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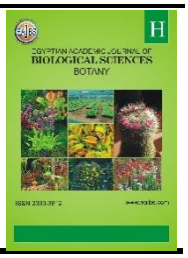

Assessment of Anti-inflammatory Activity of *Prunus dulcis* [Miller D.A. Webb (ALMOND)] SEED Aqueous Extract and Fractions

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ABSTRACT

Almonds (*Prunus dulcis*) seeds, from the Rosaceae family, have been reported to contain essential nutrients that reduced inflammation by lowering certain inflammatory markers. This study evaluated the anti-inflammatory activity of *P. dulcis* (Almond seed) extract and fractions (n-hexane and aqueous). Dried almond seed (700 g) was pulverized and soaked in distilled water for 24 hrs. The aqueous extract was filtered and the filtrate was lyophilized to obtain the seed aqueous crude extract. The crude extract portion was dissolved in water, partitioned with n-hexane and separately concentrated to dryness to obtain the aqueous and n-hexane fractions. Anti-inflammatory activity of almond seed extract and fractions were carried out via erythrocyte membrane stabilization, antitryptic and protein denaturation inhibitory assays. Results obtained shows that n-hexane fraction of *P. dulcis* seed stabilized stressed erythrocyte maximally (18.70 ± 3.23%) at 2.00 mg/mL and aqueous fraction (16.72 ± 1.97%) at 0.5 mg/mL, while aqueous extract highest % stability was 17.73± 2.32% at 2.00 mg/mL. Also, n-hexane fraction inhibited trypsin better at higher concentrations while the aqueous fraction was at a lower concentration. However, both showed better anti-tryptic activity than aqueous crude extract.Moreover, maximum percentage protein inhibitory activities for diclofenac (800 µg/ml), aqueous fraction (800 µg/ml), n-hexane fraction (1600 µg/ml) and aqueous extract (1600 µg/ml) were 72, 51, 35 and 32 respectively.This study concludes that almond seed fractions showed better anti-inflammatory activity compared to their crude extract. Therefore, the isolation of active compounds from these fractions may provide a novel anti-inflammatory drug.

INTRODUCTION

Almond is the seed of the fruit of the almond tree (*Prunus dulcis* (Mill.) (D.A. Webb) from the Rosaceae family. It is native to south-central Asia, but today is produced worldwide in hot-arid Mediterranean climate regions (Kathleen *et al*., 2022).

Beneficial nutrients of almond seed include fiber, protein, unsaturated fats, vitamins, minerals, phytosterols and pharmacological significance phytochemicals such as flavonoids, (Medzhitov, 2008; Jakumar*et al*., 2012; serhat *et al*., 2014). Almonds have been frequently demonstrated in clinical studies to lower blood glucose and cholesterol and attenuate biomarkers of inflammation and oxidative stress, all of which are risk factors for cardiovascular disease (CVD). These benefits are mainly ascribed to their nutrient composition, which is known to be low in saturated fatty acids (SFA), and rich in unsaturated fatty acids (91–94% of fats are oleic acid and linoleic acid), α-tocopherol, fiber, phytosterols, and proteins (Brzin and Kidric, 1995; Hana Jung., 2017)

Inflammation is an immune response used by the body to fight and neutralize injurious agents. Essentially, the cells capture the agent, localize and destroy the agent while guarding the body to heal. Such a response can either be acute or chronic. It is acute if the response is timely, prompt and resolved. However, if there is a breakdown in the intracellular checkpoints and the agent evades arrest, the immune cells (which are essentially the macrophages) secrete more pro-inflammatory mediators and cytokines to replace the neutrophils. The presence of macrophages which dominate the neutrophils and secrete essentially the pro-inflammatory cytokines is what characterizes chronic inflammatory conditions (Iwalewa*et al*., 2007).

Previously, the effect of different plant parts on protein denaturation have been evaluated by many scientists, for example, *Semecarpusanacardium* bark on bovine albumin (Kumar *et al*., 2013), and the ethanolic extract of *Wedeliatrilobata*on bovine albumin (Govindappa *et al*., 2011).

The study investigated the anti-inflammatory activities of *Prunus dulcis* (Almond seed) extract and fractions.

MATERIALS AND METHODS

Materials:

Chemicals: All reagents were of analytical grade.

