ORIGINAL ARTICLE

EFFECT OF GRADED LEVELS OF DACRYODES EDULIS (AFRICAN PEAR SEEDS) FLOUR ON SERUM C-REACTIVE PROTEINS OF WISTAR RATS.

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ABSTRACT

The effect of graded doses of African pear seed flour on serum C-reactive proteins was investigated in wistar rats. Twenty four (24) albino rats weighing 150-250g were randomly distributed into four groups of six (6) rats each thus; group I, II, III and IV. Group I served as control and received normal rats pellet and water ad libitum. Groups II, III and IV served as the experimental groups and were fed with respective doses of 10%, 20%, and 30% African pear seed flour in normal rat feed for 28 days. The animals also received distilled water throughout the feeding duration. At the end of the experimental period, the animals were sacrificed and blood extracted by cardiac puncture. The blood sample was collected into a centrifuge tube along with an anticoagulant and centrifuged for a period of 5 minutes at a speed of 3000rpm. The serum was obtained and used for biochemical estimation of plasma C-reactive protein levels. The results obtained showed C-reactive protein levels of 1.52 ± 1.22mg/L, 1.33 ± 0.10mg/L, 1.13 ± 0.11mg/L, and 1.15 ± 0.01mg/L in I, II, III, and IV groups respectively. From the results, it can be concluded that the fortified diets of graded doses of African pear seeds flour were safe and of nutritional benefit to the experimental animals. Thus, grinded African pear seed flour could be used to fortify diets to alleviate micronutrients malnutrition, as it appears to have immunoprotective capabilities.

INTRODUCTION

Over the years, man has progressively acquired extensive knowledge concerning the medicinal values of plants and herbs (Adesegun, 2001; Adesokan et al., 2007). Plants fruits and seed can be used for food and medicine when taken raw or during their supplementations with other food stuffs.

Dacryodes edulis (African pear plant) is an indigenous fruit tree of the Gulf of Guinea and Central African Countries (Troupin, 1950). The plant is also predominantly cultivated in Sierra Leone, Uganda, Angola, Zimbabwe and Nigeria (Anonymous, 2010). It belongs to the family Burseraceae and order Spindale. It is an evergreen tree (height 18-40 metres) of humid tropical zone (Leakey et al., 2002). It has a relatively short trunk and a deep, dense crown. The bark is pale grey and rough with droplets of resin. The fruit is ellipsoidal drupe with size of 4 to 9cm long and 5cm wide (Awono et al., 2002).

The fruits are dark blue or violet in colour with pale to light green mesocarp which is consumed raw, cooked in salt water or roasted and serves as good source of oils, vitamins and proteins (Sofowora, 2008). There are two varieties of Dacryodes; the D. edulis are larger with stout tree and ascending branches whereas, D. parvicarpa has smaller fruits and slender drooping branches (Isaac and Ekpa, 2009). D. edulis possesses a wide range of medicinal and biological attributes. It is antimicrobial, anti-inflammatory, antihypertentive, antispasmodic and antioxidant (Okunomo, 2010).

In Nigeria, the fruits are also used to remedy fever, oral problems and ear infections; the resin is used for treating parasitic skin disease and Jiggers, while the pulped bark is used to treat wounds. The fruits also possess a wide range of chemical constituents such as phytochemicals, minerals, sugars, vitamins, lipids and proteins.

PHYTOCHEMICAL COMPOSITION OF DACRYODES EDULIS RAW SEED EXTRACTS

Phytochemical screening for secondary metabolites of Dacryodes edulis raw seed extracts showed presence of free athraquinone,

steroid/triterpenes, tannin and saponin. The anti-microbial screening of methanol, ethyl acetate, chloroform and hexane extracts of D.edulis showed sensitivity against nine microbes; staphylococcus aureus, Eschericia coli, Salmonella typhi, Shigella dysenteriae, Psedomonas aeruginosa, Klebsiella pneumonia and fungi; Candida albican, Trychophytomrubrum and Microsporum spp. The chloroform extract showed the highest inhibition against the pathogens that were sensitive to the extract; Klesiella pneumonia was most inhibited (23 mm). The moderate antibacterial and antifungal activities of these extracts could not be unrelated to the presence of secondary metabolites detected in the plant. This justifies the traditional usage of the seed as remedy for stomach problems.

