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Full Length Research Paper

Influence of a homeopathic product on performance and on quality flour and cookie (Grissini) of Nile tilapia

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Animals can also benefit from homeopathic remedies by having their immunological system and organic responses to stress reduction stimulated, as well as their balance reestablished. In this context, the objective of this study was to assess the performance of Nile tilapias (*Oreochromis niloticus*) treated with food containing a homeopathic product (Homeopatila 100®), as well as the physical, chemical, technological and sensory quality of flour and cookie (grissini) based on the fish co-product. An eighty-four days assessment was conducted on two types of diet for fishes with sexual inversion: control (CT), added with 40 ml of hydroalcoholic solution (alcohol 30° GL) per kilo of food; and homeopathic treatment (HT) added with 40 ml of homeopathic product Homeopatila 100® per kilo of food. The flours (NTF) were obtained using the head and neck-ends of Nile tilapias with subsequent physical, chemical and technological analyses. In order to assess the applicability of the flours from both treatments, we formulated the grissini (cookies) added with 10% NTF substituting wheat flour; physical, chemical, technological and sensory characteristics were assessed. Considering the studied period, the fish treated with food containing the homeopathic product Homeopatila 100® presented final total weight significantly higher ($p < 0.05$) than fish in the control group. The homeopathic product had no significant influence ($p < 0.05$) on the physical and chemical of the NTF, as well as the results of the assessments on cookies (grissini) produced with the addition of 10% NTF, including the sensory test.

Key words: Homeopathy, *Oreochromis niloticus,* fish, co-product, principal component analysis.

INTRODUCTION

Aquaculture provides about one-third of fish-based products worldwide with a major role of Nile tilapia (*Oreochromis niloticus*) production. The production of this type of fish has expanded since the last decade because

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Table 1. Composition of homeopathic product *Homeopatila 100*®.

Compound	/1000 ml
Iodum 12cH[1]	250 ml
Sulphur 30cH	250 ml
Natrum muriaticum 200 cH	250 ml
Streptococcinum 30cH	250 ml
Vehicle (Ethyl alcohol 30° GL)	Q.s.f.[2]

[1]Centesimal Hahnemannian dilution. [2]Quantity sufficient for. Source: REALH Homeopathy – Brazil.

of the technological advances associated with more intensive growth practices, especially in China, Philippines, Thailand, Indonesia and Egypt (Josupeit, 2010; Yarnpakdee et al., 2014).

Nile tilapias are the most cultivated fresh-water fish in Brazil; according to the Ministry of Fishing and Aquaculture, in 2011, the production exceeded 253 thousand tons (Brazil, 2011). The increase in the fish production also increases the quantity of generated sub-products that may be used to develop processed foods due to excellent sensory and nutritional characteristics. In this context, the development of new products, based on fish, residues represent a viable technological alternative since fish are historically associated with healthy food (Brazil, 2011).

Homeopathy is a form of complementary medicine that uses high dilutions of substances derived from plants, minerals or animals based on the principle of similarity (Khuda-Bukhsh and Pathak, 2008). Bell and Koithan (2012) proposed an innovative model for the action of homeopathic remedies on living organisms; the research indicates that homeopathic remedies are constituted by nanoparticles and this model provides a basis to guide researches on the function of nanomaterial in living systems, the action mechanism of homeopathic remedies and their use in nanomedicine. Animals can also benefit from homeopathic products by having their immunological system and organic responses to stress reduction stimulated, as well as their balance reestablished. In addition to contributing with prophylaxis by reducing stress, these products may also reduce the application of chemotherapy and antibiotics, preventing environmental risks for animals and consumers (Siena et al., 2010).

The use of homeopathic products in Nile tilapias diet (40 ml kg^{-1} of food) benefited fish' survival, muscle fiber hypertrophy, reduced the hepatic lipid inclusion, hepatosomatic index and total lipids content in the muscle tissue (Valentim-Zabott et al., 2008; Júnior et al., 2012; Andretto et al., 2014). However, there is only one study approaching the development and evaluation of food products (nuggets) based on fish treated with homeopathic medicines (Lima et al., 2015). In this study the aim was to evaluate the performance of Nile tilapia treated with foods containing a homeopathic product (Homeopatila 100®), as well as physical, chemical, technological and sensory quality of flour and biscuit (grissini) produced as a co-product of this fish.

MATERIALS AND METHODS

Experimental protocol and sampling

The experiment was carried out at the experimental station for fishery of the State University of Maringá (UEM) – CODAPAR, district of Floriano, city of Maringá, state of Paraná. A hundred and sixty Nile tilapia males with sexual inversion were weighed, measured and distributed in eight fiber glass tank with capacity of 600 L, maintained at the density of 20 fish per box. The design was completely randomized with two treatments – control (CT) added with 40 ml hydro-alcoholic solution (30° GL) (vehicle as the homeopathic product) per kilo of food; homeopathic (HT) added with 40 ml Homeopatila 100® per kilo of food; each with four replicates (C1, C2, C3 and C4; H1, H2, H3 and H4, respectively). Table 1 presents the composition of the studied homeopathic product. The eighty-four day experiment was approved by the Animal Experimentation Ethics Committee of UEM (protocol 019/2013). The fish were treated with food containing 32% crude protein; the quantity provided was *ad libitum* in two daily portions (9 am and 4 pm). On a weekly basis, the homeopathic product Homeopatila 100® was incorporated into the food sprinkled with a manual sprayer; subsequently, the food was homogenized and dried at room temperature with periodical removal for 24 h. The food was stored in a dry, well-ventilated location without the presence of sunlight, chemical products or equipment emitting a magnetic field until reaching no alcohol odor and clusters. Equal inclusion process was carried out for the control treatment using hydroalcoholic solution.

At the final stage of the experiment, all fish were captured by nets, stunned with water and ice at 0°C and sacrificed through section of the spinal cord and separated per treatment. The whole fish were weighed and measured; heads were removed, washed, weighed and vacuum-packed in polyethylene bags. The neck-ends were extracted from the carcasses using High Tech® deboner (model HT C100), weighed and vacuum-packed in polyethylene bags. The heads and the neck-ends were stored at -18°C. The apparent feed conversion (AFC) was determined by the following equation:

$$AFC = (\text{Weight of meal provided during the period}) \times (\text{Final weight} - \text{Initial weight})^{-1}$$

Obtaining the Nile tilapia flour (NTF)

Four flours were obtained per treatment (one per replicates). The preparation of NTF followed the methodology by Franco et al. (2013), with modifications. Initially, heads and neck-ends were subjected to one-hour cooking process in a pressure pot; the residues were pressed in manual press for foods and grinded in a stainless steel mill (worm thread) Skymsen® (model PSEE-98MHD). Subsequently, the mass obtained was Quimis® kiln (model Q317B), allowed to remain at 60°C for 24 h. The obtained product was then grinded in cutting mill SOLAB® (model CE-430/Mini) and sieved at 60 mesh. The flours were weighed, vacuum-packed in polyethylene bags and stored at -18°C until the analyses and grissini production.

Characterizing Nile tilapia flour (NTF)

Moisture content, crude protein, ashes and total lipids were determined according to the methodology proposed by the Association of Official Analytical Chemists (AOAC, 2005). Minerals, calcium, iron and phosphorus were determined according to the analytical methods of the National Laboratory of Animal Reference (LANARA, 1981) and Institute Adolfo Lutz (IAL, 2008). Water activity (Aw) was assessed at 25°C in Aqualab® (model 4TE) equipment for the determination of water activity, according to the manufacturer's instructions. For color measurement, we used Minolta® colorimeter (model CR 400) with illuminant D65 and angle of view of 10°. The values of L (luminosity), a* (intensity of red color) and b* (intensity of yellow color) were expressed according to the color system by the Commission Internationale de L'Eclairage (CIELAB) (Minolta, 1998). The determination of the NTF yield was carried out gravimetrically through the relationship between the weight of heads and neck-ends in nature, and the weight of the extracted flours expressed in percentage. All of the analyses were carried in 3 replicates.

Cookie (grissini) formulation

In order to assess the applicability of the flours from both treatments, we formulated grissini added with 10% NTF substituting wheat flour. For the grissini production, the NTF from the four repetitions of TC (C1, C2, C3, and C4) were mixed and homogenized; equal process was employed for the HT with repetitions H1, H2, H3, and H4. The following ingredients and proportions were applied to produce the grissini: fresh yeast 2.3%; refined sugar 3%; distilled water 25%; salt 2.1%, oil 6%, wheat flour 56% and NTF (CT or HT) 5.6%. The cookies were baked in a Fisher® conventional oven at 200°C for 10 min; immediately after being cooked, the cookies were cooled at room temperature (25°C) and stored in polyethylene bags of high density and hermetically sealed glasses identified per treatment until the analyses.

