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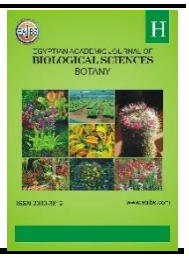

Effects of Heat and Fermentation on the Anti-inflammatory and Antioxidant Activities of *Ricinus communis* (Castor) Seeds

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ABSTRACT

This study investigated the effects of heat and fermentation on anti-inflammatory and antioxidant properties of *R. communis* seeds; determined the total protein and sugar contents of onion roots grown in seed extracts; and profiled constituents using Fourier transform infrared (FTIR) spectroscopy. De-hulled air-dried samples were grouped into five. Group 1: control (NC) was incubated at 37ºC for 72 h. Groups 2-5 were cooked for 30, 60, 90 and 120 mins respectively and incubated at 37ºC for 72 h. Fermented seeds were homogenized and used for membrane stabilizing (MS), anti-denaturant, anti-haemolytic, total antioxidant capacity (TAC), ferric reducing antioxidant power (FRAP), nitric oxide (NO), 1,1-diphenyl-2-picryl hydrazyl (DPPH), H_2O_2 radicals scavenging, total protein and sugar contents assays. Functional groups were profiled via FTIR. Results showed that the percentage MS of NC (26.62±0.0%) was significantly ($p< 0.0001$) lower than group 4 (71.79±0.02%). The percentage of anti-denaturant activity of NC (60.85±0.01%) was significantly lower than in cooked-fermented groups. The Percentage of anti-hemolytic activity in groups 4 and 5 was significantly higher than in NC. Group 2 had the highest TAC (276.43±0.00 µg AAE/g); cooked groups showed significantly high FRAP values. Group 4 had the highest NO scavenging activity (99.79±0.01%). Group 3(IC_{50} 131.36±0.04) scavenged DPPH as standard. No significant difference was observed in H_2O_2 activity. Total sugar and protein contents of NC were significantly lower than cooked-fermented groups. FTIR identified halo, alkenes, alkanes, 1,3-disubstituted, alcohols, ether, sulfonamides, ketenimines, carboxylic acids, isothiocyanate, nitro, fluoro and aromatic compounds. The study concluded that cooking *R. communis* seeds for 90-120 mins before fermentation produced robust antioxidant, anti-inflammatory and metabolic effects.

INTRODUCTION

Humans including animal cells are constantly exposed to environmental pollution that triggers free radical imbalance leading to oxidative stress conditions (Abdelmegeed and Mukhopadhyay, 2019). Free radicals are reported to interact with important biomolecules such as membrane lipids, functional proteins, and deoxyribonucleic acids (DNA) thereby

modifying their original functions or causing them to malfunction (Abdul-Latif *et al.*, 2021). Uncontrolled oxidative damage is responsible for several chronic conditions including inflammation, cancer, atherosclerosis, neurodegenerative diseases, diabetes, autoimmune disorders, etc (Agresti *et al.*, 2020). Oxidative stress and inflammation have similar pathophysiology and are often difficult to separate. Oxidative stress is the production of excess free radicals in the cells than the capacity of the antioxidant system to neutralize. The excess free radicals cross interact with membrane lipids thereby inducing lipid peroxidation and inflammatory reactions. Inflammation is an immune response of the cells against harmful *stimuli* such as free radicals or pathogens (Chen *et al*., 2018). Therefore, agents capable of scavenging free radicals or preventing free radical generation may also be useful in controlling inflammatory reactions.

Regular consumption of plant-based foods or functional foods is believed to reduce the risks of developing oxidative stress conditions like cancer and diabetes (Adefegha *et al*., 2021). The protective effects of plant-based diets are linked to the presence of phytochemicals that act as natural antioxidants or electron donors that quench the free radical chain reaction (Ademosun *et al.*, 2021). Antioxidants protect the cells against free radical production or scavenge preformed radicals by converting them into inactive metabolites (Adefegha *et al.*, 2021). Natural antioxidants such as polyphenols, vitamin C, vitamin E, carotenoids, etc have been reported in fruits, nuts, seeds, vegetables, and legumes (Goujon *et al.*, 2021).

Studies have shown that natural antioxidants are significantly lost or reduced during food processing; although, some food products may still retain some significant antioxidant properties even after being processed (Aguirre-Calvo *et al.*, 2020). This study, therefore, evaluated the effects of heat and fermentation processing on the antioxidant, anti-inflammatory and root growth-enhancing properties of *Ricinus communis* seeds.

R. communis seed, also known as castor seed, is a member of Euphorbiaceae. The seed is called castor bean, though not a true bean. Castor bean seeds are cooked and fermented traditionally for the production of "ogiri" which is used as a soup condiment in some parts of Nigeria (Barber *et al*., 2018). A brew of castor leaves is used as an eye lubricant, analgesic against stomach ache; and lactation booster in breastfeeding mothers (Andrade *et al*., 2014). The root decoction or concoction is administered for a toothache (Polito *et al*., 2019). *R. communis* leaf powder is used as mosquito and whiteflies repellant (Polito *et al*., 2019).

MATERIALS AND METHODS

Collection of Plant Material:

Dry seeds of *Ricinus communis* were collected at Ile-Ife in Osun State, Nigeria and identified at IFE HERBARIUM, Department of Botany, Obafemi Awolowo University, Ile-Ife.

Seed Sample Preparation:

Processing of *R. communis* Seeds:

The *R. communis* seeds were removed from their cover and deshelled. This was air-dried for 2 weeks; and thereafter, weighed and grouped into five as follows: Group 1 served as control (uncooked but fermented). Group 2 was boiled at 100^{o}C for 30 minutes. Group 3 was boiled at 100^{o}C for 60 minutes. Group 4 was boiled at 100^{o}C for 90 minutes; while Group 5 was boiled at 100^{o}C for 120 minutes.

Incubation and Fermentation of *R. communis* Seeds:

Both the control group and heat-treated groups were wrapped in aluminium foil (lined with blanched banana leaves) and incubated at 37^{o}C for 7 days to ferment the seeds in a controlled environment.

Preparation of *R. communis* Seed Extract:

Fermented seed samples (3 mg) were weighed and homogenized in 3 ml of distilled water or normal saline or methanol and their filtrates were used for biochemical analyses.

***In Vitro* Antioxidant Assays:**

***2, 2 Diphenyl-1-picrylhydrazyl* (DPPH) Radical Scavenging Assay:**

The DPPH radical scavenging assay was carried out using ascorbic acid as standard (Blois *et al.,* 1985). Typically, varying volumes (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml) of extract (1 mg/ml) prepared in methanol were pipetted into a set of test tubes in triplicates and the volume was adjusted to 1 ml with methanol. Thereafter, 0.3 mM DPPH (1 ml prepared in methanol) was added to the test tubes. The reaction mixture was mixed, covered with aluminium foil and incubated in the dark for 30 min. Absorbance was read at 517 nm against methanol reagent blank. The procedure was repeated for the ascorbic acid (150 µg/ml) standard. The percentage of DPPH radical scavenging activity was calculated as:

$$\% \text{ DPPH radical scavenging activity} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \text{ X } \frac{100}{1}$$

Total Antioxidant Capacity (TAC) Assay:

TAC was carried out using ascorbic acid as standard (Prieto *et al.,* 1999). Different volumes (0.0, 0.06, 0.12, 0.18, 0.24 and 0.3 ml) of ascorbic acid (100 µg/ml) were pipetted into test tubes and adjusted with distilled water. Then 3 ml of TAC reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdenate) was added and mixed. This was covered with aluminium foil and incubated at 95^{o}C and cooled. Absorbance was measured at 695 nm against a reagent blank. The procedure was repeated for the seed extract. Thereafter, the standard curve was prepared by plotting the absorbance against the concentration of ascorbic acid. The TAC of the extract was extrapolated from the standard curve and expressed as mg AAE/g extract.