The most predominant antinutrient are the trypsin inhibitors (7.33%) followed by the saponins (1.14%) and the tannins (1.05%). The polyphenols (0.35%), oxalates (0.64%) and phytates (0.77%) were also present in lesser amounts. Saponins are characterized by a bitter taste. They also inhibit nutrient transport and exhibit growth depressing action. Dietary levels of 1% phytate or more have been reported to interfere with mineral availability. Chai and Lieman has reported that a daily intake of 450 mg oxalate can interfere with body metabolism. The presence of these anti-nutrients has inhibited the use of APS as food for humans. However, it has been observed in the local communities that they are freely consumed by domestic animals.

Table 1: PROXIMATE COMPOSITION OF DACRYODES EDULIS RAW SEED EXTRACTS

COMPOSITION	Quantity (%)
Moisture	12.77
Dry Matter	87.22
Protein	18.03
Carbohydrate	39.10
Crude Fibre	3.17
Ash	3.45
Lipids	19.47
Energy (Kj/100g)	1689.9

(Akpambang VOC, Amio IA, Izuagu, 2008; Arisa and Lazarus, 2008)

A lipid content of 19.47% shows that the seed can be a good source of oil. The value falls within the range of values for most oil producing seeds like soybeans.

Table 2: Fatty acids composition of Dacryodes edulis seed oils

FATTY ACIDS	AFRICAN PEAR SEED OIL (%)
SATURATED FATTY ACIDS	
(i) Palmitic acid	38.56
(ii) Stearic acid	2.81
(iii) Palmitoleic acid	-
UNSATURATED FATTY ACIDS	
(i) Oleic acid	32.62
(ii) Linocleic acid	27.30
(iii) Linolenic acid	1.25

Umoti and Okyi (1998)

USES OF AFRICAN PEAR FRUITS:

The presence of bioactive compounds such as saponins, tannins, alkaloids and flavonoids identified in the plant has been suggested to be responsible for the various uses of D. edulis in traditional medicine to cure ringworm, wound, scabies, skin diseases and inflammation (Okwu and Nnamdi, 2008). In addition, the potential health-related functions of dietary plants were found to include antibiosis, immunostimulation, nervous system action, detoxification, anti-inflammatory, antigout, antioxidant, glycemic and hypolipidemic properties (Johns, 2001).

The oils of the plant resin were investigated for antimicrobial and antioxidant activities. The essential oil showed more potent antibacterial effect against bacteria such as Staphylococcus aureus, Bacillus cereus, Escherichia coli, Salmonella enteric amd Proteus mirabilis than antifungal effect against Candida albicans and this effect was found to be due to the presence of high content of terpinen-4-ol (19.8%) and a-pinene (17.4) (Obame et al., 2008). In another study, the antibacterial effect of the essential oil of the plant resin was confirmed to be due to the presence and high content of terpenes, but antifungal effect of the oil was reported to be lacking (Koudou et al., 2008). Since compounds such as alkanoids and saponins are known to be antimicrobial (Ajibesin

et al., 2006), their presence has been suggested to account for the antimicrobial activity of the plant (Okwu and Nnamdi, 2008).

C-REACTIVE PROTEIN STUDIES

C - reactive protein (CRP) is an annular (ring-shaped), pentameric protein found in the blood plasma, whose levels rise in response to inflammation. It is an acute-phase protein of hepatic origin that increases following interleukin-6 secretion by macrophages and T cells. Its physiological role is to bind to lysophospatidychlorine expressed on the surface of dead or dying cells (and some types of bacteria) in order to activate the complement system via C1q (Thompson et al., 1999).

