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RESEARCH ARTICLE

Fluoride level in drinking bottled waters in the Republic of Macedonia

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ABSTRACT

A vast majority of people in Macedonia use bottled water for drinking. Use of bottled water without knowing the F level may expose children to dental caries risk if the F level is lower than optimal or to dental fluorosis if the F level is too high. To determine the content of fluoride in drinking bottled water available in the country. Thirty-five commercial brands of bottled water (12 out of 23 domestic production and imported brands) were procured from bigger markets in the Republic of Macedonia. Determination of the content of fluorine is performed using ion selective electrode (Thermo Orion Ion Plus Fluoride Electrode) and Ionometer (pH/ISE meter - Thermo-Orion) of the public health Institute. The content of fluoride in packaged water from domestic production ranged from 0.035 in Spring to 1.086 in vision with an average 0.368 (± 0.305), while imported bottled water ranged from 0.032 in ordinary water ROSA to 2.220 in bottled water KOM, with an average 0.631 (± 0.497). There was a wide variation in the F levels in the different brands of bottled water. Parents that use bottled water to prepare powdered milk for babies and baby foods should be aware that it may contain higher concentrations of fluoride and put their child at risk the occurrence of dental fluorosis.

Keywords: Bottled drinking water; Fluoride; Ion-selective electrode; Macedonia.

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INTRODUCTION

Proper use of fluoride contributes to the improvement of oral health worldwide and in our country [1]. The drinking of water is a principal means by which fluorine is introduced into the human body and only 6.8% of the population receives optimally fluoridated naturally water main by the public water systems [2].

The drinking water in the country has a low content of fluorine, which is correlated with high average

DMFT score of 6.88 among the 12 year olds of our population. The concentration of fluoride in drinking water from public water utilities in the country moves from 0-0.4 mg/l [3, 4].

Water used for drinking purpose, directly or indirectly by addition into juices and food is an important source of intake of fluoride in the body. Nowadays, there is a trend of increased use of natural drinks because of the development of a passion for exercise, fitness, frequent travel and the trend of consuming food out of home. All these trends along with people's concerns about the taste and quality of water from public water supply systems, contribute more people to used bottled water.

The recent floods that occurred in the country, which were followed by a public invitation from the Institute of Public Health of the citizens not to consume drinking water from a public water supply in the flooded areas and regions, contributes to increased consumption of the bottled drinking water.

Besides that our country is rich in water [4], the amount of imported bottled water is continuously increasing in the last six years [5] (Figure 1).

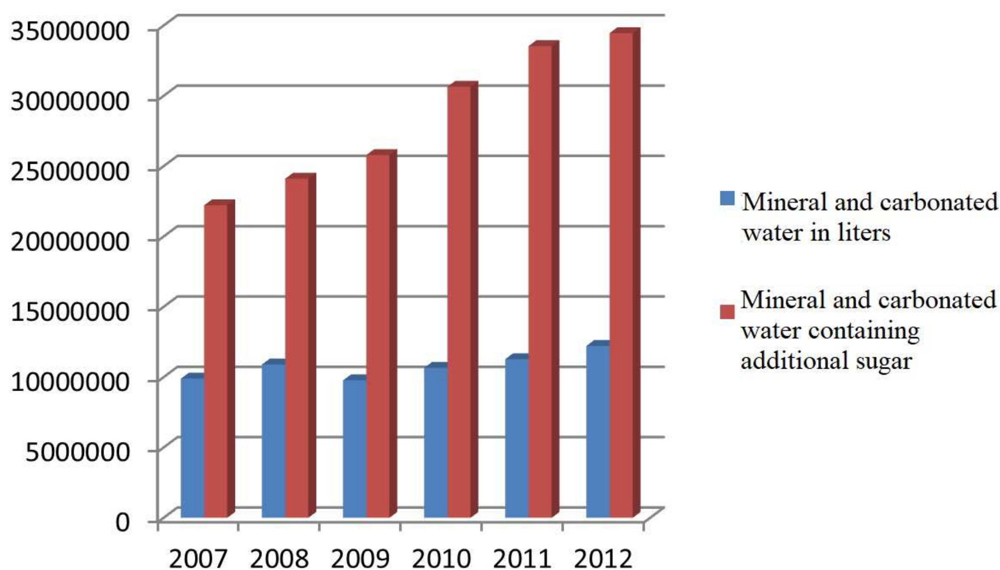

Figure 1. Imported mineral and carbonated water for 2007-2012 expressed in liters.

The concentration of some elements, such as calcium, sodium, iron, silver and aluminum in bottled water are regulated in most countries, one of which is ours. The appearance of fluorine in drinking water varies depending on the type/composition of the soil.

The content of fluoride in bottled water can be very variable and if above optimal levels may have a negative impact especially on the oral health of children who drink bottled water as the primary source of drinking water.

According to WHO recommendations [6] and also according to our current regulations [7], the content of fluorine in water drinking to 1.5 mg/l. The protective role of fluoride in drinking water from the cavities is most apparent at concentrations of 0.8-1.2 mg/l.

Because nowadays people consume more bottled drinking water, the need to determine the content of fluoride in it. Aim of the paper is to determine the content of fluoride in packaged drinking water available in the market in the country. These data will be useful as dentists dealing with clinical practice as well as those working in public health and to use when you need to implement fluoride prophylaxis of individual or mass level.

MATERIAL AND METHODS

During 2008, 35 commercial brands of bottled water were purchased from major markets in the Republic of Macedonia. After shaking the bottle with water, 1 ml of each sample is taken and mixed with 0.1 ml Total Ionic Strength Adjustment Buffer. The concentration of fluoride in all 35 samples was determined using the ion selective electrode (Thermo Orion Ion Plus Fluoride Electrode) and Ionometer (pH / ISE meter - Thermo-Orion) of the Public Health Institute. Chemical analysis was used 10% TISAB Aluminon. Fluorine solutions standards with a concentration of 0.01 to 1.00 mg/l were used to calibrate measurements.

RESULTS

From 35 packaged water, 12 (34%) was produced in Macedonia, respectively Table 1 presents the most widely used brands of bottled water in our country, the established concentration of fluoride, and tagged values for fluoride and pH of the declaration. The concentration of fluoride in bottled water from domestic production is within the lowest value of 0.035 for Spring to the highest value of 1,086 for Vizijana.