Semi-dried *Prunus dulcis* seeds were purchased from Glory-to-Glory Store, Ile Ife, Osun State.

Diclofenac Sodium was obtained from OAU Campus pharmacy.

Bovine blood was obtained from the "Odo eran" abattoir along Ede Road, Ile Ife.

Methods:

Semi-dried *P. dulcis* seeds were rinsed with distilled water and air dried on the large filter paper under shade. The seeds (700g) were blended to a fine powder with an electric blender. The powdered sample was macerated with distilled water for 24 hr and filtered using Whatman no.1 filter paper. The filtrate was lyophilized to obtain the crude extract which was divided into two portions; one was kept in the refrigerator at 4 ^{o}C for further analysis while the other half was dissolved in water and partitioned with n-hexane at a 1:2 ratio of water to n-hexane. Each solvent fraction was separately concentrated to dryness to obtain the aqueous and n-hexane fractions.

I.Evaluation of Erythrocyte Membrane Stability Activity:

A-Preparation of 2% Erythrocyte: Fresh bovine blood was collected into an anticoagulant (3.8% trisodium citrate) in a 1-liter clean plain sample bottle, mixed by inversion and transported to the laboratory in an ice bucket. The blood was washed with normal saline (0.85% w/v NaCl), centrifuged at 3000 rpm for 10 min at room temperature and the supernatant was carefully removed with a Pasteur pipette. The process of washing and centrifuging was repeated until a clear supernatant was obtained. The clear supernatant was decanted and 2% (v/v) red blood cells were prepared from the packed erythrocytes with normal saline and kept in the refrigerator for further analysis (Oyedapo *et al*. 2010)

B-Membrane Stabilizing Activity Assay: The assay was carried out according to the method of Oyedapo *et al*. (2010). The assay mixtures were made up of 1 ml of hyposaline [(0.25% w/v) sodium chloride], 0.5 ml of 0.15 M phosphate buffer (pH 7.4), 0.5 ml of 2% (v/v) bovine erythrocyte suspension, varying concentrations of extract/fractions of almond seed and final reaction mixtures were made up to 3.0 ml with isosaline. Drugs were not added to the blood control reaction mixture while the test control did not contain the erythrocyte suspension. The reaction mixture was incubated at 56 °C for 30 min in a water bath, followed by centrifugation at 3000 rpm for 10 min at room temperature. The absorbance of the released haemoglobin in the supernatant was read at 560 nm. The percentage membrane stability was calculated using the expression:

$$100 - [\frac{\{\text{Abs of test drug} - \text{Abs of drug control}\}}{\{Absofbloodcontrol\}} x100]$$

Blood control - 100% lysis.

Protein Denaturation Inhibitory Assay:

Protein denaturation inhibitory assay was carried out according to the procedure of Mizushima and Kobayashi (1968) as reported by Akinpelu *et al*., (2018). Bovine serum albumin (BSA) and diclofenac sodium served as standard protein and drug respectively. The reaction mixture contained 0.5 ml (BSA, 0.25mg/ml) with a varying concentration of the extract/fractions of almond seed (100, 200, 400,800, and 1600 µg/ml) in a final reaction volume of 3.0 ml. The mixture was incubated at 37ºC for 20 minutes, and thereafter, was heated at 57ºC for 3 minutes. After cooling, 2.5 ml phosphate buffer (0.5 M, pH 6.3) was added to the mixture. Thereafter, 1.0 ml of the reaction mixture was pipetted into a clean test tube followed by the addition of 1.0 ml alkaline copper reagent and 1.0 ml of (10% v/v) Folin – Ciocateu's reagent. The reaction mixture was then incubated at 55ºC for 10 min and allowed to cool. The absorbance of intact protein was measured at 650 nm against the reagent blank. The quantity of un-denatured protein left and % inhibition was calculated using the equation below:

Total protein = $\frac{Abs_{sample}\ @650\ nm \times Concentration_{standard}}{Abs_{standard}\ @650\ nm}$

Quantity of protein left = $\frac{Absorbance_{test} - Absorbance_{control}}{Absorbance_{test}}$

Percentage inhibition (%) = $\frac{\text{Quantity of protein left} \times 100}{\text{Total protein}}$

The absorbance of control represents 100% denaturation.

