Original Research Article

Amino Acid Compositions and *In Vitro* Anti-inflammatory Properties of Cookies produced from Wheat and Soybean Composite Flour

ABSTRACT

Composite flours were formulated from wheat and soybean and mixed together to formulate four blends WSY 1 (100%), WSY 2 (80%-20%), WSY 3 (70%-30%), WSY 4 (60%-40%), respectively to bake cookies. The cookies were investigated for their amino acid profiles, *in vitro* anti-inflammatory properties and consumer acceptability of the cookies using standard methods. The amino acid profiles of the cookies were well established with high biological values (>70%) in terms of their essential, non-essential and hydrophobic amino acids while glutamic acid served as the most abundant amino acid (19.15 – 28.69%) present in each of the cookies. Moreso, the *in-vitro* anti-inflammatory properties of the cookies showed more potency with low IC_{50} (~20 μg/ml) than the standard sodium diclofenac, a well-known non-steroidal anti-inflammatory drug. Overall, the cookie sample from the 60% wheat and 40% soybean composite flour was acceptable to the consumers as shown by their ratings and perceptions, which could be highly found useful as potential bioactive antioxidant, anti-diabetic and anti-inflammatory agents in the management of chronic inflammations.

***Keywords*:** Cookies; soybean; biological value; anti-inflammatory; hydrophobic amino acid

1. INTRODUCTION

Inflammation is a biological response of the immune system that can be triggered by a variety of factors, including pathogens, damaged cells and toxic compounds [1]. These factors may induce acute and/or chronic inflammatory responses in the heart, pancreas, liver, kidney, lung, brain, intestinal tract and reproductive system, potentially leading to tissue damage or disease. Important microcirculatory events that occur during the inflammatory process include vascular permeability changes, leukocyte recruitment and accumulation, and inflammatory mediator release [1]. Composite flour was generally considered to be blends of wheat flour with other flours for production [2]. Thus, recent approach has shown the composite flour to contain two or more edible flours blended together at different ratios for novel food production, such as cookie, with one positive health benefit or the other [3-4]. Thus, cookie is produced as nutritive snacks from unpalatable dough that is transformed into appetizing products through the application of heat in the oven [5]. Cookie is generally acceptable food product and could be used as an excellent and convenient food item for protein fortification to improve the nutritional well-being/health of the people, and in nutritional programs which would enhance reduction in protein malnutrition that is prevalent in Nigeria as well as other developing countries [5]. Cookie is universally accepted as a very convenient form of food that is important for population. It is a good source of nutrients, such as macronutrients (carbohydrates, protein, and fat) and micronutrients (minerals and vitamins) that are essential for human health [6]. Legumes are an important part of the traditional diets around the world but were often neglected in the typical Western diets. They are inexpensive, nutrient-dense sources of protein that could be substituted for dietary animal protein [7]. They are rich in

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protein and essential minerals but contained small quantities of fats that were mostly unsaturated. Since the legume is well adapted to tropical regimes and insufficient good quality protein is a limiting factor in developing countries, appropriate processing to improve the utilization is of great importance. Attempts have been made to improve its utilization in human diet due to increasing need for cheaper and available plant proteins, especially amongst Nigerian populace. Soybean (*Glycine max*) is relatively cheap and contained a high amount of protein (23%) that is rich source of lysine but is usually deficient in sulphur-containing amino acids especially methionine and cystine. It is relatively high in protein and could be used to fortify cookies [8]. Soybean contained complex phytochemicals, beta-carotene (a pro-vitamin A carotenoid), vitamins C, which were major and well-known antioxidants [9]. It contained protein alongside with all essential amino acids [9]. In Nigeria, wheat production is limited and wheat flour is imported to meet local flour needs for bakery products. Thus, a huge amount of foreign exchange is used every year for importing wheat. Efforts have been made to promote the use of composite flours in which flour from locally grown crops and high protein seeds replaced a portion of wheat flour for the use in cookie, thereby helping in producing protein-enriched cookie [10]. Supplementation with legumes is one way to meet the need of carbohydrate foods, particularly baked food [5]. Therefore, it is envisaged that a blend of wheat and soybean flours would result in enriched baked products with a good balance of some of the essential amino acids meant to serve as potent antioxidant and anti-diabetic agents.