Table 1. The content of fluorine in 12 packaged water from domestic production.

Brand of bottled water	Source of water	Type of water	Concentration of fluoride	Concentration of fluoride marked on the declaration	pH value
Vizijana	Klechovce village	Natural	1,086	1.00	not indicated
Kozuvcanka	Mrezicko village	Spring	1,046	not indicated	6.5
DobraVoda	Topolovic	Natural	0,81	not indicated	not indicated
Akva Kokino	Nagoricane village	Natural	0,34	not indicated	not indicated
Pela Rosa	Kremenica	Natural	0,32	0.14	6.6
Germina	Germijan village	Natural	0,3	not indicated	not indicated
Pelisterka	Medjitlija village	Natural	0,149	not indicated	7
Davina	Skopje		0,105	0.4	7.5
Gorska	Trnskot	Spring	0,084	not indicated	6
Ladna	Breza-Lisec	Spring	0,080	not indicated	not indicated
Aqua Kiss	Kochani		0,071	not indicated	not indicated
Izvorska	Vasov Grad	Spring	0,035	not indicated	not indicated

From 12 branded packaged water (domestic production), only three had pointed the content of fluoride on its declaration, from which two did not correspond to the content that we have determined.

Table 2 presents imported packaged water with the concentration of fluorine which we have determined and it ranged from the lowest value of 0.032 mg/l in bottled water Rosa originating from Serbia to 2.220 in water Kom originating from Bulgaria.

As for the quality of labeling of packaged water, 17 (48.5%) packaged water not emphasized fluorine content on their label, while 18 packaged waters emphasized contents of fluorine, in 8 that does not correspond to the content we have determined.

Table 3 presents the average, maximum and minimum values that we received for mineral waters and those imported from domestic production.

Table 2. Content of fluoride in 23 imported packaged water.

Brand of bottled water	Source of water	Country of origin	Concentration of fluoride in the water	Concentration of fluoride marked on the declaration	pH value marked on the declaration
Kom	Barzia village	Bulgaria	2,22	2.2	9.0
Zlatibor	Spring on the mountain Zlatibor	Serbia	1,52	not indicated	not indicated
Knjaz Milos	Arangelovac	Serbia	1,41	1,3	not indicated
Heba	"HEBAA" Bujanovac Spa	Serbia	1,28	1,45	not indicated
Klokoti	Vitina	Kosovo	1,15	not indicated	not indicated
Studenac	Earls springs Lipik	Croatia	1,07	1,5	not indicated
Ozkainak	YaŞam Kaynaği	Turkey	0,935	0,72	6.5
Uludag	Caybasi Koyu	Turkey	0,81	not indicated	not indicated
Bursa	Bursa	Turkey	0,79	not indicated	not indicated
Jamnica	Spring Jamnica, on the river Kula, near the Karlovac city	Croatia	0,77	0,9	not indicated
Voda Voda	Spring on the river in the mountain Suvobor	Serbia	0,71	0,48	7.23
Radenska klasik	Kraljevi Vrelec	Slovenia	0,47	0,5	not indicated
Lutraki	Lutraki Spa	Greece	0,325	not indicated	8.31
Studena	Psunj, spring Studena	Croatia	0,227	0,25	not indicated
Gala	Maljen mountain	Serbia	0,25	not indicated	not indicated
Akva Viva	"PARK" Arangelovac	Serbia	0,123	0,18	not indicated
Prolom	Prolom Spa	Serbia	0,12	0,12	9.2
Korpi	Akarnan mountains in Western Greece	Greece	0,075	not indicated	7.3
Evian	From the heart of the Alps	France	0,069	0,07	7.2
Jana	Sv.Jana source of the river Kupa	Croatia	0,06	0,04	not indicated
San Benedeto	Dolomiti, San Pellegrino, thermal springs	Italy	0,05		7.68
Vikos	Near the canyon Vikos	Greece	0,05		7.62
Rosa	Vlasina-Topli Do	Serbia	0,032		not indicated

Table 3. The average, minimum and maximum content of fluoride in bottled water.

Bottled water	Average value	Standard deviation	Minimum value	Maximum value
Imported water	0,631	± 0,497	0,032	2,220
Domestically produced water	0,368	±0,305	0,035	1,086

DISCUSSION

Several authors have dealt with the determination of the concentration of fluoride in the water packaged in their countries [8-12]. The concentration of fluorine in packaged Evian water 0.15 mg/l in the study of Tombaet al. [8] at the study Zohouri et al. [12] as in our study it is 0.06 mg/l.

Because the method for analyzing fluorine is similar in all studies, differences between measured concentrations of fluoride in different studies may be explained as a variation of the content of fluoride over time, depending on the source of bottled water, as well as seasonal fluctuations.

Healthcare professionals should be aware that the value of the tags cannot be credible as this study demonstrated in 44.4% of the branded packaged water no alignment between the determined value of fluorine and that pointed on labels. The large number of domestic manufacturers of packaged water does not emphasize the content of fluoride on their declaration because they are legally required to highlight the content of fluoride on the label if it is greater than 1 mg/l.

According to the Rulebook on the special requirements for natural mineral water [16] Article 14 states that if the natural mineral water contains more than 1 mg/l should be appointed to the product or another prominent position "contains fluoride". If natural mineral water contains more than 1.5 mg/l fluoride should be indicated that the product is not suitable for regular / repeated use of infants and children under 7 years old. According to our analyzes we conducted on natural mineral water from home production only Kozuvcanka (1.046 mg/l), vision (1.086 mg/l) and Good Water (0.810 mg/l) contain higher values of fluoride that are within the recommended values of WHO and our legislation.

It is also very important water consumers to have accurate information on the concentration of fluoride in the water, which they consume. Having in mind this situation, we recommend packaged water be analyzed at least twice a year at the Institute of Public Health of the Republic of Macedonia or in other accredited independent laboratory.

The natural mineral waters with low or very low content of fluorine, like type of Gorska, Aqua Kokino, Ladna could be used by the general population including child population. It is worth noting that the first Cold bottled water is recommended for the youngest by the Association of Psychiatrists of Macedonia.

