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RESEARCH ARTICLE

THE *IN VITRO* ANTIOXYDANT ACTIVITIES OF DEFATTED ROSELLE (*HIBISCUS SABDARIFFA L.*) SEED PROTEIN HYDROLYSATES

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Abstract

The aim of this study was to produce natural antioxidants from Roselle seed proteins. In recent years, the potential application of natural antioxidants as substitutes as drawn much interest due to their safety and wide distribution. Literature indicated that Roselle whole seeds contained high amounts of protein, oil and carbohydrate However, There is scanty scientific documentation on the protein hydrolysis and the study of antioxidant activities of the hydrolysates.Roselle seed protein isolate and Roselle seed protein fractions namely globulin, albumin and glutelin were digested using pepsin followed by pancreatinat different time in order to produce hydrolysates with good antioxidant activity suitable for conversion to high-value products. . The antioxidant activities of the hydrolysates were investigated using different *in vitro* methods. The prepared samples were as effective as antioxidants in model systems, in scavenging of free radicals, and acting as reducing agents. This effect was concentration-dependent and was also influenced by the type of proteins. Among all the hydrolysates, the 3 hours samples and particularly those of protein isolate showed the highest antioxidant activity followed by albumin fraction, Glutelin and Globulin hydrolysates respectively (Protein isolate>Albumin >Glutelin> Globulin hydrolysates)..The study confirmed that fraction with strong antioxidant activities from Roselle seed protein can be successfully provided through enzymatic digestion.

Introduction:-

Free radicals are constantly generated in the body tissues as a result of oxidative metabolism. Studies suggest that free radicals play a critical role in a variety of pathological conditions, including the processes of aging, cancer, multiple sclerosis, inflammation, coronary heart and cardiovascular diseases, senile dementia, arthritis and atherosclerosis (Kehrer, 2015; Halliwell and Gutteridge, 2015). In foods, oxidation also directly affects food quality, and this is commonly associated with changes in food flavor and texture. An antioxidant is defined as any substance that significantly delays or inhibits oxidation of a substrate when present at low concentrations compared to that of an oxidizable substrate (Lewis, 1993; Halliwell and Gutteridge, 2015). In order to provide protection against serious diseases and to prevent foods from undergoing (spoilage) deterioration, many chemicals with strong

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antioxidant activity such as butylated hydroxyanisole, butylated hydroxytoluene and *n*-propyl gallate are used as food additives. Moreover, their use in foodstuffs is restricted or prohibited in some countries because of the *in vivo* potential risks of artificial antioxidants (Goli et al., 2005).

In recent years, the potential application of natural antioxidants as substitutes as drawn much interest due to their safety and wide distribution (Zhang et al., 2006; Hettiarachy et al., 1996). A vast number of studies have been conducted on the preparation of hydrolysates (Kim et al., 2001; Wang and Xiong, 2005).

In general, the results show that the solubility of proteins varies, depending on protein composition and the degree of hydrolysis. Hydrolysis is a process by which shorter chain compounds with lower molecular mass are released. The produced protein hydrolysates may possess some physicochemical characteristics and bioactivities, such as antioxidant activity which are not found in original proteins.

In addition to antioxidant activity and angiotensin I converting enzyme (ACE) inhibitory activity was also reported in bovine skin gelatin hydrolysates (Wang and Xiong, 2005). Hydrolysis has also been proposed to be of better use in rapeseed meal. Subsequent studies were carried out to evaluate the hydrolysis products. Studies by Vioque*et al*. (1999) and Vioque*et al*. (2000) reported that rapeseed protein hydrolysates can be produced under different conditions.

Roselle seed is recognized as a potential source of protein (El- Adawy And Khalil, 1994; Tounkara et al., 2011). But until now, scanty reports are available on the hydrolysis of Roselle seed protein and on the investigation of antioxidant activity of the hydrolysates. In this study, the antioxidant activity of different Roselle seed protein hydrolysates prepared using pepsin and pancreatin, was evaluated and compared in the purpose to select the more suitable protein for production of hydrolysates with higher antioxidant activities.

Material And Methods:-

Materials:-

Hibiscus Sabdariffa seeds were obtained from Koutiala, southern region of Republic of Mali. All enzymes used were of food grade. Pepsin, pancreatin and DPPH were purchased from Sigma Chemical Co. (St. Louis, USA). All the other chemicals used in the experiments were from commercial sources and were of analytical grade.

Methods:-

Sample Preparation

Roselle seeds were winnowed and destoned. The seeds were milled using a laboratory scale hammer miller and the resulting powder was sieved through a 60 mesh screen until fine powder was obtained. Thereafter the powder was defatted twice with n-hexane, following a small-scale hexane extraction method described by (Tounkara *et al*., 2014b). The experiment was repeated twice as described above. The oil-free flours was desolventized and stored at -20°Cfor subsequent uses.

