relative effects of regression coefficient for total phenols accepted models in a) Methanol b) Ethanol c) Acetone for NE36. Vertical line represents $p < 0.05$.

was the cross product of solid volume and mix time, which showed a linear positive relationship for TP yields. For ethanol extractions, both solid volume and solvent polarity (linear coefficients) negatively influenced the TP yields (Figure 7b). For acetone extractions, there were no significant interactions so higher models may be used. However, extraction of line NE40 with acetone showed linear and quadratic positive relationship with solvent polarity and solid volume on yield of TP. Similarly cross product interaction of solid volume/solvent polarity and solid volume/mix time also showed a positive relationship (Figure 8a). However**,** the mix time, solvent polarity and solid volume negatively affected the TP yields via a quadratic relationship. The extractions with methanol (Figure 8b) on the other hand showed a cross product interaction of solvent polarity /solid solvent and solid solvent/mix time and quadratic solid solvent via positive relationship and linear negative relationship with solid solvent and solvent polarity and quadratic solvent polarity.

D.1.2.e *Final optimized TP values and processing factors*: Based on the model, and the factors tested, optimal processing factors were determined that are expected to produce the highest TP yields (Table 14, 15 and Figure 9 a,b). A comparison of the optimum yields suggest that the phenols present in red beans are more non-polar as the acetone system yielded high TP. However, a high proportion of water was needed considering a coded value of -0.22 (or ~45:55 solvent: water). These results indicated that solvents with different polarity had significant effects on total phenolic contents, extracted components and antioxidant activities (Xu et al). For both lines, optimal

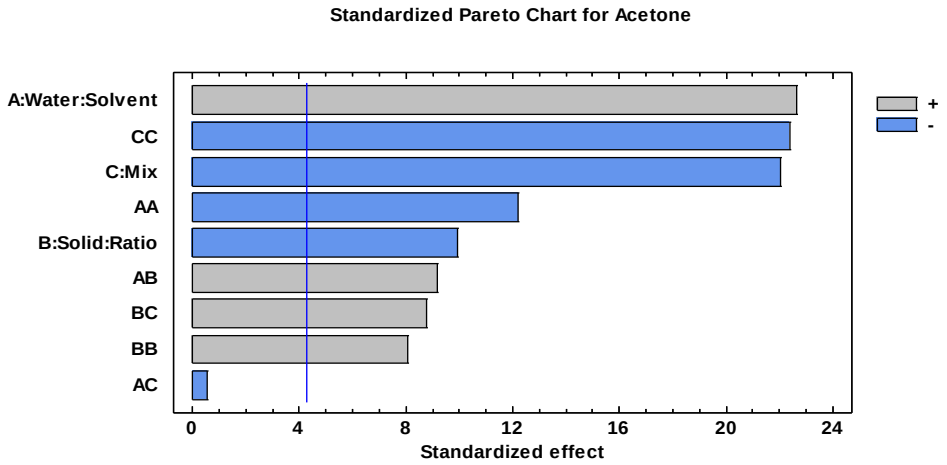


Figure 8a

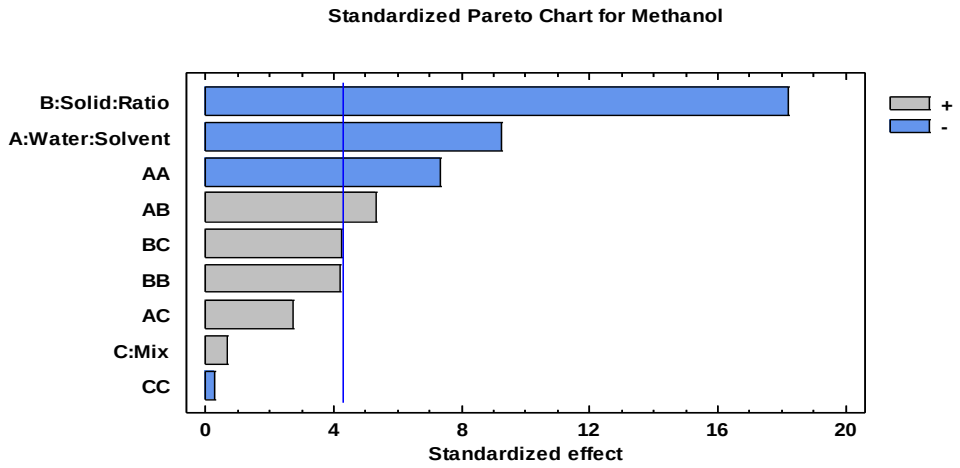


Figure 8b

Figure 8: Pareto charts showing relative effects of regression coefficient for total phenols accepted models in a) Acetone b) Methanol for NE40. Vertical line represents $p < 0.05$.

Table 14: Optimized factors (in coded value) required to produce optimum TP yield for the cited system for NE36.

Optimum value = 3.44129 mg/g of beans

Factor	*Low*	*High*	*Optimum*
Solvent: Water	-1.0	1.0	0.239012
Solid: Volume	-1.0	1.0	-1.0
Mix	-1.0	1.0	-0.0380243

Table 15: Optimized factors (in coded value) required to produce optimum TP yield for the cited system for NE40.

Optimum value = 3.68734 mg/g of beans

Factor	*Low*	*High*	*Optimum*
Solvent: Water	-1.0	1.0	0.268188
Solid: Volume	-1.0	1.0	-1.0
Mix	-1.0	1.0	-0.369724

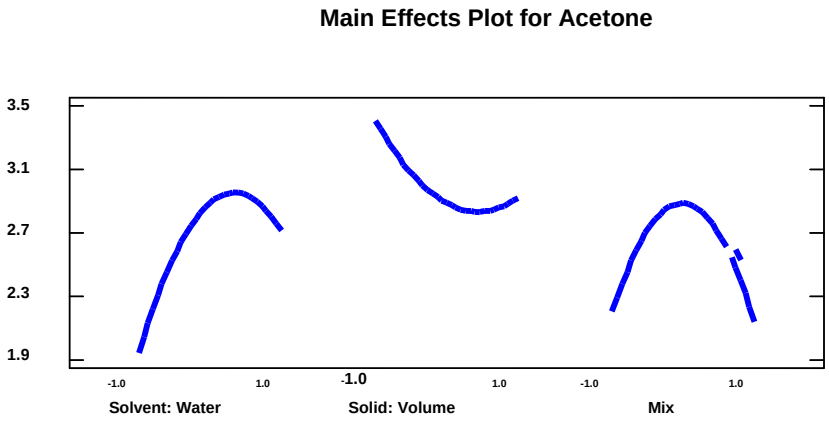


Figure 9a: Main effects plot for Acetone (NE36)

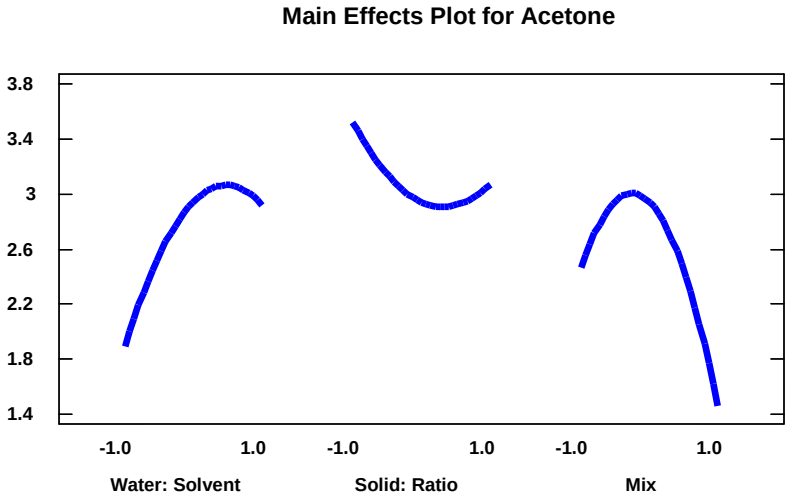


Figure 9b. Main effects plot for Acetone (NE40**)**

yields were expected for a solid ratio of 10%, and because the optimal coded value for this factor is -1 the lowest volume ratio actually tested. As such, even lower solid volume ratios may increase TP yields. Pompeau et al, (2009) determined that solid to liquid ratio had a significant effect on extraction of phenolics from *Euterpe oleracea* using an acidified aqueous alcoholic solution .The yield of phenolic compounds increased with the decrease in the solid-to-liquid ratio. A plateau in the mass transfer was, however, reached in the solid-to-liquid ratio. These results most likely occurred because the extraction solvent was able to make more contact with the anti-oxidative components and thus increase extraction)as the amount of solvent increased. However, further solvent increase may dilute the extracting solution and result in lower antioxidant activity per volume. Prasad et al (2011) reported that solid liquid ratio played a significant role in the yield of phenolics for the extraction of *magnifera pajang* peels.

In terms of mixing time, a review of the literature has shown that longer extraction times have minimal effects on phenolic levels from various types of natural products. For example, water extraction of phenolics from pistachio hulls showed a dramatic increase in TP levels from 5-20 min but then plateaued from 20 min to 100 min (Rajaei et al, 2010). Liyana-Pathirana, (2005) also reported that mix time had no significant effect on phenolic compound extractions, but rather ethanol composition and temperature did have an effect. Deshpande et al, (1985) demonstrated that the optimum extraction time required for dry bean phenolic was 50–60 min, which is similar to the values obtained in this study for small red beans (Figure 9a,b.) Longer or shorter time

periods resulted in decreased TP, which most likely is due to limited phenol contact for shorter time periods and degradation of the phenols with higher time periods.

Interestingly, the TP were similar for both NE36 and NE40 when using acetone as were the parameters needed for optimal extraction (Figure 9a,b; Table 14a,b). However, the models differed for the acetone extracted lines (Figure 7c-NE36; Figure 8a-NE40). At this time, we have no explanation for the phenomenon, except for the possibility that different types of phenols were extracted or other matrix components that differed between the lines affected the model.

D.2 *Total Flavonoids (TF):*

D.2.1.a *TF results obtained by face centered composited design (FCCD):* The TF results for each extraction defined by the FCCD-RSM are shown in Table 16 (NE36) and Table 18 (NE40) as the mean +/- standard deviation of three replicates. TF levels in terms of high, low, and overall ranges are also presented in Table 17 (NE36) and 19 (NE40). The highest TF levels were extracted with acetone (5.60 mg/g NE36, extraction 10; and 4.00 mg/g NE40, extraction 3) followed by ethanol (0.78 mg/g NE36, extraction 14; and 1.08 mg/g NE40, extraction 3) and methanol (0.89 mg/g NE36, extraction 10; and 0.77 mg/g NE40. extraction 9).