Cookie (grissini) characterization

The moisture content, crude protein, ashes, total lipids, Aw, color and minerals (calcium, iron, phosphorus) were determined according to the description for NTF. Total carbohydrates were calculated by difference (100 g – total grams of moisture, crude protein, total lipids, and ashes). The content of soluble and insoluble dietary fibers was determined through the enzymatic-gravimetric method 985.29 by the AOAC (Prosky et al., 1985); total dietary fiber was calculated using the sum of these two fractions. All of the analyses were carried out in triplicate. The characteristics of the cookies were assessed according to method 10 - 50D (AACC, 2000); the dough was determined using pre- and post-heat weight expressed in grams. The width, thickness and length of the cookies were determined using millimeter scale ruler with pre- and post-heat values expressed in centimeters. The specific volume was calculated from the relationship between the apparent volume (determined using millet displacement method) and the post-heat weight of the cookies (Pizzinatto et al., 1993). The yield was determined through the difference between pre- and post-heat weight of the cookies. The results represented an arithmetic mean of 10 determinations derived from samples of a single test. The tensile strength was determined using Stable Micro Systems Texture Analyser TA.XT2 texture meter, with probe 3-Point bending Rig (HDP/3PB) and platform HDP/90. Results were expressed in Newton (N) representing an arithmetic mean of 15 determinations of tensile strength for samples derived from a single test randomly selected. The following parameters were employed: pretest velocity = 1.0 mm s^{-1}; test velocity = 3.0 mm s^{-1}; post-test velocity = 10.0 mm s^{-1}; distance 5 mm, with measure of compression force (Clerici et al., 2013).

Sensory evaluation

The sensory evaluation was approved by the Human Research Ethics Committee of UEM (623.527/2014). For the grissini acceptance test for both treatments, we applied a nine-point hedonic scale (1 = dislike extremely to 9 = like extremely) assessing the attributes of color, aroma, texture, taste and overall evaluation with the collaboration of 120 non-trained tasters – 56 female, 64 male, ages ranging from 15 and 57 years old, all employees and students at the Federal Technological University of Paraná (Dutcosky, 2013). After the sensory evaluation, we calculated the Acceptance Index (AI) for the attribute of overall evaluation by using the following expression:

$$AI (\%) = A \times 100 / B$$

Where A = average score for the attribute, and B = maximum score for the attribute (Lawless and Heymann, 2010).

Statistical and multivariate analyses

Fish performance results, the physical, chemical and technological analyses of the NTFs and grissini as well as the sensory evaluation were submitted to the t-Student means difference test ($p<0.05$). The multivariate analysis indicated the individual values for each replicate in the four NTF repetitions (n=24) for both treatments – CT and HT; the grissini had one repetition (n=6) and analyses in triplicate for both cases divided into data matrices. The first matrix contained the data of the NTF characterizations, and two matrices for the results obtained for the grissini. The data were pre-processed through self-scaling; in this case, data are centered on the mean and each individual value is divided by the standard deviation. The procedure is to guarantee that all of the variables have equal importance, that is, equal weight. Posteriorly, we applied the principal component analysis (PCA) using NIPALS algorithm with the scores (samples) and loadings (variables) decomposed in a dimensional graph. Statistical software, version 8.0 (StatSoft, 2007) was applied using 5% significance level ($p<0.05$) for the rejection of the null hypothesis and selection of the principal component.

RESULTS AND DISCUSSION

Fish performance

Table 2 presents the mean values of fish performance during the experimental period. No significant difference was observed between the treatments in relation to initial total weight, initial total length, final total length, head and neck-end weight, and apparent feed conversion (AFC); however, significant difference between the treatments ($p < 0.05$) was observed in relation to final total weight. The weight gain after 84 days for the tilapias treated with homeopathy (HT) was 207.16 g, while those in the control treatment (CT) presented 192.05 g. According to Rocha et al. (2002), weight gain is a very important factor when growing fish and determinant for the economic exploration of the area.

Intensive growth systems impose animals with

Table 2. Mean values of Nile tilapia performance for the different treatments.

Parameter	Treatments	
	Control (CT)	Homeopathy (HT)
Initial total weight (g)	101.12±17.73[a]	99.73±19.85[a]
Initial total length (cm)	18.52±6.00[a]	18.05±1.11[a]
Final total weight (day 84) (g)	293.17±56.76[b]	306.89±58.30[a]
Final total length (day 84) (cm)	26.42±1.81[a]	26.22±1.79[a]
Head weight (day 84) (g)	99.41±18.52[a]	100.86±19.81[a]
Neck-end weight (day 84) (g)	5.28±0.78[a]	5.99±1.59[a]
AFC	1.33[a]	1.27[a]

Mean ± standard deviation. Means followed by different letters (a-b) on a single line indicate significant differences through t-Student test ($p < 0.05$). AFC = Apparent Feed Conversion.

conditions that are opposite to the one found in their natural environment, such as high density and constant handling, factors that damage their well-being and have a negative influence on production. Stressed fish generally have their development compromised and possible hematological alterations followed by hyperglycemia and hemoglobin increase caused by the higher release of cortisol, which induces hepatic glycogenesis boost (Mazeaud et al., 1977; Carneiro, 2001).

Siena et al. (2010) treated Nile tilapia (*O. niloticus*) with food containing four levels of the homeopathic product Homeopatila 100® in hydroalcoholic solution per kilo of food (0.20, 40 and 60 ml) concluding that the fish treated with 40 ml kg^{-1} of food presented higher survival rate and lower hepatosomatic index than the control group. Braccini et al. (2013) demonstrated adding Homeopatila 100® (40 ml kg^{-1} of food) to young Nile tilapias' diet reduced the hepatosomatic index, increased the amount of hepatocytes and the percentage of intracellular glycogen compared with fish in the control group.

The formulation of the studied homeopathic product has four compounds (iodum, sulphur*, Natrum muriaticum* and Streprococcinum); according to Vijnovsky (2003), they have different indications for the treatment of behavior disorders. Therefore, the product was developed in order to reduce production stress by acting on the vital energy and maintaining the organic homeostasis of the fish.

Characterizing Nile tilapia flour (NTF)

According to the t-Student test ($p<0.05$), no minimum significant difference was observed between the NTF treatments in relation to the physical, chemical analyses (Table 3). The results of ashes and crude protein indicate that the NTF present high content of minerals in addition to being excellent sources of animal protein. Franco et al. (2013) studied the flour of Nile tilapia (*O. niloticus*) carcass and found contents of moisture, ashes and crude protein similar to those indicated in this study – 2.15, 38.03, and 45.32%, respectively. The chemical composition of fish-based products may vary according to the source of acquisition of heads and neck-ends, the food provided to the fish and the applied filleting method. Another factor that may have influence on the proximate composition of fish-based flour is the methodology employed in the production. Monteiro et al. (2012) developed a flour based on the head and carcass of Nile tilapia and verified contents of 4.01% moisture, 56.15% crude protein, 30.14% total lipids, and 3.89% ashes.

The residues of Nile tilapia (*O. niloticus*), especially head and bones, present high levels of calcium, iron and phosphorus (Oetterer, 2002). Calcium was the most abundant mineral present in the flours developed in this study followed by phosphorus and iron (Table 3). Petenuci et al. (2008) also found higher amount of calcium followed by phosphorus and iron when developing a flour based on Nile tilapia neck-end –2715.9 mg 100 g^{-1}, 1132.7 mg 100 g^{-1} and 1.3 mg 100 g^{-1}.

The intensity of yellow color (b*) in the NTF for the different treatments in this study was higher than the Brazilian catfish flour (*Brachyplatystoma vaillantii*) studied by Oliveira et al. (2015), while the luminosity parameter (L) and the intensity of red color (a*) presented lower values. The color of a certain food is influenced mostly by the employed raw materials and possible chemical and biochemical alterations occurring during processing and storage (Ribeiro et al., 2007).

Principal component analysis

The principal component analysis (PCA) was carried out to enable a wider exploration of the results, considering the weight of all of the obtained experimental measures. The data obtained from the four repetitions of NTF of the CT (C1, C2, C3 and C4) and the HT (H1, H2, H3 and H4) enabled the achievement of the significant principal components ($p<0.05$): PC1 and PC2, explaining 20.65 and 17.10%, respectively, of the results variability. We observe the formation of two distinguished groups (Figure

Table 3. Physical and chemical composition and quality of Nile tilapia flour (NTF) and grissini cookie for the different treatments.