When we evaluate the effect of the consumption of bottled water on total intake of fluoride in one individual, three factors should be considered:

1. The amount of bottled water consumed daily.
2. The intake of fluoride from other sources, such as swallowing toothpaste with tooth brushing and food with high content of fluorine.
3. The use of bottled water for preparing milk feeds and dissolution of the pulp food for newborns.

CONCLUSIONS

1. Manufacturers of bottled water did not emphasize the content of fluorine labels or hoist that is incorrect.
2. Dentists should be aware of the concentration of fluoride in the water, which patients drink from their childhood, whether it is packaged or tap water.
3. Parents who use bottled water to prepare the milk meal for their babies, should be aware about the fluoride level in water used for meal preparation. Using water that contains high concentrations of fluoride, they will expose their children to the increased risk for dental fluorosis.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

Immunological and biochemical characterization of chitinase with protease activity against Rapither AB

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Ethical considerations: All these studies were conducted under IBSC guidelines and approved by IBSC committee of Savitribai Phule Pune University, Pune, India.

ABSTRACT

The objective of our study is to confirm and isolate the enzyme chitinase from bacterial samples that are present in different soil samples of Baramati region, Maharashtra and determined its antimicrobial activity of chitinase with protease activity against Rapither AB in virally infected human whole blood samples. In this study, soil samples of different regions of Baramati were collected and identified chitinase enzyme on the basis of zone inhibition in chitin agar plates and finally confirmation through Bergey manual method. For these studies, quantifying the protein content including crude enzyme of bacterial colonies using Nanodrop method. In addition, protease activity of chitinase crude enzyme was determined against Rapither AB and estimated its proliferation rate including total cellular content in virally infected human whole blood samples. After confirmation of chitinase enzyme through various biochemical tests (protein content) and showed higher or rich amount of protein including crude enzyme at a very low concentration. In contrast, protease at higher concentration showed declined in proliferation rate including total cellular content in virally infected human whole blood samples. Overall studies indicated its antimicrobial activity of chitinase with protease activity against Rapither AB.

Keywords: Chitinase; Protease; Rapither AB; Proliferation.

INTRODUCTION

Chitin (polymer of N-acetylglucosamine) is one of the main structural component of the cell wall of fungi. It is crystalline in structure and is generally observed as well as studied through X-ray diffraction technique [1]. This chitin is directly bound to polysaccharides including protein components as well. As per the literature, three forms of arrangement of chitin i.e. α, β and γ are reported [1, 2]. Normally, these chitin components does not able to accumulate in the environment because of the presence of various bacterial chitinases which are present abundantly especially in soil. These enzymes are one of the most reliable candidates that are responsible for degrading chitin molecules that are present in the cell walls of fungi as well as the exoskeletons of insect. One of the useful aspects of chitinase enzyme is for bioconversion of chitin into active pharmacological products especially N-acetylglucosamine and chitooligosaccharides [1-3]. These products are

generally required for various immunopharmacological activities (antioxidants, wound healing, antimicrobials, immunoenhancers etc.) and also activates host defense system including controlling the level of blood cholesterol [3-4].

Chitinases are widely distributed in bacteria such as *Serratia, Chromobacterium, Klebsiella, Bacillus, Pseudomonas, vibrio, Arthrobacter* etc. Number of bacterial species are reported and claimed as good source of bioactive compounds i.e. antibiotics, enzymes etc. The production of chitinolytic enzymes has been identified in various bacterial species [5, 6]. Now a day, scientists focused on chitinase enzymes for various industrial, clinical, and pharmaceutical purposes [5-8]. In this regard, we tried to explore the effect of protease extracted from chitinase enzyme containing Rapither AB.

One of the parasitic diseases especially Malaria is spread to people through the bites of infected female anopheles mosquitos. Till now, only four main strains of malaria are reported, out of these the most serious strain is falciparum. In comparison with other three strains (*vivax, ovale* and *malariae*) are less serious as compared to falciparum. But we still need to be prevented and treated promptly [9, 10]. In Asian and African countries, Malaria is considered as one of the major life threatening disease [11]. Recently, Rapither AB used as anti-malaria segment or used in the form of injection as medicine for the treatment of malaria and other conditions [12]. In this regard, we determined its protease activity of chitinase enzyme containing Rapither AB in virally infected human whole blood samples.

MATERIALS AND METHODS

Microorganisms and culture conditions

In this study, we identified various bacterial (i.e. *Bacillus* species, *Pseudomonas* species, *Streptomyces* species etc.) samples that are reported in soil and showed high chitinase activity as mentioned in the literature. These bacterial species were identified as well as isolated in our lab from various soil samples (rhizosphere of pomogranate, jawar, ground and fish market) collected aseptically from different regions of Baramati, Maharashtra, India.

Colloidal chitin

For the preparation of colloidal chitin using concentrated hydrochloric acid (HCl, 120 ml) and it was dissolved slowly in chitin powder (10 g). This sample was put in rotary incubator (180 rpm) at 37 ºC for 1h. Filter the solution through glass wool/muslin cloth and then dissolved in equal quantity of ethanol (50%). Mix properly and then transfer through filter paper. Finally, add distilled water and then finally reached its final concentration with pH 7. Collect the samples and store at 4 ºC. Finally, this solution was used for media preparation [13].

Colloidal chitin agar medium and purification of chitinase enzyme

For screening of chitinase producing bacteria, the agar medium amended with colloidal chitin was used. The medium consists of disodium hydrogen phosphate (6 g), potassium dihydrogen phosphate (3 g), ammonium chloride (1 g), sodium chloride (0.5 g), yeast extract (0.05 g), agar (15 g) and colloidal chitin (1%) dissolved in one litre of distilled water that are prepared aseptically. The confirmation of these bacterial colonies showing clearance zones on a creamish background and were considered as chitinase-producing bacteria [13].

After confirmation, inoculate chitinase producing colonies in colloidal chitin broth for 48 h rotatory incubation. Thereafter, centrifuging at 10,000 rpm for 20 min, supernatant was collected and then add ammonium sulfate in small quantity. Incubate these samples overnight at 4ºC. Again, centrifuging the samples at the same speed, again collect the supernatant and undergoes dialysed for 24 h at 4°C with continuous stirring and occasional changes of the buffer. The resultant dialysate was chitinase crude extract.