2. MATERIALS AND METHODS

2.1 Materials

The commercial wheat flour, which have been commonly used for all baking processes, was obtained from a commercial baking ingredients store in Ado Ekiti, Nigeria. The soybean seeds were obtained from the King's market, Ado Ekiti, Nigeria and authenticated at the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure, Nigeria. All chemicals used were of analytical grade and obtained from Sigma-Aldrich, London, UK.

2.2 Production of soybean flour and composite cookies from flour blends

The Soybean flour was obtained according to the described methods [11]. Briefly, the raw seeds were sorted and cleaned, roasted in an oven for 20 min at 180 ^{o}C, dehulled and milled using a Binatone kitchen blender (mode BLG 4O2, Zhongshan, Haishang) and the resultant flour sieved to obtain a uniform size of 400 μm. The individual flours were now formulated into composite flour blends as WSY 1 (100%), WSY 2 (80%-20%), WSY 3 (70%-30%), WSY 4 (60%-40%), respectively. Cookies were produced as previously described [5] with the following ingredients, composite flour, margarine, baking powder, salt, beet, eggs and water. The dried ingredients were thoroughly mixed in a bowl for few minutes followed by adding the margarine and eggs and kneaded to form batter. The batter was then rolled on a rolling board sprinkled with flour for a uniform thickness and cut with a 50 mm-diameter cookie cutter. The cookies were placed in baking trays leaving a 25 mm space in between and baked at 180 ^{0}C for 10 min in the baking oven. After baking, the cookies were cooled at ambient temperature, packaged in polyethylene bags and stored prior to subsequent analysis.

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2.3 Amino acid analysis

The amino acid profiles of the cookies were determined using the High-performance liquid chromatography (HPLC) method as previously described [10]. Briefly, food sample was placed in hydrolysis ampoule, then dried under vacuum using a Savant SpeedVac. Approximately 100 µL of 6 N HCL was placed in the lower part of the ampoule, freezed in a dry ice/ethanol bath, attached to a vacuum system via ¼" ID x 5/8" OD Tygon tubing, then slowly thawed and evacuated to <150 mtorr. Oxygen/methane flame was used to seal the neck of the tube at the constriction. After hydrolysis and acid removal, samples that contained 0.5-10 µg of protein were reconstituted with 60-200 µgof Na-S sample buffer and the amino acid composition then finally analyzed. The cysteine and methionine contents were determined after performic acid oxidation and the tryptophan content was determined after alkaline hydrolysis.

2.4 Determination of *in vitro* anti-inflammatory properties

Determination of anti-proteinase

The test was performed according to the modified method [10]. The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1ml test sample of different concentrations (100 – 500 µg/ml). The mixture was incubated at 37 ^{0}C for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank.

Inhibition of protein denaturation

The inhibition of protein denaturation was evaluated by the method [10] with slight modification. 500 µL of 1% bovine serum albumin was added to 100 µL of plant extract. This mixture was kept at room temperature for 10 min, followed by heating at 51 °C for 20 min. The resulting solution was cooled down to room temperature and absorbance was recorded at d660 nm. Acetyl salicylic acid was taken as a positive control.

Inhibition of membrane separation

The membrane stabilization inhibition was determined by the method [10]. Briefly, a volume of 100 µL of 10% RBC was added to 100 µL of the extract. The resulting solution was heated at 56 °C for 30 min followed by centrifugation at 2500 rpm for 10 min at room temperature. Supernatant was collected, and absorbance was read at 560 nm. Acetyl salicylic acid was used as a positive control.

Nitric oxide (NO) scavenging activity

The NO scavenging activity of sample was determined by adding 400 µL of 100 mM sodium nitroprusside, 100 µL of PBS (pH - 7.4) and 100 µL of different concentration of plant extract [10]. This reaction mixture was kept for incubation at 25 °C for 150 min. To 0.5 mL of above solution, 0.5 mL of Griess reagent was added (0.1 mL of sulfanilic acid and 200 µL

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naphthylethylenediamine dichloride (0.1%) w/v). This was kept on incubation at room temperature for 30 min, and finally absorbance was observed at 540 nm.