Ferric Reducing Antioxidant Power (FRAP) Assay:

The assay procedure was carried out according to the method of Oyaizu (1986) using ascorbic acid (300 µg/ml) as standard. Varying volumes (0.0, 0.05, 0.10, 0.15, 0.20 and 0.25) of the extracts were pipetted in triplicates and adjusted to 1 ml with distilled water. Then 0.2 M phosphate buffer (2.5 ml) and 1 ml of 1% [$K_3Fe(CN)_6$] (1 ml) were added and incubated at 50^{o}C for 20 min. After cooling, 2.5 ml trichloroacetic acid (10%) was added and centrifuged at 3000 rpm for 10 min. The supernatant (2.5 ml) was pipetted into clean test tubes and 2.5 ml of distilled water was added followed by 1.0 ml of ferric chloride (0.1%). Absorbance was measured at 700 nm after 10 min. the procedure was repeated for the ascorbic acid (300 µg/ ml) standard. The absorbance was plotted against concentrations of both the extract and ascorbic acid.

Hydrogen Peroxide Radical Scavenging Assay:

The assay was carried out according to the procedure of Archana and Vijayalakshmi (2016) using ascorbic acid as standard. Different volumes (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml) of ascorbic acid (1 mg/ml) were adjusted to 1 ml with distilled water. Then, 0.5 ml phosphate buffer (20 mM) and 0.4 ml hydrogen peroxide (2 mM) were added and incubated at room temperature for 5 min. Thereafter, 2 ml dichromate acid (5%) was added, and absorbance was measured at 570 nm against blank. The same procedure was followed for the sample extract. The percentage inhibition of hydrogen peroxide radical was calculated as:

$$\% \text{ Inhibition of hydrogen peroxide} = \frac{Absorbance\ of\ test - Absorbance\ of\ control}{Absorbance\ of\ test} \text{ X } \frac{100}{1}$$

Nitrite Oxide (NO) Radical Scavenging Assay:

The nitric oxide radical scavenging assay was carried out using Griess' reagent method (Green *et al*., 1982) as modified by Marcocci *et al.* (1994). The reaction mixture

consisted of 0.1 ml of the extract (0.3125, 0.625, 1.25, 2.5, 5 and 10 mg/ml) and 0.9 ml of sodium nitroprusside (2.5 mM) in phosphate buffer saline. This was incubated under light for 150 min. Then, 0.5 ml of 1% sulphanilamide in 5% phosphoric acid was added and incubated in the dark for another 10 min. Finally, 0.5 ml of 0.1% NED (N-1-napthylethylenediamine dihydrochloride) was added and the absorbance was measured at 546 nm. The NO scavenging activity was calculated as:

% Inhibition = [(Ablank-Asample)/Ablank] x 100

A blank = absorbance of control; A sample = absorbance of test compound

***In Vitro* Anti-Inflammatory Assays:**

Preparation of 2% (v/v) Red Blood Cells:

Fresh (whole) bovine blood was collected from Abattoir in Ile-Ife into anti-coagulant (3.8% trisodium citrate) and rocked gently to prevent lysis. The blood was centrifuged at 3000 rpm for 10 min. The packed red cells were washed severally with normal saline (0.85% NaCl) until a clear supernatant was obtained. The supernatant was removed and 2 ml of the packed red cells were made up to 100 ml with normal saline to give 2% (v/v) bovine erythrocyte (Oyedapo *et al*., 2010).

Membrane Stabilization Assay:

The membrane stabilization assay was carried out based on the modified procedure of Oyedapo *et al*. (2010) using the 2% (v/v) erythrocyte suspension diclofenac sodium (1 mg/ml) as standard. The assay mixture consisted of 1.0 ml of hyposaline (0.25% w/v NaCl), 0.5 ml phosphate buffer (0.15 M, pH 7.4), varying concentrations of the extract/drug (0-300 µg/ml) which were made up to 3.0 ml with normal saline (0.85% w/v NaCl) and 0.5 ml of 2% (v/v) red blood cells. The blood control was prepared without the drugs; while the drug control did not contain the erythrocyte suspension. The reaction mixture was incubated at 56 ^{o}C for 30 min and centrifuged at 3000 rpm for 10 min. The supernatant was collected and the absorbance of the released haemoglobin was read at 560 nm against the reagent blank. The experiment was performed in triplicates and percentage membrane stability was expressed as:

$$\% \text{ stability} = \left[100 - \left\{\frac{\text{Absorbance of test drug-Absorbance of drug control}}{\text{Absorbance of blood control}}\right\}\right] \times 100$$

The blood control without the drug represented 100% lysis.

Inhibition of Albumin Denaturation Assay:

The Inhibition of albumin denaturation assay was carried out according to the procedure reported by Aina and Oyedapo (2013) with slight modification. Varying volumes (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml) of extracts (1 mg/ml) were pipetted and adjusted to 1.0 ml with distilled water. Exactly 0.5 ml of bovine serum albumin (2.5 mg/ml) was added and incubated at 37 ^{o}C for 20 min. Thereafter, the mixture was heated at 57 ^{o}C for 3 min and cooled. After the addition of 0.5 M phosphate buffer (2.5 ml), the reaction mixture (1.0 ml) was pipetted into a clean test tube and alkaline copper reagent (1.0 ml) and 10% Folin's reagent (1.0 ml) were added. The solution was mixed and incubated at 55 ^{o}C for 10 min. The absorbance was read at 650 nm against blank. Diclofenac was used as a reference drug and treated like an extract. The percentage Inhibition of albumin denaturation was calculated as:

$$\% \text{ Inhibition} = \frac{Absorbance\ of\ test - Absorbance\ of\ control}{Absorbance\ of\ test} x100$$

Anti-haemolysis Assay:

The assay was carried out as described by Thephinlap *et al*. (2013) using diclofenac as standard. Typically, varying volumes (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml) of extracts (1 mg/ml) were pipetted and adjusted to 1.0 ml using isosaline**.** Then 0.5 ml erythrocyte (2%) and 0.5 ml of phosphate buffer (0.15 M, pH 7.4) were added and incubated at room temperature for 5 min. Thereafter, 2.5 mM iron (III) sulphate (0.25 ml) was added and incubated at room temperature for 30 min and centrifuged at 2500 rpm for 5 min.

Absorbance was measured at 540 nm. The same procedure was followed for the diclofenac standard (1 mg/ml). The percentage Inhibition of hemolysis was calculated as:

$$\% \text{ Inhibition of Haemolysis} = \left[\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \right] x\ 100$$

Root Growth Assay:

Allium cepa:

Fermented *R. communis* seed extracts were investigated for root growth inhibitory properties using *Allium cepa* model (Akinpelu *et al*., 2018). *Allium cepa* bulbs, purchased from Ile-Ife, were sundried for 2 weeks. The *A. cepa* bulbs were prepared and planted in distilled water to initiate rooting for 48 h in the dark. The best-rooted bulbs were planted separately in 1 mg/ml of *R. communis* seed extract for 24 hours while the control group was planted in distilled water. Thereafter, the roots were harvested and used for biochemical analyses of total sugar and protein.