Protein isolates preparation

Roselle seed protein isolates (RSPI) were obtained from defatted flour as reported by (Tounkara *et al*., 2014b) with some modifications. The defatted flour was dispersed in distilled water at flour to water ratio of 1:10 (W/V); the pH was adjusted with 1 M NaOH and stirred at room temperature. The residues were re-extracted twice as described above. The extracts were combined and the protein was precipitated before centrifugation. The protein isolate was washed twice with distilled water. It was then resuspended in distilled water and the pH was adjusted to 7.0 with 1 M NaOH prior to freeze-drying. The dried protein (protein isolates) was stored in desiccator at room temperature for subsequent analyses. The protein content was determined by the Kjeldahl method, AOAC (2000).

Protein Fractionation:

Proteins were extracted from defatted Roselle flour based on their solubility at room temperature (25^{o}C) in water, 5% NaCl and 0.1 M NaOH using the procedure described by (Osborne et al., 1909) but with minor modifications. The defatted flour was extracted using 400 mL of distilled water stirred and centrifuged at 3000 x g for 30 min to obtain the albumin fraction. The obtained residue was extracted with 400 mL of 5% NaCl to obtain a globulin fraction. Thereafter the resulting residue was then extracted with 400 mL of 0.1 M NaOH (1 h) to obtain the glutelin fraction. All the extractions were carried out twice. The albumin, globulin and glutelin fractions were then subjected

to isoelectric precipitation and washed with distilled water. All fractions were freeze dried using a Christ – Alpha 1-4 Freeze dryer (Biotech International, Germany). The quantity of proteins in various fractions was determined using a micro- Kjeldahl method, AOAC (2000).

Protein Hydrolysate Preparation:
Enzymatic hydrolysis was performed by sequential treatment with pepsin and pancreatin according to (Lo et al., 2006) under their optimal conditions. Whole Roselle seeds were ground and defatted using hexane as described in the method above. The defatted Roselle meal samples were vacuum-packed and stored at -20ºC prior to protein extraction as described above. The crude protein content of RSPI, globulin, albumin and glutelin was determined using Kjeldahl method as described in AOAC (2000). Quantifying the amount of sample required for the hydrolysis process, based on enzyme/protein ratio. Hydrolyzed samples were prepared by dividing into three groups (each containing 20 g and 400 mL of water) in a 500mL reactor with temperature and pH control devices. The first hydrolysis was with pepsin (1%) (PH 2.0) and was stopped by increasing the pH to 7.0. Then, the pancreatin (2%) was added (pH 7.0) at the same temperature 37°C. Sample 1 was hydrolyzed by pepsin for 30 min followed by pancreatin for 1 h and sample 2 hydrolyzed by pepsin for 1 h followed by pancreatin 1 h. Finally, sample 3 was hydrolyzed; using pepsin for 1 h followed by pancreatinfor another 2 h.

All Conditions were constantly monitored and maintained throughout the process. Upon completion of the hydrolysis, the enzymes were deactivated by heating under boiled water bath for 10 min, centrifuged at 7000 x g for 10 min, and supernatant was collected. The Roselle seed protein isolate hydrolysates (RSPH1.5, RSPH2, RSPH3), Roselle seed globulin hydrolysates (RSGBH1.5, RSGBH2, RSGBH3), Roselle seed albumin hydrolysates (RSALH1.5, RSALH 2, RSALH 3) and the Roselle seed glutelin hydrolysates (RSGTH1.5, RSGTH2, RSGTH3) obtained were freeze-dried and stored at -20ºC for subsequent analysis. The degree of hydrolysis was determined by measuring the nitrogen content soluble in 10% trichloroacetic acid as described by (Kim et al., 2001b).

Scavenging Effect on DPPH radical:
The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging activity of enzymatically prepared Roselle seed protein hydrolysates was determined, as described by (Shahidi et al., 2006) with minor modifications. Freeze-dried hydrolysate samples were dissolved in 95% ethanol at varying concentrations (0.5, 1, 1.5, 2, 2.5, 3, 3.5 mg/mL). A sample aliquot (0.1 ml) was mixed with 1.9 mL of ethanolic DPPH solution (50 µM) and the mixture was allowed to stand for 30 min at room temperature. An absorbance reading was taken at 517 nm using a spectrophotometer and the scavenging effect on DPPH due to protein hydrolysates was calculated using the following equation Scavenging effect (%) = 100% x [$Abs_{control} - (Abs_{sample} - Abs_{blank}) / Abs_{control}$].

Where, Abs_{sample} is the absorbance of protein hydrolysates with DPPH, $Abs_{control}$ is the absorbance of DPPH without any protein hydrolysate, while Abs_{blank}represents absorbance of protein hydrolysates without DPPH, since proteins absorb at this wavelength.