Trypsin Inhibitory Assay:

The anti-tryptic activity of the extracts was assayed as described by Oyedapo and Famurewa, (1995) and Shivraj *et al.*, (2013) with slight modifications. The reaction mixture made up of 0.06 ml trypsin, 0.5 ml Tris HCl buffer (20 mM, pH 7.4) and 0.5 ml of the extracts (100-500 µg/ml) was incubated at 37^0C for 5 minutes. This was followed by the addition of 0.8% (v/v) BSA (0.5 ml) and incubated further 20 minutes. The reaction was terminated by the addition of 1 ml of 5% (w/v) trichloroacetic acid and centrifugated at 3500 rpm for 5 min. The absorbance of the supernatant was read at 280 nm against a buffer blank. Aspirin was used as a standard drug. Percentage anti-tryptic activity was calculated using the formulae below:

$$\%\ TrypsinINHIBITION = \frac{Abs.oftest - Abs.ofcontrol}{Absoftest} X\ 100$$

Abs. – Absorbance

BSA – Bovine serum albumin

Abs. of control represents 100% hydrolysis of BSA.

RESULTS

A-Membrane Stabilization Activities:

Figures (1 A.-1 C.) show the membrane stabilizing profiles of the crude extract and fractions of *Prunus dulcis* on bovine red blood cells exposed to heat and hypotonic-induced lyses. The N-hexane fraction of *P. Dulcis seeds* exhibited maximum erythrocyte membrane stability of 18.70 ± 3.23% at 2.00 mg/mL closely followed by the aqueous fraction of *P. Dulcis seeds* at 16.72 ± 1.97% at a concentration of 0.5 mg/mL while aqueous extract exhibited 17.73 2.32 % maximally at 2.00 mg/mL.

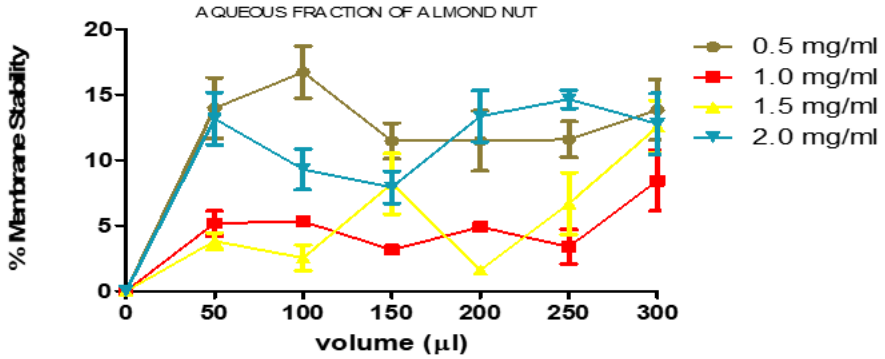

Fig. 1 A.: Membrane stabilization activity of aqueous fraction of almond seed.

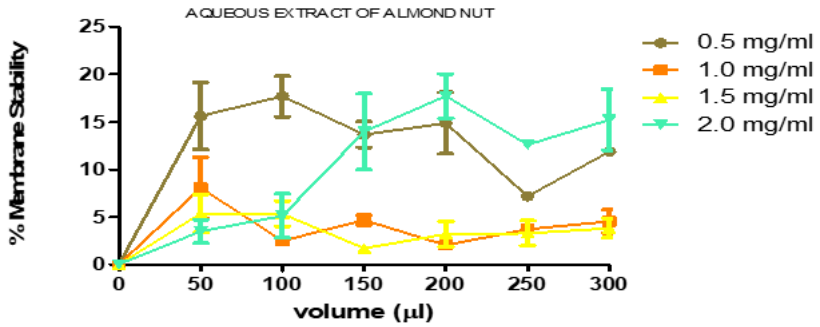

Fig. 1 B.: Membrane Stabilization Activity of Aqueous Extract of Almond Seed

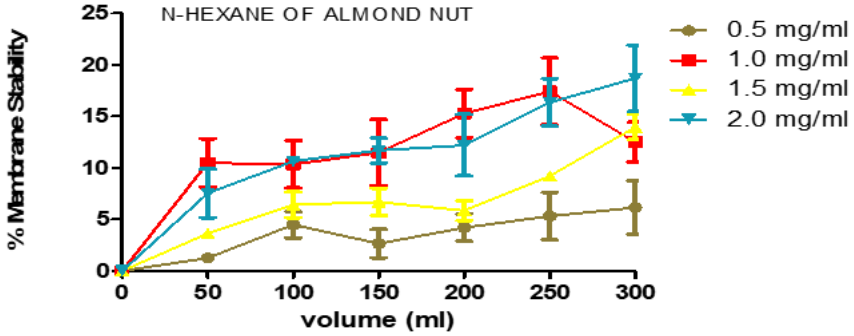

Fig. 1 C.: Membrane Stabilization Activity of Hexane Fraction of Almond Nut.