Estimation of Total Soluble Protein:

The total protein concentration in the *A. cepa* meristemic roots were determined following the method of Schacterk and Pollack (1973). *A. cepa* roots (0.5 g) were homogenized separately in 5 ml normal saline (0.85%, w/v) with a mortar and pestle. Homogenates were centrifuged at 3000 rpm for 15 min. on a table centrifuge. The supernatants were used for the estimation of total soluble protein. The supernatant (0.2 ml adjusted to 1 ml with distilled water) was pipetted into clean test tubes. Then 1 ml alkaline copper reagent [(10% (w/v) Na_2CO_3, 0.1% (w/v) K-Na tartrate and 0.05% $CuSO_4.5H_2O$ in 0.1 M NaOH) was added and mixed. The mixture was left to stand for 10 min. at room temperature undisturbed after which 2 ml of Folin-Ciocalteu's Phenol reagent (1:10 dilution) were added. It was then incubated at 55°C for 15 min in a water bath, tubes were then collected and allowed to cool. The blank was treated similarly with distilled water in place of extract. The absorbance was read at 650 nm against the blank. A standard calibration curve was prepared using bovine serum albumin 0, 20, 40, 60, 80 and 100 µg/ml. Protein concentrations were extrapolated from the standard calibration curve and expressed as mg/g fresh weight of root sample (µg/g FW).

Estimation of Total Soluble Sugar:

The total soluble sugar in the *A. cepa* roots was estimated as described (Dubois *et al.*, 1956) with slight modification. *Allium cepa* roots (0.5 g) were homogenized separately in 5 ml of ethanol (80%) using a pestle and mortar. Homogenates were centrifuged at 3000 rpm for 15 min. on a table centrifuge. The supernatants were collected and used for total soluble sugar analysis. The sample supernatant (1 ml) was measured in test tubes and 4 ml of Anthrone reagent (0.2% in concentrated H_2SO_4) was added. This mixture was heated in boiling water for 10 min and allowed to cool. Optical density was measured at 620 nm using 1.0 ml distilled water and 4 ml of Anthrone reagent as blank. A standard calibration curve was prepared using D-glucose 0, 20, 40, 60, 80 and 100 µg/ml. The concentration of soluble sugar in *A. cepa* root samples was extrapolated from the glucose calibration curve and expressed as µg/g of fresh weight of plant tissue (mg/g FW).

Fourier Transform Infrared Spectroscopy (FTIR) Analysis:

The FTIR analysis of fermented *R. communis* seed extracts was carried out based on the standard method of FTIR spectrometer (Khyade *et al*., 2015; Janakiraman *et al*., 2011). Samples (1 mg/ml) were homogenized in distilled water and used for FTIR analysis at the Central Research Laboratory, Redeemer University, Ede, Nigeria. Peaks were examined in the region 4000 – 400 cm^{-1} and peak values were recorded.

Statistical Analysis:

Data were analyzed using Microsoft Excel 2013 and expressed as Mean ± SEM (n=3). Differences were determined by ANOVA and values were considered significant at $p \leq 0.05$.

RESULTS

In Vitro Antioxidant Activity:

DPPH Radical Scavenging Activity of Fermented *R. communis* Seed Extracts:

The DPPH radical scavenging activity of fermented *R. communis* seed extracts as compared with L-ascorbic was shown in Table 1. Results showed that the IC_{50} value of the Control Group (uncooked-fermented seeds) was significantly higher than other groups (cooked-fermented seeds). Group 2 (IC_{50} 131.36 ± 0.04) and Group 5 (IC_{50} 144.12 ± 0.05 µg/ml) had lowest IC_{50} values comparable with ascorbic acid standard (IC_{50} 136.68 ± 0.00 µg/ml). Since the lower the IC_{50} value, the higher the radical scavenging activity; Groups 2 and 5 therefore, have the highest DPPH radical scavenging activity.

Table 1: DPPH radical scavenging activity of fermented *R. communis* seeds

***R. communis* Seed Sample**	**IC_{50} (µg/ml)**
Group 1 (Control)	1688.36 ± 0.02
Group 2	1589.80 ± 0.01[b]
Group 3	131.36 ± 0.04 [b]
Group 4	599.59 ± 0.02 [b]
Group 5	144.12 ± 0.05 [b]
Ascorbic Acid	136.68 ± 0.00 [b]

Values were expressed as Mean ± SEM (n = 3). The letter (b) is significantly lower than the Control Group. Group 1 – Raw (uncooked) *R. communis* seeds but fermented; Group 2 – *R. communis* seeds cooked for 30 minutes before fermenting; Group 3 – *R. communis* seeds cooked for 60 minutes before fermenting; Group 4 – *R. communis* seeds cooked for 90 minutes before fermenting; Group 5 – *R. communis* seeds cooked for 120 minutes before fermenting.

Total Antioxidant Capacity (TAC):

The results of TAC were presented in Table 2. TAC values of various seed extracts showed no regular pattern. However, Group 2 (seeds cooked for 30 minutes before fermenting) had TAC values (276.43 ± 0.00 µg AAE/g extract) significantly higher than the control group (232.14 ± 0.08 µg AAE/g extract). Meanwhile, Group 3 (seeds cooked for 1 h before fermenting) showed the least TAC values (166.79 ± 0.00 µg AAE/g extract). The TAC values suggest that *R. communis* seeds harbour compounds capable of donating hydrogen ions to free radicals to terminate free radical-mediated reactions.

Table 2: Total antioxidant capacity of fermented *R. communis* seeds

***R. communis* Seed Sample**	**TAC (µg AAE/g Extract)**
Group 1 (Control)	232.14 ± 0.08
Group 2	276.43 ± 0.00 [a]
Group 3	166.79 ± 0.00 [b]
Group 4	232.14 ± 0.08
Group 5	219.64 ± 0.07

Values were expressed as Mean ± SEM (n = 3). Letter (a) is significantly higher than Control Group; while (b) is significantly lower than Control Group. Group 1 – Raw (uncooked) *R. communis* seeds but fermented; Group 2 – *R. communis* seeds cooked for 30 minutes before fermenting; Group 3 – *R. communis* seeds cooked for 60 minutes before fermenting; Group 4 – *R. communis* seeds cooked for 90 minutes before fermenting; Group 5 – *R. communis* seeds cooked for 120 minutes before fermenting.

Ferric Reducing Antioxidant Power (FRAP) Values:

The FRAP values of various *R. communis* seeds were shown in Table 3. FRAP measures the ability of a compound to reduce Fe^{3+} to Fe^{2+}. A higher value denotes higher

reducing power and vice versa. Group 5 (seeds cooked for 120 minutes before fermenting) and Group 4 (seeds cooked for 90 minutes before fermenting) had FRAP values of 924.17 ± 0.05 and $8.91E^{+02}$ ± 0.02 µmol AAE/g which were significantly higher than Control Group (uncooked-fermented seeds). This suggests that heat had a significant effect on the ferric reducing the antioxidant power of *R. communis* seeds.

Table 3: FRAP Values of fermented *R. communis* seeds

***R. communis* Seed Sample**	**% Inhibition**
Group 1 (Control)	53.70 ± 0.0
Group 2	53.89 ± 0.0
Group 3	54.08 ± 0.0
Group 4	54.65 ± 0.0
Group 5	55.04 ± 0.0
Ascorbic acid	7.19 ± 0.0 [b]

Values were expressed as Mean ± SEM (n = 3). Letter (a) is significantly higher than Control Group; Group 1 – Raw (uncooked) *R. communis* seeds but fermented; Group 2 – *R. communis* seeds cooked for 30 minutes before fermenting; Group 3 – *R. communis* seeds cooked for 60 minutes before fermenting; Group 4 – *R. communis* seeds cooked for 90 minutes before fermenting; Group 5 – *R. communis* seeds cooked for 120 minutes before fermenting.

Hydrogen Peroxide Radical Scavenging Activity:

Hydrogen peroxide radical scavenging activity of fermented *R. communis* seeds was presented in Table 4. There was no significant difference in the hydrogen peroxide radical scavenging activity of *R. communis* seeds.