The TF range for acetone (Table 17 and 19) extraction solvent was also much larger for both lines compared to the methanol or ethanol extractions, indicating the ruggedness of using this solvent. More specifically, these results indicated that multiple parameters could be used to obtain TF at levels higher than the limited parameters needed for ethanol and methanol. The lowest TF levels for NE36 were even comparable to the

higher levels for methanol and ethanol, but differed for NE40. It also should be noted that different extraction methods (Table 16 (NE36) and 18 (N340)) resulted in the highest TF levels from both lines. This could be due to different types of flavonoids present in the lines, or slightly different matrix components (levels and types) present in each, affecting the extraction.

A similar study conducted by Madhujith et al (2006) used RSM to optimize the extraction parameters for recovering phenolic compounds from six lines of barley. Again, methanol, ethanol and acetone served as the solvent systems and mix time was yet another variable; while the third variable was temperature. The researchers reported that out of the six varieties tested, three were more effective antioxidants, which they attributed to the higher TP content. No other explanation was given except that they were different lines.

D.2.1.b Fitting *the TF models:* Multiple regression coefficients were again determined for each of the three solvent systems used to extract TF from the small red bean lines (Table 21 (NE36) and 22 (NE40)). The R^2 values for NE40 (Table 22) were 96.13 for methanol, 87.54 for ethanol and 96.45 for acetone translating into a variability of ~85 to 95% of the TF that could be predicted by the models. In the case of for NE36 (Table 21), the R^2 values were at 81.96, 87.52, and 86.17, for methanol, ethanol and acetone, respectively, which were slightly lower than NE40. Nonetheless, these values are highly acceptable as Le Behera et al, (2010) and Chauhan et al, (2004) have emphasized the acceptance of any model with $R^2 > 75.0$. The ANOVA of the quadratic model was adequate for all the three solvent systems for both the lines NE36 and NE40

that is $p < 0.05$ (Tables 21 and 22). As the results for all TF extraction obtained from each solvent adequately described the model, the solvents were assessed for lack of fit.

D.2.1.c *Adequacy of the TF models and corresponding regression equations:* The solvent systems that complied with the lack of fit for NE40 were methanol and acetone, whereas only acetone was acceptable for NE36, as each had p value > 0.05 (Table 19 and 20). These results could be due to non-uniform particle size of the bean powder used for extraction, as some studies have shown that this parameter affects extraction efficiencies (Stalikas, 2007; Luthria et al., 2011; Brewer et al., 2014). It is also possible that a higher order model with more complex interactions between the different parameters could better explain the extractions. Preliminary analysis currently in-progress in our lab indicate that many of these extracts are indeed better suited to a cubic model (data not shown).

Table 16: Total flavonoid response (in mg/g) of red beans extracts under different extraction conditions and solvent system for line NE36

Std Order	Methanol	Ethanol	Acetone
1	0.26 ± 0.01	0.41 ± 0.02	2.7 ± 0.08
2	0.72 ± 0.05	0.56 ± 0.02	1.96 ± 0.06
3	0.72 ± 0.06	0.61 ± 0.07	2.66 ± 0.08
4	0.20 ± 0.03	0.29 ± 0.01	2.83 ± 0.19
5	0.33 ± 0.01	0.51 ± 0.00	2.73 ± 0.14
6	0.40 ± 0.04	0.60 ± 0.00	2.97 ± 0.18
7	0.52 ± 0.01	0.77 ± 0.03	2.95 ± 0.16
8	0.20 ± 0.03	0.35 ± 0.01	2.40 ± 0.11
9	0.25 ± 0.04	0.58 ± 0.06	3.89 ± 0.29
10	0.89 ± 0.02	0.27 ± 0.02	5.60 ± 0.16
11	0.42 ± 0.03	0.58 ± 0.03	2.93 ± 0.12
12	0.21 ± 0.03	0.63 ± 0.03	1.79 ± 0.04
13	0.25 ± 0.03	0.57 ± 0.00	2.81 ± 0.18
14	0.89 ± 0.02	0.78 ± 0.02	2.05 ± 0.28
15	0.21 ± 0.02	0.48 ± 0.05	0.71 ± 0.08
16	0.39 ± 0.01	0.61 ± 0.01	2.98 ± 0.16
17	0.26 ± 0.01	0.27 ± 0.00	1.32 ± 0.11

* Data are shown as the mean ± standard deviation (n=3).

Table 17: Ranges of total flavonoid for each solvent system (NE36)

Extraction Solvent	Total Flavonoid (mg/g)	Range (mg/g)
Methanol	0.20-0.89	0.69
Ethanol	0.27-0.78	0.51
Acetone	0.71-5.60	4.89

Table 18: Total flavonoids (in mg/g) of red beans extracts under different extraction conditions and solvent systems for line NE40

Std Order	Methanol	Ethanol	Acetone
1	0.32 ± 0.02	0.57 ± 0.00	0.68 ± 0.02
2	0.59 ± 0.04	0.63 ± 0.04	1.94 ± 0.10
3	0.11 ± 0.00	1.08 ± 0.13	4.00 ± 0.14
4	0.03 ± 0.00	0.61 ± 0.04	3.09 ± 0.27
5	0.40 ± 0.04	0.56 ± 0.01	0.70 ± 0.05
6	0.52 ± 0.02	0.69 ± 0.01	0.86 ± 0.06
7	0.69 ± 0.02	0.85 ± 0.05	0.72 ± 0.04
8	0.38 ± 0.02	0.34 ± 0.03	1.96 ± 0.11
9	0.77 ± 0.07	0.62 ± 0.06	1.98 ± 0.21
10	0.49 ± 0.03	0.83 ± 0.01	2.79 ± 0.16
11	0.49 ± 0.03	0.65 ± 0.01	0.67 ± 0.03
12	0.29 ± 0.03	0.83 ± 0.01	1.00 ± 0.04
13	0.04 ± 0.01	0.72 ± 0.08	3.31 ± 0.57
14	0.13 ± 0.00	0.59 ± 0.03	3.00 ± 0.39
15	0.03 ± 0.00	0.20 ± 0.03	1.30 ± 0.11
16	0.47 ± 0.02	0.68 ± 0.02	0.72 ± 0.03
17	0.33 ± 0.02	0.32 ± 0.05	0.17 ± 0.02

* Data are shown as the mean ± standard deviation (n=3).

Table 19: Ranges of total flavonoid for each solvent system (NE40)

Extraction Solvent	Total Flavonoid (mg/g)	Range(mg/g)
Methanol	0.03-0.77	0.74
Ethanol	0.20-1.08	0.88
Acetone	0.17-4.00	3.88

Table 20: Regression coefficients (coded) predicted by the quadratic polynomial model for flavonoids when extracted with the cited solvent systems (NE36)

Coefficient	Methanol	Ethanol	Acetone
b_o	0.366	0.570	2.625
Linear			
b_1 (SP)	0.052*	-0.045*	0.559
b_2 (S:S)	-0.227*	-0.021**	-0.747**
b_3 (MT)	-0.012	-0.055	0.467
Quadratic			
b_{11} (SP x SP)	-0.090**	-0.246	-0.004*
b_{22} (S:S x S:S)	-0.017	0.219	0.080*
b_{33} (MT x MT)	0.187*	-0.014	-0.024
Cross product			
b_{12} (SP x S:S)	-0.056*	0.0364	0.049*
b_{13} (SP x MT)	0.094*	-0.062	-0.049*
b_{23} (S:S x MT)	0.053**	0.060	0.512*
R^2	81.96	87.52	86.17
p values			
Model	0.0551	0.0180	0.0247
Lack of Fit	0.0023	0.0031	0.2422

SP – Solvent Polarity, S:S – Solid: Solvent, MT – Mix Time

* Significant at 1% **,** **Significant at 5%

Table 21: Regression coefficients (coded) predicted by the quadratic polynomial model for flavonoids when extracted with the cited solvent systems (NE40).

Coefficient	Methanol	Ethanol	Acetone
b_o	0.472	0.622	0.690
Linear			
b_1 (SP)	-0.024	0.096*	0.528*
b_2 (S:S)	-0.104*	-0.131*	-0.421*
b_3 (MT)	0.218*	0.005	-0.501*
Quadratic			
b_{11} (SP x SP)	-0.134**	-0.137*	-0.218
b_{22} (S:S x S:S)	0.084**	0.121*	-0.065
b_{33} (MT x MT)	-0.146**	0.035	1.870*
Cross product			
b_{12} (SP x S:S)	0.0502**	-0.083*	0.146
b_{13} (SP x MT)	-0.023	-0.161*	-0.155**
b_{23} (S:S x MT)	-0.052**	0.057**	0.072
R^2	96.13	87.54	96.45
p values			
Model	0.0004	0.0178	0.0003
Lack of Fit	0.0977	0.0194	0.0638

SP – Solvent Polarity, S:S – Solid: Solvent, MT – Mix Time

* Significant at 1% ,**Significant at 5%

D.2.1.d *Regression coefficients equations and Pareto charts:* Regression equations that fit the model and passed the lack of fit test are shown in Table 22, with the coefficients that were determined to be significant (Table 20 and 21). Based on the Pareto charts for NE36 (Figure 10a), which show the relative effects of the interaction parameters, the solid:solvent ratio had a linear, negative influence for the acetone TF extraction. For NE40, the acetone TF extractions (Figure 10b) showed a positive influence with mix time (quadratic) and solvent polarity (linear), while mix time and solid:solvent had a significant negative influence on the TF levels at the linear level. Methanol TF extractions of NE 40 were not affected by the mixing time (linear), but the cross product of solid ratio and solvent polarity along with quadratic solid volume had a positive influence. Lastly, a negative relationship occurred for the solid:volume ratio (linear), mix time and solvent polarity (quadratic) and cross product interaction for mixing time with solvent polarity (Figure 10c). In general, these results demonstrate that the solid:solvent ratio and solvent polarity significantly influenced the TF yields.

In a study conducted by Shenget et al (2014) on extraction of TF from *flos populi* using ethanol, it was reported that the choice of solid:solvent ratio and solvent polarity played a critical role. If the solid:solvent ratio was too high, incomplete extraction of TF could occur. Another study on extraction of TF from *fructus* showed that the order of factors influencing the yield of TF as ethanol concentration > extraction time > temperature> the solid: liquid ratio.

Table 22: Regression equations that fit the model and passed lack of fit test.