B-Inhibition of Albumin Denaturation:

The % inhibition of protein denaturation of almond seed extract and fractions was within the range from 6.55% - 56.35% at the concentration range of 100-1600 µg/ml (Fig. 2). Maximum inhibition was observed from almond aqueous fraction at 100 µg/ml lower than that of others.

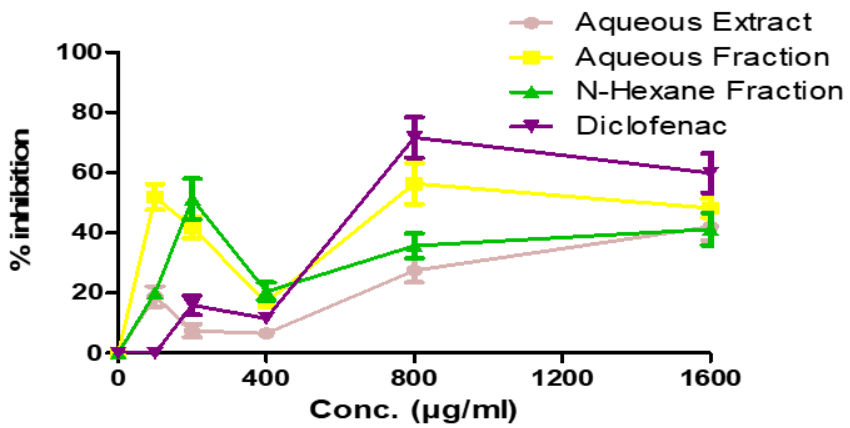

Fig. 1: Effect of Almond Seed *Extract* and *Fractions o*n BSA Denaturation Inhibitory Activity.
Almond seed aqueous fraction, n-hexane fraction, aqueous extract and diclofenac inhibited protein denaturation maximally by 56%, 51%, 42% and 71% at 100, 200, 1600 and 800 µg/ml respectively.

C-Antitryptic Activities

As presented in Figures 3 n-hexane and aqueous fractions of *P. dulcis* are competitive inhibitors of trypsin (about 60%) as much as aspirin while the aqueous fraction is a weak inhibitor of trypsin activities.

N-hexane inhibited trypsin better at higher concentrations while aqueous fraction inhibits better at lower concentrations but both showed better activity than aqueous extract. The 3 are less competitive than aspirin.

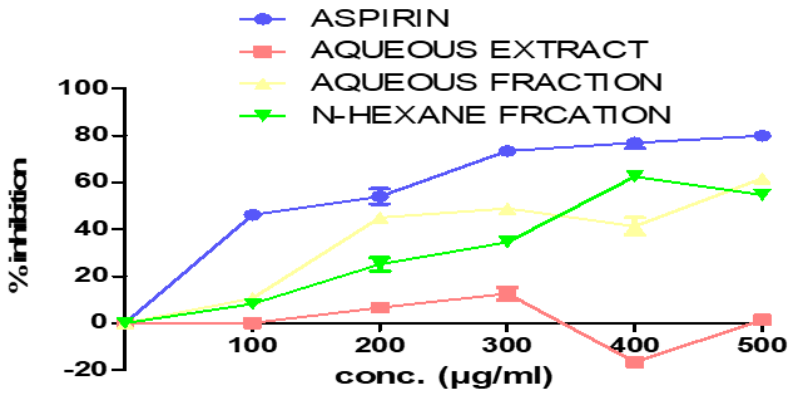

Fig. 3: Antitryptic activities of almond seed extract and fraction.