Anti-lipoxygenase and anti-cyclooxygenase activity

The anti-lipoxygenase activity was studied using linoleic acid as substrate and lipoxygenase as enzyme [10]. Test samples were dissolved in 0.25 ml of 2 M borate buffer pH 9.0 and added 0.25 ml of lipoxidase enzyme solution (20,000 U/ml) and incubated for 5 min at 25 ^{0}C. After which, 1.0 ml of linoleic acid solution (0.6 mM) was added, mixed well and absorbance was measured at 234 nm. Indomethacin was used as reference standard. The percentage inhibition was calculated from the following equation; % inhibition= [{Abs control- Abs sample}/Abs control] x 100. The anti-cyclooxygenase activity was measured using the assay mixture containing Tris- HCl buffer, glutathione, hemoglobin& enzyme. The assay started by the addition of arachidonic acid and terminated after 20 min incubation at 37 ºC by addition of 0.2 ml of 10% TCA in 1N HCl, mixed and 0.2 ml of TBA was added and contents heated in a boiling water bath for 20 min, cooled and centrifuged at 1000 rpm for 3 min. The supernatant was measured at 632 nm for COX activity.

2.5 Evaluation of sensory attributes

The cookies were coded and presented to twenty (20) semi-trained panelists to be evaluated for their appearance, texture, taste, aroma, mouth feel, crumbling, overall acceptability using the Hedonic scale of 1 to 9, where 1 = dislike extremely and 9 = like extremely as previously described by Malomo and Udeh (2018).

2.6 Statistical analysis

All determinations were carried out in triplicates. Data was subjected to analysis of variance (ANOVA) using SPSS (version 21, USA), while means was separated using New Duncan Multiple Range Test (NDMRT) at 5% level of significance ($p<0.05$).

3. RESULTS AND DISCUSSION

3.1 Amino acid profile of cookie samples

Table 1 showed the amino acid profile of the cookie samples. The samples' ranges for total essential amino acids (TEAA) and non-essential amino acids (TNEAA) were 30.06 to 37.65 and 47.47 to 56.28%, respectively. Comparing the WSY 3 sample to the other samples, it was found that the total essential amino acids were considerably ($p>0.05$) greater in that sample. This could be explained by the ratio's 30% soybean flour content. The current findings' total essential amino acids, however, were significantly ($p<0.05$) greater than the FA recommendations for adults and children, which are 26 and 39% of essential amino acids, respectively [5]. Thus, it's possible that the essential amino acids in the cookies will be sufficient to promote healthy growth and development in infant, children, and adults. The body is unable to produce the important amino acids, including phenylalanine, tryptophan, and tyrosine, which make up the hydrophobic amino acids (HAA), hence diets are the only way to obtain them. As a result, compared to other samples (35.42-38.15%), the WSY 3 showed greater amounts of HAA (39.85%). The majority of these

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HAA are body-found serum aromatic amino acids, which have been connected to elevated insulin secretion in type-2 diabetic patients [12].

Table 1 – Amino acid profiles of cookies samples **(%)**

Amino acids/ Samples	WSY 1	WSY 2	WSY 3	WSY 4	Average	±Std	#LSD (p<0.05)
Leucine	5.76	7.05	7.11	7.59	6.88	0.68	0.37
Lysine	2.88	2.96	3.12	2.98	2.99	0.09	1.00
Isoleucine	2.95	3.03	3.21	3.88	3.27	0.37	0.33
Phenylalanine	4.54	4.71	4.48	4.57	4.58	0.08	1.00
Valine	3.82	3.04	3.91	3.97	3.69	0.38	1.00
Methionine	1.90	1.96	1.99	1.71	1.89	0.11	0.15
Tryptophan	1.02	1.29	1.36	1.34	1.25	0.14	1.00
Threonine	3.28	3.65	3.01	3.30	3.31	0.23	1.00
Tyrosine	2.21	2.37	3.35	2.72	2.66	0.44	1.00
Cystine	1.67	1.90	2.21	2.37	2.04	0.27	1.00
Histidine	2.07	2.18	2.30	2.30	2.21	0.10	0.09
Alanine	3.22	3.62	3.29	5.21	3.84	0.81	1.00
Proline	8.33	8.69	7.94	7.79	8.19	0.35	1.00
Glutamic	19.15	20.19	23.87	28.69	21.98	3.74	1.00
Serine	4.33	4.94	3.66	3.41	4.09	0.60	1.00
Aspartic acid	5.17	5.24	5.45	5.30	5.29	0.10	1.00
Glycine	3.26	4.03	3.34	4.61	3.81	0.55	1.00
Arginine	4.01	4.20	4.85	5.19	4.56	0.48	1.00
TEAA	30.03	33.69	35.38	37.65	34.19	2.78	1.00
TNEAA	47.47	51.36	53.07	56.28	52.05	3.18	1.00
HAA	35.42	37.66	38.15	39.85	37.77	2.58	1.00
BV (%)	71.37	81.11	86.27	89.04	81.95	6.75	1.00