Estimation of biochemical tests (protein content)

In this study, colonies of chitinase were collected and estimated the protein content. Colonies of chitinase were added in test tube and then add extraction buffer (i.e. 20 mM Tris HCl) dissolved in PBS (pH 7.4). Incubate chitinase colonies along with extraction buffer for 5 minutes at room temperature and then centrifuged (5500 rpm; 8 minutes at 4°C). Finally, the supernatant was collected after centrifugation and then add similar volume of ice cold acetone.

Incubate the solution for 10-15 minutes at room temperature and then centrifuging at the same speed. Thereafter, pellet was taken and washed with ice cold acetone in order to remove the pigments including lipid contents as well. Finally, protein concentration of chitinase colonies was determined by using Nanodrop method [14].

Estimation of protease content

After getting the protein from chitinase colonies that are dissolved in PBS. For crude enzyme collection, centrifuging the protein content (10000 rpm at 4°C for 30 minutes) and then estimate its crude enzyme and then measured its protease content against Rapither AB.

Calorimetric assay was performed for protease estimation using Rapither AB as substrate. For these studies, add equal volume of Rapither AB and crude enzyme extract of chitinase in 2 ml Eppendorf tube. Incubate the test solution for 2-3 h at room temperature. After incubation, trichloroacetic acid (TCA) solution was added in order to stop enzymatic reaction. Centrifuging these samples (crude enzyme containing Rapither AB) and supernatant was collected finally and then add similar volume of sodium hydroxide (NaOH) solution in comparison with trichloroacetic acid (TCA) solution. Incubate it another 20 minutes at room temperature. Thereafter, addition of Folins Colin reagent (0.5 ml) and the intensity of blue color was measured at 700 nm within half an hour using spectrophotometer [15].

Total cellular content

Infected EDTA human whole blood (virally infected) samples (n=5; 50 µl) were collected from Mangal laboratory (Pathology lab, Baramati) was pipetted directly into a eppendorf tube containing variable concentration of protease dissolved in PBS and then incubated at carbon dioxide incubator (37°C, 5% CO_2) for 2 h. After incubation, add red cell lysis buffer (ammonium chloride, potassium bicarbonate and ethylene diamine tetra acetic acid) and then centrifuged. Pellet was taken and washed 2-3 times with PBS. After washing, pellet (leucocytes) dissolved in PBS and studied total cellular content. All these samples were measured at 570 nm using UV spectrophotometer [14-15].

Proliferation assay

In this study, lysed infected human whole blood samples (100 µl) were added in 96 well plate (flat bottom tissue culture plate) and treated with variable concentration of protease isolated from chitinase, enzyme containing Rapither AB as antigen. For 24 h incubation at carbon dioxide incubator, lysed blood samples along with variable concentration of proteases dissolved in PBS containing 10% FBS (Fetal bovine serum). After incubation, add MTT solution (2.5 mg/ml; 10 µl; dissolved in PBS) and then incubated again at carbon dioxide incubator for 4 h. Afterwards, formazon crystals were appeared and settled at the bottom. These crystals were dissolved in DMSO (dimethyl suphoxide) and then its optical density (OD) was measured at 570 nm [14-15].

Statistical analysis

Values are represented in the form of Mean ± standard error. The difference between the control and treated groups of proteases is determined by one way ANOVA test (Bonferroni multiple comparison test).

RESULTS

Estimation of protein and protease content

The results of these studies related to protein content including crude enzyme isolated from the chitinase-producing colonies as shown in Fig. 1 and 2. The results showed that chitinase-producing colonies showed lot of protein content at a very low concentration (10 µl containing 5.342 mg/ml) including crude enzyme (10 µl containing 79-85 mg/ml). In addition, crude enzyme of chitinase-producing colonies containing Rapither AB showed enormous production of protease i.e. 57.81 mg/ml (Fig. 3) as compared to Rapither AB (33.82 mg/ml).

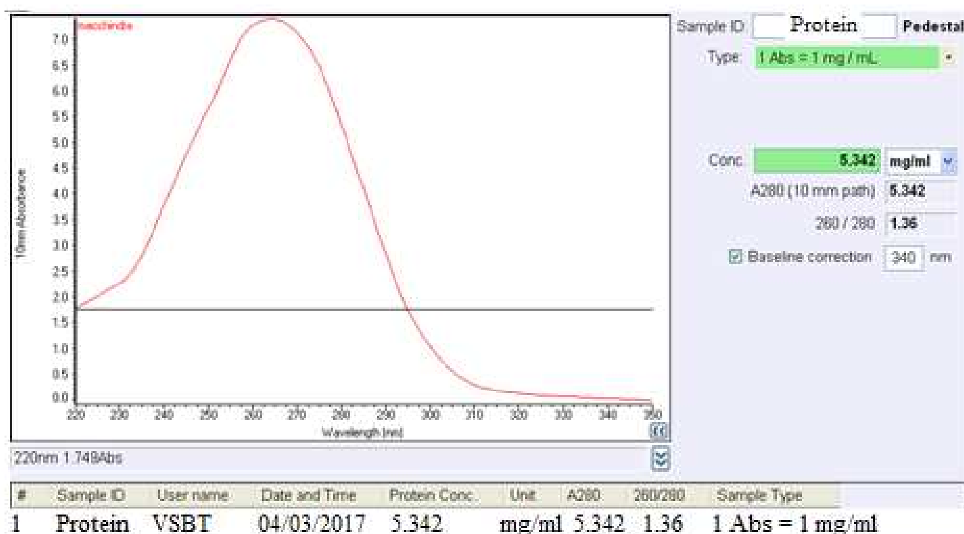

Figure 1. Estimation of protein content from chitinase-producing colonies.