DISCUSSION

Membrane stabilization is a procedure of measuring the integrity of biological membranes against mechanical, (e.g heat, protein aggregation), chemical (e.g reactive oxygen species, amphiphilic molecules), microbial (e.g pore-forming toxins, viroporings) immune (e.g complement, perforing) and intracellular (ferroptosis, necroptosis) stressors (Oyedapo 2010; Ammendolia *et al* 2021).

Screening techniques for probable anti-inflammatory agents include erythrocyte membrane stabilization, fibrinolytic assay, inhibition protein denaturation, lysosomal membrane stabilization, platelet aggregation, etc. The erythrocyte membrane is similar structurally and chemically to the lysosomal membrane; hence, the stability of the erythrocyte membrane could thus be extended to the stabilization of the lysosomal membrane (Oyedapo *et al*., 2010). Lysosomal constituents are bactericidal enzymes and

proteinases which, upon extracellular release, cause further tissue inflammation and damage (Chou, 1997).

The result of this study revealed that *P. dulcis* seed extract and fractions stabilized stressed erythrocytes. N-hexane fraction stabilized stressed erythrocyte maximally (18.70 ± 3.23%) at 2.00 mg/mL and aqueous fraction (16.72 ± 1.97%) at 0.5 mg/mL while aqueous extract maximum % stability was 17.73 2.32% at 2.00 mg/mL. Both aqueous extract and fraction exhibit a biphasic mode of action while n- hexane fraction mode of stabilization is monophasic (except at 1.0 mg/ml)

Proteolytic enzymes(proteases) play vital roles in the normal physiological functions of cells. Diverse diseases such as cancer, pulmonary emphysema, muscular dystrophy, arthritis, pancreatitis *etc* are associated with the excessive activity of proteases. Thus, inhibitions of protease activity have been employed in the treatment of various diseases (Atta-ur-Rahman *et al*., 2005).

Protein denaturation is a process in which proteins lose their biological function when denatured as a result of the application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent, or heat. The denaturation of protein molecules is well documented in the literature, and the production of autoantigens or self-antigens in certain arthritic conditions was reported to be attributed to the denaturation of tissue proteins *in vivo* (Umapathy *et al*., 2010).

The result of the protein denaturation inhibitory profile on heat-treated bovine serum albumin (Figure 2) showed that the almond seed aqueous fraction elicits the highest percentage inhibitory capacity (56.35% at 100 µg/ml), followed by n-hexane fraction (51.24% at 200 µg/ml) then the aqueous extract (42.05% at 1600 µg/ml). The standard anti-inflammatory drug (diclofenac sodium) had 71.14% at 800 µg/ml. Comparatively, almond seed extract and fractions inhibited protein denaturation at a lower percentage than the standard drug. The order of the inhibition of the extract and fractions of almond seed was AAF > AHF > AAE. An increase in the absorbance of the test samples with respect to the control indicates stabilization of protein i.e. inhibition of heat-induced protein denaturation by the seed (Jagtap *et al*., 2011).

The phenolic compounds and phytosterols present in the nuts have also been reported to exhibit anti-inflammatory activity (Rahman *et al*., 2006). Therefore, the protein denaturation inhibitory capacity of almond seed observed in this study supports its anti-inflammatory property.

Furthermore, the study showed almond seed extract and fractions as potent inhibitors of trypsin that compete favourably with aspirin. However, the fractions showed better antitryptic activity than the aqueous extract. The n-hexane fraction inhibited trypsin better at higher concentrations while the aqueous fraction was at a lower concentration. The health implication is that the consumption of almonds should be regulated to avoid unregulated blood clotting factors that may arise during bleeding most especially in a patient with fibrinogen disorder.

Previous studies have shown the anti-protease activities of almond seeds (Nohwar *et al*., 2015). Alpha-1 antitrypsin (A1AT) is a broad-spectrum protease inhibitor that takes part more in modulating various inflammatory responses. A1AT has been reported to modulate and/ or avert tissue damage in certain experimental animal models of human diseases such as inflammatory bowel disease, rheumatoid arthritis, atherosclerosis, etc. (Janciauskiene, S., 2017). Consequently, the trypsin inhibitory activity of almond seed extract suggests the seed may serve as a source of the bioactive agent(s) for the treatment of A1AT deficiency.

Conclusion: This study concludes that almond seed fractions showed better anti-inflammatory activity compared to their crude extract. Therefore, the isolation of active compounds from these fractions may provide a novel anti-inflammatory drug.

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