TF acetone (NE36)	**= 2.625 - 0.747*Xss***
TF acetone (NE40)	**= 0.690 + 0.528*Xsp* -*0.501Xmt* - *0.421Xss*+ 1.870*XmttXmt.***
TF methanol (NE40)	**= 0.472+ 0.218*Xmt*+0.0502*XssXsp*+0.084*XssXss*-0.104*Xss*-0.146*XmtXm*t-0.134*XspXsp*-0.052*XssXmt***

Xsp: solvent polarity ,Xmt: mixing time ; Xss: solid solvent

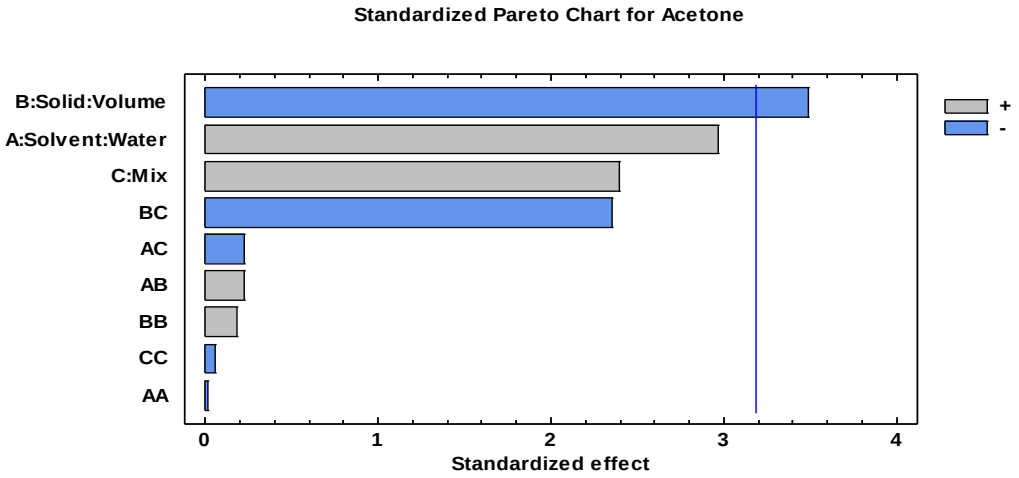


Figure 10a

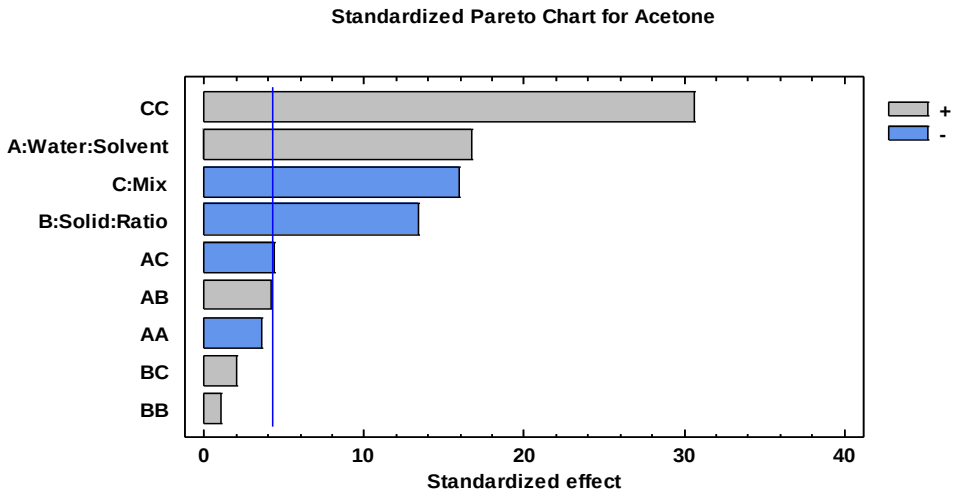


Figure 10 b

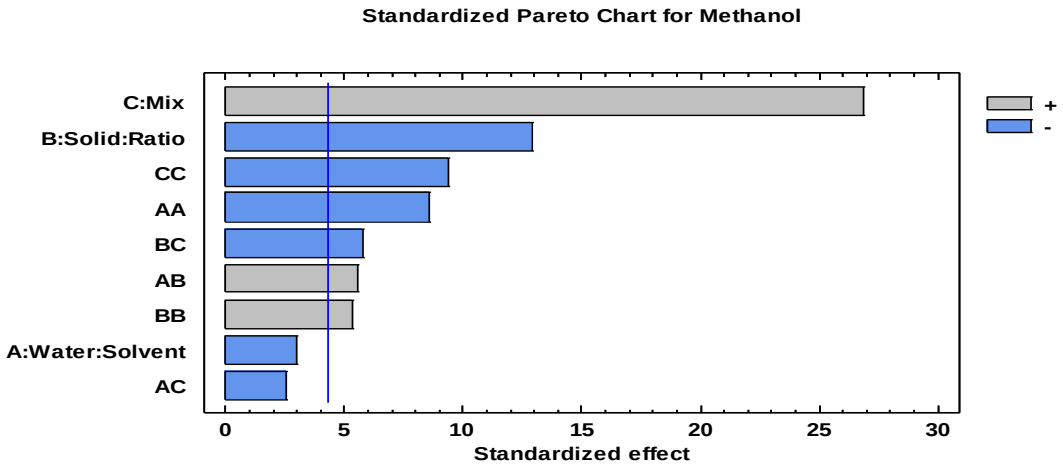


Figure 10 c

Figure 10 (a, b and c): Pareto charts showing relative effects of regression coefficient for total flavonoids accepted models for (a) Acetone for NE36, (b) Acetone for NE40, and (c) Methanol for NE40. Vertical line represents $p < 0.05$.

D.2.1.e *TF final optimized parameter and processing effects:* Optimized values obtained from the accepted TF model were determined (Table 23 (NE36) and 24 (NE40)) According to the model, optimum TF yields will be obtained with 75% acetone water for both the NE36 and NE40 red bean lines (coded value of 1), indicating that the components are non-polar probably due to the absence of conjugates, such as glycosides. Moreover, tannins, which are polymers of anthocyanins, may also be contributing to the assay, albeit this hypothesis has yet to be verified. Nonetheless, tannins are extracted more readily in acetone than methanol / ethanol, as confirmed in our laboratory for multiple bean market classes (data not shown). Considering that the optimal coded value for the parameter solid volume was -1, the lowest solid volume ratio actually tested, even lower solid volume ratios may increase TF yields for both NE36 and NE40 (Figure 11,12). In terms of extraction time, a study conducted by Sheng et al (2013) on TF extraction showed that the yield increased markedly with the mix time increasing from 0.5 hr to 2 hr, with the yield only decreasing slightly at after?2 hr. This might be due to the decomposition of active compounds during the prolonged extraction time (Li et al, 2009; Sheng et al, 2011; Sun et al, 2010). An optimum TF extraction time of 180 min also occurred for NE36, but could increase considering that that the coded number was 1.0 (Table 23). Also, the linear positive line did not show any indication of plateauing (Figure 11). More research is thus needed to increase the upper mixing time to determine the optimal value. It should be noted that the experimental data is not available as we have not applied these conditions to a real sample, which is part of our future work.

Table 23: Optimized factors (in coded value) required to produce optimum TF yield for the cited system for NE36

Optimum value = 4.86376 mg/g of beans

Factor	*Low*	*High*	*Optimum*
Solvent: Water	-1.0	1.0	1.0
Solid: Volume	-1.0	1.0	-1.0
Mix	-1.0	1.0	1.0

Table 24: Optimized factors (in coded value) required to produce optimum TF yields for the cited system for NE40

Optimum value Acetone = 3.94033 mg/g of beans

Factor	*Low*	*High*	*Optimum*
Water: Solvent	-1.0	1.0	1.0
Solid: Ratio	-1.0	1.0	-1.0
Mix	-1.0	1.0	-1.0

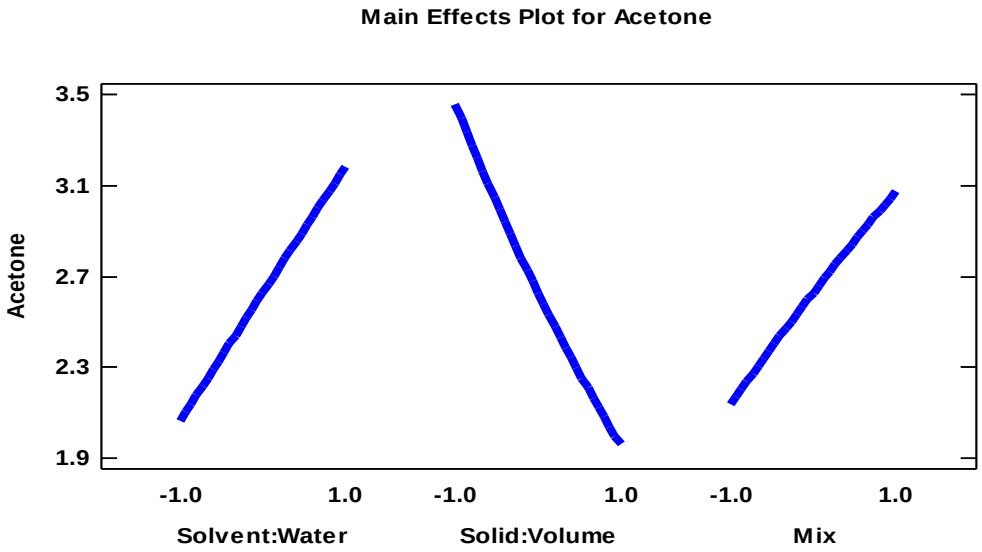


Figure 11. Main effects plot for Acetone (NE36**)**

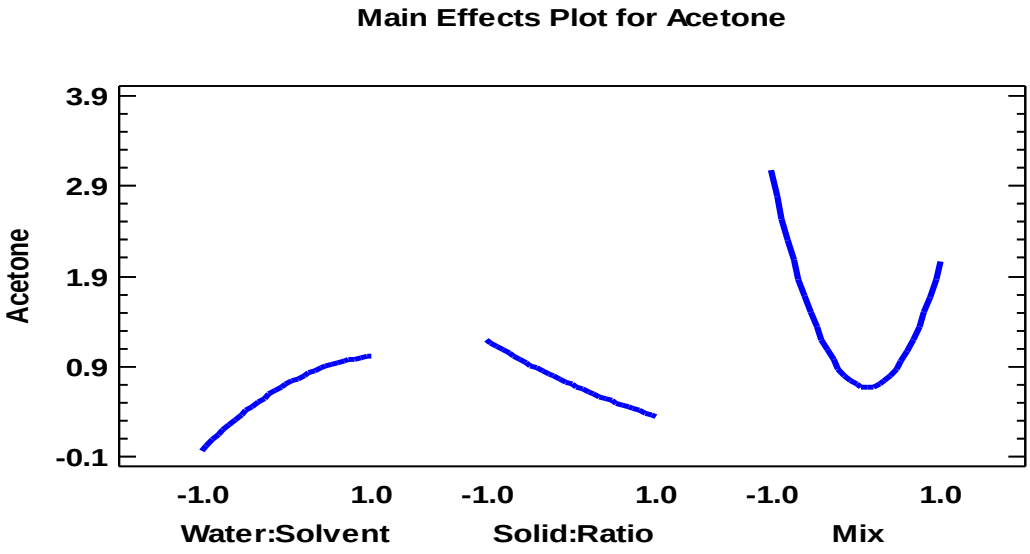


Figure 12. Main effects plot for Acetone (NE40**)**

Interestingly, the optimum mix time for NE40 was 60 min with optimum coded value of -1 indicating that a different mix of TF was extracted in a short time. It should be noted that the experimental data is not available as we have not applied these conditions to a real sample which are part of our future work. Interestingly although the optimum mix time for NE40 was 60 min with optimum coded value of -1 indicating that a different mix of TF was extracted in a shorter duration, with even shorted times possible producing higher TF using the other cited variables.