TEAA- Total Essential amino acids, **TNEAA**- Total Non-essential amino acid, **HAA**– Hydrophobic amino acids, **BV**- Biological value, **#LSD** = Least significant difference. **Key: WSY 1** = 100% wheat flour; **WSY 2** = 80% wheat flour + 20% soybean flour; **WSB 3** = 70% wheat flour + 30% soybean flour; **WSY 4** = 60% wheat flour + 40% soybean flour

Therefore, high value of aromatic amino acids obtained in the present study, as a result of high HAA and concise biological values (~90%) is found beneficial in prevention and management of type-2 diabetes. Previous studies have also reported that the plasma concentrations of branched chain amino acids (BCAA), the good exampls of HAA, were prognostic for the onset and progress

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of Type 2 diabetes [13-14]. Hence, the BCAA (a good proportion of HAA) recorded in present study is potentially advantageous to the management of type-2-diabetes mellitus.

3.2 Anti-inflammatory properties of cookies

The inhibition of protein denaturation of composite cookies at 50% inhibition (IC_{50}) is presented in Fig 1. It was observed that the composite cookie WSY 3 has low IC_{50} (22 μg/ml) when compared to the control sample (70 μg/ml), which is the 100% wheat cookies (WSY 1). The main cause of inflammation is denaturation of protein. Inflammation, which is generally referred to as a complex biological response of vascular tissues to harmful stimuli, has been associated with pain, and it involved in an increase of protein denaturation, an increase of vascular permeability, and membrane alteration, proteinase, among others [15]. Therefore, this result showed that sample WSY 3 is able to inhibit denaturation of protein more, which caused inflammation, when compared to the control. Fig 2 showed the inhibition of trypsin activities of composite cookies at 50% inhibition. It was observed that the composite cookies especially WSY 3 has low IC_{50} (23 μg/ml) when compared to the samples WSY 1, 2 and 4 (45, 30 and 32 μg/ml), respectively. Inflammation, such as anthritis (degeneration or breakdown of body joints) has also been associated with increase in trypsin activities [1, 15]. This result showed that sample WSY1 is able to inhibit trypsin activities, which mostly caused inflammation of body joints leading to arthritis. Fig 3 showed membrane separation activities of the cookie with the potency of composite cookie WSY 3 having lower IC_{50} (25 μg/ml) than the samples WSY 1, 2 and 4 (44.01, 30 and 28 μg/ml), respectively. Inflammation has been strongly associated with increase in membrane alteration. Hence, sample WSY 3 is less altered compared to the control group and this gave the sample more membrane stability, which helped against inflammation than the control group. Nitric oxide (NO) has been one of the indicators used when there existed inflammation in the body, for instance, an inflammation in the body could be a product of NO formation [10]. The result presented in Fig 4 revealed that WSY 3 has significant ($p<0.05$) low IC_{50} (15 μg/ml) when compared to sodium diclofenac (21 μg/ml), a well-known and common non-steroidal anti-inflammatory drug (NSAID) and the control sample WAYB 1 (39 μg/ml), respectively. The current study showcased the cookie sample WSY 3 as a potent functional agent available to organically (without any negative health side effect) inhibit or scavenge the NO production (that has been associated with metabolic impact of serious inflammation in the body system) more than the common NSAID (having lots of side effects, such as nausea, vomiting, itches, etc.) and samples WSY 1, 2 and 3 (39, 23.40 and 32.03 μg/ml), respectively. Cyclo-oxygenase and Lipoxygenase were the dual enzymes that catalyzed the primary oxidation of unsaturated fatty acids or unsaturated fats by oxygen, leading to inflammation in the body [1, 15, 16]. It was observed from the results presented in Figs 5 and 6, that the activities of the dual cyclo-oxygenase and lipoxygenase were being able to be checkmated through the obtained significant ($p<0.05$) low IC_{50} (12.85 and 17.09 μg/ml, respectively) when compared to sodium diclofenac (13.04 and 18.60 μg/ml) and the samples WSY 1, 2 and 4 (45.54-46.01, 20.01-20.06 and 22-23 μg/ml), respectively. Hence, the cookie WSY 3 could be potential agent in the inhibition of activities of the two enzymes implicated in the inflammatory reactions in the body [16].