Table 4: Hydrogen peroxide radical scavenging activity of fermented *R. communis* seeds

***R. communis* Seed Sample**	**µmol AAE/g**
Group 1 (Control)	$-4.35E^{+04}$ ± 0.02
Group 2	$-1.18E^{+03}$ ± 0.01
Group 3	$1.25E^{+03}$ ± 0.04 [a]
Group 4	$8.91E^{+02}$ ± 0.02 [a]
Group 5	924.17 ± 0.05 [a]

Values were expressed as Mean ± SEM (n = 3). Letter (b) is significantly lower than Control Group; Group 1 – Raw (uncooked) *R. communis* seeds but fermented; Group 2 – *R. communis* seeds cooked for 30 minutes before fermenting; Group 3 – *R. communis* seeds cooked for 60 minutes before fermenting; Group 4 – *R. communis* seeds cooked for 90 minutes before fermenting; Group 5 – *R. communis* seeds cooked for 120 minutes before fermenting.

Nitric Oxide (NO) Radical Scavenging Activity of *R. communis* Seeds:

NO radical scavenging activity of various *R. communis* seed extracts was shown in Table 5. There was a significant difference in the NO radical scavenging activity between the control group and heat-treated groups. The NO radical scavenging activity of *R. communis* seed extracts was found to be heat-dependent; the longer the heat, the higher the scavenging activity.

Table 5: Nitric oxide radical scavenging activity of fermented *R. communis* seeds.

***R. communis* Seed Sample**	**% Inhibition**
Group 1 (Control)	-94.88 ± 0.002
Group 2	67.84 ± 0.031 [a]
Group 3	92.27 ± 0.014 [a]
Group 4	99.79 ± 0.001 [a]
Group 5	90.39 ± 0.005 [a]
Ascorbic Acid	55.32 ± 0.004 [a]

Values were expressed as Mean ± SEM (n = 3). Group 1 – Raw (uncooked) *R. communis* seeds but fermented; Group 2 – *R. communis* seeds cooked for 30 minutes before fermenting; Group 3 – *R. communis* seeds cooked for 60 minutes before fermenting; Group 4 – *R. communis* seeds cooked for 90 minutes before fermenting; Group 5 – *R. communis* seeds cooked for 120 minutes before fermenting.

In Vitro Anti-Inflammatory Activity:

Membrane Stabilizing Activity of *R. communis* Seeds:

The result of membrane stabilizing activity of *R. communis* seeds was shown in Figure 1**.** Both heat and fermentation had significant effects on the membrane stabilizing activity of the seed extracts. There was a significant difference ($P < 0.05$) between the control group and the diclofenac standard. The membrane-stabilizing activities of Group 2 (72.58 ± 0.0%) and Group 4 (71.79 ± 0.02%) were significantly higher than the Control Group (26.62 ± 0.0%). The levels of significant difference were found to vary across different concentrations tested. The various seed extracts exhibited a biphasic mode of protection while diclofenac sodium showed a monophasic mode of protection.

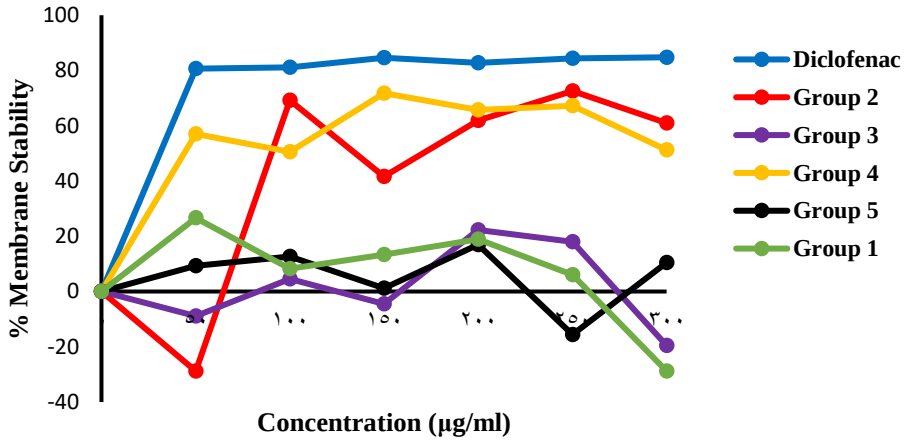

Fig. 1: Effects of heat and fermentation on the erythrocyte membrane stabilisation of *R. communis* seeds.

Values were expressed as Mean ± SEM (n = 3). Group 1 – Raw (uncooked) *R. communis* seeds but fermented; Group 2 – *R. communis* seeds cooked for 30 minutes before fermenting; Group 3 – *R. communis* seeds cooked for 60 minutes before fermenting; Group 4 – *R. communis* seeds cooked for 90 minutes before fermenting; Group 5 – *R. communis* seeds cooked for 120 minutes before fermenting.

Inhibition of Albumin Denaturation Activity of *R. communis* Seeds:

The anti-denaturant activity of *R. communis* seeds was shown in **Figure 2.** There was a significant difference ($P < 0.05$) between the control group and seed samples cooked for 30, 60 and 90 minutes, respectively. Group 5 anti-denaturant activity (78.44 ± 0.01%) was significantly higher than other groups including the Control Group (60.85 ± 0.01%). The anti-denaturant effects of group 5 and diclofenac sodium were concentration-dependent and increased even as concentration was increased.

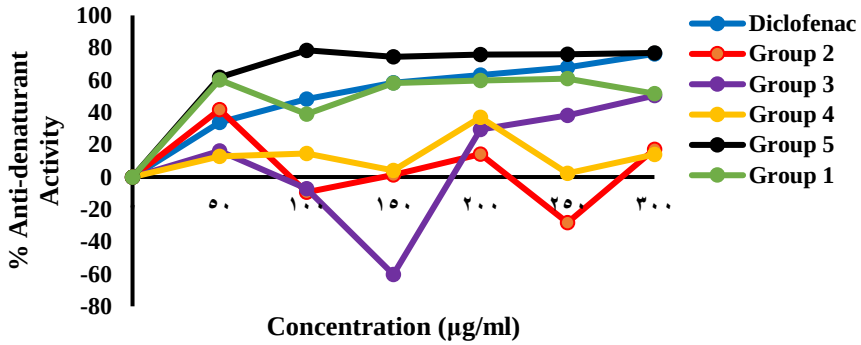

Fig. 1: Effects of heat and fermentation on the inhibition of albumin denaturation of *R. communis* seeds.
Values were expressed as Mean ± SEM (n = 3). Letter (a) is significantly higher than Control Group; Group 1 – Raw (uncooked) *R. communis* seeds but fermented; Group 2 – *R. communis* seeds cooked for 30 minutes before fermenting; Group 3 – *R. communis* seeds cooked for 60 minutes before fermenting; Group 4 – *R. communis* seeds cooked for 90 minutes before fermenting; Group 5 – *R. communis* seeds cooked for 120 minutes before fermenting.

Anti-hemolytic Activity of *R. communis* Seeds:

The anti-hemolytic activity of *R. communis* seeds was presented in Figure 3. Results showed that optimum percentage inhibition of haemolysis was obtained between 50 and 150 µg/ml extract concentration. At concentrations higher than 150 µg/ml, the *R. communis* seed extracts were found to significantly lose their anti-hemolytic activity; suggesting that high consumption of *R. communis* seeds may impair red blood cell function. The anti-hemolytic activity of the control group was significantly ($P < 0.05$) lower than Groups 2, 4, and 5 including the diclofenac reference drug.