Analyses [1]	Treatments – Flour (FTN) [2]		Treatments – Grissini	
	Control (CT)	Homeopathy (HT)	Control (CT)	Homeopathy (HT)
Yeld (%)	12.97±0.03	12.98±0.01	1.43±0.20	1.46±0.06
Moisture (g $100g^{-1}$)	2.53±0.53	2.60±0.49	8.35±0.01	8.17±0.16
Ashes (g $100g^{-1}$)	33.00±2.39	31.23±2.12	6.03±0.6	5.90±0.10
Total lipids (g $100g^{-1}$)	15.05±1.79	16.76±2.76	9.61±0.35	9.62±0.44
Crude protein (g $100g^{-1}$)	48.70±1.70	47.68±0.74	11.81±0.19	11.83±0.25
Carboidrato (g $100g^{-1}$)	-	-	64.33±0.59	64.35±0.42
Calcium (mg $100g^{-1}$)	2427.22±30.82	2447.60±31.95	167.65±7.30	170.11±4.50
Iron (mg $100g^{-1}$)	225.12±4.52	222.34±4.10	8.28±0.07	8.08±0.85
Phosphorus (mg $100g^{-1}$)	842.46±6.69	838.11±5.28	264.75±3.85	260.48±3.12
Aw	0.09±0.02	0.11±0.03	0.57±0.01	0.57±0.002
L	42.79±1.72	41.73±1.71	65.66±0.31	66.08±0.50
a*	1.47±0.54	1.80±0.46	3.46±0.09	2.93±0.52
b*	19.42±0.58	20.11±1.06	31.23±0.39	31.00±0.20
Soluble dietary fiber (g $100g^{-1}$)	-	-	1.77±0.13	1.81±0.13
Insoluble dietary fiber (g $100g^{-1}$)	-	-	8.89±0.29	9.44±0.29
Total dietary fiber (g $100g^{-1}$)	-	-	10.66±0.30	11.26±0.42
PreHD (g)	-	-	5.23±0.45	5.29±0.32
Post-HD (g)	-	-	3.80±0.25	3.83±0.33
PreHW (cm)	-	-	1.34±0.16	1.35±0.12
Post-HW (cm)	-	-	1.60± 0.25	1.65±0.12
PreHT (cm)	-	-	0.81±0.10	0.78±0.08
Post-HT (cm)	-	-	1.50±0.15	1.47±0.08
PreHL (cm)	-	-	4.82±0.28	4.82±0.11
Post-HL (cm)	-	-	4.97±0.33	4.95±0.19
SV (ml g^{-1})	-	-	3.36±0.51	3.52±0.43
TS (N)	-	-	46.55±6.25	48.66±9.60

[1]Means ± standard deviation. [2] Means for the four repetitions C1, C2, C3 and C4 (CT); H1, H2, H3 and H4 (HT). L = luminosity. a* = intensity of red color. b* = intensity of yellow color. PreHD = preheat dough. Post-HD = post-heat dough. PreHW = preheat width. Post-HW = post-heat width. PreHT = preheat thickness. Post-HT = post-heat thickness. PreHL = preheat length. Post-HL = post-heat length. SV = specific volume. TS = tensile strength – absent analysis.

1): the group formed by the blue ellipse containing the CT repetitions and the one formed by the red ellipse containing the HT repetitions. The HT repetitions obtained higher influence of the following loadings: water activity (0.8001), total lipids (0.7521) and moisture (0.6186). The loadings of crude protein (0.8464) and phosphorus (0.6619) (PC2) presented higher weight when distinguishing the CT repetitions (PC1); a possible explanation would be higher values of water activity, total lipids and moisture for the HT, and crude protein and phosphorus for the CT (Table 3).

Characterizing the (cookie) grissini

The results of the physical, chemical and technological analyses of the grissini for the different treatments are presented in Table 4. When statistically analyzed (t-Student $p<0.05$), the results demonstrated no significant difference between the treatments. Grissini are classified as cookies prepared with wheat flour, fat, water, salt and other food substances in the shape of thin, short cylinders (Brazil, 1978). Following the pattern in the NTF, calcium was the most abundant mineral found in the grissini followed by phosphorus and iron. The NTF has a dark color, consequently, the cookies presented more intense color than cookies produced exclusively with wheat flour (Sharmas and Gujral, 2014). Preheat weight, width, thickness and length of the grissini for both treatments presented no statistical differences ($p<0.05$), which indicated a high level of homogeneity during the processing. This allows the assumption that any differences possible to be observed after the cooking would derive from the NTF behavior in the dough. We observed that the width, thickness and length of the grissini in both treatments increased after the heat, while weight decreased; similar results were found by Fasolin et al. (2007) in cookies produced with banana flour, and

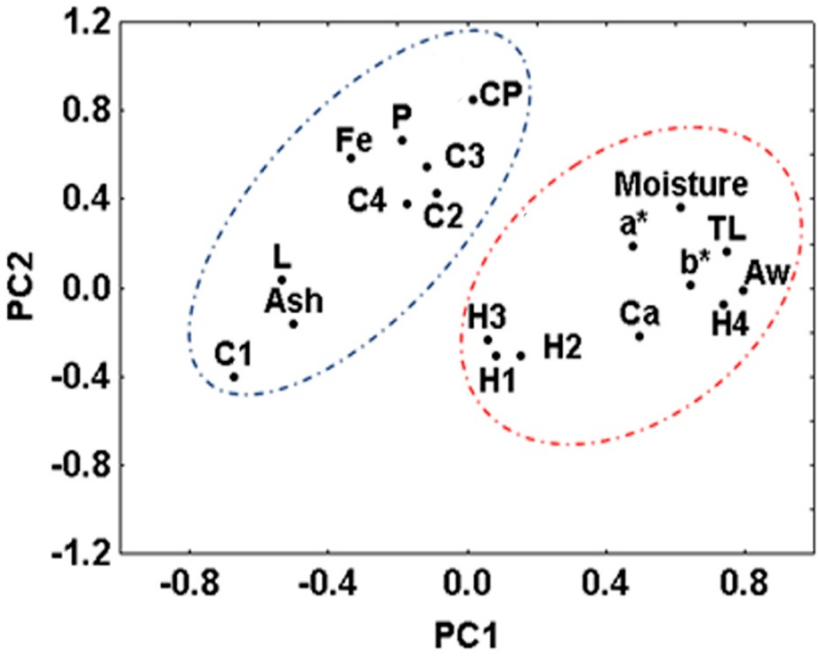

Figure 1. Principal component analysis for Nile tilapia flour (NTF) physical, chemical and technological analyses regarding control treatment (CT blue ellipse) and homeopathy (HT red ellipse). C1, C2, C3 and C4 = repetitions of CT. H1, H2, H3 and H4 = repetitions of HT. CP = Crude protein. P = Phosphorus. Fe = Iron. L = Luminosity. L = Total lipids. a* = intensity of red color . b* = intensity of yellow color. Aw = Water activity. Ca = Calcium.

Table 4. Mean values of the acceptance scores for grissini produced using Nile tilapia flour (NTF) for the different treatments.

Attributes	Treatments	
	Control (CT)	Homeopathy (HT)
Color	7.40±1.69	7.74±1.98
Aroma	7.06±1.65	7.17±1.78
Texture	6.74±1.90	7.15±1.07
Taste	7.02±1.83	7.30±1.90
Overall evaluation	6.96±1.90	7.00±1.97
AI (%)	77.33	77.78

Mean based on the 120 tasters. [1]Hedonic Scale: (9) like extremely, (8) like very much, (7) like moderately, (6) like slightly, (5) neither like nor dislike, (4) dislike slightly, (3) dislike moderately, (2) dislike very much, (1) dislike extremely. AI = Acceptance Index.

Moraes et al. (2010) in cookies with variations in the contents of sugar and lipids.

Sensory evaluation

No minimum significant difference ($p<0.05$) was observed among the means of the evaluation of attributes referent to the acceptance of the products (Table 4). All of the evaluations were within categories 'like slightly' and 'like regularly'. The attribute color received the most satisfactory score for both treatments; color is a major parameter to be considered when adding different types of flour into the cookies formulation since it may influence its appearance, and consequently its acceptance. Lima et al. (2015) carried out a sensory evaluation on nuggets containing fillet and mechanically separated meat of Nile tilapia treated with homeopathic product Homeopatila 100® and also found no significant differences ($p<0.05$) compared to the control treatment, for all attributes.