Otherwise, the EC_{50} (meaning the median effective concentration that causes a decrease in the initial radical concentration by 50%) is a parameter widely used to measure the antiradical efficiency. The lower the EC_{50} is, the higher the free radical-scavenging ability is (Tounkara et al., 2014b).

Scavenging Effecton Hydroxyl (HO•) Radical:
The hydroxyl radical scavenging effects of the Roselle protein hydrolysates were assayed using the method of (Halliwell et al., 1987). The reagents were added to a test tube in the following order: 0.4 mL KH_2PO_4–KOH buffer (pH 7.4), 0.1 mL sample solution with concentrations (10 mg/mL), and 0.1 mL of 1 mM EDTA, 10 mM H_2O_2, 60 mM 2-deoxy-Dribose, 2 mM ascorbic acid, and 1 mM $FeCl_3$ (0.1 mL distilled water was used as control instead of $FeCl_3$). The reaction solution was incubated at 37 ºC for 1 h. Then, 1 mL of 20 % TCA was added to stop the reaction. The color was developed by addition of 1 mL of 1 % TBA into the reaction tubes, which were placed in boiling water for 15 min. The tubes were cooled to room temperature and then the absorbance was read at 532 nm. For each concentration of the hydrolysates from one batch, samples were prepared in triplicate and the antioxidant activity of each was measured in duplicate. The scavenging effects were calculated according to Equation: Radical scavenging activity (%) = (1- Ac/As) x 100 .

Determination of Reducing Power:
The reducing power of the different hydrolysates was measured as described by (Duh et al., 2001) with some

modifications. Briefly, samples (RSPH, RSGBH, RSALH and RSGTH) were dissolved into 0.2 M phosphate buffer (pH 6.6) at varying concentrations of 0.5, 1, 2, 4, and 5 mg/mL. A sample aliquote of 2.5ml was then added to 2.5 mL of a 10 mg/mL potassium ferricyanide solution and incubated at 50ºC for 20 min followed by addition of deionized water (2.5 mL) and a ferric chloride solution (1.0 mg/mL, 0.5 mL) . Absorbance readings at 700 nm were immediately recorded. The trichloroacetic acid addition step was omitted since it would precipitate out the protein whose antioxidant activity was to be assessed. A control, devoid of any hydrolysates and a blank, containing only hydrolysate samples, were used because proteins also absorb at the same wavelength. Increased absorbance of the reaction mixture indicated increased reducing power.

Statistical Analysis:

All experiments were conducted at least in triplicate with SPSS software (version 16.0, the predictive Analytics Company, Chicago, U.S.A.). The data were subjected to a one way analysis of variance (ANOVA), followed by Duncan's multiple range test.

Results and Discussion:-

Scavenging Effect on DPPH radical:

The DPPH radical is a stable organic free radical with its maximum absorbance in the range at 515-520 nm. DPPH radical scavenging activity test system can be used for the primary characterization of the scavenging potential of compounds (Zhang et al, .2006; Zhu et al., .2006; Nagai el., 2002). Therefore, the antioxidant activities of different hydrolysates were evaluated using the DPPH free radical scavenging activity test system. All the hydrolysates resulting from various protein fractions were capable of scavenging DPPH radicals (Fig 1). The DPPH radical scavenging activities of all the samples were influenced by concentration. Among the different samples, the 3 h hydrolysates exhibited the highest radical scavenging activity value. Among the 3 h hydrolysates, the RSPH3 was the more potent hydrolysates followed by RSALH3, RSGTH3, while the lowest DPPH radical-scavenging activity was obtained with RSGBH3 (44.01% at 2 mg/ml). The DPPH scavenging activity of the protein hydrolysates in the decreasing order was, RSPH3> RSALH3 > RSGTH3> RSGBH3. From these findings we concluded, that the differences in the radical scavenging ability may be attributed to the difference in amino acid composition and/or molecular weight of peptides within protein hydrolysates. The obtained results confirmed that the 3 hours hydrolysis time is more suitable for production of hydrolysates with higher antioxidant activity and that RSPH3 is the indicated hydrolysate for the purpose. From the results, we concluded that the RSPH3 contained some effective antioxidative peptides possibly, which could convert DPPH free radical to more stable products and terminate the radical chain reaction.