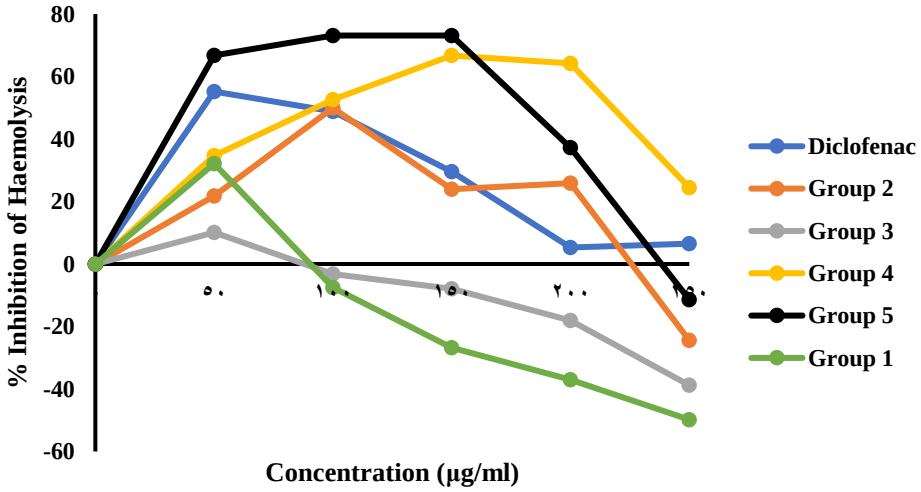

Fig. 1: Effects of heat and fermentation on the inhibition of hemolysis of *R. communis* seeds.
Values were expressed as Mean ± SEM (n = 3). Group 1 – Raw (uncooked) *R. communis* seeds but fermented; Group 2 – *R. communis* seeds cooked for 30 minutes before fermenting; Group 3 – *R. communis* seeds cooked for 60 minutes before fermenting; Group 4 – *R. communis* seeds cooked for 90 minutes before fermenting; Group 5 – *R. communis* seeds cooked for 120 minutes before fermenting.

Effects Of *R. Communis* Seed Extract on *A. Cepa* Root Growth Metabolites

Total Soluble Protein and Total Soluble Sugar

The soluble sugar and protein contents of *A. cepa* exposed to *R. communis* seed extracts were presented in Table 6. Both the sugar and protein contents of the control group were significantly lower than other groups save for the total sugar content in group 2. This

suggests that heat and fermentation concertedly enhanced the root growth metabolites (sugar and protein) to aid the actively dividing cells.

Table 6: Total soluble sugar and protein contents of *A. cepa* roots exposed to *R. communis* seed extracts

Sample Extract	Total Sugar (mg/g)	Total Protein (mg/g)
Group 1	94.34±0.02	124.23±0.01
Group 2	94.34±0.0	127.80±0.03
Group 3	107.07±0.0 [a]	144.47±0.0 [a]
Group 4	110.70±0.04 [a]	152.80±0.0 [a]
Group 5	107.07±0.0 [a]	148.04±0.0 [a]

Values were expressed as Mean ± SEM (n = 3). Group 1 – Raw (uncooked) *R. communis* seeds but fermented; Group 2 – *R. communis* seeds cooked for 30 minutes before fermenting; Group 3 – *R. communis* seeds cooked for 60 minutes before fermenting; Group 4 – *R. communis* seeds cooked for 90 minutes before fermenting; Group 5 – *R. communis* seeds cooked for 120 minutes before fermenting. Letter (a) is significantly higher than Control Group.

FTIR Identified Compound Class:

The FTIR spectra of different seed extracts of *R. communis* were shown in Plates 1-5; while the compound classes were presented in Tables 7-11. Group 1 had 15 peaks with 2 peaks (418.57 and 1560.46 cm^{-1}) unknown. Group 2 had 19 peaks; peaks 418.57 and 1560.46 cm^{-1} were unknown. Group 3 had 15 peaks. Group 4 had 17 peaks with peaks of 925.86 and 1560.46 cm^{-1} unknown. Group 5 had 14 peaks and only 1560.46 cm^{-1} was unknown. The unknown peak (1560.46 cm^{-1}) was common in all the seed extracts except in sample group 3. Functional groups identified in *R. communis* seed samples were halo compounds, alkenes, alkanes, 1,3-disubstituted compounds, alcohols, alkyl aryl ether, sulfonyl chloride, sulfonamides, ketenimines, carbon dioxide, carboxylic acids, esters, isothiocyanate, aliphatic ether, nitro, fluoro and aromatic compounds.

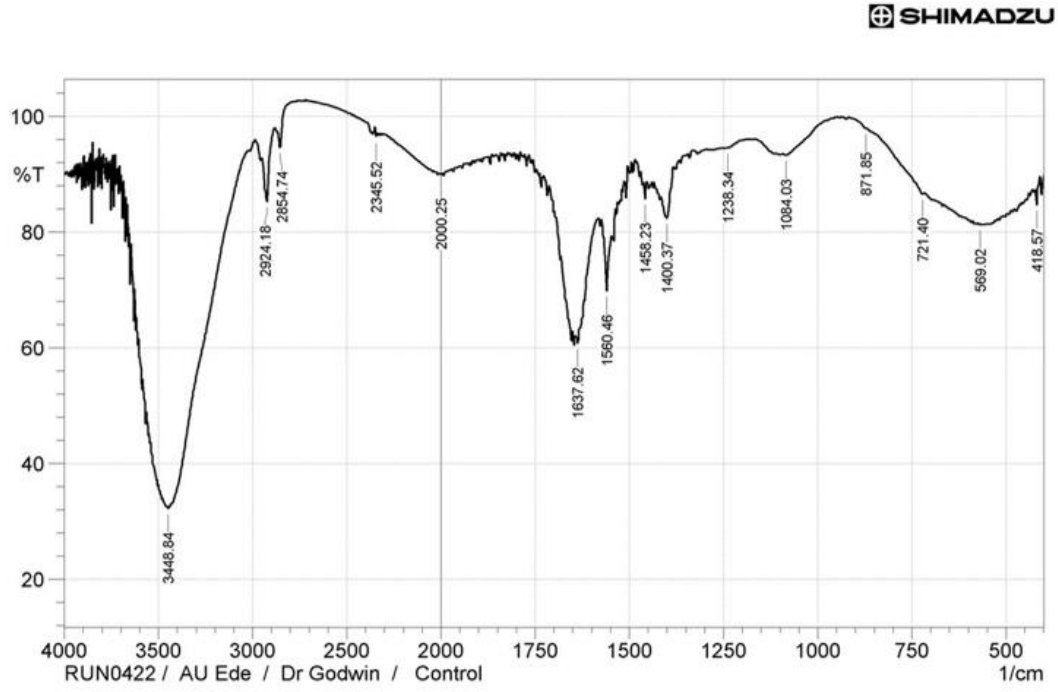

Plate 1: FTIR spectrum of uncooked fermented *R. communis* seed extract (Group 1).

Table 7: Functional Groups identified in Group 1 (Uncooked but fermented *R. communis* seeds).