D.3 Anti-Oxidative Capacity (AC):

D.3.1.a *AC results obtained face centered composited design (FCCD):*

The results of AC capacity are shown as mean +/- standard deviation of three replicates for each solvent extraction and each line, i.e., NE36 (Table 25) and NE40 (Table 27). The coded and actual values used for characterizing the extraction procedures as they apply to AC are shown in Table 6. The high, low, and overall range for AC in each of the solvent systems is also listed in Table 26 (NE36) and 28 (NE40). The highest AC was 144.33 μmole Trolox/g (extraction 9) for line NE36 and 164.96 μmole Trolox/g (extraction 10) for line NE40. For this response, the results showed that ethanol produced the highest overall AC results. Limited literature exists comparing the free radical scavenging ability of solvent based extracts from the bean based systems, but research has been reported on aqueous alcoholic extracts from on other natural systems. For example, Singh et al (2002) tested the AC of methanol and water extracts of pomegranate seeds using DDPH method, which resulted in greater AC with methanol extracts. Filho et al (1998) also reported higher AC of ethanolic extracted samples compared to water only in

cinnamon extracts. Pinelo et al (2004) determined that methanol extracts showed high AC for almond hulls while ethanol extracts resulted in higher AC for pine saw dust.

As stated previously, this study also showed that ethanol based extracts for both NE36 and NE40 have higher AC (Table 25 and 26). In combination, the results demonstrate that phytochemicals recovered from methanol or ethanol based extraction systems were better suited to scavenge free radicals. It must be noted that other radical scavenging molecules may have been extracted with both the methanol and ethanol system, and not acetone, most notably ascorbic acid. As shown in Table 1, small red beans contain relatively high levels of this vitamin.

Table 25: Data for ORAC (μmoletrolox/g) of red bean extracts under different extraction conditions and solvent systems for line NE36.

Std Order	Methanol	Ethanol	Acetone
1	48.15 ± 8.66	51.04 ± 4.04	94.56 ± 3.03
2	139.68 ± 9.08	78.83 ± 3.93	87.26 ± 2.56
3	138.75 ± 8.29	89.52± 5.16	72.99 ± 10.18
4	12.11 ± 2.77	55.91 ± 707	69.11 ± 4.71
5	47.80 ± 4.80	47.71 ± 0.63	60.78 ± 2.94
6	63.35 ± 5.59	61.28 ± 2.59	47.14 ± 6.60
7	82.41 ± 6.34	97.12 ± 1.03	95.38 ± 9.64
8	76.20 ± 4.48	51.00 ± 3.49	61.38 ± 0.74
9	29.55 ± 6.99	144.33 ± 18.15	95.67 ± 11.61
10	43.23 ± 6.91	89.48 ± 1.97	55.95 ± 4.86
11	73.01 ± 1.28	60.40 ± 6.71	79.40 ± 10.47
12	39.23 ± 4.14	41.74 ± 1.57	50.46 ± 6.12
13	27.74 ± 3.63	58.63 ± 2.85	47.38 ± 2.38
14	62.95 ± 8.98	69.86 ± 14.96	32.33 ± 6.45
15	10.93 ± 1.46	51.84 ± 1.12	44.24 ± 8.28
16	69.52 ± 8.43	45.91 ± 6.61	89.76 ± 7.21
17	39.96 ± 4.49	29.53 ± 4.19	84.41 ± 3.80

* Data are shown as the mean ± standard deviation (n=3).

Table 26: Ranges of Anti-oxidative capacity AC for each solvent system (NE36)

Extraction Solvent	AC (μmole Trolox/g)	Range (μmole Trolox/g)
Methanol	10.93 – 139.68	128.75
Ethanol	29.53– 144.33	114.8
Acetone	32.33-95.67	63.34

Table 27: Data for ORAC (μmole trolox/g) of red beans extract under different extraction conditions and solvent system for line NE40.

Std Order	Methanol	Ethanol	Acetone
1	45.28 ± 3.60	54.01 ± 4.80	82.15 ± 1.80
2	48.60 ± 5.02	62.16 ± 5.72	71.00 ± 8.08
3	101.79 ± 7.78	6.47± 0.73	51.79 ± 3.86
4	70.61 ± 0.92	14.81 ± 1.30	53.74 ± 4.66
5	54.72 ± 6.66	50.78 ± 1.35	62.21± 0.74
6	79.49 ± 3.39	72.76 ± 7.59	84.79 ± 10.62
7	97.13 ± 9.53	98.96 ± 12.77	117.67 ± 3.98
8	25.59 ± 5.28	50.25 ± 7.01	62.88 ± 2.91
9	120.40 ± 11.84	75.43 ± 12.90	96.90 ± 21.60
10	71.66 ± 10.15	164.96 ± 4.34	69.20 ± 11.73
11	67.95 ± 2.44	75.30 ± 3.30	91.49 ± 9.55
12	36.79 ± 4.43	36.03 ± 5.24	54.00 ± 5.30
13	40.50 ± 1.68	59.48 ± 1.72	58.79 ± 1.89
14	97.06 ± 1.00	11.27 ± 0.79	129.35 ± 13.55
15	39.94 ± 4.30	11.56 ± 1.56	36.98 ± 7.23
16	63.34 ± 3.99	75.00 ± 3.24	93.27 ± 4.05
17	36.61 ± 7.79	45.56 ± 7.04	50.56 ± 4.35

* Data are shown as the mean ± standard deviation (n=3).

Table 28: Ranges of Anti-oxidative capacity AC for each solvent system (NE40).

Extraction Solvent	AC (μmole Trolox/g)	Range(μmole Trolox/g)
Methanol	25.59– 120.40	94.81
Ethanol	11.27– 164.96	153.69
Acetone	36.98 – 129.35	92.37

D.3.1.b *Fitting the AC models:* Multiple regression coefficients for the AC in each of the three solvent systems are summarized in Tables 29 (NE36) and 30 (NE40). After the experimental data were fitted to the second-order polynomial model, the equation obtained was tested to determine the variability in the responses by evaluating the coefficients of regression and performing ANOVA. The ANOVA showed that the quadratic model was adequate for methanol and ethanol for line NE36, but methanol only for NE40 ($p < 0.05$, $R^2 > 75$). For acetone higher models (cubic model) were determined to be adequate giving evidence for a low dispersion of the experimental data. (More complex interactions can be explained by higher models.) However, analysis of more points or center points may account for the low R^2 value and failure to satisfy the model.

D.3.1.c *Adequacy of the AC models and corresponding regression equations:* Evaluation by ANOVA of the three solvents for the lack of fit test showed compliance of

Table 29: Regression coefficients (coded) predicted by the quadratic polynomial model for Anti-oxidative capacity when extracted with the cited solvent system (NE36)

Coefficient	Methanol	Ethanol
b_o	63.77	54.55
Linear		
b_1 (SP)	6.211	0.539
b_2 (S:S)	-2.60	-23.652*
b_3 (MT)	3.684	4.974
Quadratic		
b_{11} (SP x SP)	-12.006	-7.647
b_{22} (S:S x S:S)	20.097**	19.637
b_{33} (MT x MT)	-30.743*	7.938
Cross product		
b_{12} (SP x S:S)	7.476	6.408
b_{13} (SP x MT)	6.694	-9.000
b_{23} (S:S x MT)	13.769**	-11.524
R^2	94.08	86.44
p values		
Model	0.0016	0.0232
Lack of Fit	0.577	0.2385

SP – Solvent Polarity, S:S – Solid:Solvent, MT – Mix Time

* Significant at 1% **,** **Significant at 5%

Table 30: Regression coefficients (coded) predicted by the quadratic polynomial model for Anti-oxidative capacity when extracted with the cited solvent system (NE40)

Coefficient	Methanol	Ethanol	Acetone
b_o	63.32	65.90	82.86
Linear			
b_1 (SP)	-4.59	15.53*	-4.29
b_2 (S:S)	-29.05*	-14.89*	-19.00*
b_3 (MT)	-4.68	28.52	2.33
Quadratic			
b_{11} (SP x SP)	-17.18	-9.78*	-11.25
b_{22} (S:S x S:S)	17.79	15.30	12.32**
b_{33} (MT x MT)	1.48	-21.70*	-15.24**
Cross product			
b_{12} (SP x S:S)	4.17	-2.82	16.99**
b_{13} (SP x MT)	-8.15	7.58*	4.61
b_{23} (S:S x MT)	-1.40	-25.92	4.51
R^2	90.53	86.93	78.65
p values			
Model	0.0075	0.0207	0.0896
Lack of Fit	0.2789	0.0031	0.046

SP – Solvent Polarity, S:S – Solid:Solvent, MT – Mix Time

* Significant at 1% **,** **Significant at 5%

methanol and ethanol for NE36 (Table 31) and methanol and acetone for NE40 ($p > 0.05$). Interestingly, the ethanol system for NE40 had R^2 value > 0.75 but failed lack of fit tests (Table 30), again indicating that a higher model may be needed to describe the more complex interactions between the process parameters.

D.3.1.d *Regression coefficients equations and Pareto charts:* The regression equation derived by the AC data using methanol and ethanol based systems for line NE36 and methanol and acetone for NE40 are shown in Table 31. These equations are based on the significance of individual regression coefficients only ($p < 0.05$). The associated Pareto charts are illustrated in Figure 13a-c. For line NE36 (Figure 13a), the methanol AC extracts were mainly affected by mix time with higher amounts negatively affecting AC. The next parameter that affected AC was the cross product of solid:solvent ratio and mix time and solvent polarity. Alternatively, a linear positive relationship for AC and ?occurred while ethanol extraction (Figure 13b) was negatively affected by linear solid:solvent ratio. The methanol AC extractions of NE40 showed a linear and quadratic negative relationship with solid:solvent ratio (Figure 13c), whereas the acetone extractions showed a positive quadratic relation for solid:solventratio and cross product interaction of solid solvent:water ratio, and negative relationship via quadratic mix time and linear solid solvent ratio (Figure 13d).

D.3.1.e *Final optimized AC values and processing factors*: The optimized factors predicted to produce the highest AC values are respectively shown in Table 32 (NE36) and 33 (NE40), while schematic representations are provided in Figures 14 (NE36) and 15 (NE40). Considering that the optimal coded value for the parameter solid:solvent ratio was -1 for both NE36 and NE40, even lower solid volume ratios than 10%

Table 31: Regression equations that fit the model and passed lack of fit test.