CRP is synthesized by the liver (Pepys and Hirschifield, 2003) in response to factors released by macrophages and fat cells (adipocytes) (Lau et al., 2005). It is a member of the pentraxin family of proteins. It is not related to C-peptide (insulin) or protein C (blood coagulation) C-reactive protein was the first pattern recognition receptor (PRR) to be identified (Mantovani et al., 2008).

HISTORY AND NUMENCLATURE OF C-REACTIVE PROTEIN

Discovered by Tillett and Francis in 1930 (Tillett and Francis, 1930), it was initially thought that CRP might be a pathogenic secretion since it was elevated in a variety of illnesses, including cancer (Pepys and Hirschfield, 2003). The later discovery of hepatic synthesis (made in the liver) demonstrated that it is a native protein (Pincus et al., 2007; Ratey et al., 2008; Kennelly et al., 209). Initially, CRO was measured using Quellung reaction which gave a positive or a negative reaction. More precise methods nowadays is using dynamic light scattering on antibodies specific to CRP (Bray et al., 2016).

CRP was so named because it was first identified as a substance in the serum of patients with acute inflammation that reacted with the antibody against the somatic capsular polysaccharide (C-polysaccharide) of pneumococcus (Ananthanarayan and Paniker, 1978; Levine, 2011).

FUNCTIONS OF C-REACTIVE PROTEIN

CRP binds to the phosphocholine expressed on the surface of dead or dying cells and some bacteria. This activates the complement system, promoting phagocytosis by macrophages, which clears necrotic and apoptotic cells and bacteria (Bray et al., 2016).

This so-called acute phase response occurs as a result of a rise in the concentration of IL-6, which is produced by macrophages (Pepys and Hirshfield, 2003) as well as adipocytes (Lau et al., 2005) in response to a wide range of acute and chronic inflammatory conditions such as bacterial, viral, or fungal infections; rheumatic and other inflammatory diseases; malignancy; and tissue injury and necrosis. These conditions cause release of interluken-6 and other cytokines that triggers the synthesis of CRP and fibrinogen by the liver.

CRP binds to phosphocholine on microorganism. It is thought to assist in complement binding to foreign and damaged cells and enhances phagocytosis by macrophages (opsonin-mediated phagocytosis), which express a receptor for CRP. It plays a role in innate immunity as an early defense system against infections (Bray et al., 2016).

SERUM LEVELS OF C-REACTIVE PROTEIN

In healthy adults, the normal concentrations of CRP varies between 0.8mg/L to 3.0mg/L. However, some healthy adults show elevated CRP at 10mg/L. The rate of CRP production increases with inflammation, infection, trauma, necrosis, malignancy, and allergic reaction. The CRP level also increases with age, possible due to increasing subclinical condition. There is also no seasonal variations of CRP levels. Gene polymorphism of interleukin-1 family, interleukin-6, and polymorphic GT repeat of the CRP gene do affects the usual CRP levels when a person does not have any medical illnesses (Pepys and Hirschfield, 2003). Other inflammatory mediators that can cause a rise in CRP are TGF beta 1, and tumor necrosis factor alpha. In acute inflammation, CRP can raise as much as 50 to 100mg/dL within 4 to 6 hours in mild to moderate inflammation or insult

such as skin infection, cystitis, or bronchitis. It can double every 8 hours and reaches its peak at 36 to 50 hours following injury or inflammation. CRP concentrations between 2 to 10mg/dL are considered as metabolic inflammation (metabolic pathways that causes arteriosclerosis and type II diabetes mellitus). Once inflammation subsides, CRP level falls quickly because of its relatively short half-life (Bray et al., 2016).

MATERIALS AND METHODS

SAMPLE COLLECTION AND PREPARATION

African pear fruits were purchased from Watt market in Calabar, Cross River State. The pulps of the fruits were removed by cutting with a knife, the seeds were cleaned by washing with distilled water and dried at ambient temperature for 7 days. The dried seeds were then milled with a corona traditional mill (1.00mm particle size) prior to formulation of the feed.

Milled dried seeds were then mixed with normal rat chow at graded doses of 10 per cent, 20 per cent and 30 per cent, respectively. Thereafter, the various formulations were stored in a cool dry place for use as feeds for the experimental animals.