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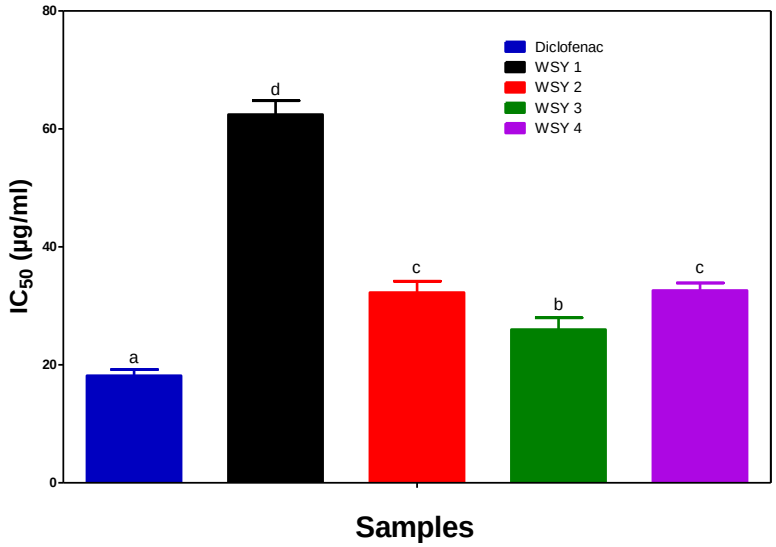

Fig 1: Inhibition of protein denaturation activity of different cookies at 50% level of inhibition concentration (IC_{50})
Bars (n=3) with different letter are significantly different ($p<0.05$).
Key: WSY 1 = 100% wheat flour; WSY 2 = 80% wheat flour + 20% soybean flour; WSY 3 = 70% wheat flour + 30% soybean flour; WSY 4 = 60% wheat flour + 40% soybean

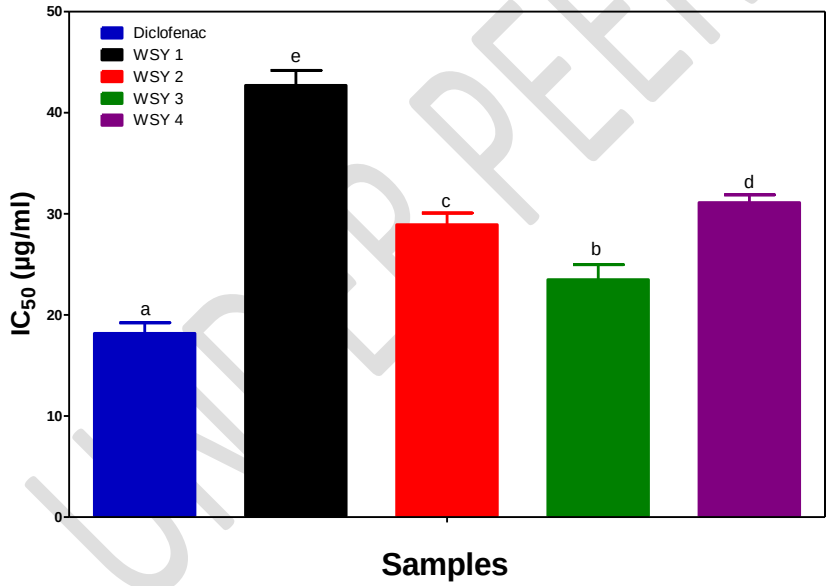

Fig 2: Inhibition of proteinase (trypsin) activity of different cookies at 50% level of inhibition concentration (IC_{50})
Bars (n=3) with different letter are significantly different ($p<0.05$).
Key: WSY 1 = 100% wheat flour; WSY 2 = 80% wheat flour + 20% soybean flour; WSY 3 = 70% wheat flour + 30% soybean flour; WSY 4 = 60% wheat flour + 40% soybean