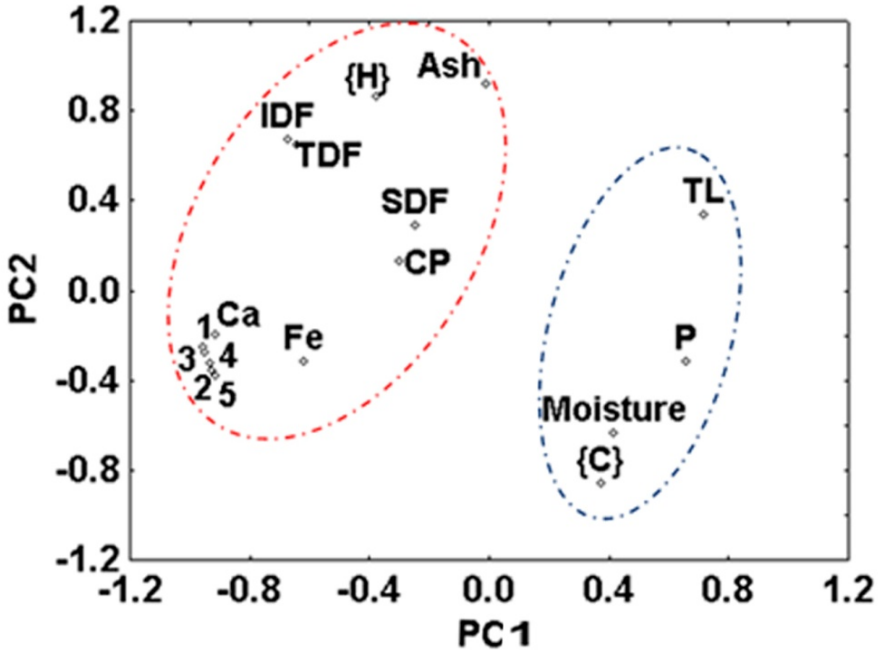

Figure 2. Principal component analysis of the proximate composition and sensory attributes of the control treatment grissini (CT blue ellipse) and homeopathic treatment (HT red ellipse). H = Homeopathic treatment. C = Control treatment. CP = Crude protein. IDF = Insoluble dietary fiber. TDF = Total dietary fiber. SFD = Soluble dietary fiber. Ca = Calcium. Fe = Iron. P = Phosphorus. Sensory attributes: 1 Color, 2 Aroma, 3 Texture, 4.Taste and 5 Overall evaluation.

Rebouças et al. (2012) produced salted cookie added with protein concentrate of Nile tilapia and obtained means for the sensory evaluation close to 5 ('indifferent') for all of the attributes. Rocha (2011) carried out a sensory evaluation on salted cookie containing Nile tilapia flour and obtained 56.66% acceptance with scores within 'like regularly' and 'like extremely'. Neiva et al. (2011) produced low commercial value cookies based on fish meat mechanically separated (*Menticirrhus americanus* and *Umbrina corides*), and verified that the samples presented good scores, varying from 6 ('like slightly') to 9 ('like extremely') for all of the evaluated attributes.

According to Dutcosky (2013), for a product to be considered well-received in consumers market in terms of sensory properties, it is required an Acceptance Index (AI) of at least 70%. As observed in Table 4, the grissini IA for both treatments was satisfactory.

Principal component analysis

The grissini developed with NTF from both the CT and the HT were grouped in two PCA dimensional graphs (Figures 2 and 3). Figure 2 presents the data on proximate composition (moisture, ashes, crude protein and total lipids), minerals as well as the sensory attributes, explaining a data variance of 73.78%. It was possible due to the selection of the PC1 with 47.12%, and PC2, responsible for 26.85% of the results variability presenting 5% significance level. According to Figure 2, the grissini containing NTF from the HT (red ellipse) obtained higher contribution in all of the evaluated organoleptic properties – color (-0.9534), odor (-0.9263), texture (-0.9512), taste (-0.9312) and overall impression (-0.9147) through PC1. The loadings ashes (0.9150), insoluble dietary fiber (IDF) (0.6681) and total dietary fiber (TDF) (0.6495) in PC2 also had an influence on the characterization of the HT grissini. The multivariate analysis enabled the identification of the differences between the treatments indicating better sensory preference for the grissini containing NTF from the HT (Figure 2), even though such results did not present minimum significant difference (p<0>05) through t-Student test (Table 4).

By conducting a multivariate analysis on the data of the technological characterization of the grissini, we observed a region with common loadings (Figure 3) for both treatments (CT and HT): pre- (0.9784) and post-heat thickness (-0.9852); pre- (0.9925) and post-heat width (0.9678); pre- (0.9733) and post-heat length (0.9949), and tensile strength (0.9005) in PC1 (48.54%). These parameters are used in the quality control during and after the processing of this type of product; it implies that,

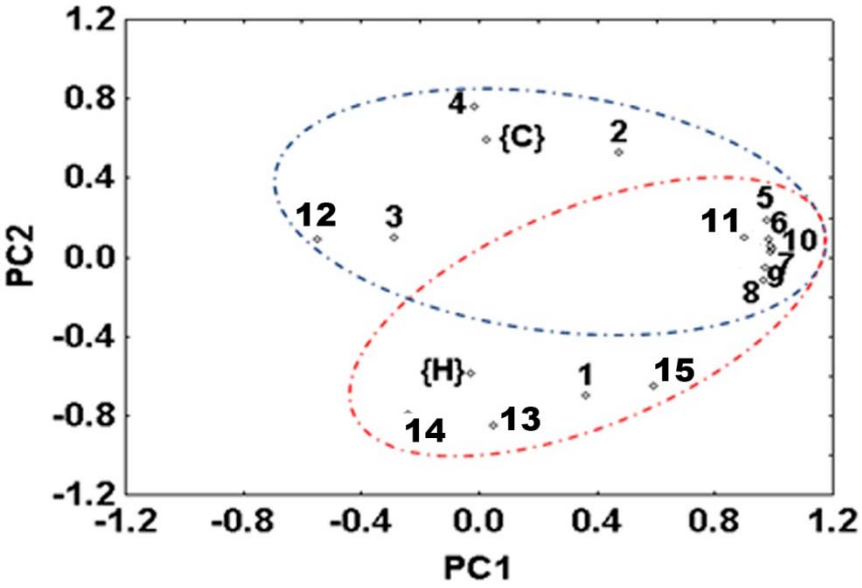

Figure 3. Principal component analysis of the control treatment grissini (CT: blue ellipse) and homeopathic treatment (HT: red ellipse) regarding the technological analyses results. C = Control treatment. H = Homeopathic treatment. 1 = L (luminosity). 2 = a* (intensity of red color). 3 = b* (intensity of yellow color). 4 = Aw. 5 = pre-heat thickness. 6 = post-heat thickness. 7 = pre-heat width. 8 = post-heat width. 9 = pre-heat length. 10 = post-heat length. 11 = Tensile strength. 12=Specific volume. 13 = pre-heat dough. 14 = post-heat dough. 15 = yield.

according to the statistical multivariate analysis, the use of NTF in both treatments had no influence on the grissini processing.

The HT grissini was characterized (red ellipse) by the following loadings: pre- (-0.8548) and post-heat dough (-0.7998); luminosity – L (-0.7010) and performance (-0.6555) in PC2 (21.91%, Figure 3). The CT grissini (blue ellipse) obtained better contribution of loadings a* (0.5292) and water activity (0.7624) in PC2 (Figure 3). According to studies carried out by Pagamunici et al. (2014) and Souza et al. (2014), the application of the PCA enables food products to be distinguished and characterized regarding their loading contributions.

Conclusion

During the studied period, the Nile tilapias treated with food containing the homeopathic product Homeopatila 100® presented final total weight significantly higher ($p<0.05$) than the fish in the control group. The homeopathic product had no significant influence ($p<0.05$) on the physical and chemical properties of the NTF, as well as the results of the assessment on the cookies (grissini) produced with addition of 10% NTF, including the sensory test. The multivariate analysis enabled an improved grouping and distinction of the samples through the respective physical, chemical compositions and technological and sensory characteristics. The obtained results suggest that the homeopathic product Homeopatila 100® used in Nile diet (40 ml kg^{-1} of food) is able to improve weight gain without compromising the physical, chemical, technological and sensory quality of the fish flour and cookie (grissini).

Conflict of Interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Securinega virosa (Euphorbiaceae) root bark extract inhibits glioblastoma multiforme cell survival *in vitro*

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Securinega virosa **Roxb (Ex Willd) is a commonly used medicinal plant in the management of inflammatory conditions and tumor in African traditional medicine. In this study, the effects of the crude methanol root bark extract and its fractions were evaluated on the survival of Glioblastoma mutiforme (GBM) tumor cells (U-1242) *in vitro*. The effects of the crude extract and its fractions on epidermal growth factor receptor (EGFR) which is overexpressed in about 40 to 60% of GBM patients were also evaluated. Their activities on the phosphorylation of downstream mediators of tumor cell growth and proliferation such as Akt and mitogen activated protein kinase (MAPK) were also studied using western blot analysis. The crude methanol root bark extract and the various fractions; namely, residual aqueous, n-butanol soluble and chloroform fractions significantly ($P < 0.0001$) and concentration-dependently (31.25 to 500 µg/ml) reduced tumor cell growth with IC_{50} ranging from 4.87 µg/ml for the chloroform fraction to 58.5 µg/ml for the butanol fraction. The crude methanol root bark extract and the residual aqueous fraction blocked EGFR phosphorylation at tyrosine 1068 and 1045 sites. They also significantly inhibited platelet derived growth factor receptor (PDGFR) phosphorylation. In addition, crude methanol root as well as the residual aqueous and butanol fractions blocked phorbol myristate acetate (PMA) and PDGFR-induced transactivation of the EGFR at tyrosine 1068. Similarly, the crude methanol root bark extract reduced the phosphorylation of MAPK. In contrast, Akt was not significantly altered. These findings showed that the crude methanol root bark extract of *Securinega virosa* possesses significant activity against GBM cells which may be mediated partly via EGFR and some other downstream mediators of cell survival.**

Key words: *Securinega virosa*, glioblastoma multiforme, epidermal growth factor receptor (EGFR), mitogen activated protein kinase (MAPK).

INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive of the gliomas, a collection of tumors arising from glia or

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their precursors within the central nervous system. It accounts for 12 to 15% of all intracranial tumors and 50 to 60% of astrocytic tumors (Chang et al., 2014). It is characterized by features of vascular proliferation, hypercellularity, pleomorphism, and pseudopalisading necrosis (Huse and Holland, 2010; Ostrom et al., 2014). It is clinically classified into primary and secondary with the former accounting for more than 90% of all cases and arises as a *de novo* process (Chang et al., 2014). It has an average incidence rate of 3.19 in every 100,000 population, affecting more men and individual of white race and non-Hispanic ethnicity (Thakkar et al., 2014). Most patients with GBMs die of their disease in less than a year and essentially none has long-term survival (Holland et al., 2000). The standard treatment protocol for patients with this cancer involves surgical tumor resection followed by radiation with or without chemotherapy with alkylating agent, temozolamide (Stupp et al., 2005). Unfortunately, most of the treatment is palliative for most patients (Zahonero and Sánchez-Gómez, 2014). Sadly also, the use of this combination has not produced a significant improvement in GBM response rates, and the prognosis for GBM patients has remained dismal (Rao et al., 2003). The efficacy of these therapeutic approaches often is limited by non-specific toxicity due to non-exclusivity of their activity (Sampson et al., 2009). Epidermal growth factor receptors (EGFR) are involved in glioma formation, proliferation and invasion. EGFR has been reported to be amplified or over expressed in about 60% of GBM. It is a receptor tyrosine kinase which involved in cell proliferation, differentiation, and migration as well as apoptosis modulation (van der Geer et al., 1994). EGFR is of significant prognostic value in predicting survival (Shinojima et al., 2003) and upregulation has been found to be positively correlated with GBM malignancy while its signaling may be involved in resistance to irradiation therapy.

Traditional medicines have been used for ages for the management of human ailments including cancers (Corson and Crews, 2007). Medicinal plants constitute the cornerstone of traditional medicines. It is a fact that a number of anticancer agents in use today have their origin traced to medicinal plants (Shoeb, 2006; Balunas and Kinghorn, 2005). *Securinega virosa* is one of the great African medicinal plants described as a true "cure all", of which all parts are used as remedies, particularly the root. It is a dense, low branching, many branched shrub, sometimes a small spreading tree up to about 6 m high, although, more commonly 2 to 3 m, evergreen or deciduous (Neuwinger, 1996). The plant has enjoyed a wide patronage in traditional medicines for the management of a number of conditions including cancer (Tatematsu et al., 1991). The leaves of the plant are used in the management of cancer in the South Western Nigeria (Sholadoye et al., 2010). The decoction of the root plant and some other plants is used in North-Western Nigeria in the management of "*daji*", the most common term used to describe cancers by the Hausa speaking people of Northern Nigeria (Abubakar et al., 2007). The root extract of *S. virosa* has been reported to be active in bioassay against *Pyricularia oryzae* fungus, a preliminary screening of antineoplastic and antifungal agents (Tsuruo et al., 1986; Hu et al., 2001). This study, therefore, aims at evaluating the effect of the methanol root bark extract of *S. virosa* and its fractions on GBM cells survival.

MATERIALS AND METHODS

Collection of plant and preparation of extract and fractions

The plant material was collected in February, 2009, in Basawa town, Sabon Gari Local Government Area of Kaduna State, Nigeria and was identified by Umar Gallah of the herbarium section of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria by comparing it with existing specimen (Number 918). A voucher specimen was subsequently deposited for future reference.

Extraction and fractionation

The root bark of the plant was removed, air dried and size-reduced. The powdered root bark of *S. virosa* (1000 g) was extracted with 4 L of absolute methanol using a Soxhlet apparatus over a period of 72 h. The resultant extract was concentrated *in vacuo,* yielding 9.5% of brownish residue subsequently referred to as crude methanol extract (CRE). The dried crude methanol extract (50 g) was dissolved in water and filtered. The filtrate was successively partitioned into chloroform and n-butanol. The fractions were separately concentrated *in vacuo* affording the following yields: chloroform (0.28 g), n-butanol (7.32 g) and residual aqueous (30.82 g) fractions. They were subsequently stored in desiccators before use.

Preliminary phytochemical screening

The CRE and fractions of *S. virosa* were screened for the presence of alkaloids, tannins, saponins, flavonoids and steroids/triterpenes using standard protocols (Silva et al., 1998).

Materials for anticancer study

Phorbol myristate acetate (PMA, 100 nM), EGF, PDGF and anti-tubulin antibody were purchased from Sigma Aldrich (USA). The phospho-specific antibodies directed against the EGFR at Tyr_{1068} and Tyr_{1045} and anti-EGFR antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Cell cultures

The human U-1242 MG cell line was generously provided by Prof. Isa M. Hussani. U-1242 MG is one of the well characterized glioblastoma cell lines with mutant p53. The cell lines were originally isolated from astrocytic tumors that were designated as

Table 1. Preliminary phytochemical screening of the crude extract and fractions of *Securinega virosa*.

Constituent	CRE	CCF	NBF	RAF
Tannins	+	-	+	+
Saponins	+	-	+	+
Flavonoids	+	-	+	+
Alkaloids	+	+	+	+
Resins	+	+	+	+
Steroid/Terpenoids	+	+	-	+

+: Present; –: absent; CRE: crude methanol root bark extract; CCF: chloroform fraction; NBF: n-butanol fraction; RAF: Residual Aqueous fraction.

glioblastomas, and their characteristics have been described previously by Hussaini et al. (2000). Cell lines were regularly determined to be free of mycoplasma with reagent from Gen-Probe Inc. (San Diego). Cells were grown in modified α-minimal essential medium with 10% defined fetal bovine serum (Hyclone, Logan, UT) and 20 µg/ml bovine zinc insulin (25.7 IU/mg, Sigma). The cells were cultured to 100% confluence, passaged every 4 to 5 days from an initial concentration of 6 to 8 × 10^3 cm^{-2} in T flasks or 6- or 24-well plates, and cultured in astrocyte growth medium, 5% fetal bovine serum at 37°C in 5% CO_2 and 90% relative humidity. Prior to assays, cultures that were 80 to 100% confluent were washed three times with serum-free medium.

Cell survival assay

Cells in 6 well dishes were treated with dimethyl sulphoxide (DMSO), the crude methanol root bark extract or its fractions (CCF, NBF and RAF) at concentrations of 31.5 to 250 µg/ml; and incubated at 37°C for 48 h. Cells were subsequently washed three times with deionized water and stained with crystal violet dye. The cells were subsequently photographed. Adherent cells were released from the plate with 0.05% w/v trypsin-EDTA (0.53 mM) for 2 min. The trypsin activity was halted by addition of equal volume of serum-free α-MEM. 30 µl of the trypsinized cells was then collected in a cuvette and the viable cell count was determined using the O_2 coulter counter instrument (Beckman Coulter Inc.). The experiment was repeated three times and the values presented represent the mean number of cells.

In another study, cells were treated with DMSO, the CRE or its fractions (31.5 to 250 µg/ml). One hour later, EGF (25 ng/µl) was added to each plate. The cells were incubated for 48 h and the same procedures for cell count were carried out as described earlier.

Western blot analysis

Cells in 100 ml dishes were serum-starved for 24 h after reaching 80 to 100% confluence by replacing the medium with serum-free α-minimal essential medium. The cells were treated with the crude extract (CRE) and it fractions (CCF, NBF and RAF) at concentration of 250 ng/µl or DMSO (1 µl/ml) and allowed to stand for 1 h in the incubator. Thereafter, EGF (25 ng/ml), PMA (10 µM) or PDGF (20 ng/µl) were added separately and allowed to stand for 30 min in the incubator. After the different treatments, the cells were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 1.5 mM KH_2PO_4, pH 7.4) containing 0.2 mM sodium orthovanadate and extracted with 1% Triton X-100 (containing 2 mg/ml sodium orthovanadate and 5 mg/ml DTT) . Cells were centrifuged at 14,000 × *g* for 1 min at 4°C. The protein concentration of the supernatant was determined by the BCA protein assay (Bio-Rad). Proteins were boiled for 5 min in SDS-PAGE buffer. Proteins (200 µg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) polyacrylamide gels (8% for EGF and PMA; and 10% for PDGF) and then electroblotted onto nitrocellulose and reacted with mouse or rabbit antibodies specific for the EGFR, platelet derived growth factor (PDGF), mitogen activated protein kinase (MAPK) and Akt. Immunoblotted proteins were subsequently detected using the ECL reagents as described by the manufacturer with horseradish peroxidase-conjugated secondary antibodies (Hussaini et al., 2000).