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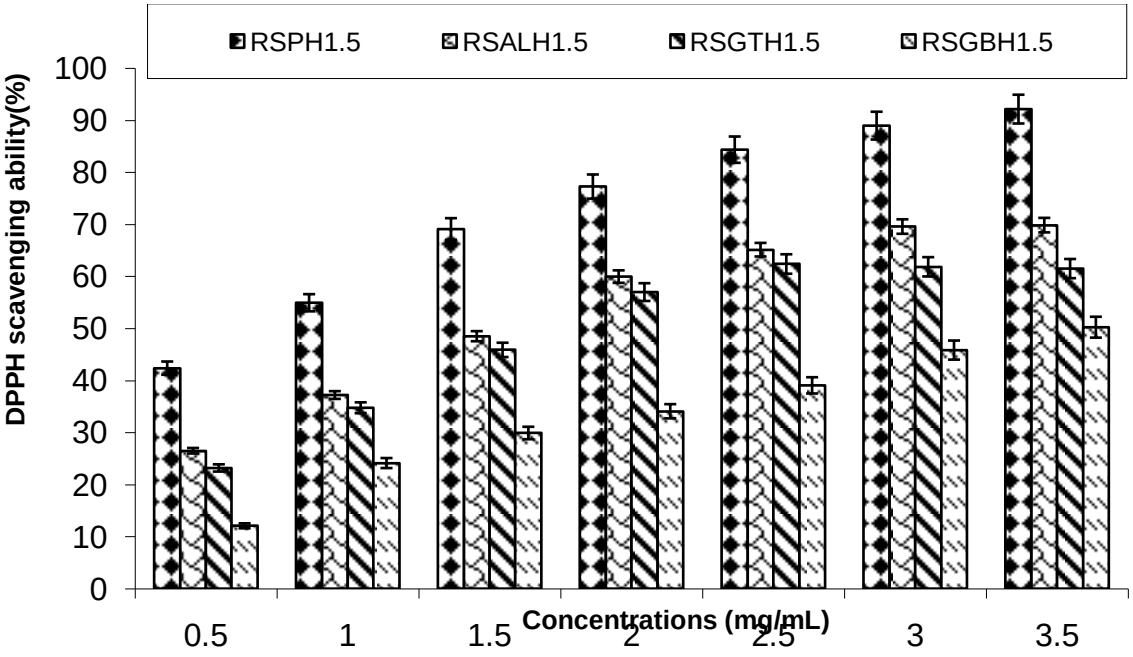

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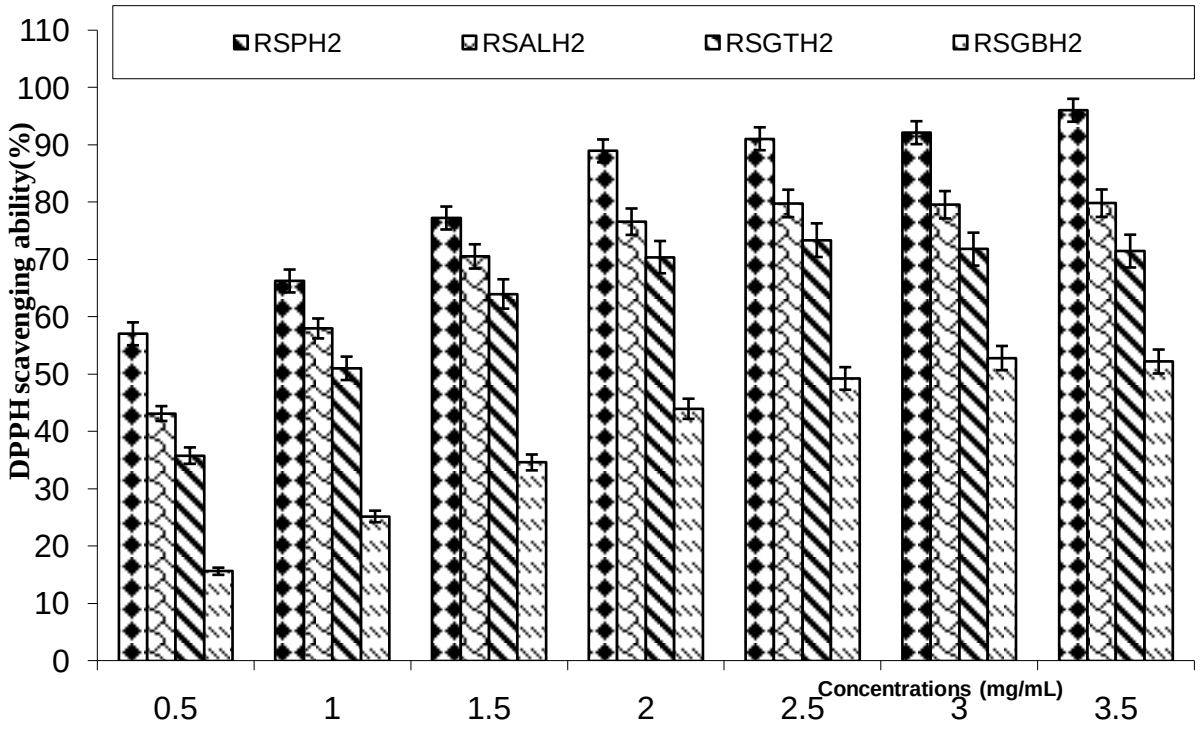