METHODS

ANIMAL EXPERIMENTATION

24 (twenty-four) albino rats of Wistar strain weighing between 150-250 grams, obtained from the animal house of the Department of physiology, university of Calabar, Calabar, were used. The animals were acclimatized for one week and their weights noted before, during and on the last day of experimental treatments. Groups of 6 animals each were housed in cages with the normal day lighting pattern of about 12 hours light (0630-1830 hours) and 12 dark. Animals had free access to standard livestock feed for the normal control as well as the formulated feeds for the experimental groups and distilled water ad libitum throughout the experimental period of 28 days.

EXPERIMENTAL PROCEDURES

The twenty four albino Wistra rats weighing 150-250g were randomly grouped into four (4) experimental groups of six (6) rats each. The rats had free access to standard livestock feed for the normal control as well the formulated feeds for the experimental groups and distilled water ad libitum throughout the experimental period of 28 days as follows:

GROUPS	ADMINISTRATION
I.	Control, fed standard livestock feeds (n=6)
II.	10% African pear seed flour in normal feeds (n=6)
III.	20% African pear seed flour in normal feeds (n=6)
IV.	30% African pear seed flour in normal feeds (n=6)

The experimental feeding lasted for a period of twenty eight (28) days. On the twenty ninth day (29th) day, the animals were sacrificed and the tissue of interest were collected and stored accordingly for analysis.

COLLECTION AND PREPARATION OF TISSUES FOR ANALYSES

At the end of the experimental treatments the animals were sacrificed and the blood collected was centrifuged at 3000rpm for 5 minutes and the serum decanted for analysis.

PRINCIPLES AND PROCEDURES FOR BIOCHEMICAL ESTIMATION

ESTIMATION OF PLASMA C-REACTIVE PROTEIN LEVELS

Principles:

The cohesion Bioscience Human C-reactive protein ELISA (Enzyme-Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of Human C-reactive protein in Cell Culture Supernatants, Serum, Plasma. This assay employs an antibody specific for Human C-Reactive protein coated on a 96-well plate. Standards and samples are pipetted into the wells and C-Reactive protein present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-Human C-Reactive protein antibody is added. After washing away unbound biotinylated antibody, HRP conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and colour

develops in proportion to the amount of C-Reactive protein bound. The stop solution changes the colour from blue to yellow, and the intensity of the colour is measured at 450 nm.

COMPONENT	VOLUME
96-well Plate Coated With Anti-Human C-Reactive Protein Antibody	12x8Strips
Human C-Reactive Protein Standard	10 ng x 2
Biotin-Labelled Detection Antibody (100X)	120 ul
Streptavidin-HRP (100X)	120 ul
Standard/Sample Diluent	30ul
Detection Antibody Diluent	12 ml
Streptavidin-HRP Diluent	12 ml
Wash Buffer (20X)	30 ml
TMB Substrate Solution	12 ml
Stop Solution	12 ml
Plate Adhesive Strips	3 Strips
Technical Manual	1 Manual
Storage and Stability	

All kits components are stable at 2 to 8 ^{0}C. Standard (recombinant protein) should be stored at -20 ^{0}C or -80 ^{0}C (recommended at -80 ^{0}C) after reconstitution. Opened microplate wells or reagents may be stored for up to 1 month at 2 to 8 ^{0}C. Return unused Wells to the pouch containing dessicant pack, reseal along entire edge.

Note: The kit can be stored at -20 ^{o}C. Avoid repeated freeze-thaw cycles.

Materials Required:

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Adjustable pipette and pipette tips to deliver 2 ul to 1 ml volumes.
3. Adjustable 1-25 ml pipettes for reagent preparation.
4. 100 ml and 1 lier graduated cylinders.
5. Absorbent paper
6. Distilled or deionized water
7. Computer and software for ELISA data analysis
8. Tubes to prepare standard or sample dilutions.

Reagent Preparations

1. Sample preparation: Store samples to be assayed within 24 hours at 2-8^{0}C. For long-term storage, aliquot and freeze samples at -20^{0}C. Avoid repeated freeze-thraw cycles.