NE36
AC methanol = 63.77-30.74 *XmtXmt* +13.76*Xss Xmt* +20.097*Xss Xss*
AC ethanol =54.55-23.52Xss
NE40
AC methanol = 63.32-29.05Xss
AC acetone= 82.86+16.99*XssXsp*+12.32*XssXss*-19.0*Xss*-15.24*XmtXmt*

Xsp: solvent polarity, Xmt: mixing time ; Xss: solid solvent

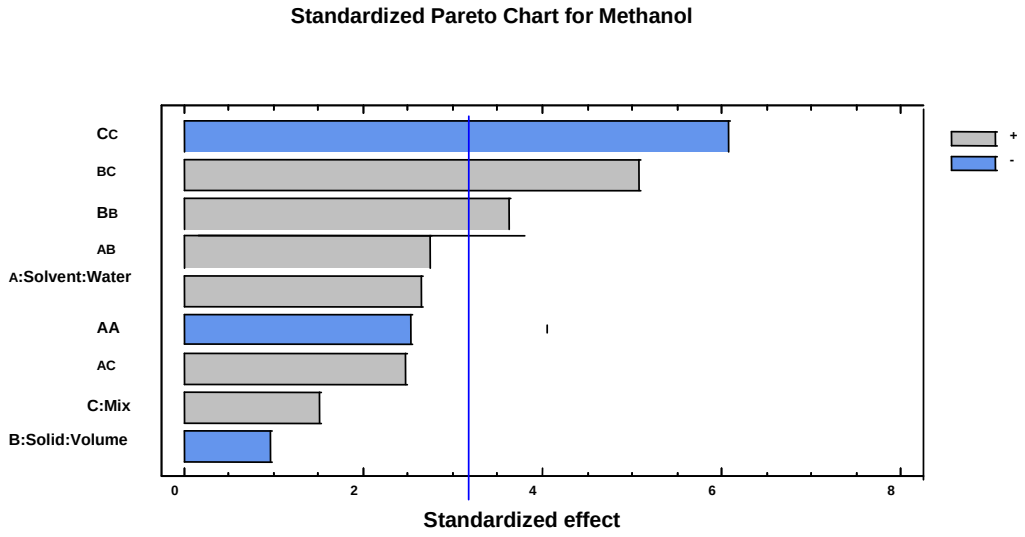


Figure 13 a

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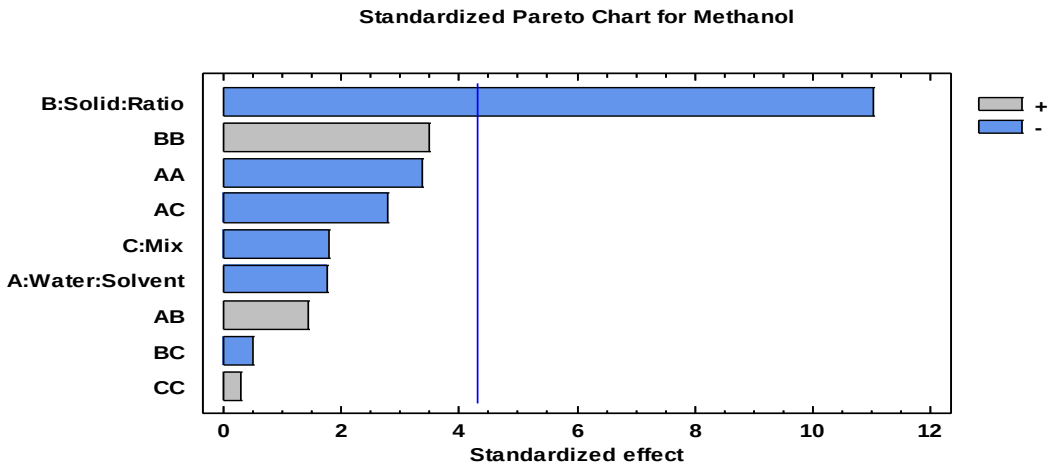


Figure 13 b

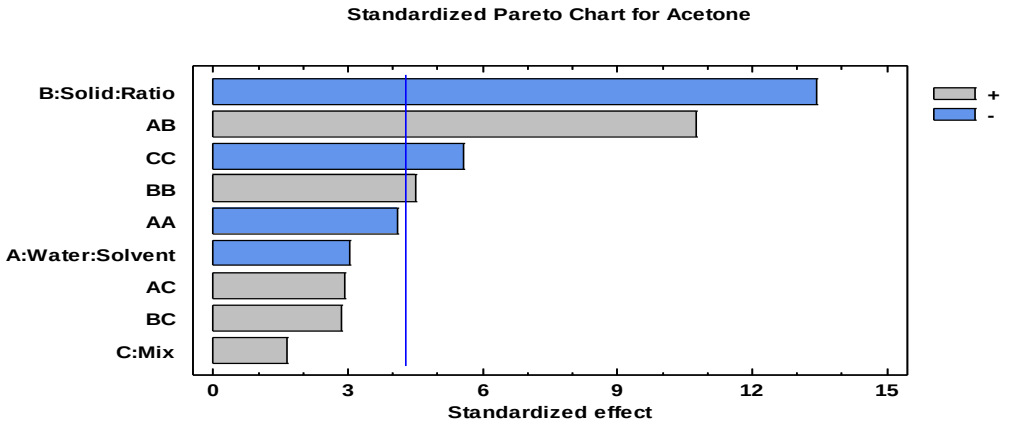


Figure 13 c

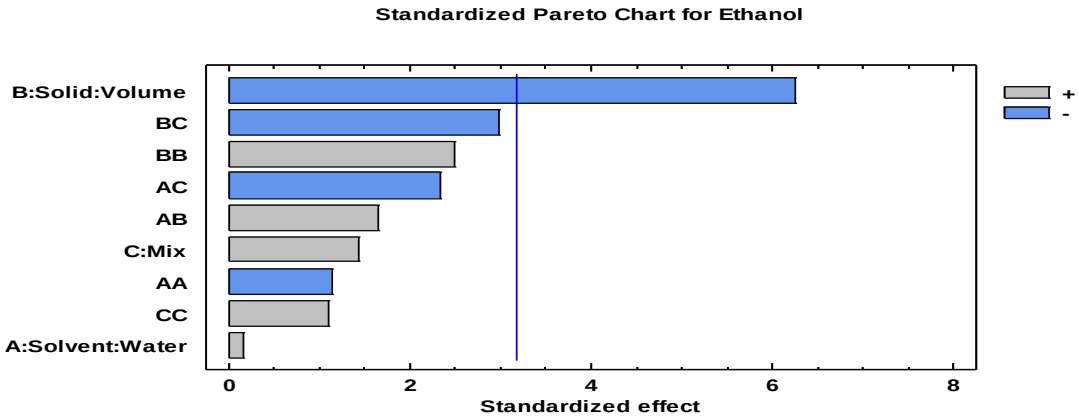


Figure 13 d

Figure 13: Pareto charts showing relative effects of regression coefficient for AC accepted models by (a) methanol (NE36) (b) ethanol (NE36) and (c) methanol (NE40) (d) acetone (NE40). Vertical line represents $p < 0.05$

Table 32: Optimized factors (in coded value) required to produce optimum AC yield for ethanol for NE36

Optimum value = 129.483(μmole Trolox/g)

Factor	*Low*	*High*	*Optimum*
Solvent: Water	-1.0	1.0	-0.968764
Solid: Volume	-1.0	1.0	-1.0
Mix	-1.0	1.0	1.0

Table 33: Optimized factors (in coded value) required to produce optimum AC yield for ethanol for NE36

*Optimum value for Ethanol = 145.633***(**μmole Trolox/g)

Factor	*Low*	*High*	*Optimum*
Solvent: Water	-1.0	1.0	1.0
Solid:Ratio	-1.0	1.0	-1.0
Mix	-1.0	1.0	1.0