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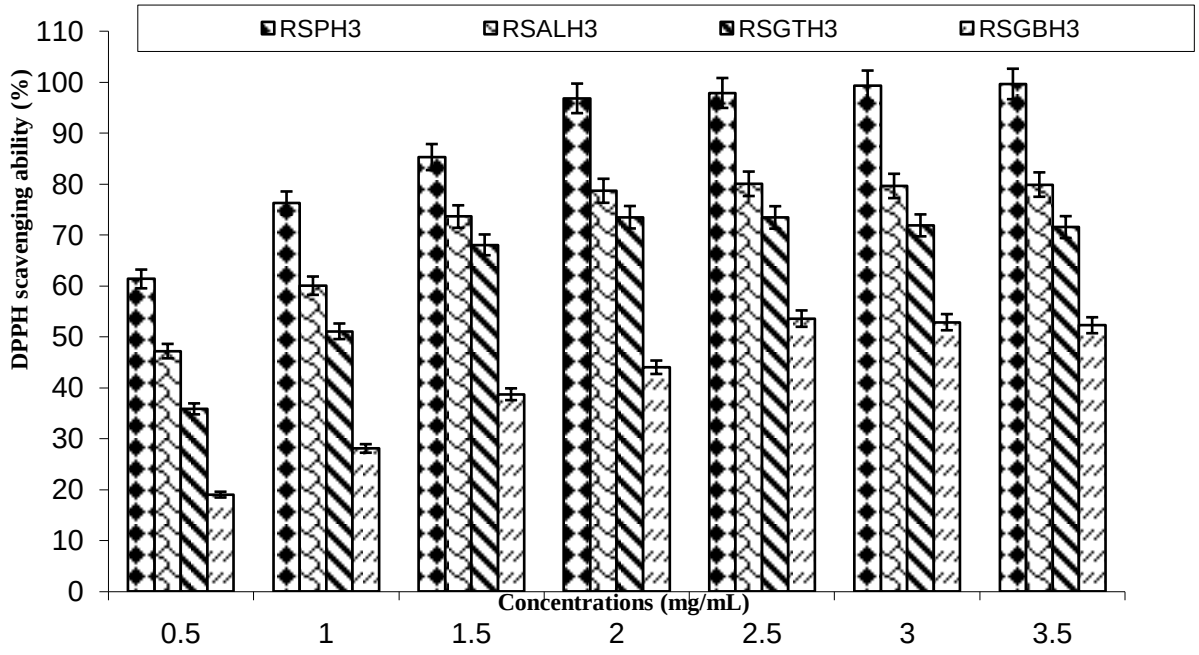

Fig. 1:- The scavenging capacity of DPPH radicals (%) by various concentrations of the 1.5 h RSPH (a), the 2 h RSPH (b) and 3 h RSPH (c). Each value is expressed as mean ± SD of three determinations.

Scavenging Effect on Hydroxyl Radical:

The radical system used for the antioxidant activity evaluation may influence the experimental results, hence two or more radical systems are required to investigate the radical-scavenging capacities of a selected antioxidant (Yu et al., 2002; Cacciuttolo et al.,1993). Therefore hydroxyl radical (HO•) scavenging capacities of the different

hydrolysates were also measured. Hydroxyl radicals are extremely reactive species and induce severe damage to adjacent biomolecules, resulting in lipid peroxidation in biological systems. Therefore removal of hydroxyl radicals is probably one of the most effective defenses mechanism through which living body defends its self against various diseases. Fig.2 indicates hydroxyl scavenging effect of the hydrolysates. Among all samples, the 3 h protein hydrolysates exhibited the strongest scavenging ability compared to the 1.5 h and 2 h hydrolysates (Fig 2). The result in the decreasing order was RSPH3 > RSPH2 >RSPH1.5 (Fig 2). However, the more potent activity was observed in the RSPH3 (57.09%), RSALH3, RSGTH3 and RSGBH3 at the same concentration (10 mg/mL) exhibited 52.15, 44.325 and 40.35% hydroxyl radical-scavenging activity respectively(Fig 2). These results revealed that all tested samples possessed hydroxyl radical scavenging activity. Admittedly, the hydroxyl radical possesses the strongest chemical activity among the ROS, and easily reacts with biomolecules such as amino acids, proteins, and DNA (Cacciuttolo et al., 1993). Theantioxidant activity of hydrolysates from many kinds of food proteins has been studied in recent years. Peng et al. (2009) reported that whey protein hydrolysate and its peptide fractions showed antioxidant properties against hydroxyl radical similar to the results of this study.