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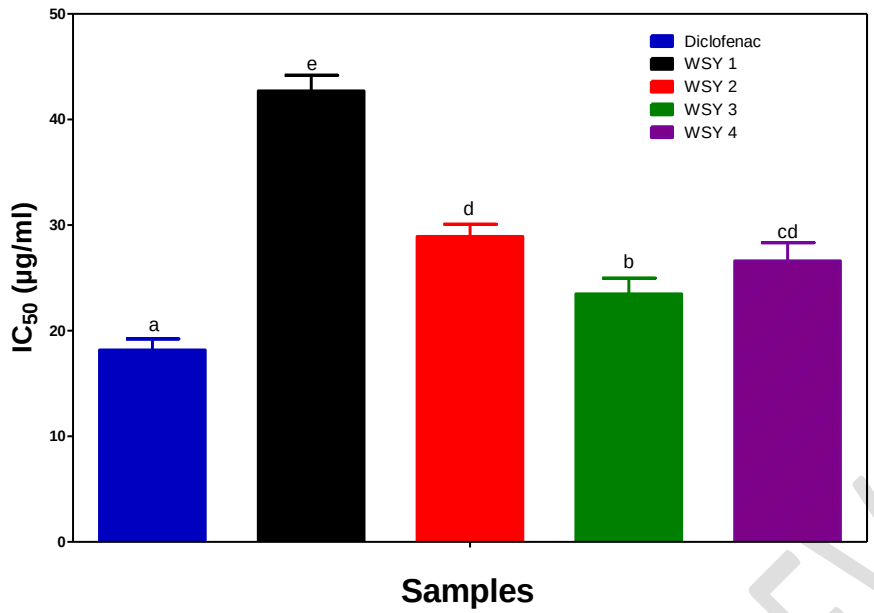

Fig 3: Membrane stabilization activity of different cookies at 50% level of inhibition concentration (IC_{50})
Bars (n=3) with different letter are significantly different ($p<0.05$).
Key: WSY 1 = 100% wheat flour; WSY 2 = 80% wheat flour + 20% soybean flour; WSY 3 = 70% wheat flour + 30% soybean flour; WSY 4 = 60% wheat flour + 40% soybean

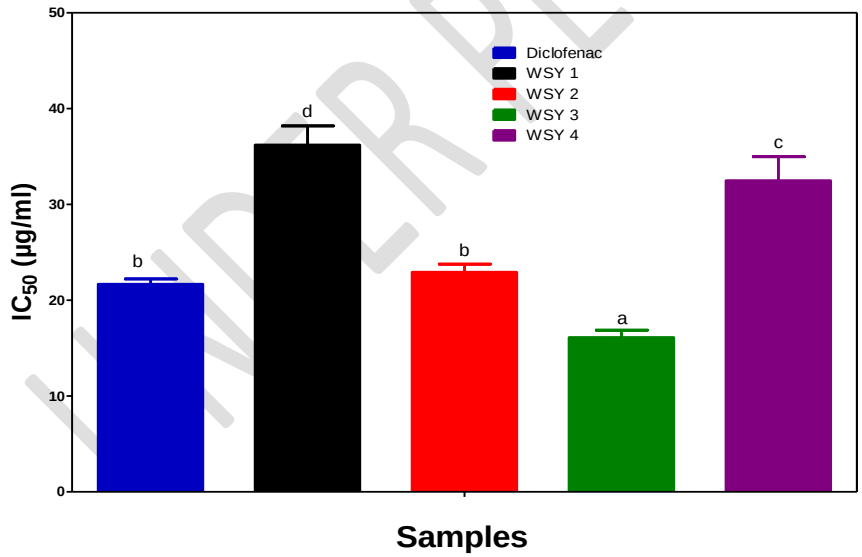

Fig 4: Nitric oxide (NO) scavenging activity of different cookies at 50% level of inhibition concentration (IC_{50})
Bars (n=3) with different letter are significantly different ($p<0.05$).
Key: WSY 1 = 100% wheat flour; WSY 2 = 80% wheat flour + 20% soybean flour; WSY 3 = 70% wheat flour + 30% soybean flour; WSY 4 = 60% wheat flour + 40% soybean