Standard Peak Position	Sample Peak	Compound Class	Functional Group	Peak Detail
-	418.57	Unknown	-	
515-690	569.02 s	Halo compound	C-Br	Stretching
665-730	721.4 s	Alkene	C=C	Stretching
860-900	871.4 s	1,3-Disubstituted	C-H	Stretching
1050-1085	1084.03 s	Primary alcohol	C-O	Stretching
1200-1275	1238.34 s	Alkyl aryl ether	C-O	Stretching
1380-1410	1400.37 s	Sulfonyl chloride	S=O	Stretching
1450-1465	1458.23 s	Alkanes	C-H	Stretching
-	1560.46	Unknown	-	-
1626-1662	1637.62 m	Alkene	C=C	Stretching
2000	2000.25 s	Ketenimine	C=C=N	Stretching
2349	2345.52 s	Carbon dioxide	O=C=O	Stretching
2840-3000	2854.74 m	Alkane	C-H	Stretching
2840-3000	2924.18 m	Alkane	C-H	Stretching
3200-3550	3448.84 m	Alcohol	O-H	Bending

S = strong; m = medium

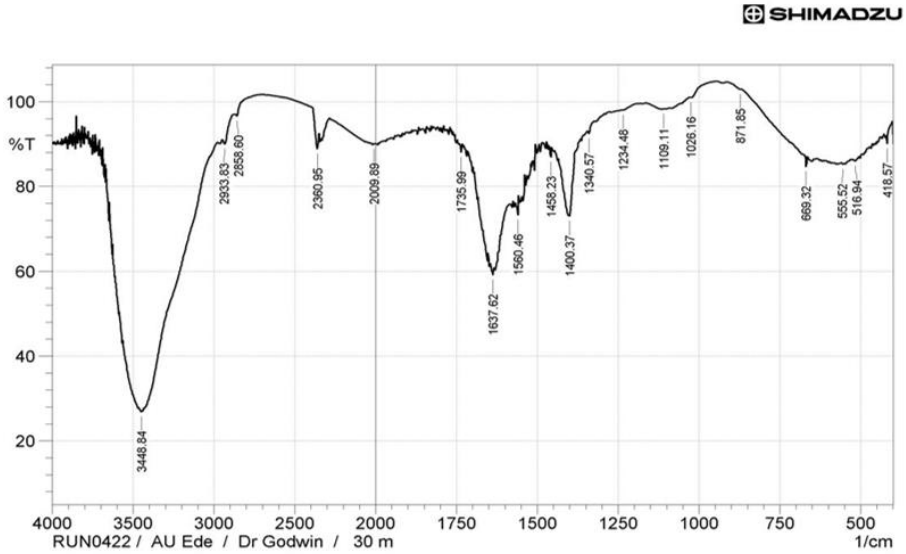

Plate 2: FTIR spectrum of *R. communis* seed cooked for 30 min before fermentation (Group 2).

Table 8: Functional groups identified in Group 2 (*R. communis* seed cooked for 30 min before fermentation.

Standard Peak Position	Sample Peak	Compound Class	Functional Group	Peak Detail
-	418.57	Unknown	-	
515-690	516.94 s	Halo compound	C-Br	Stretching
550-850	555.52 s	Halo compound	C-Cl	Stretching
665-730	669.32 s	Alkene	C=C	Stretching
860-900	871.85 s	1,3-Disubstituted	C-H	Stretching
1020-1250	1026.16 s	Amine	C-N	Stretching
1050-1085	1109.11 s	Secondary alcohol	C-O	Stretching
1200-1275	1234.48 s	Alkyl aryl ether	C-O	Stretching
1335-1370	1340.37 s	Sulfonaminde	S=O	Stretching
1000-1400	1400.37 s	Fluoro compound	C-F	Stretching
1450	1458.23 s	Alkane	C-H	Stretching
-	1560.46	Unknown	-	-
1626-1662	1637.62 m	Alkene	C=C	Stretching
1735-1750	1735.99 s	Ester	C=O	
1990-2140	2009.89 s	Isothiocyanate	N=C=O	Stretching
2349	2360.95 s	Carbon dioxide	O=C=O	Stretching
2840-3000	2858.6 m	Alkane	C-H	Stretching
2840-3000	2933.83 m	Alkane	C-H	Stretching
3200-3550	3448.84 m	Alcohol	O-H	Bend

S = strong; m = medium

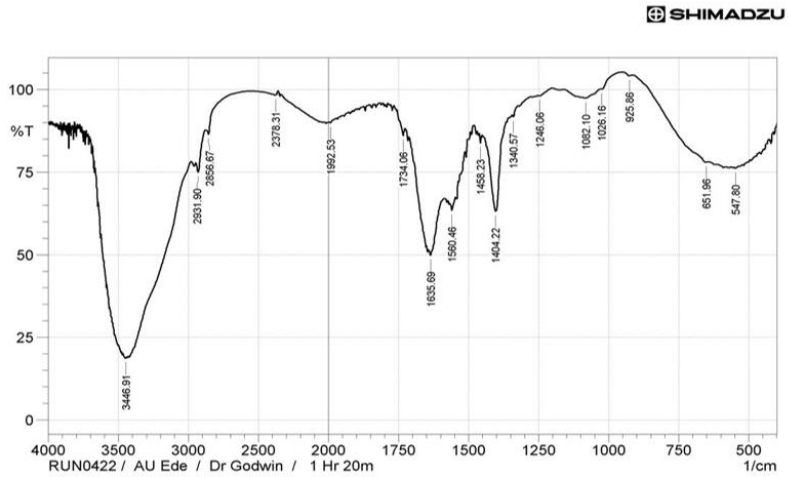

Plate 3: FTIR spectrum of *R. communis* seeds cooked for 60 minutes before fermentation (Group 3).

Table 9: Functional groups identified in Group 3 (*R. communis* seeds cooked for 60 minutes before fermenting).

Standard Peak Position	Sample Peak	Compound Class	Functional Group	Peak Detail
500-600	424.35 s	Halo compound	C-I	Stretching
515-690	516.94 s	Halo compound	C-Br	Stretching
515-690	584.45 s	Halo compound	C-Br	Stretching
665-730	669.32 s	Alkene	C=C	Stretching
1085-1150	1085.96 s	Aliphatic ether	C-O	Stretching
1087-1124	1112.96 s	Secondary alcohol	C-O	Stretching
1020-1250	1234.48 s	Amine	S=O	Stretching
1395-1440	1400.37 s	Carboxylic acid	O-H	Stretching
1500-1550	1550.82	Nitro compound	N-O	Stretching
1638-1648	1639.55 m	Alkene	C=C	Stretching
1650-2140	1871.01 s	Aromatic compound	C-H	Stretching
1990-2140	1994.46 s	Isothiocyanate	N=C=S	Stretching
2349	2360.95 m	Carbon dioxide	O=C=O	Stretching
2840-3000	2939.61 m	Alkane	C-H	Stretching
3200-3550	3448.84 m	Alcohol	O-H	Bend

S = strong; m = medium

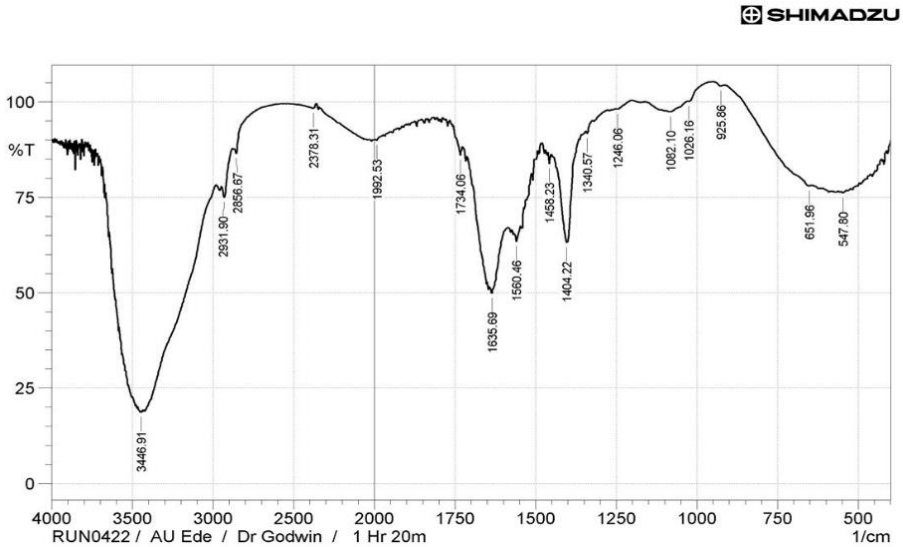

Plate 4: Spectrum of *R. communis* seeds cooked for 90 minutes before fermentation (Group 4)