   Cell culture supernates: Remove particulates by centrifugation, assay immediately or aliquot and store samples at -20^{0}C.

   Serum: Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature. Centrifuge at approximately 1000 x g for 15 minutes. Analyze the serum immediately or aliquot and store samples at 20^{0}C.

   Plasma: Collect Plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1500 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at -20^{0}C.

   Cell Lysates: Collect cells and rinse cells with PB. Homogenize and lyse cells thoroughly in lysate solution. Centrifuge cell lysates at approximately 10000 X g for 5 minutes to remove debris. Aliquots of the cell lysates were removed and assayed.

   Bone Tissue: Extract demineralized bone samples in 4m Guanidine-HCl and protease inhibitors. Dissolve the final sample in 2m Guanidine-HCl.

   Tissue Homogenates: Rinse tissue with PBS to remove excess blood, chopped into 1-2mm pieces and homogenize with a tissue homogenizer in PBS or in lysate solution, lysate solution: tissues net weight=10ml : 1g (i.e Add 10ml lysate solution to 1g tissue). Centrifuge at approximately 5000 X g for 5 minutes. Assay immediately or aliquot and store homogenates at -20^{0}C. Avoid repeated freeze-thaw cycles.

   Urine: Urinary samples should be cleared by centrifugation and then can be used directly without dilution. Storage at -20^{0}C.

2. Human C-Reactive Standard protein preparation: Reconstitute the lyophilized Human C-Reactive protein standard by adding 1ml of Standard/Sample diluent to make the 10,000pg/ml standard stock solution. Allow solution to sit at room temperature for 5 minutes, then gently vortex to mix completely. Use within one hour of reconstituting. Two tubes of the standard (10 mg per tube) are included in each kit. Use one tube for each experiment. Perform 2-fold serial dilutions of the top standards to make the standard curve within the range of this assay (156 pg/ml – 10000 pg/ml) as below.

Standard/Sample Dilution Buffer serves as the zero standard (0pg/ml)

Standard	Add	Into
10,000pg/ml	500ul of the Standard (10,000pg/ml)	500ul of the Standard/Sample Diluent
5,000pg/ml	500ul of the Standard (5,000pg/ml)	500ul of the Standard/Sample Diluent
2,500pg/ml	500ul of the Standard (2,500pg/ml)	500ul of the Standard/Sample Diluent
1,250pg/ml	500ul of the Standard (1,250pg/ml)	500ul of the Standard/Sample Diluent
625pg/mg	500ul of the Standard (625pg/ml)	500ul of the Standard/Sample Diluent
313pg/ml	500ul of the Standard (313pg/ml)	500ul of the Standard/Sample Diluent
156pg/ml	500ul of the Standard (156pg/ml)	500ul of the Standard/Sample Diluent
0ng/ml	1 ml of the Standard/Sample Diluent	

Note: The standard solutions are best used within 2hours. The 10,000 pg/ml standard solution should be stored at 4^0C for up to 12 hours, or at -20^0C for up to 48 hours. Avoid repeated freeze-thraw cycles.

2. Biotin-Labelled Detection Antibody Working Solution Preparation: The Biotin Labelled Detection Antibody should be diluted in 1:100 with the Detection Antibody Diluent and mixed thoroughly. The solution should be prepared no more than 2 hours prior to the experiment.
3. Streptavidin-HRP should be diluted in 1:100 with the Streptavidin-HRP Diluent and mixed thoroughly. The solution should be prepared no more than 1 hour prior to the experiment.
4. Wash Buffer Working Solution Preparation: Pour entire contents (30ml) of the Wash Buffer Concentrate into a clean 1,000 ml graduated cylinder. Bring final volume to 600ml with glass-distilled or deionized water (1:20).

Assay Procedure

The Streptavidin-HRP working solution and TMB substrate solution must be kept warm at 37^0C for 30 minutes before use. When diluting samples and reagent, they must be mixed completely and evenly. Standard detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of protein amount in samples.