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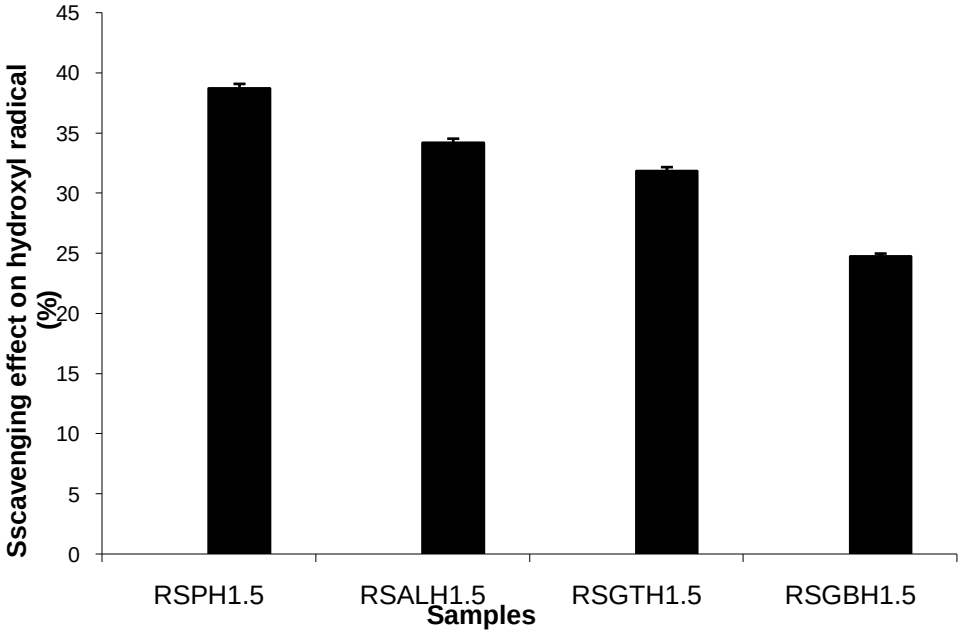

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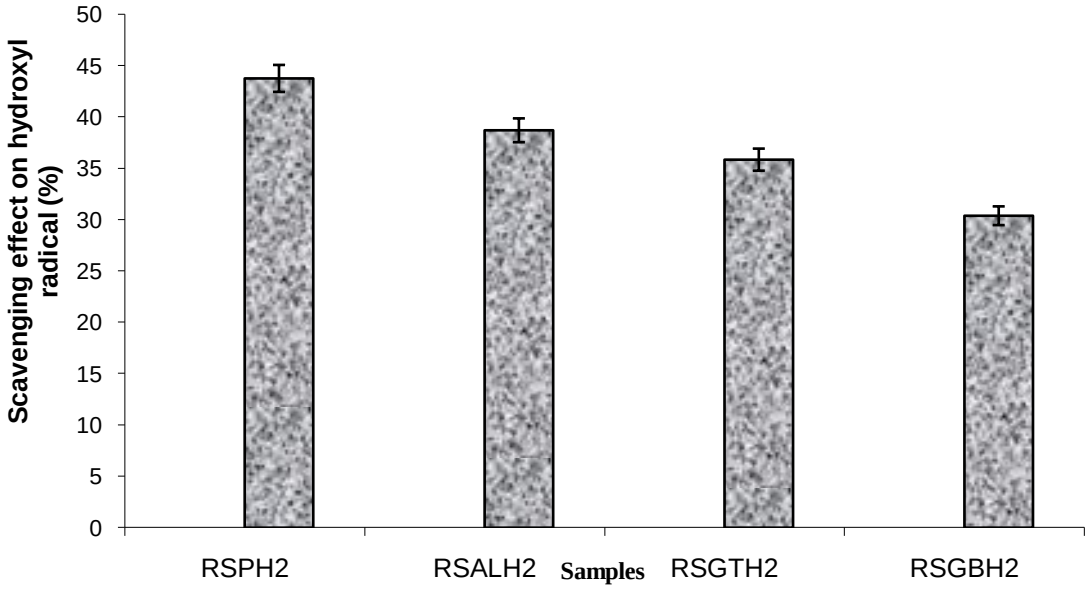