Table 10: Functional groups identified in Group 4 (*R. communis* seeds cooked for 90 min before fermenting

Standard Peak Position	Sample Peak	Compound Class	Functional Group	Peak Detail
515-690	547.8 s	Halo compound	C-Br	Stretching
515-690	651.96 s	Halo compound	C-Br	Stretching
-	925.86	-	-	-
1020-1250	1026.16	Amine	C-N	Stretching
1050-1085	1082.1 s	Primary alcohol	CO	Stretching
1020-1250	1246.06 s	Amine	C-N	Stretching
1335-1370	1340.57 s	Sulfonamide	S=O	Stretching
1395-1440	1404.22	Carboxylic acid	O-H	Stretching
1450	1458.23 s	Alkane	C-H	Stretching
-	1560.46	-	-	-
1626-1662	1635.69 s	Alkene	C=C	Stretching
1720-1740	1734.06	Aldehyde	C=O	Stretching
1990-2140	1992.53	Isothiocyanate	N=C=S	Stretching
2349	2378.31	Carbon dioxide	O=C=O	Stretching
2840-3000	2856.67 s	Alkene	C-H	Stretching
2840-3000	2931.9 s	Alkene	C-H	Stretching
3200-3550	3446.91 s	Alcohol	O-H	Bending

S = strong; m = medium

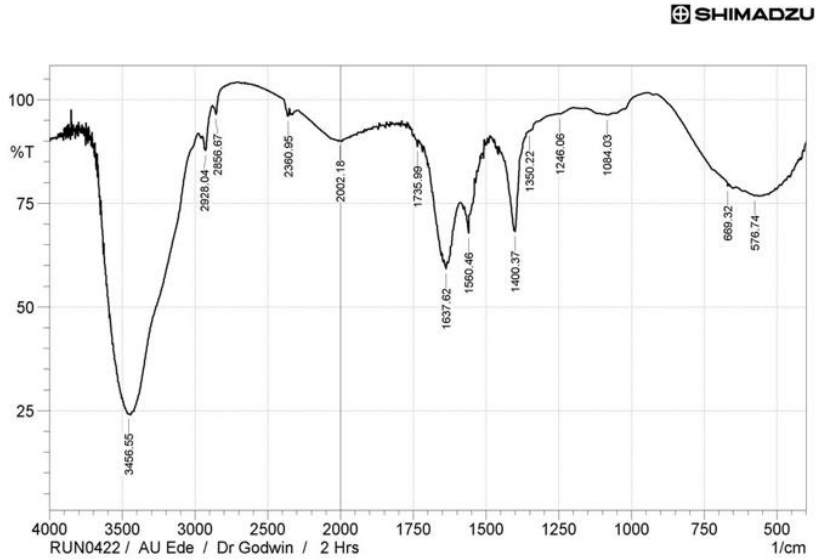

Plate 5: FTIR spectrum of functional groups identified in *R. communis* seeds cooked for 2 h prior to fermentation (Group 5).

Table 11: Functional groups identified in Group 5 (*R. communis* seeds cooked for 120 min before fermenting.

Standard Peak Position	Sample Peak	Compound Class	Functional Group	Peak Detail
500-690	576.74 s	Halo compound	C-Br	Stretching
500-690	669.32 s	Halo compound	C-Cl	Stretching
1050-1085	1084.03 s	Primary alcohol	C-O	Stretching
1020-1250	1246.06 s	Amine	C-N	Stretching
1335-1370	1350.22 s	Sulfonaminde	S=O	Stretching
1380-1410	1400.37	Sulfonyl chloride	S=O	Stretching
-	1560.46	-	-	-
1626-1669	1637.62 m	Alkene	C=C	Stretching
1735-1750	1735.99 s	Aldehyde	C=O	Stretching
1990-2140	2002.18 s	Isothiocyanate	N=C=O	Stretching
2349	2360.95 s	Carbon dioxide	O=C=O	Stretching
2840-3000	2856.67 m	Alkene	C-H	Stretching
2840-3000	2928.04 m	Alkene	C-H	Stretching
3200-3550	3456.55 m	Alcohol	O-H	Bend

S = strong; m = medium

DISCUSSION

This study investigated the effects of heat and fermentation on the antioxidant, anti-inflammatory and root growth-enhancing properties of raw or cooked fermented *R. communis* seeds; and profiled their functional groups using Fourier transform infrared (FTIR) spectroscopic technique. Human systems are constantly exposed to environmental and oxidative stress conditions caused by free radicals (Ademosun *et al*., 2021). The latter interacts with vital biomolecules thereby causing them to malfunction (Abdul-Latif *et al.*, 2021). If uncontrolled, oxidative stress can lead to inflammatory and other chronic conditions (Agresti *et al.*, 2020). Natural antioxidants obtained from plant-based foods or functional foods can scavenge free radicals thereby combating the development of oxidative stress conditions including inflammatory conditions (Adach *et al*., 2020).

This study has shown that heat and fermentation have significant effects on the antioxidant, anti-inflammatory and growth-enhancing properties of *R. communis* seeds. As shown in Table 1, the DPPH radical scavenging activity of raw fermented *R. communis* seed extracts was significantly lower than seed groups cooked at various time intervals before fermentation. Suggesting that heat was an important factor that enhanced the antioxidant property of the fermented seeds. Seed extract group 2, boiled for 30 minutes before fermentation, demonstrated the highest DPPH radical scavenging activity. This was followed by seed extract group 5 which was boiled for 120 minutes before fermentation. This suggests that short-term or long-term cooking is required to enhance the radical scavenging activity of *R. communis* seeds. It was observed that fermentation of raw *R. communis* seeds without cooking diminished antioxidant properties. However, when the heat was applied and then fermented, the antioxidant activity was significantly increased. DPPH is globally employed as a free radical to investigate substances with reducing properties (Khoudja *et al*., 2014).

Similarly, total antioxidant capacity TAC (Table 2) showed that Group 2 seed extracts cooked for a short time (30 minutes) before fermenting had TAC values significantly higher than uncooked fermented seed extract. TAC is a globally recognised technique for assessing compounds or plant extracts with the antioxidant property through the reduction of molybdenum (vi) to molybdenum (v); hence, the description as ammonium molybdenum method (Prieto *et al*., 1999). The higher TAC values of cooked-fermented *R. communis* seeds suggest that the heat-processed seeds contain higher groups of phytochemical compounds with antioxidant properties.

Furthermore, the FRAP values of *R. communis* seeds (Table 3) showed that Groups 4 and 5 seeds cooked for 90 and 120 minutes before fermenting had FRAP values significantly higher than the uncooked control group. FRAP is an *in vitro* antioxidant model used globally to measure the ability of a compound to reduce Fe^{3+} to Fe^{2+}; with high FRAP values denoting high antioxidant power (Akinpelu *et al*., 2019). This study has shown that heat had a significant effect on the ferric reducing the antioxidant power of *R. communis* seeds. Therefore, cooking the seeds is a prerequisite for enhancing the antioxidant property. Since oxidative stress conditions are mediated via complex mechanisms (Abdelbaky *et al*., 2021), it is, therefore, insufficient to determine an antioxidant property of a compound or herbal preparations using only a single test method (Khyade *et al*., 2015). More than one antioxidant assay method is required to provide supportive proof of the acclaimed antioxidant property. Therefore, the antioxidant property of raw-fermented and heat-fermented *R. communis* seeds was also investigated via the hydrogen peroxide radical scavenging method. The result showed no significant difference between raw-fermented and cooked-fermented *R. communis* seeds. In addition, nitric oxide (NO) radical scavenging activity of various *R. communis* seed extracts showed that seeds cooked before fermentation had high NO scavenging activity while the control group showed no activity. Also, the NO

radical scavenging activity was heat-dependent; the longer the heat duration, the higher the NO scavenging activity.