Statistical analysis

The difference between the control and the test groups were analyzed for statistical difference using one way analysis of variance (ANOVA) followed by Dunnett post hoc test for multiple comparisons. P values less than 0.05 were considered significant.

RESULTS

Preliminary phytochemical screening

The preliminary phytochemical screening revealed that the residual aqueous fraction contained similar constituents with the CRE. The chloroform fraction was deficient in tannins, saponins and flavonoids (Table 1).

Cell survival assay

Treatment of the cells with EGF resulted in 38% increase in cell count compared with DMSO treated cells (Figure 2). The crude methanol extract (CRE) and its fractions significantly ($P < 0.001$) and concentration- dependently

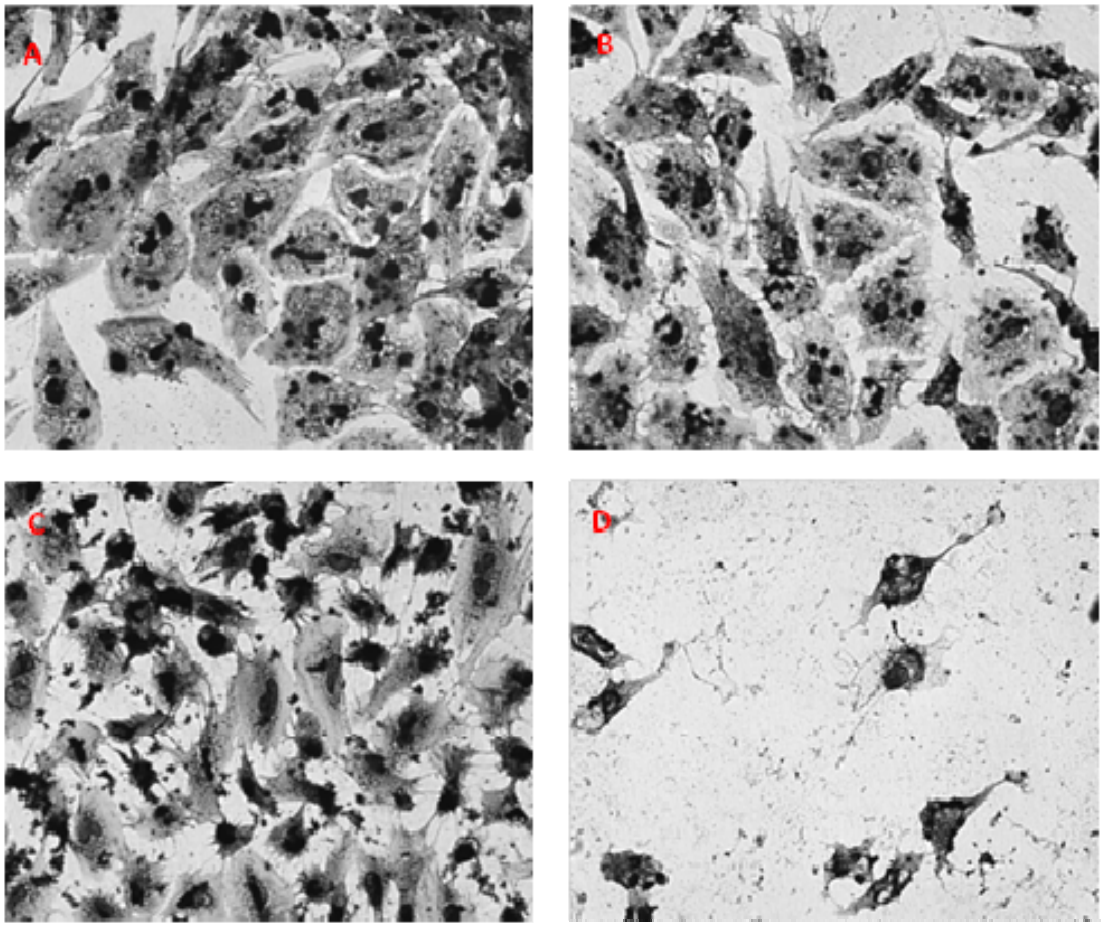

Plate 1. GBM U-1242 MG cells treated with either DMSO or the crude extract. After 48 h cells were stained with crystal violet dye and observed under microscope (×20) A: DMSO; B: CRE 62.5 µg/ml; C: CRE 125 µg/ml; D: CRE 250 µg/ml.

Table 2. IC_{50} values of the Crude methanol root bark extract of *Securinega virosa* and its fractions against Glioblastoma muliforme cells

Extract/Fraction	IC_{50} value (µg/ml)
CRE	41.035
RAF	29.809
NBF	58.502
CCF	4.872

CRE: Crude methanol root bark extract; CCF: chloroform fraction; NBF: n-butanol fraction; RAF: residual aqueous fraction.

decreased cell viability of the U-1242 MG cells in the presence and absence of EGF (Plate 1, Figures 1 and 2). The highest activity was observed with the chloroform fraction with an IC_{50} value of 4.87 µg/ml (Table 2).

Western blot analysis

The crude methanol root bark extract (CRE) of *S. virosa* blocked the phosphorylation of EGFR at tyr 1068 site. It

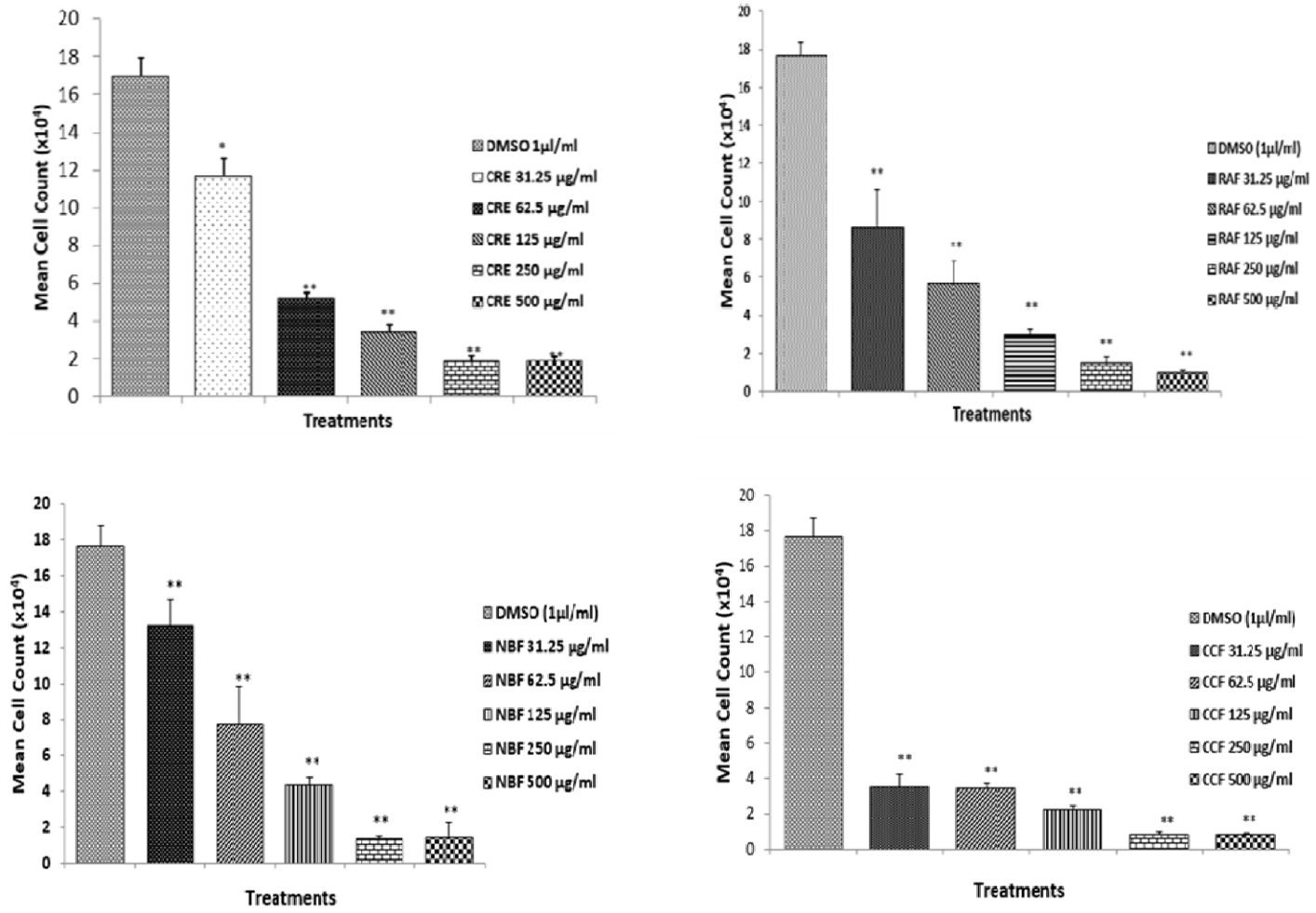

Figure 1. The effect of crude methanol root bark extract of *Securinega virosa* and its fractions on cell survival of GBM cells U-1242 MG. DMSO: Dimethyl suphoxide; CRE: crude methanol root bark extract; RAF: residual aqueous fraction; CCF: chloroform fraction. Data presented as mean ± SEM; *P < 0.05; **P < 0.001; n=4.