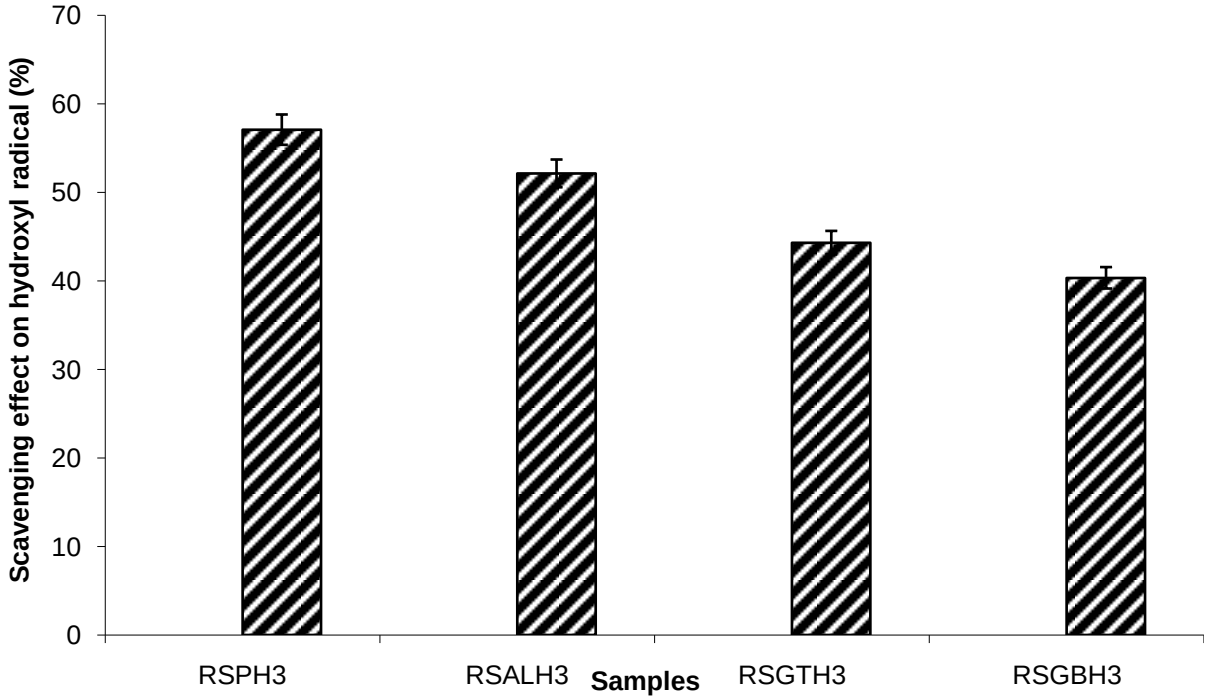

Fig. 2:- The scavenging capacity of hydroxyl radicals (%) of the different Roselle seed protein hydrolysates: 1.5 h RSPH (a), the 2 h RSPH (b) and 3 h RSPH (c). Each value is expressed as mean ± SD of three determinations.

Determination of Reducing Power:

The results of the reducing power of the hydrolysates correlated well with those of the DPPH scavenging assay, as shown in (Fig 3). From the results, the presence of antioxidants caused the reduction of the the Fe^{3+} ferricyanide complex to the ferrous form, and the yellow color of the test solution changed to various shades of green and blue depending on the reducing power of each compound. The Fe^{2+} was then monitored by measuring the formation of Perl's Prussian blue at 700 nm (Dinis et al., 1994). Reducing power of Roselle seed protein hydrolysates appeared to be concentration-dependent thus increases with increasing concentration. Reducing power determination is used to measure the potential antioxidant activities of bioactive compounds in different products, including peptides (Mitsuta, et al., 1996; Huda-Faujan, et al., 2007). Like the radical-scavenging capacity, the reducing power of Roselle seed protein hydrolysates varied, depending on the protein fractions used for hydrolysis. Among all samples, the 3 h hydrolysates showed considerable amount of reducing power (Fig 3c). However, the RSPH3 showed the highest reducing power among all samples tested, followed by the RSALH3, RSGTH3 and RSGBH3. The data indicated that all the hydrolysates are capable of donating electrons, which can react with free radicals to convert them to stable products, and strongly inhibiting radical chain reaction. A similar observation has been reported by other investigators (Amadou, et al., 2011; Wang et al., 2007).

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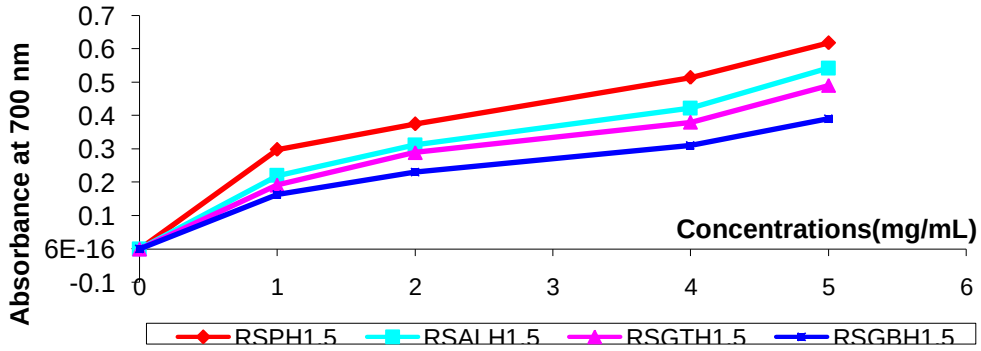