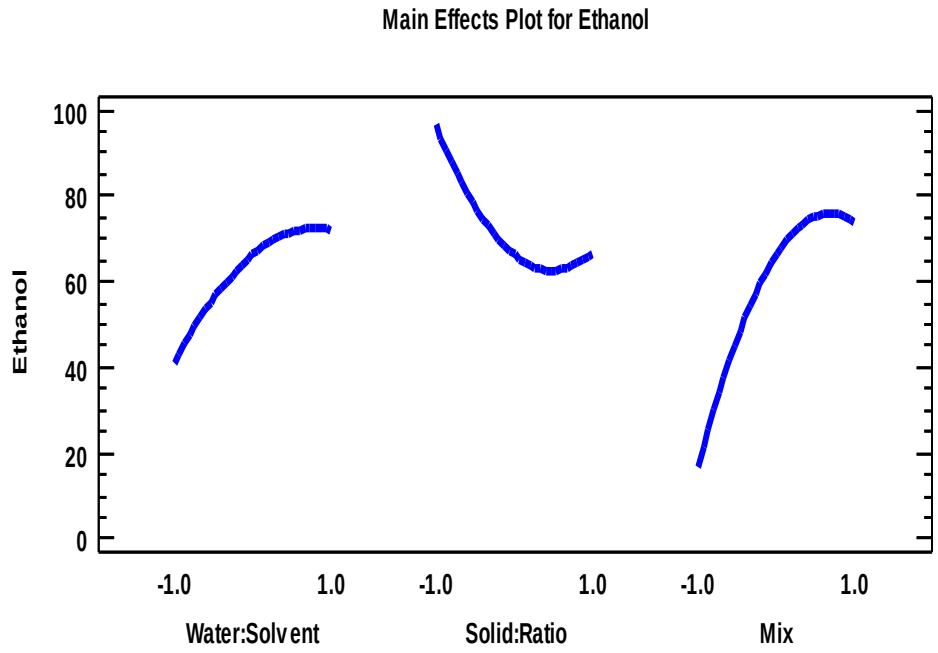


Figure 14. Main effects plot for Ethanol for NE36

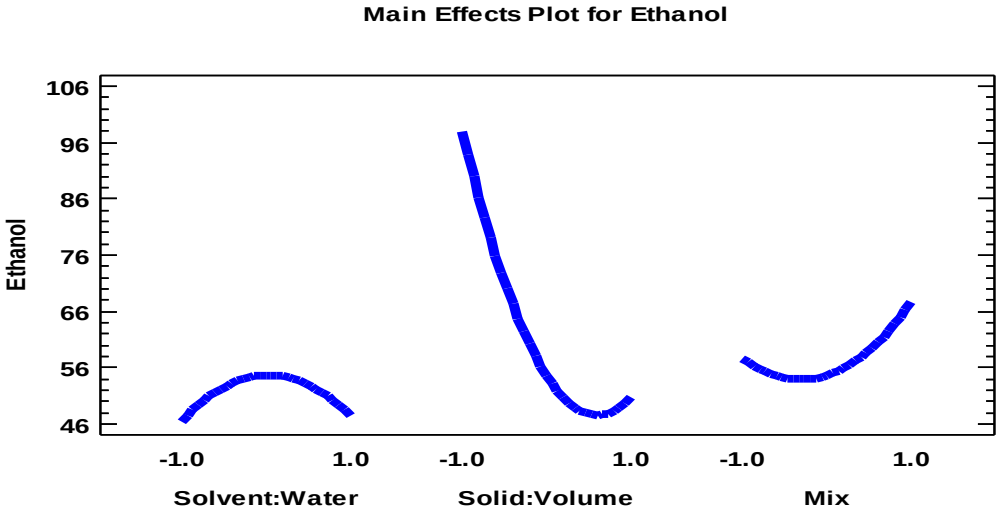


Figure 15. Main effects plot for Ethanol for NE40

(-1 coded) may increase AC (Figure 14 (NE36) and 15 (NE40)). With respect to solvent:water ratio, the optimum value for NE36 was 25% and 75% for NE40. Using RSM, Karacabey et al (2010) showed that the AC of grape cane extracts was significantly affected by ethanol:water optimal ratio of 50.5%. The different ethanol:water polarities may be due to differences in the composition of phenolic compounds obtained from different solvent concentrations, which will cause a difference in the AC of that extract (Karacabey et al, 2010). Anti-oxidative capacity may be dependent on solvent polarity due to structural differences of extracted phenolics or the presence of other antioxidants, as described above. Moreover, mix times higher than 180 min may be required to obtain AC compounds from NE40, as evidenced by Table 17. Extended extraction time can favor the extraction of polyphenolic compounds due to longer exposure of the solute to specific solvent the dissolution into the liquid phase (Gan, et al 2011). Alternatively, AC samples obtained from NE36 was starting to decrease after a mix time of ~ 120 min. Again, different types of and amounts, of phenols as well as other antioxidants, may be the reason for this effect. Additional work is occurring in our laboratory to more thoroughly characterize these fractions.

D4. *Specific Aim 2*: Anti -Inflammatory Evaluation

Iinflammation is initiated by complex processes triggered by microbial pathogens and other repair signals (West et al, 1995). Macrophages are just one of the multiple immune cells involved in the inflammatory process, but are major players for sustaining chronic inflammation (González-Gallego et al, 2006; Middleton et al, 2000; Monterio et al, 2010; Shewry et al, 2010). When macrophages are exposed to bacterial products, such as endotoxin lipopolysaccharides (LPS), LPS binds to Toll-like receptor 4 (TLR4) that

activates two major signaling pathways, myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adaptor inducing IFN-β (TRIF) (Sachithanandan et al, 2011; Hwang et al, 2014) This cascade event then activates the transcription factor nuclear factor –kappa β (NF-κβ), which in turn induces the upregulation of the inducible nitric oxide synthase (iNOS) enzyme. Activation of the iNOS signaling pathway produces nitric oxide (NO), which is a marker of the pro-inflammatory M1 phenotype (Mills et al, 2000), and indirectly multiple pro-inflammatory cytokines and adhesion molecules (Lee et al, 2011; Lee et al, 2012). The pharmacological reduction of LPS induced inflammatory mediators (e.g., NO, TNF-R, and IL) is regarded as one of the most important factors to alleviate a variety of disorders caused by activated M1 macrophages (Karpurapu et al, 2011). RAW 264.7 macrophage (an immortal cell line from an animal model) provides an excellent cell system for anti-inflammatory screening of natural plant extracts as they contain the iNOS pathway.

Studies have shown the effect of phenolic acids, such as chlorogenic acid, on pro-inflammatory cytokines and the adhesion molecule (Ninj1) regulated by the NFκB pathway on LPS-stimulated RAW264.7 cells (Hwang et al, 2014). It was determined that chlorogenic acid inhibited LPS induced inflammation in RAW 264.7 cells resulting in decreased NO production caused by the NFκB down regulation of iNOS. Therefore, the inhibitory effects of red beans on the production of NO in response to LPS induced RAW 264.7 macrophages is an important part of this research and is described in this section.

Samples from the two small red beans lines (NE36 and NE40) that showed the three highest TP, TF and AC values were prepared with each of the three solvent systems

and corresponding extraction methods as determined from Specific Aim 1. For convenience in Tables 34 (NE36) and 35 (NE40) the specific extraction procedures are provided again with each of level of TF, TP, and AC. Also included in these tables are the corresponding flavonoids, phenols yields, and anti-oxidative capacities obtained with the same extraction, although not the highest based on the RSM studies. The effects on the NO production in response to these extracts were examined using LPS (200 ng/ml) stimulated RAW 264.7 cells. Experiments were also completed using only the extracts exposed to the cells but without LPS activation to ensure that these test samples did not induce inflammation, which did not occur.

D4.1 *Effect of bean extracts on RAW 264.7 cell viability:*

The effect of the extracts on cell viability is important to distinguish between their toxicity and biological activity. This is especially important when using the NO assay as cell death can alter NO concentration, as can inflammatory events (Kassim et al,2010). Cell viability was thus measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. All the extracts cited in Table 34 and 35 were subjected to MTT assays at 8-10 concentrations. The concentrations that were not toxic to the cells (>80% viability) were selected for NO assays. Figure 16 and 17 shows the MTT data from methanol extracts for high TF for NE36 and NE40 as an example of these experiments. As is evident from Figure 18, NE36 extract levels of 3.125, 1.56, 0.78, and 0.39 µg/ml were not toxic to the cells; whereas 6.25, 3.125, 1.56 and 0.78 µg/ml of NE40 extracts did not negatively impact cell viability (Figure 17). These extract levels were also comparable in terms of cell viability to the other extraction solvents and thus used for all the anti-inflammatory experiments.

Table 34: Red bean extracts from NE36 showing high TP, TF and AC

Extract	Solvent Composition	Solid solvent ratio	Mix time	Phenols	Flavonoid	AC
High TP		%	min	mg/g	mg/g	μmole* / g
A	Acetone (50:50)	10	120	3.45±0.17	2.95 ± 0.16	95.38 ± 9.64
B	Methanol(75:25)	10	180	1.16±0.06	0.89 ± 0.02	43.23 ± 6.91
C	Ethanol (25:75)	10	60	1.64±0.07	0.78 ± 0.02	69.86 ± 14.96
High AC						
D	Methanol (50:50)	20	180	0.84 ±0.09	0.72 ± 0.05	139.68±9.08
E	Ethanol (25:75)	10	180	1.25 ± 0.03	0.58 ± 0.06	144.33±18.5
F	Acetone (25:75)	10	180	1.72 ± 0.15	3.89 ± 0.29	95.67±11.61
High TF						
P	Methanol(75:25)	10	180	1.16 ± 0.07	0.89±0.02	43.23 ± 6.91
Q	Ethanol (25:75)	10	60	1.64 ± 0.07	0.78±0.02	69.86 ± 14.96
R	Acetone (75:25)	10	180	2.51 ± 0.25	5.60±0.16	55.95 ± 4.86

* μmole Trolox/g of product

Table 35: Red bean extracts from NE40 showing high TP, TF and AC

Extract	Solvent Composition	Solid solvent ratio	Mix time	Phenols	Flavonoid	AC
High TP	%	%	min	mg/g	mg/g	μmole* / g
G	Methanol (25:75)	10	60	1.50±0.02	0.13 ± 0.00	97.06 ± 1.00
H	Ethanol (25:75)	10	180	1.69±0.17	0.62 ± 0.06	75.43 ± 12.90
I	Acetone (50:50)	10	120	3.52±0.09	0.72 ± 0.04	117.67 ± 3.98
High AC						
J	Methanol (25:75)	10%	180	1.29 ± 0.10	0.77 ± 0.07	120.40 ± 11.84
K	Ethanol (75:25)	10%	180	1.28 ± 0.05	0.83 ± 0.01	164.96 ± 4.34
L	Acetone (25:75)	10%	60	2.37 ± 0.37	3.00 ± 0.39	129.35 ± 13.55
High TF						
M	Methanol(25:75)	10	180	1.29 ± 0.10	0.77± 0.07	120.40 ± 11.84
N	Ethanol (75:25)	10	60	1.13 ± 0.16	1.08± 0.13	6.47± 0.73
O	Acetone (25:75)	10	60	2.37 ± 0.37	3.00 ± 0.39	129.35 ± 13.55

* μmole Trolox/g of product

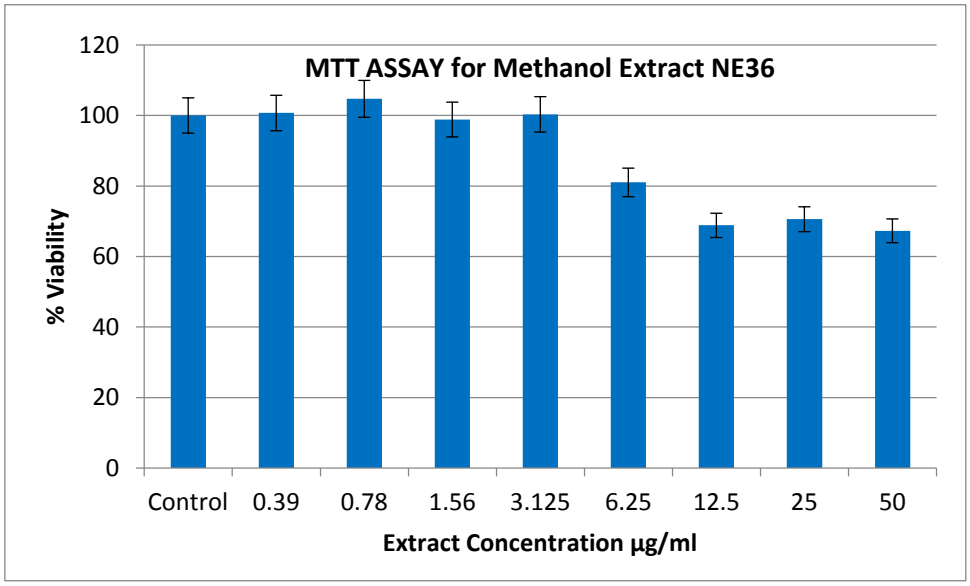


Figure 16: MTT data for red bean extract in methanol for NE36 (High TF). Data is expressed as viability (%) relative to control that was not incubated with red bean extract