1 Add 100 ul of each standard and sample into appropriate wells.

2 Cover well and incubate for 90 minutes at room temperature or overnight at 4^0C with gentle shaking.

3 Remove the cover, discard the solution and wash plate 3 times with Wash Buffer Working Solution, and each time let Wash Buffer Working solution stay in the wells for 1-2 minutes. Blot the plate onto paper towels or other absorbent material. Do not let the wells completely dry at any time.

4 Add 100ul of Biotin-Labbeld Detection Antibody Working Solution into each well and incubate the plate at 37^0C for 60 minutes.

5 Wash plate 3 times with Wash Buffer Working Solution, and each time let Wash Buffer Working Solution stay in the wells for 1-2 minutes.

6 Discard the Wash Buffer Working Solution and blot the plate onto paper towels or other absorbent materials.

7 Wash plate 5 times with Wash Buffer Working Solution and each time, let the wash buffer stay in the wells 1-2 minutes. Discard the wash buffer and blot the plate onto paper towels or other absorbent materials.

8 Add 100ul of TMB Substrate Solution into each well and incubate plate at 37^0C in dark for 30 minutes.

9 Add 100ul of stop solution into each well. The colour changes into yellow immediately.

10 Read the O.D absorbance at 450nm in a microplate reader within 30 minutes after adding the Stop Solution.

For Calculation,
(the relative O.D.450) = (the O.D.450 of each well)- (the O.D450 of Zero well).

The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the

respective concentration of the standard solution (X). the concentration of the samples can be interpolated from the standard curve.

Note: If the samples measured were diluted, multiply the dilution factor to the concentration from interpolation to obtain the concentration before dilution.

STATISTICAL ANALYSIS

The data obtained were analyzed statistically using analysis of variance (ANOVA) and student's Ttest. Data were expressed as ±mean standard deviation. Values of $p<0.05$ were regarded as being significant.

RESULTS AND DISCUSSION

Table 3: Shows levels of C-Reactive protein in the serum experimental animals fed with graded doses of African Pear seed flour in normal rat chow.

Experimental Groups	CRP (mg/L)
I	1.52±1.22
II	1.33±0.10
III	1.13±0.11
IV	1.15±0.01

values are expressed as mean ±SEM, n = 6

Keys:
CRP – Serum C-reactive protein Levels

I Control group fed normal rat chow
II Experimental group fed 10% African pear seed flour in normal rat chow
III Experimental group fed 20% African pear seed flour in normal rat chow
IV Experimental group fed 30% African pear seed flour in normal rat chow

DISCUSSION

C-Reactive protein (CRP) is a major acute phase reactant synthesized primarily in the liver hepatocytes. CRP shows the strongest association with cardiovascular events. It is detectable on the surface of about 4% of normal peripheral blood lymphocytes. Acute phase reactant CRP is produced in the liver. Displays several functions associated with host defense: it promotes agglutination, bacterial capsule swelling, phagocytosis and complement fixation through it calcium-dependent binding to phosphorylcholine. Can interact with DNA and histones and may scavenge nuclear material released from damaged circulating cells. Results show that experimental animals in groups II (1.33±0.09mg/L) exhibited slightly lower levels of C-reactive protein in their serum than the animals in group I-control (1.52±0.05mg/L) significant at $p<0.05$. However, groups III and IV experimental animals (1.18±0.11 and 1.14±0.01 mg/L respectively) recorded much lower levels than the control group (significant at $p<0.05$). This is a strong indication that African pear seed flour (within the administered doses) did not result into inflammatory risk factors, thus no potential secretion of phagocytic agents were triggered.

CONCLUSION

This research has led to the conclusion that the fortified diets of graded doses of African pear seeds flour were safe and of nutritional benefit to the experimental animals. Thus ground African pear seed flour could be used to fortify diets to alleviate micronutrients malnutrition, as it appears to have immunoprotective capabilities.

RECOMMENDATIONS

Further research should be conducted to understudy the mechanism of action of the African pear seeds flour on the entire immunological survey.

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