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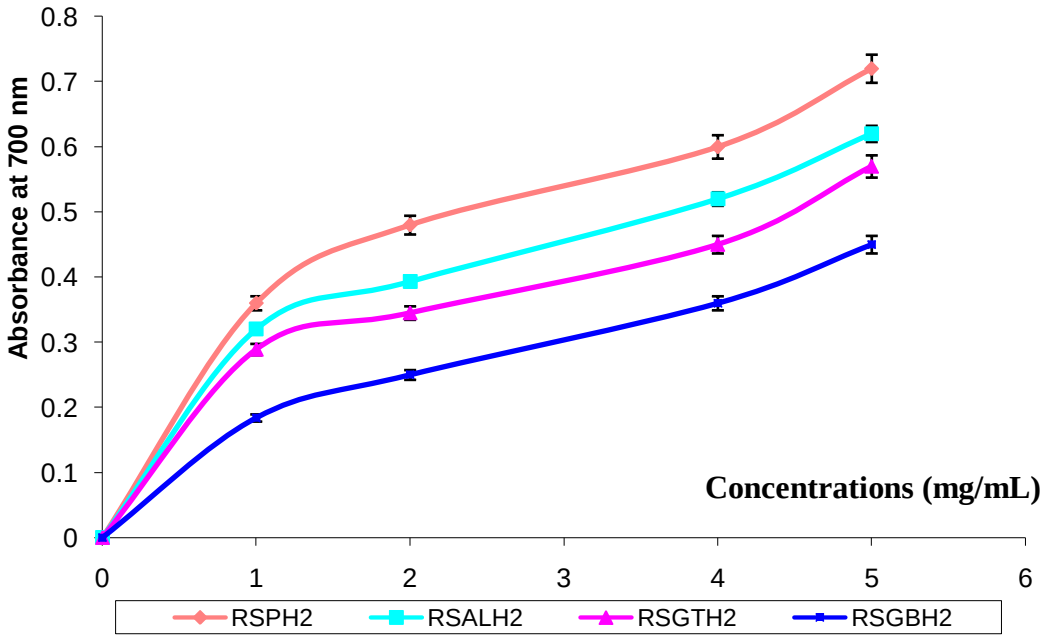

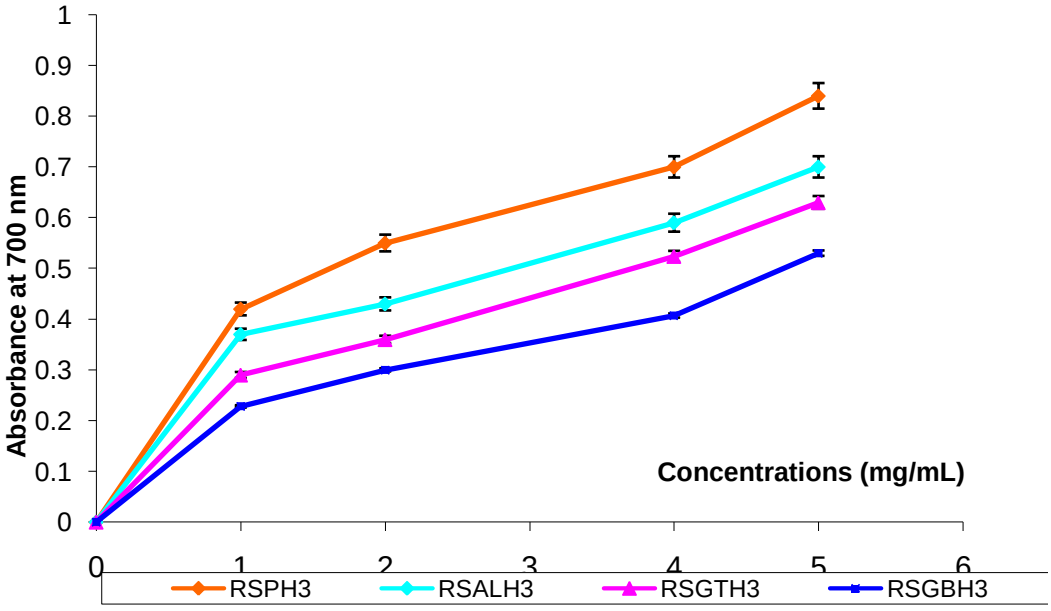

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Fig. 3:- Reducing power of the of different Roselle seed protein hydrolysates: 1.5 h RSPH (a), the 2 h RSPH (b) and 3 h RSPH (c), at various concentrations measured as absorbance at 700 nm. Each value is expressed as mean ± SD of three determinations.

Conclusion:-

Roselle seed protein hydrolysates were found to possess antioxidant activity. Among the hydrolysates examined, those prepared using RSPI showed the highest antioxidant activities by scavenging DPPH and hydroxyl radicals, also by acting as reducing agent. Further studies are needed to identify the peptide fractions responsible for the antioxidant potential of the products.

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