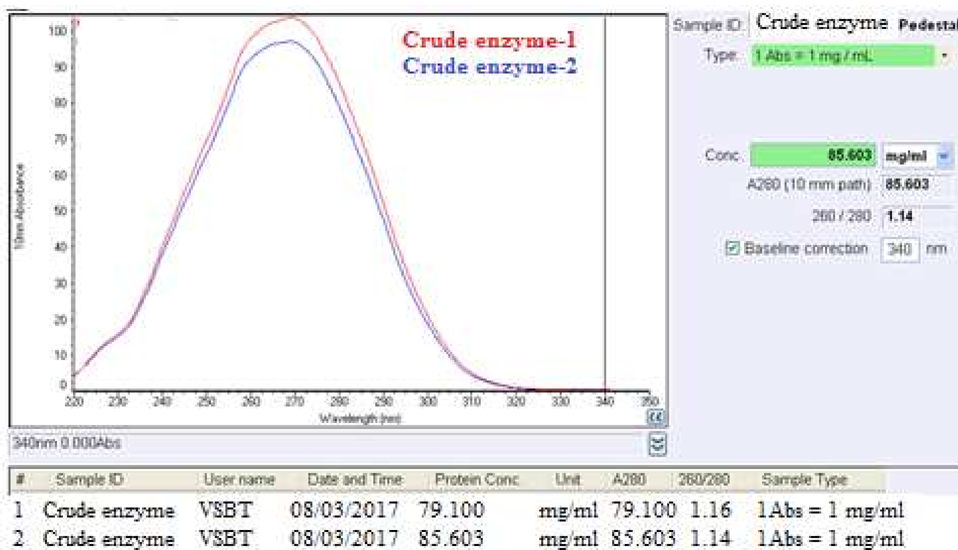

Figure 2. Estimation of crude enzyme extracted from protein content of chitinase-producing colonies.

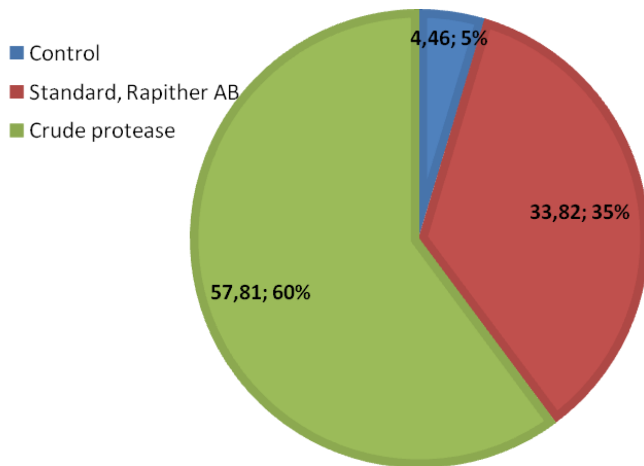

Figure 3. Estimation of protease content from crude enzyme of chitinase colonies containing Rapither AB.

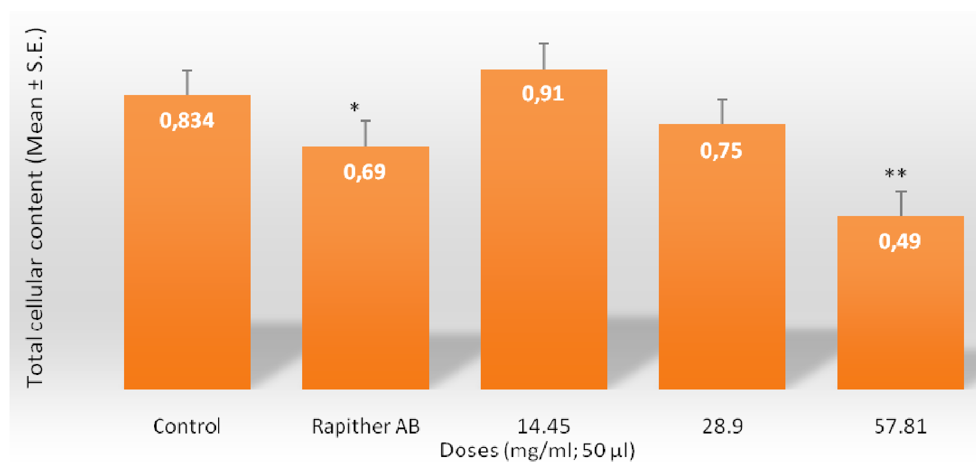

Figure 4. Estimation of total cellular content of protease extracted from chitinase colonies containing Rapither AB on virally infected human whole blood samples. Lysed virally infected human whole blood was cultured with variable doses of proteases. Values are expressed as Mean ± S.E. The difference between control and variable doses of medicinal plant products is controlled by one way ANOVA test (Bonferroni multiple comparison test). *P<0.05; **P<0.01.

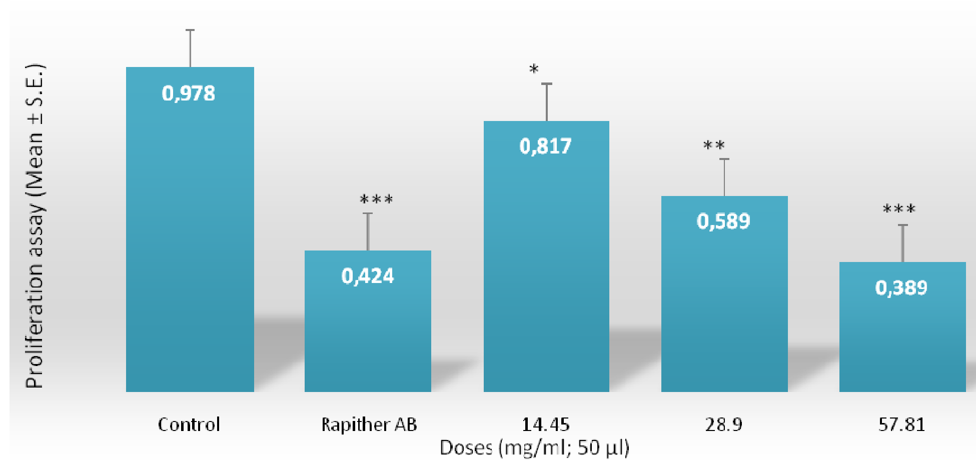

Figure 5. Proliferation assay. Virally infected lysed human whole blood was cultured with variable concentration of proteases. After incubation, centrifuge the samples and add MTT solution (5 mg/ml, 10 µl). Fresh formazan crystals were appeared and settled at the bottom and then finally dissolved in dimethyl sulphoxide (DMSO) in a final volume of 0.2 ml. The optical density (OD) was measured at 570 nm. The difference between control and variable doses of proteases is determined through one way ANOVA test (Bonferroni multiple comparison test). *P < 0.05; **P < 0.01 and ***P < 0.001.