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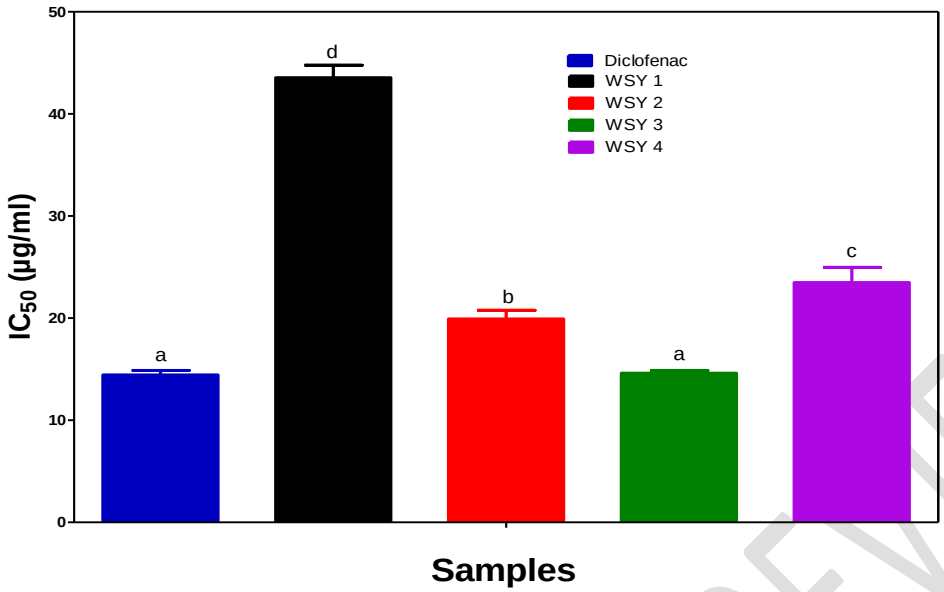

Fig 5: Anti-cyclooxygenase activity of different cookies at 50% level of inhibition concentration (IC_{50})
Bars (n=3) with different letter are significantly different ($p<0.05$).
Key: WSY 1 = 100% wheat flour; WSY 2 = 80% wheat flour + 20% soybean flour; WSY 3 = 70% wheat flour + 30% soybean flour; WSY 4 = 60% wheat flour + 40% soybean

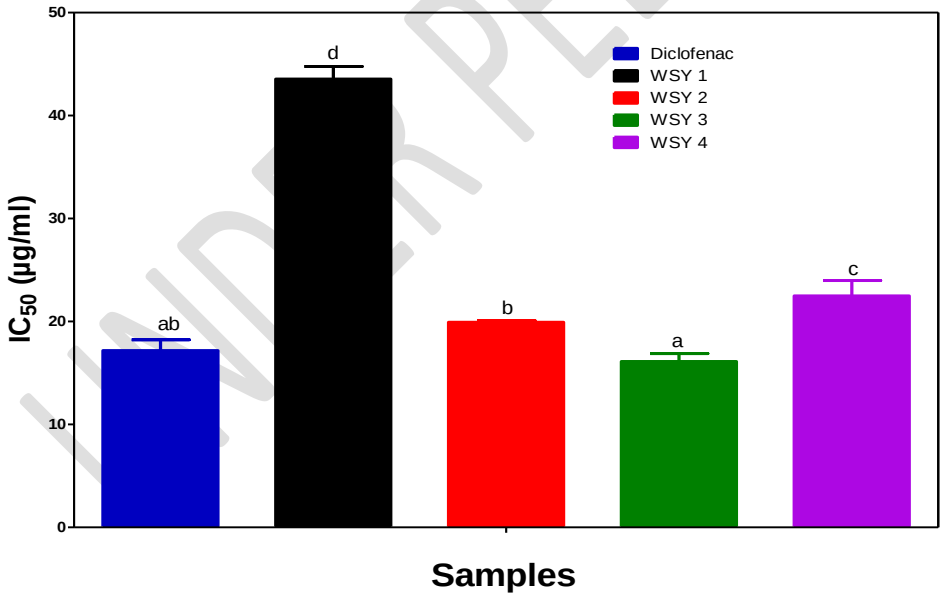

Fig 6: Anti-lipoxygenase activity of different cookies at 50% level of inhibition concentration (IC_{50})
Bars (n=3) with different letter are significantly different ($p<0.05$).
Key: WSY 1 = 100% wheat flour; WSY 2 = 80% wheat flour + 20% soybean flour; WSY 3 = 70% wheat flour + 30% soybean flour; WSY 4 = 60% wheat flour + 40% soybean