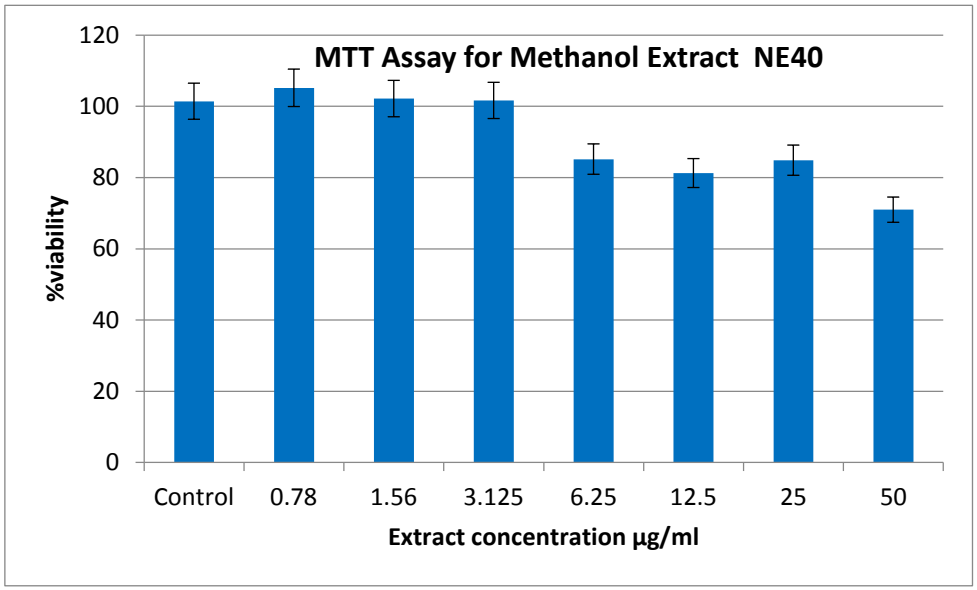


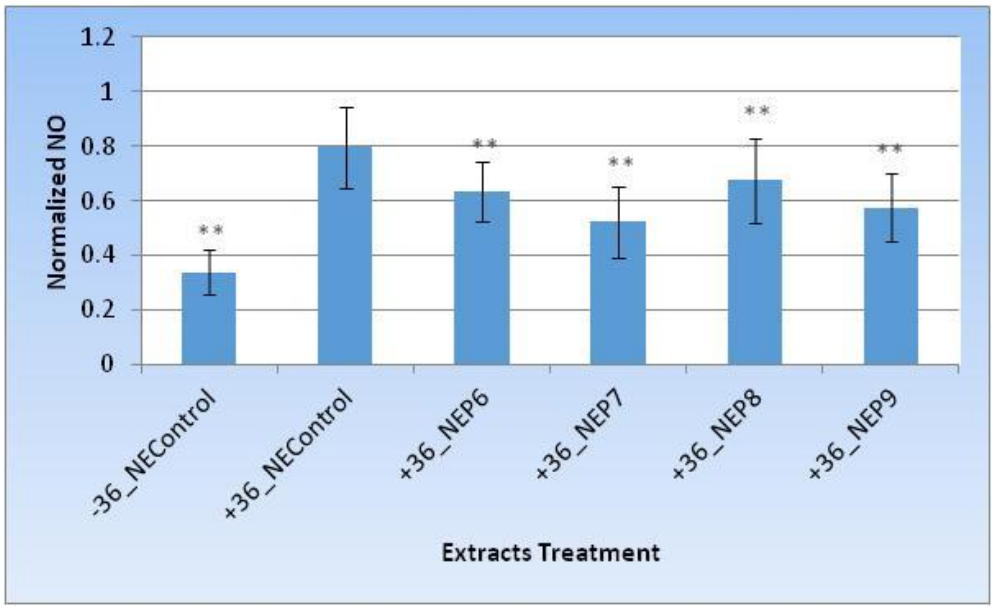
Figure 17 : MTT data for red bean extract in methanol for NE40 (High TF). Data is expressed as viability (%) relative to control that was not incubated with red bean extract.

D 4.2 *Effect of TP, TF, and AC rich bean extracts on NO production in RAW264.7 cell induced with LPS*

As many phenolic compounds have shown potent pharmacological attributes due to their anti-inflammatory, antioxidant and antitumor properties (Soobrattee et al, 2005), it was expected that the phenol rich extracts obtained from small red beans would also demonstrate anti-inflammatory activity. The extracts that produced the highest TF, TP, and AC for NE36 and NE 40 that did not cause cell toxicity, as cited previously, were incubated with cells already exposed to LPS. It is important to note that this approach is unique to many other anti-inflammatory research studies cited in the literature. In those studies, the natural system of interest is first exposed to the cells followed by LPS exposure. The ability of the natural system or an isolated component to "prevent" inflammation is thus evaluated (Kobuchi et al, 1997; Kim et al, 1999, Wadsworth et al 1999, Číž et al, 2008).

Since inflammation is critically needed to repair tissue and protect against bacterial infections, prevention is not an acceptable alternative. Rather, remediation of the inflammation is needed after the acute event has occurred to stop chronic inflammation that if left unchecked leads to other diseases, (as described in the Literature Review looks Section). Initially exposing the cells to LPS, followed by the small red bean extracts used in these studies has provided information on remediation instead of prevention.

As shown in Figures 18 and 19, NO increased significantly in RAW264.7 cells supernatants after 24 hour treatment with 100 ng/ml of LPS (positive control) compared to the sample without LPS (negative control). It must also be emphasized that experiments



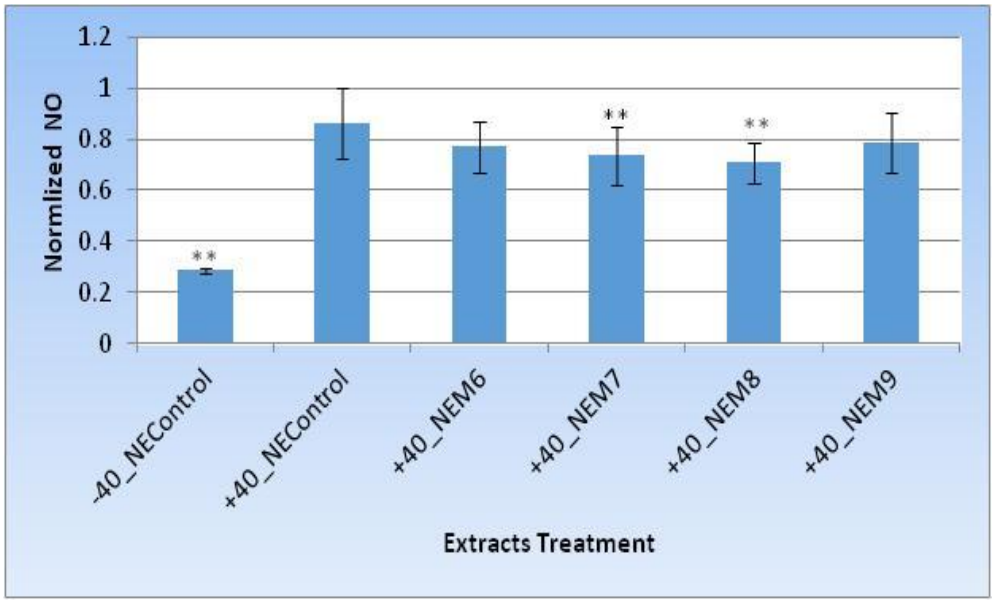
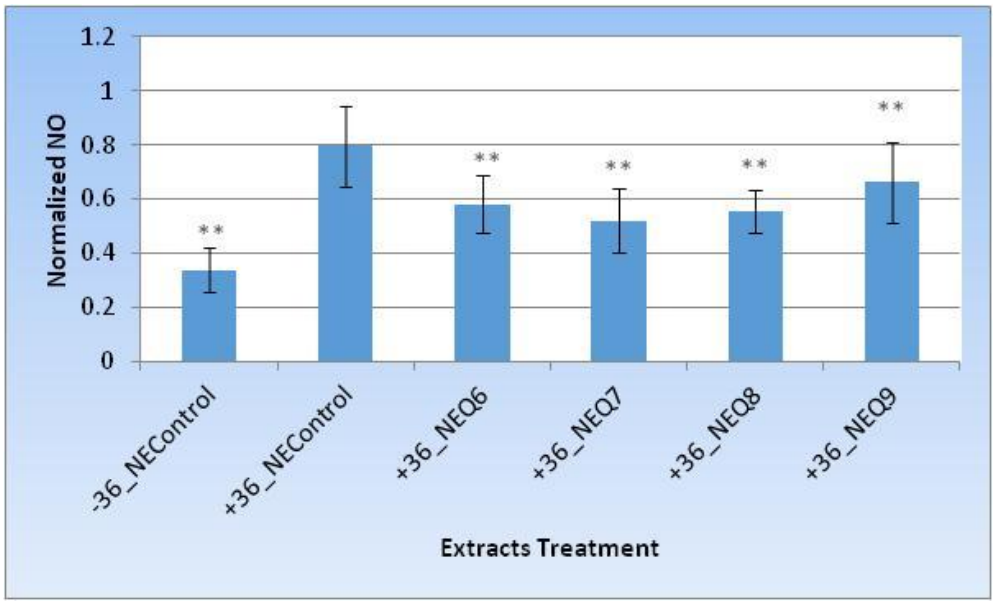


Figure 18: Effect of Methanolic Extracts of Red Beans on NO inhibition in RAW264.7 macrophages. Results significant at *p<0.1,**p<0.05 compared to LPS activated cells (+36_NEControl). The extracts P6,P7,P8,P9 are 3.125,1.56,0.78 and 0.39 µg/ml for NE36 AND M6,M7,M8,M9 are 6.25,3.125,1.56 and 0.78 µg/ml (High TF). The data represent three biological replications blocked by day. Error bars represent the mean +/- standard error of the mean. +36_NEControl and +40_NEControl represents cells + LPS without extract treatment (positive control); -36_NEControl and -40_NEControl represent cells – LPS without extract treatment (negative control).