Total cellular content

The results of these studies related to protease isolated from chitinase enzyme containing Rapither AB as shown in Fig. 4. The results showed that protease at higher concentration showed reduction in total cellular content in virally infected human whole blood samples. On the other hand, virally infected sample showed enormous production of total cellular content.

Proliferation assay

The effect of protease using variable concentration on virally infected human whole blood samples and determined its effect on proliferation assay using MTT as shown in Fig. 5. The results showed that protease at higher concentration showed remarkable reduction in proliferation assay as compared to control.

DISCUSSION

As per the literature, chitinases producing organisms have been identified and isolated from various sources i.e. air, water, soil, marine water etc. [16]. In addition, chitinases also identified and isolated from the stomach of certain mammals including humans. As per its applications, chitinases are used as fungicides, bacteriocides, biopesticides, against mosquitoes etc. In the present study, numerous bacteria samples were reported and identified in soil samples and is able to produce various chitinases in order to supply various nutrients especially nitrogen and carbon. These chitinases were generally used for degrading chitin molecules and utilized as an energy source. One of the most dominant and prevalent bacteria i.e. *Serratia marcescens* that is responsible for producing chitinases and showed various immunopharmacological properties e.g. chitinases/chitinase-like proteins that played a crucial role in innate as well as adaptive immune responses [17, 18]. Although nonspecific type of immune stimulatory functions of chitin and its derivatives were reported and considered as potent immunoadjuvant candidate that enhances type 2 adaptive immune response. In this regard, we focused on chitinases especially its crude enzyme against Rapither AB in virally infected human whole blood samples.

From these soil samples, chitinase producers were isolated on colloidal chitin agar medium and its result was totally similar [19]. In addition, chitin acts as a sole carbon source for highest chitinase production [18, 19]. In this study, we isolate chitinase with protease activity containing Rapither AB and testing on virally infected human whole blood samples that will help us in order to understand its anti-viral properties. Firstly, estimated its total protein including amino acids content from chitinase colonies dissolved in PBS and results showed some enhancement at a very low concentration (i.e. 10 µl containing 5.345 mg/ml) using Tris HCl and ice cold acetone. After getting the protein content from chitinase colonies, centrifuging these samples and collect supernatant in the form of crude enzyme and further dissolved in similar volume of injection i.e.Rapither AB. For estimation of protease content using Folin Colins reagent and determined through Nanodrop method. In this study, we determined the interaction between protease and antigen (virally)-specific human whole blood that provides an important signals for the efficient activation or inhibition of T cell response. In order to determine its protease activity by means of proliferation and total cellular content in infected human whole blood samples. The results of these showed that protease at higher concentration showed remarkably decline in total cellular content and proliferation rate in infected lysed human whole blood samples.

These proteases are showed some potential or ability to reduce the burden of various infectious diseases. Recently, proteases derived from micro-organisms including medicinal plant products are the major source for drug development. Most of proteases derived from micro-organisms that are available or still under clinical trials [20]. In view of this, similar results were observed in case of protease extracted from chitinase crude enzyme containing Rapither AB and showed fascinating immunopharmacological property i.e. antimicrobial. Hopefully, this protease also involved in various applications especially for biotechnology. However the isolation and purification of proteases will assist us to recognize the mechanism of various disease models.

CONCLUSION

Immunological finding of proteases extracted from crude enzyme of chitinase containing Rapither AB was very interesting with respect to decline in proliferation rate and total cellular content in virally infected human whole blood samples. Further studies are required pertaining to isolation, characterization and elucidation of structures of these proteases.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between four authors. AG, MP and SS designed the study, wrote the protocol and interpreted the data where MP anchored the field study, gathered the initial data related to his M.Sc. Microbiology dissertation work under AG guidance and performed preliminary data analysis. AG, MP, SS and NP managed the literature searches whereas AG and MP produced the initial draft. The final manuscript has been read and approved by all authors.

CONFLICT OF INTERESTS

Authors have declared that no conflict of interests exists.

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RESEARCH ARTICLE

Evaluation of HPLC-UV-DAD and antiproliferative characteristics of the leaf infusion of *Ximenia americana* Linn.

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ABSTRACT

Ximenia americana (*X. americana*) is reputable for the treatment of various ailments in Nigeria. The hot aqueous extract of *X. americana* leaf (XA) was obtained by infusion. The antiproliferative potential of XA was evaluated employing *Sorghum bicolor* seed radicle as test subject over the period of 48-96 hours. The mean radicle lengths (mm), percentage inhibition and percentage growth were determined. XA was chemically characterized using colour reactions and high performance liquid chromatography with UV-diode array detector (HPLC-UV-DAD). Phytochemical investigation indicated the presence of tannins, saponins and flavonoids. HPLC analysis revealed thirteen peaks with rutin and ferullic acid eluting at 6.886 and 7.796 minute respectively. XA significantly ($p < 0.0001$) inhibited *S. bicolor* seed growth over a period of 48-96 h against the control seeds. At 96 h, XA dose-dependently inhibited seed growth, giving percentage inhibition of 23.24, 29.06, 30.68, 38.27, 49.57, 50.39, 64.60, 79.67 and 82.01% for seeds treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml 16 mg/ml, 24 mg/ml, 32 mg/ml, 40 mg/ml and 48 mg/ml respectively with IC_{50} of 24 mg/ml. Methotrexate 0.167 mg/ml used as positive control gave inhibition of 92.76% at 96 h. This result revealed the potential of XA to inhibit the growth of fast proliferating cells of *S. bicolor* seed radicle.

Keywords: *Ximenia americana*; Antiproliferative; *Sorghum bicolor*; Caffeic acid; Rutin.