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3.3. Sensory attributes of cookies

Sensory analysis is carried out using untrained panelists to evaluate sensory characteristics, like appearance, aroma, taste, texture and overall acceptability of the food product. Mean score for sensory evaluation of cookie given in Table 2 revealed that there are significant differences ($p \leq 0.05$) between the samples in terms of their attributes like taste, mouth feel crumbling, appearance, texture and overall acceptability. Sensory rating of cookie for appearance showed that sample WSY 2 was ranked high (7.60) when compared to the commercial cookie (8.00). This result agreed with the past report that baked goods using soybean at minimal level as ingredient, provided one of the most attractive possibilities because it increased dough yield and contributed to attractive crumb and crust (Bunde *et al.,* 2010). Moreso, noticeable change in colour from light brown to darker shades of brown could be associated to non-enzymatic browning reactions (Maillard reactions) between reducing sugar molecules and lysine. Soybeans is reported to be rich in lysine, which produces darker shades of brown colours (Bunde *et al.*, 2010). There was no significant difference in the taste of the composite cookies, mainly might be as a result of presence of soybean because it has a characteristic bland flavour that is neither bitter nor sweet. In contrast, the mouthfeel characteristics decreased with increasing levels of soybean flour (from 0 to 40%), which could also be due to the difference in particle size of the composite flours as observed in the higher substitution. The remarked feeling grits in cookie sample WSY 2 could be associated to its small particle size. The overall acceptability of the cookie revealed that the sample WSY 4 (60% wheat: 30% soybean) had higher acceptability than the others and corresponds closely to the commonly known commercial samples.

Table 2: Sensory attributes of the cookies

SAMPLES	APPEARANCE	TASTE	TEXTURE	MOUTHFEEL	CRUMBLINGS	AROMA	OVERRALL ACCEPT.
WSY 1	7.10^{bc}	7.16^{b}	6.50^{c}	7.91^{a}	7.10^{bc}	7.40^{c}	7.80^{c}
WSY 2	7.60^{ab}	7.23^{b}	7.81^{b}	7.80^{a}	7.64^{b}	8.70^{a}	7.72^{c}
WSY 3	7.35^{b}	7.34^{b}	7.64^{b}	7.10^{ab}	7.38^{b}	7.15^{cd}	7.76^{c}
WSY 4	7.05^{bc}	7.12^{b}	8.05^{a}	6.65^{c}	6.85^{d}	8.15^{ab}	8.14^{a}
Commercial product	8.00^{a}	8.00^{a}	8.00^{a}	7.00^{ab}	8.00^{a}	8.88^{a}	8.87^{a}

Means (n=50) with different letter in the column are significantly different ($p<0.05$).

Key: WSY 1 = 100% wheat flour; WSY 2 = 80% wheat flour + 20% soybean flour; WSY 3 = 70% wheat flour + 30% soybean flour; WSY 4 = 60% wheat flour + 40% soybean

4. CONCLUSION

This study concluded that cookie sample produced from mixtures of 60% wheat flour: 40% soybean flour gave the best products due to its high hydrophobic amino acid, improved amino

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profiles and enhanced inhibition of cyclooxygenase and lipoxygenase enzymes. These properties and bioactivities of the cookie showed that it would be helpful and potentially useful in reducing inflammation-related diseases in numerous age-associated individuals. Moreover, consumption of functional cookie samples made from these composite flour blends could be said to be nutritionally more superior to those from whole-wheat flour in terms of improving the nutritional status of the consumers as well as serving as vehicular means for protein fortification and other nutritional improvement in Nigeria.

Patents: Not Applicable.

Data Availability Statement: Data are available upon request by contacting the authors.

Abbreviations:
DPPH = 2, 2-diphenyl-1-picrylhydrazyl (DPPH); FRAP = Ferric reducing antioxidant power; GAE = Gallic acid equivalents; HCl = Hydrochloric acid; HPLC = High performance liquid chromatography**;** TCA = Trichloroacetic acid.

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