Because uncontrolled free radical generation can also lead to inflammatory disorders, the anti-inflammatory property of the various seed extracts of *R. communis* was investigated via *in vitro* methods. The result of membrane stabilization activity of *R. communis* seeds (Figure 1) showed that cooked-fermented seeds had higher membrane stabilizing activity than the control group. Also, the levels of significant difference were found to vary across different concentrations tested. All the seed extracts exhibited a biphasic mode of protection while diclofenac sodium had a monophasic mode of protection. Membrane stabilization is the way cells preserve the integrity of their membrane against osmotic shock or induced lysis (Anosike *et al*., 2019). Maintenance of membrane integrity or structure is important in achieving cell health. Agents that stabilize cell membranes are known to inhibit the early phase of inflammation including phospholipase A_2 inhibition (Nigam and Pace-Asciak, 2012). Also, by stabilizing the cellular membrane, anti-inflammatory agents may also prevent the release of lysosomal contents (e.g. neutrophils) which drive tissue and protein damage (Shalini *et al*., 2015; Akinpelu *et al*., 2018).

Results of anti-denaturant activity of *R. communis* seeds (Figure 2) showed that cooked-fermented seeds exhibited higher activity than the uncooked-fermented control Group; and competed favorably with the diclofenac standard in a concentration-dependent manner. Tissue protein denaturation and inflammation have the same pathophysiology (Mizushima and Kobayashi, 1968; Aina *et al*., 2013; Akinpelu *et al*., 2018). Examples of heat, strong alkalis and bases cause tissue protein denaturation; these factors have also been known to cause inflammation. Therefore, agents or drugs, or herbal preparations capable of inhibiting protein denaturation can also serve as anti-inflammatory agents. Some nonsteroidal anti-inflammatory drugs have been shown to elicit some of their anti-inflammatory mechanisms via inhibition of tissue protein denaturation (Mizushima and Kobayashi, 1968; Aina *et al*., 2013; Akinpelu *et al*., 2018).

Furthermore, transition metals (such as iron) induce rapid production of reactive oxygen species through the Fenton reaction (Thephinla *et al*., 2013); resulting in oxidative damage and loss of tissue functions. In fact, iron overload has been reported in persons with sickle cell β-thalassemia (Finberg, 2012) making them susceptible to oxidative stress and several disease conditions (Nematbakhsh *et al*., 2013). Studies have also shown that certain flavonoids (such as quercetin, myricetin, kaemferol) of plant origin are capable of chelating transition metals (Cu^{2+} and Fe^{2+}) using their functional carbonyl group (Kazazic *et al*., 2006). Such chelating activity makes the transition metals unavailable to induce hemolysis through the Fenton reaction. Therefore, having plant extracts that can bind excess iron and remove them from the system is considered valuable in antioxidant drug discovery (Thephinla *et al*., 2013). Interestingly, this present study has shown that cooked-fermented seed extracts of *R. communis* (Figure 3) elicited higher anti-hemolytic activity than uncooked fermented seeds (control group) and completed iron-binding within 5 min of incubation time. The iron-binding activity might be associated with structures of compounds present in the plant seed extracts. Ak *et al*. (2008) reported that polyphenols chelate iron using C=O and C-OH functional groups. Coincidentally, these functional groups have also been identified by FTIR analysis of the seed extracts; suggesting that the extracts also contain some polyphenols and flavonoids. Results also that the cooked seed extracts protected the bovine red blood cells from lysis at a very narrow concentration between 50 and 150 μg/ml. At concentrations higher than 150 μg/ml, the various *R. communis* seed extracts significantly lost the anti-hemolytic property. This suggests that high consumption of *R. communis* seeds may impair red blood cell function. Thus, the cooked fermented seeds of *R. communis* should be consumed in moderation to prevent adverse effects.

Also, studies have shown that cells undergoing harsh environmental conditions (salinity, water deficit stress, or pollution) employ different adaptive mechanisms to alleviate the stress (Azeez *et al*., 2021; Akinpelu *et al*., 2018). During metabolic adjustment, metabolites like sugars, proteins, proline, polyols, glycine betaines, organic acids, calcium, potassium and chloride ions accumulate in the cells (Farooq *et al.*, 2009). Some of these metabolites have been shown to possess strong antioxidant properties against oxidative damage in stressed cells (Bacanlı *et al*., 2015). An increase in proline and protein levels in stressed plants is associated with increased biosynthetic activity and decreased mitochondrial function. In contrast, decreases in proline and protein levels are associated with reduced biosynthetic activity and rapid mitochondrial oxidation. An increase in sugar contents in plant cells undergoing harsh environmental stress conditions is well documented as plants use the sugars to offset the stress condition (Anjorin *et al*., 2016). An increase in soluble sugar level is attributed to an increase in photosynthetic activities and stomatal opening. In this study, however, both soluble sugar and protein contents of *A. cepa* roots exposed to raw-fermented seed extracts (control group) of *R. communis*, were significantly lower than *A. cepa* roots exposed to cooked-fermented seed extracts. Suggesting that heat (and not fermentation) exclusively enhanced biosynthetic activity such as photosynthesis while reducing the oxidative mitochondrial function. The implication is that consumption of cooked-fermented *R. communis* seeds may help to offset or relieve oxidative stress conditions in actively metabolising cells.

With recent advances in science and technology, many research scientists can now employ sophisticated techniques such as FTIR to differentiate closely related functional groups present in sample extracts (Janakiraman *et al*., 2011; Khyade *et al*., 2015). In this study, FTIR results showed that raw (uncooked) fermented *R. communis* seeds contained 15 compound classes. Group cooked for 30 minutes (Group 2) showed the presence of 19 compound classes. Increasing the cooking hour to 60 minutes (Group 3) reduced the compound class to 15 groups. Cooking the seed longer for 90 minutes (Group 4) revealed 17 compound classes. Then when the seeds were cooked for 120 minutes (Group 5), some constituents became degraded thereby reducing the number to 14 functional groups. The functional groups identified in *R. communis* seed samples were halo compounds, alkenes, alkanes, 1,3-disubstituted compounds, alcohols, alkyl aryl ether, sulfonyl chloride, sulfonamides, ketenimines, carbon dioxide, carboxylic acids, esters, isothiocyanate, aliphatic ether, nitro, fluoro and aromatic compounds. All biological and pharmacological properties of any known compound are attributed to the functional groups they contain. Many such functional groups have been identified in this present study. Therefore, cooked-fermented seed extracts of *R. communis* have shown immense potential for the discovery of new pharmaceutical leads.

CONCLUSION

In conclusion, the study has shown that cooking *R. communis* seeds (at least 90-120 minutes) before fermentation produced potent antioxidant, anti-inflammatory and metabolic effects. Also using the raw fermented seeds was associated with a weak or toxic response suggesting the presence of toxic constituent(s) in the uncooked fermented seeds. The higher medicinal values of the cooked-fermented seeds may have been due to the possible degradation of toxic constituents as supported by FTIR spectra.

Author's Contributions: MAE, TAO and GA – conceptualization; AMD, IOO – analysis, GA -data interpretation and manuscript writing, TAO, MAE and GA – critical revisions and final approval of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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