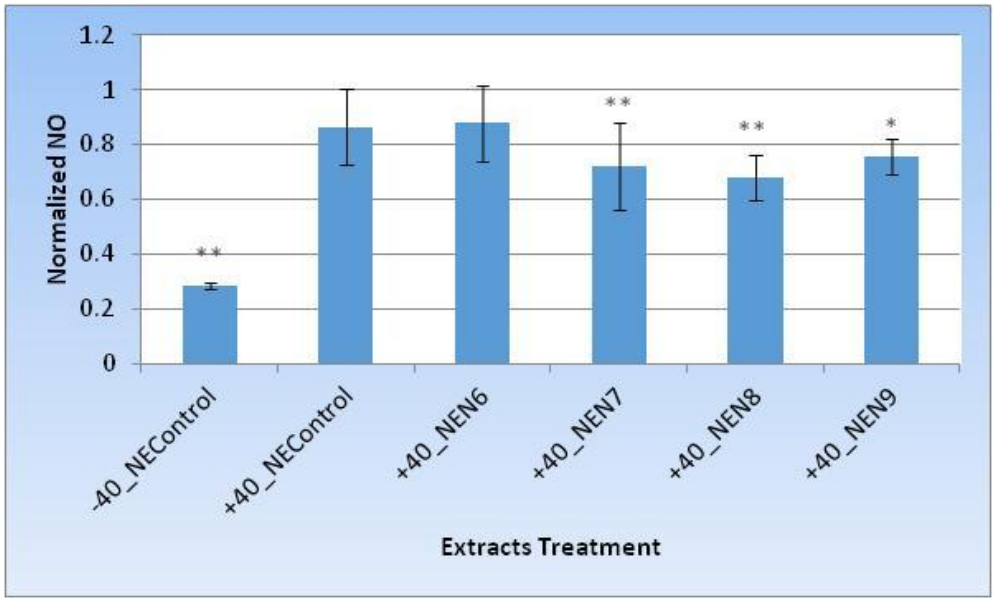
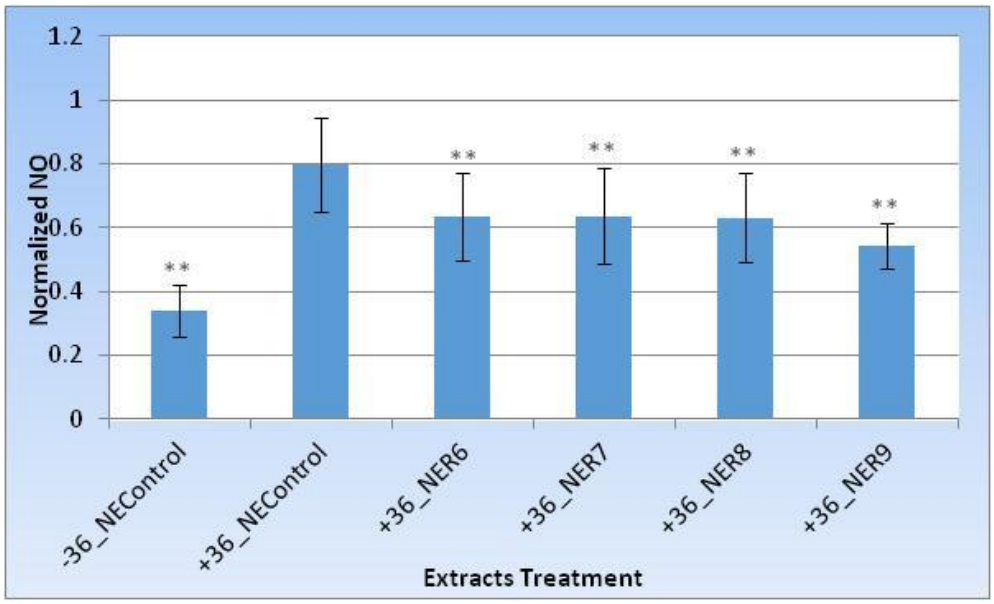


Figure19: Effect of Ethanolic Extracts of Red Beans on NO inhibition in RAW264.7 macrophages. Results significant at *p<0.1,**p<0.05 compared to LPS activated cells (+36_NEControl). The extracts Q6,Q7,Q8,Q9 are 3.125,1.56,0.78 and 0.39µg/ml for NE36 AND N6,N7,N8,N9 are 6.25,3.125,1.56 and 0.78 µg/ml. (High TF) The data represent three biological replications blocked by day. Error bars represent the mean +/- standard error of the mean. +36_NEControl and +40_NEControl represents cells + LPS without extract treatment (positive control); -36_NEControl and -40_NEControl represent cells – LPS without extract treatment (negative control).



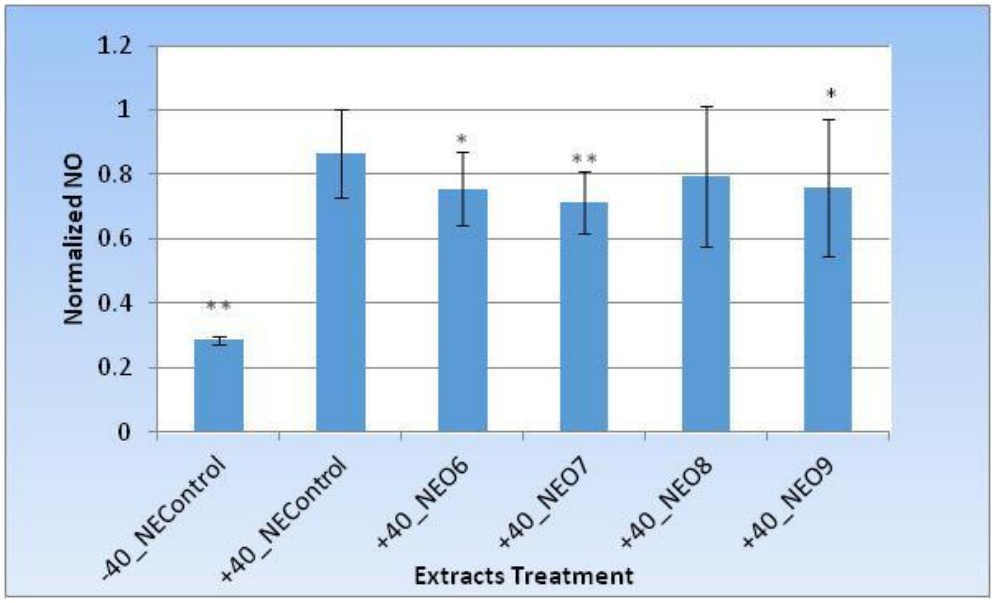


Figure20: Effect of Acetone Extracts of Red Beans on NO inhibition in RAW264.7 macrophages. Results significant at *p<0.1,**p<0.05 compared to LPS activated cells (+36_NEControl). The extracts Q6,Q7,Q8,Q9 are 3.125,1.56,0.78 and 0.39µg/ml for NE36 AND N6,N7,N8,N9 are 6.25,3.125,1.56 and 0.78 µg/ml. (High TF) The data represent three biological replications blocked by day. Error bars represent the mean +/- standard error of the mean. +36_NEControl and +40_NEControl represents cells + LPS without extract treatment (positive control); -36_NEControl and -40_NEControl represent cells – LPS without extract treatment (negative control).

were also completed using only the cells treated with the extracts without initial LPS activation to ensure that these test samples did not induce inflammation. It was determined that no difference was induced by treatment in cells relative to the negative control,regardless of the extract (data not shown). However, the AC extracts showed no inflammatory remediation effect in LPS induced cells, while only one concentration from the TP extract was efficacious (data not shown). On the other hand, the majority of the TF extracts remediated inflammation as shown in Figure 18 , Figure 19 and Figure 20 (NE36 and NE40). The anti-inflammatory effect of the TF treatments was thus statistically analyzed at $p < 0.1$ and 0.05 vs the positive control for both NE36 and NE40.

Flavonoids possess anti-inflammatory and immunomodulatory activities *in vitro* and *in vivo*. The cellular action mechanisms of flavonoids for these pharmacological activities have been reported partly by inhibiting cyclooxygenase / lipoxygenase due to their anti-oxidative nature (Bauman et al, 1980; Havsteen et al, 1983). Wang et al (2006) showed that the flavanols, kaempferol, fisetin and quercetin inhibited NO production in LPS-stimulated RAW264.7 macrophages, in a dose dependent manner. Another study conducted by Kim et al (1999), studied the effect of naturally occurring flavonoids on NO production in RAW 264.7 macrophages, which showed that the inhibitory effect may be due to reduction of iNOS enzyme expression. Other studies have shown that flavonoids and condensed tannins suppressed the expression of pro-inflammatory targets in pain and inflammatory diseases (Iwalewa et al, 2007).

For these studies, the acetone extracted TF samples resulted in significant results at $p<0.05$, indicating that the more non-polar TF extracts were more potent than the more polar TF extracts. Other components, such as short chain sugars, minerals, proteins,

amino / organic acids, etc., that may be present in the methanol /ethanol based extracts also may be negatively impacting the overall effect. Along these lines, the acetone extracts also contain more condensed tannins as determined in our laboratory (data not shown), which may be contributing to the anti-inflammatory effect (Iwalewa et al, 2007). Although the TP and AC extracts did not reduce inflammation, they also did not induce this response causing no harm, which is the first rule for any health benefiting component (first do no harm).

F. FUTURE WORK:

As an outcome of this work, other future studies became evident and are cited below.

- The extracts from each of the bean lines require further characterization to identify the amounts and types of phytochemicals present in each, which most likely resulted in different extraction models for TF, TP, and AC.
- The extracts must be characterized for individual TP and TF, to determine if the phenols are acting alone, synergistically, or additively to impact (negatively and positively) both AC and anti-inflammatory effects.
- The crude extracts of beans may contain carbohydrates, proteins and minerals along with the phenolic compounds, necessitating further purification to remove these impurities for further studies and characterization of individual phenolic compounds.
- Other inflammation markers such as inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF-α), interleukin-1b

(IL-1b), interleukin-6 (IL-6), and chemokine (C-X-C motif) ligand 1(CXCL1) can be studied to further understand the mechanism of this bioactivity (anti-inflammatory activity) of red bean extracts.

E. CONCLUSIONS:

- The response surface methodology was successfully used for obtaining critical information relative to extraction of phenolic-rich extracts from red beans. The most effective factors that resulted in overall maximum yields for TP were: acetone: water composition of 50%, a solid:solvent ratio of 10% and a mix time of 60 min for both lines. For optimal TF extractions, an acetone:water composition of 75%, solid:solvent ratio of 10% and mix time of 180 min or mix time of 60 min for NE40 were required. Maximum AC values were obtained for 25% ethanol: water composition, solid solvent ratio of 10% and mix time of 180 min for both NE36 and for NE40 while all other parameters remain same.
- Acetone was most effective for extracting TP and TF and ethanol for AC for both lines of red beans. In most cases a second-order polynomial model could be used to optimize extraction of TP from red beans with the exception where the data did not fit the models, which could be due to variability in the assay. A higher order model may better explain the complex interactions occurring or non-uniform particle size of the red bean powder may also be a contributing factors.
- Two lines of red beans (NE36 and NE40) were tested, and it was determined that there were significant differences in the amounts of phenolic compounds

extracted from each for different solvents used and subsequently in the resulting bioactivity (anti-inflammatory properties).

- The bean extracts in different solvents may show higher AC, but that does not necessarily translate into more potent anti-inflammatory activity, owing most likely to different types of phenolic compounds present in each extract.
- The results of this study indicate that different extraction methods and solvents will yield different concentrations of phenolic compounds in different lines of red beans. In addition, the two different lines show varying anti-inflammatory activities in the in vitro model. This bioactivity may be attributed, at least in part, to the phenolic compounds within the extracts.
- Lastly, consumption of a diet rich in beans may reduce the harmful effects of nitric oxide in chronic inflammatory conditions.

This study showed the potential therapeutic value of red beans and its extracts in inflammatory conditions, thus highlighting the nutritional value of this food. In conclusion, red beans have a potential for prevention of chronic inflammatory diseases, which may be due to the additive and synergistic effects of phytochemicals responsible for their biological functions.

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