OPEN ACCESS

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INTRODUCTION

Cancer generally refers to a group of diseases that cause cells in the body to change and grow out of control. It can spread to other parts of the body through lymph and blood. Breast cancer has been described as the most commonly diagnosed malignancy and the leading cause of cancer-related deaths of women worldwide [1]. Incidence of cancer is on the increase worldwide, with estimated 14.1 million new cancer cases in 2012; female

breast, colorectal and stomach cancers accounted for over 40% of all cases diagnosed globally. Lung cancer accounted for 16.7% of all new cases in men [2].

Ximenia americana belongs to the family Olacaceae. The genus *Ximenia* has eight different species namely *Ximenia roiigi, Ximenia aegyptiaca, Ximenia parviflora, Ximenia coriaceae, Ximenia aculeate, Ximenia caffra, Ximenia americana and Ximenia aegyptica* It is commonly referred to as wild plum and tallow nut. It is a shrub or small tree that grows to about 7 m high, with zigzag branches. The bark is black or grey-brown, smooth when young but becomes rough with age having stiff axillary spines. The leaves are alternate or clustered on spur shoots. The seed morphology is variable. Flowers are green white to greenish yellow, scented and 5-10 mm long in small branched inflorescences [3]. Native names in Nigeria: Hausa (Tsada), Fulani (Chabbull), Tiv (Anomadze) ,Yoruba (Igo) and Benin (Alimo-mamiwota) [4].

The methanolic extract of leaves of *Ximenia americana* has been proven to have anti-diabetic effect in rats [5]. The ethanolic extract of the bark of *X. americana* revealed the presence of the following metabolites; flavonoids, steroids, tannins, alkaloids, phenolic compounds, saponin, terpenoids, and glycosides. It also has activity against *Staphylococcus aureus* and low activity against *Pseudomonas aeruginosa*. Other investigations led to isolation of two compounds. The first one which is new is 3-methyl-1-oxoisochroman-8-carboxylic acid and the second compound which is a known steroid is ergosta-4,6,8,22-tetraen-3-one [6].

The plant has also been reported to have anti-HIV/AIDS related diseases effects such as abscesses, skin rashes, diarrhea and gonorrhea. The anti-HIV/AIDS effect of *Ximenia americana* is due to the fact that it contains oleic, ximenic (hexacos-17-enoic), linoleic, linoleric and stearic acids. Its oil consists of very long chain fatty acids with up to 40 carbon atoms used to manage STIs including gonorrhoea [7].

Literature review reveals that the plant *Ximenia americana* has been alleged to have antineoplastic activities, antimicrobial and anti-inflammatory activities and lots more. The aqueous plant extract of the leaves is used in the treatment of cancer in African traditional medicine [8]. *X. Americana* has a wild varieties of phytochemical compounds which among them are the following secondary metabolites; saponins, flavonoids, tannins, terpenoids, sterols, quinines, alkaloids, cyanogenetics glycosides, cardiac glycosides and carbohydrates in the form of sugars and soluble starch [10]. The essential oils of the leaves of *X. americana* using GC-MS and identified 33 components representing 98% of the total oil. The main constituents analysed are benzaldehyde 63.5%, hydroxybenzylcyanide 13%, isophorone 3.5%. The hydroxybenzylcyanide is known as a primary breakdown products of glucosinolates found mainly in the Brassicaceae family [11]. The fixed oil present in the seeds of *X. americana* [12].

The ethanolic extract was reported to contain polyphenols, cyanogenic acid, glycoside sambunigrin, gallic acid, gallotannins-β-glucopyranose, flavonoids, quercetin, quercitrin (quercetin3-0-α-rhamnopyranoside), avicularin (quercetin-3-0-α-arabinofuranoside), Quercetin-3-0-β-xylopyranoside, quercetin-3-0-(6-galloyl)-β-glycopyranoside. The flavonoids were active both as enzymes inhibitors and DPPH radical scavengers [13]. The aim of this study is to evaluate the phytochemical, high performance liquid chromatography profile and antiproferative activity of the leaves of *Ximenia americana*, a reputable herbal medicine grown in Nigeria.

MATERIALS AND METHODS

Chemicals and reagents

Unless otherwise stated, all chemicals and reagents were of analytical grade and purchased from Sigma Aldrich (Germany). All the solvents for chromatographic purpose were HPLC grade, purchased from Sigma Germany.

Experimental plant

The leaves of *Ximenia americana* used for this study were collected from Bamburu-Gwagwalada Abuja by Mallam Muazzam of the National institute for Pharmaceutical Research and Development Idu Abuja, Nigeria.

It was identified and authenticated by Mr. Akeem Lateef at the Herbarium of the National Institute for Pharmaceutical Research and Development Idu Industrial Area Abuja.

Preparation of plant extract

The fresh leaves of *Ximenia americana* were air-dried at room temperature. The dried sample was pulverized. Then 50 g of powdered sample was weighed and extracted by hot water (1000 ml) infusion in an air tight container for 24 h. The resultant mixture was filtered using a funnel whose exit was tightened with cotton wool. The filtrate was dried over a water bath to yield *Ximenia americana leaves* aqueous extract [14].

Phytochemical analysis

Phytochemical screening was conducted on the aqueous extract for secondary metabolites and the following was present; carbohydrates, tannins, saponins and flavonoids [15].

High performance liquid chromatography analysis

The bioactive constituents of XA was analysed by high performance liquid chromatography (HPLC) with UV diode array detector (UV-DAD). The HPLC consisted of Ultra-Fast LC-20AB equipped with SIL-20AC auto-sampler; DGU-20A3 degasser; SPD-M20A UV-diode array detector; column oven CTO-20AC, system controller CBM-20Alite and Windows LC solution software (Shimadzu Corporation, Kyoto Japan); column, 5µm VP-ODS C_{18} and dimensions (4.6 x 150 mm). The chromatographic conditions included mobile phase: 0.2% v/v formic acid and acetonitrile (20:80); mode: isocratic; flow rate 0.6 ml/min; injection volume 10 µl of 100 mg/ml solution of extract in water; detection UV 254 nm. The HPLC operating conditions were programmed to give solvent B: 20%. Column oven temperature was 40 ^{o}C. The total run time was 30 minutes. Flavonoids and phenolic acid standards such as apigenin, rutin, quercetin, caffeic acid, ferulic acid were employed for the identification of the phytoconstituents of XA by comparing the retention time under similar experimental conditions [16]