also blocked the phosphorylation of MAPK (Figure 3). The CRE, residual aqueous fraction and n-butanol fraction blocked the phosphorylation of EGFR at tyr 1045. However, the activity was more remarkable with the CRE and residual aqueous fraction. Only the crude extract and the residual aqueous fraction remarkably blocked EGFR phosphorylation at Tyr 1068 site (Figure 4). The CRE significantly blocked PMA-induced transactivation of EGFR at Tyr 1068 site. The effects produced by the residual aqueous and n-butanol fractions were less remarkable (Figure 5). The CRE significantly blocked the phosphorylation of platelet derived growth factor receptor (PDGFR). The extract and the residual aqueous fraction remarkably blocked PDGF-induced transactivation of EGFR at Tyr1068. Conversely, the extract as well as its fractions produced no effect on the phosphorylation of Akt (Figure 6).

DISCUSSION

In the present study, the effect of the methanol root bark extract of *S. virosa* and its fractions on GBM cell survival was evaluated. The extract and its fractions possess significant activity against the GBM cells viability in the presence of EGF. GBM is the most common and aggressive primary CNS tumor with a median survival of 15 months and an average incidence rate of 3.19 in every 100,000 population (Thakkar et al., 2014). GBM cells are highly proliferative and diffusely invade surrounding brain

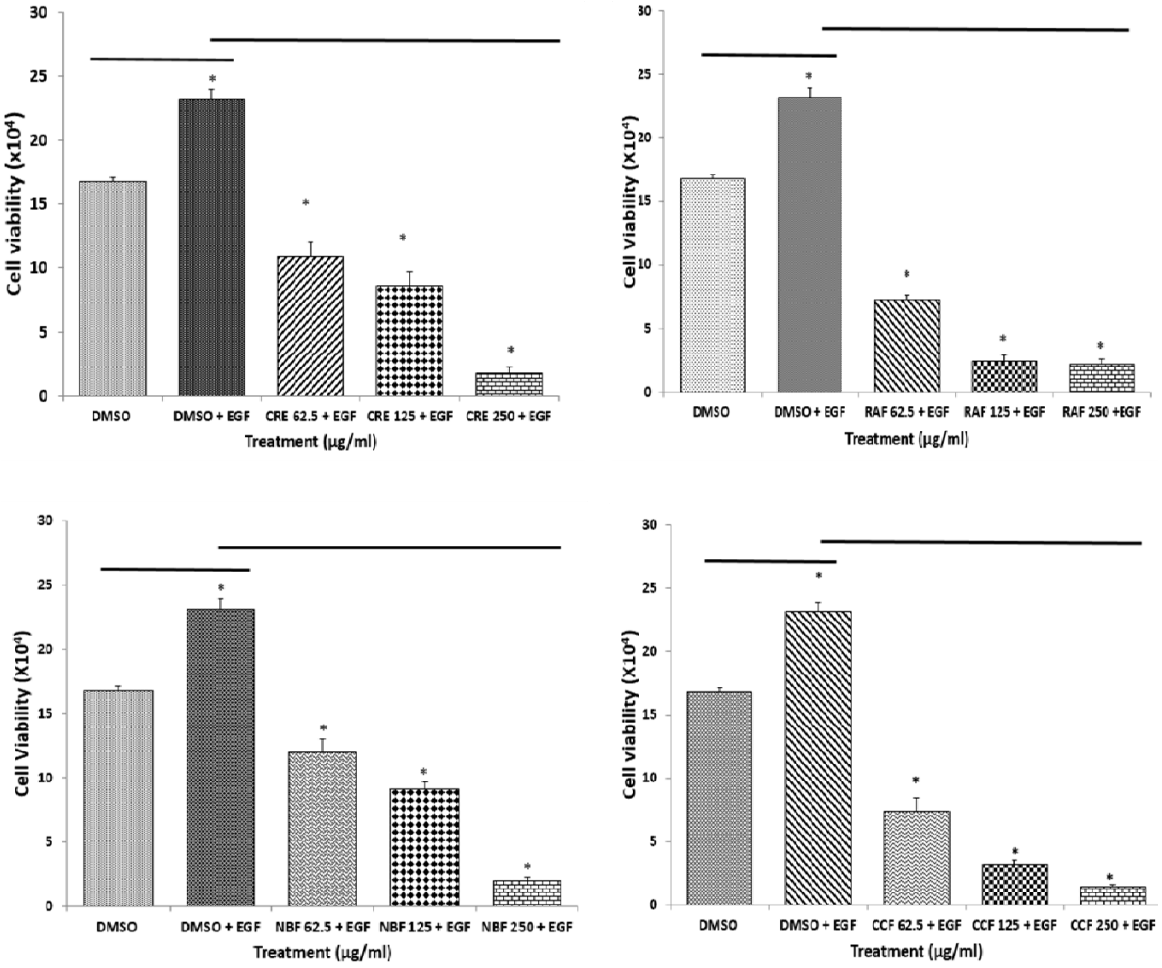

Figure 2. The effect of crude root bark extract of *Securinega virosa* and its fraction on viability of U-1242 MG GBM cells treated with epidermal growth factor. Cell count was carried out 48 h after the treatment. DMSO: Dimethyl sulphoxide; EGF: epidermal growth factor; CRE: crude methanol root bark extract; RAF: residual aqueous fraction; NBF: n-Butanol fraction; CCF: chloroform fraction. Data presented as mean ± SEM; *P < 0.001; n=4.

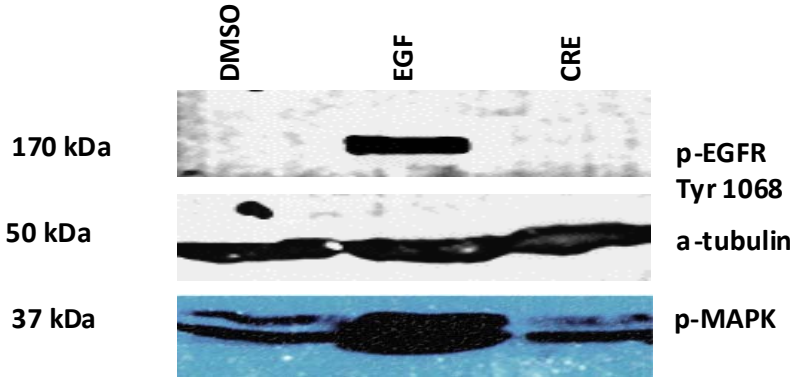

Figure 3. The effect of methanol root bark extract (CRE) of Securinega virosa on phosphorylation of EGFR receptor at tyr 1068 site and mitogen protein kinase (MAPK).

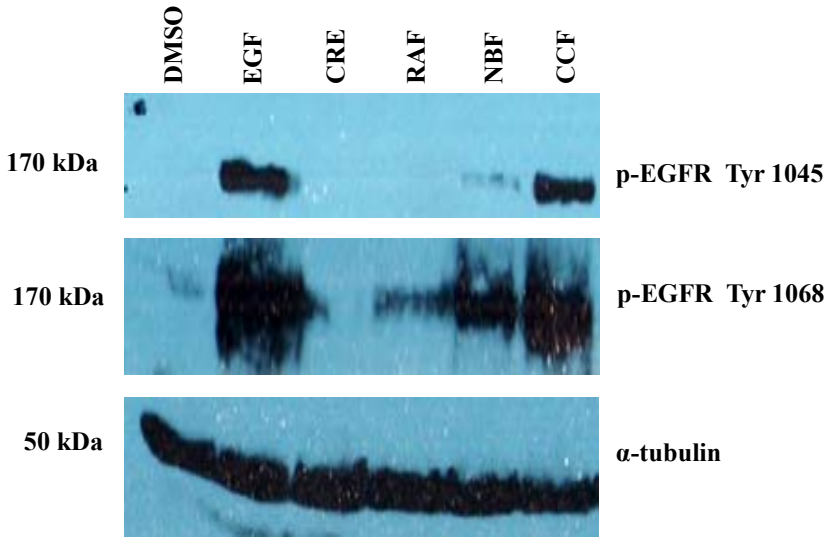

Figure 4. Effect of crude methanol root bark extract of *Securinega virosa* and its fractions on EGF induced phosphorylation of EGFR at tyr 1045 and 1068 sites.

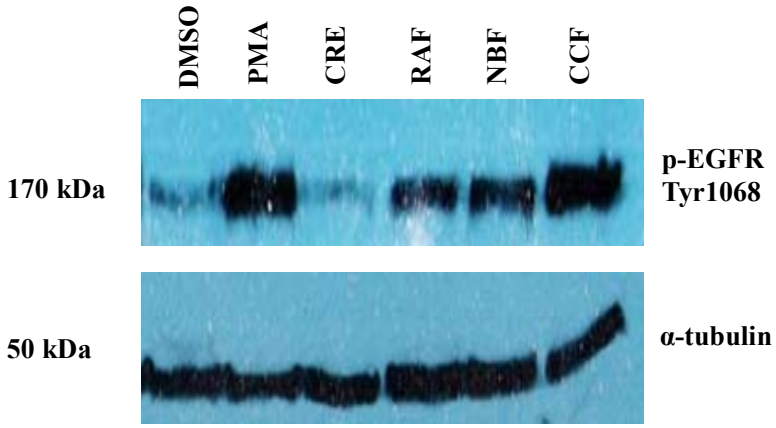

Figure 5. Effect of methanol root bark extract of *Securinega virosa* and its fractions on PMA- induced transactivation of EGFR at tyr 1068 site.

structures, thereby making complete surgical resection practically impossible (Barcellos-Hoff et al., 2009). Furthermore, majority of GBMs are intrinsically resistant to most forms of radiation and chemotherapy, thus rendering the standard arsenal of anticancer treatmentsrather ineffective (Reardon and Wen, 2006). The failure of the conventional therapies for GBM to target tumor cells exclusively, make their efficacy limited by non-specific toxicities (Sampson et al., 2009). Therefore, killing tumor cells efficiently and with less toxic effect is the ultimate target of GBM chemotherapy and will require the adoption of targeted therapeutic approach as against the deployment of cytotoxic arsenal (Zahonero and Sánchez-Gómez 2014).

GBMs commonly overexpress oncogenes such as EGFR and PDGFR and contain mutations and deletions of tumor suppressor genes such as phosphatase and tensin homolog (PTEN) and tumour suppressor protein 53 (TP53), all of which can have an impact on the activation state of signal transduction pathways that influence their biological behaviour (Mischel and Cloughesy, 2003). EGFR is a tyrosine kinase receptor which is over-expressed in cancer cells, and has been identified as a target of tumor therapy. 50 to 60% and

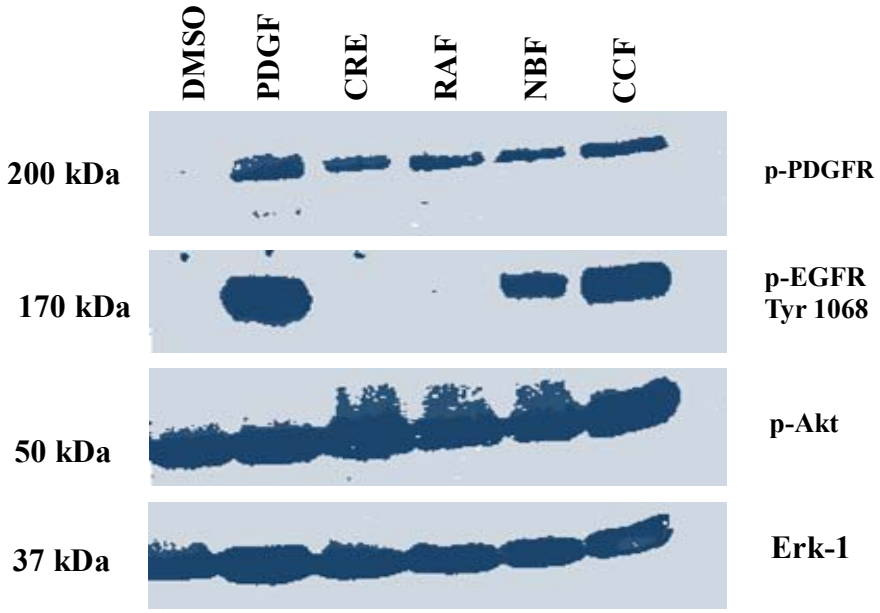

Figure 6. Effect of methanol root bark extract of *Securinega virosa* and its fraction on PDFR-induced phosphorylation of PDGFR, transactivation of EGFR at tyr 1068 receptor site and phosphorylation of Akt.

40% of GBMs have overexpression and amplification of the EGFR, respectively (Ohgaki et al., 1995). The EGFR or ErbB family belongs to subclass I of the superfamily of the receptor tyrosine kinases. Receptor tyrosine kinases represent an important subclass of these transmembrane proteins, with the EGFR being the most prominent representative. The EGFR controls a wide variety of biological processes such as cell proliferation, differentiation, and migration and modulation of apoptosis (van der Geer et al., 1994).

The EGFR variant III is the most commonly occurring mutation of the EGFR in the glioblastoma and is present in 25 to 33% of all GBM (specifically in those showing EGFR receptor amplification and overexpression) (Aldape et al., 2004). This variant is a product of the deletion of exons 2 to 7 and consists of an in frame deletion of 801 bp from the extracellular domain of the EGFR that splits a codon and produces a novel glycine at the fusion junction, rendering the mutant receptor incapable of binding any known ligand (Gan et al., 2013). The mutation encodes a constitutively active tyrosine kinase which enhances tumorigenic, cell migration and confers resistance to radiation and chemotherapy (Chu et al., 1997; Sampson et al., 2009). The absence of this mutation in normal tissues makes it a potential target for tumor specific therapy (Pelloski et al., 2007). Therefore, concerted efforts are been channeled towards the development of anti-EGFR III agents including strategy such as immunotherapy and antibody neutralization (Reardon et al., 2014).

The methanol root bark extract and its residual aqueous fraction blocked the phosphorylation of the EGFR at tyrosine 1045 and 1068 sites. This suggests the involvement of these sites in the activities of the extracts. The blockage of PDGFR phosphorylation is an indication that the activity of the extract may not be limited to EGFR. Similarly, PMA induced transactivation of EGFR at tyr 1068 were also blocked. PMA has been reported to phosphorylate the EGFR at Tyr1068 through a PKC/c-Src-dependent pathway resulting in the activation of MAPK and increase in cell proliferation in glioblastoma cells (Amos et al., 2005).

MAPK phosphorylation was also blocked by the crude methanol root bark extract. The blockade of MAPK phosphorylation by the crude methanol extract suggests that its activity may involve some downstream mediators. However, the extract and its fractions did not affect the phosphorylation of Akt suggesting that their activity may not involve PI3k/Akt pathway.

Although the chloroform fraction gave the highest activity in terms of reduction in cell viability with an IC_{50} of 4.87 µg/ml, its mechanism of action may not involve EGFR, PDGFR, MAPK or Akt; but may be a necrotic phenomenon.

The ability of the extract and fractions to block the phosphorylation of EGFR and PDGFR suggests that they

may be acting via tyrosine kinase receptors. However, there more pronounced activity on EGFR, there blockade of PMA and PDGFR mediated transactivation of EGFR at Tyr1068 suggest that they may be more specific for the EGFR.

Alkaloids, tannins, saponins and flavonoids have been reported to possess anticancer activities (Stevigny et al., 2005; Miyamoto et al., 2003; Kaskiw et al., 2009; Ren et al., 2003). Virosecurinine and viroalloscurinine alkaloids isolated from the leaves of *S. virosa* have been shown to possess some cytotoxic activity (Tatematsu et al., 1991). Interestingly, ethyl acetate fraction which was found to be deficient in alkaloids did not show significant anti-GBM activity (data not shown), suggesting that alkaloids may be responsible for the observed activity. It is therefore plausible to suggest that these phytochemical constituents found to be present in the CRE and fractions of *S. virosa* may be responsible for the anticancer potential of the root of *S. virosa*.

The findings of this study lend some credence to the ethnomedical use of the root of the *S. virosa* in the management of cancer. Further work will involve toxicity evaluation of the extract and its fractions in non-cancerous cell lines as well as isolation of the bioactive principle(s) responsible for these activities, particularly, the alkaloids which have been previously documented to exhibit anticancer property.

Conflict of Interest

The authors have not declared any conflict of interest.

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