Determination of growth inhibitory effect

The modified methods of Ayinde et al. [17] and Chinedu et al. [18] were used for this study. *Ximenia americana* hot aqueous extract (300 mg) was dissolved in 60 ml of distilled water to obtained 50 mg/ml stock solution. Various concentrations (1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml 16 mg/ml 24 mg/ml 32 mg/ml 40 mg/ml and 48 mg/ml) of the extract were prepared. Methotrexate was made to a concentration of 0.167 mg/ml as positive control. Petri dishes were layered with cotton wool and filter paper (Whatman No. 1). Twenty seeds (n = 20) of *S. bicolor* were placed in each of the Petri dishes. The control seeds were treated with 15 ml distilled water. The test seeds were treated with the different preparations of the extract as the seeds in each specific Petri dish received 15 ml of a particular concentration (the seeds in the eleven different Petri dish were treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml, 16 mg/ml, 24 mg/ml, 32 mg/ml, 40 mg/ml and 48 mg/ml concentration respectively. The seeds were incubated in a dark room and observed for growth after 24 h. The mean lengths (mm) of radicle emerging from the seeds were measured after 48, 72 and 92 h. The percentage inhibition was calculated as [(mean radicle length control – mean radicle length treated)/mean radicle length control] ×100. Percentage growth was calculated as 100 – % inhibition. Percentage inhibition and percentage growth at 48, 72 and 92 h for seeds treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml, 16 mg/ml, 24 mg/ml, 32 mg/ml, 40 mg/ml 48 mg/ml, negative control and the positive control methotrexate at 0.167 mg/ml are as shown in Table 1.

Statistical Analysis

The data obtained were expressed as mean ± standard error of mean and analyzed using Graph pad prism (version 7). Two way analysis of variance was used to test for significance. *P*<0.0001 was considered to be significant.

RESULTS

Phytochemical analysis

Extraction of 50 g of *Ximenia americana* powdered sample by hot water infusion yielded 5.6 g (11.2%) of the dried extract. Phytochemical screening indicated the presence of tannins, saponins, flavonoids, and carbohydrates.

High performance liquid chromatography analysis

The HPLC chromatogram of XA shown in Figure 1, showed that thirteen peaks were detected as the constituents with retention times in minutes of 3.530, 4.070, 5.896, 6.886, 7.796, 10.767, 11.823, 13.790, 15.431, 17.898, 18.928, 22.019 and 25.752. Compounds with retention time in minute of 6.886 and 7.796 minute corresponded to rutin and ferullic acid respectively.

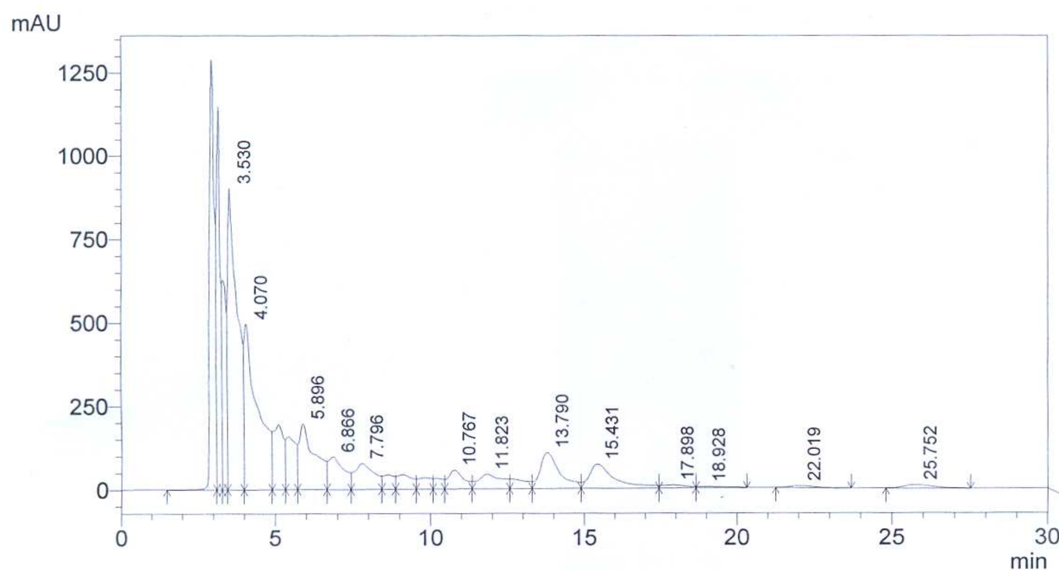

Figure 1. HPLC spectrum of leaf hot aqueous extract of *Ximenia americana* (XA).

The HPLC spectrum showed thirteen peaks with retention times in minutes of 3.530, 4.070, 5.896, 6.886, 7.796, 10.767, 11.823, 13.790, 15.431, 17.898, 18.928, 22.019 and 25.752. Compounds with retention time in minute of 6.886 and 7.796 minute corresponded to rutin and ferullic acid respectively.

Growth inhibitory effects of XA on *Sorghum bicolor* seed

There was an appreciable reduction on the length of radicles of *Sorghum bicolor* seeds treated with the various concentration of the extract. The seed radicle lengths increased with the incubation period of 48-96 h. A rapid and progressive growth was observed in the water control seeds radicle lengths. At 48 h, percentage seed

growth inhibition was 28.43% for seeds treated with 1 mg/ml of HC. Then at 96 h, the mean radicle lengths of the control seeds was 69.50±4.06 mm while the mean radicle length of the seeds treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml 16 mg/ml, 24 mg/ml, 32 mg/ml, 40 mg/ml and 48 mg/ml were 53.35±1.34 mm, 49.30±2.48 mm, 48.18±1.21 mm, 42.90±0.84 mm, 35.05±3.53 mm, 34.48±3.20 mm, 24.60±0.38 mm, 14.13±1.23 mm, and 12.50±1.32 mm as shown in Fig. 2, corresponding to percentage inhibitions of 23.24%, 29.06%, 30.68%, 38.27%, 49.57%, 50.39%, 64.60%, 79.67% and 82.01%, respectively.

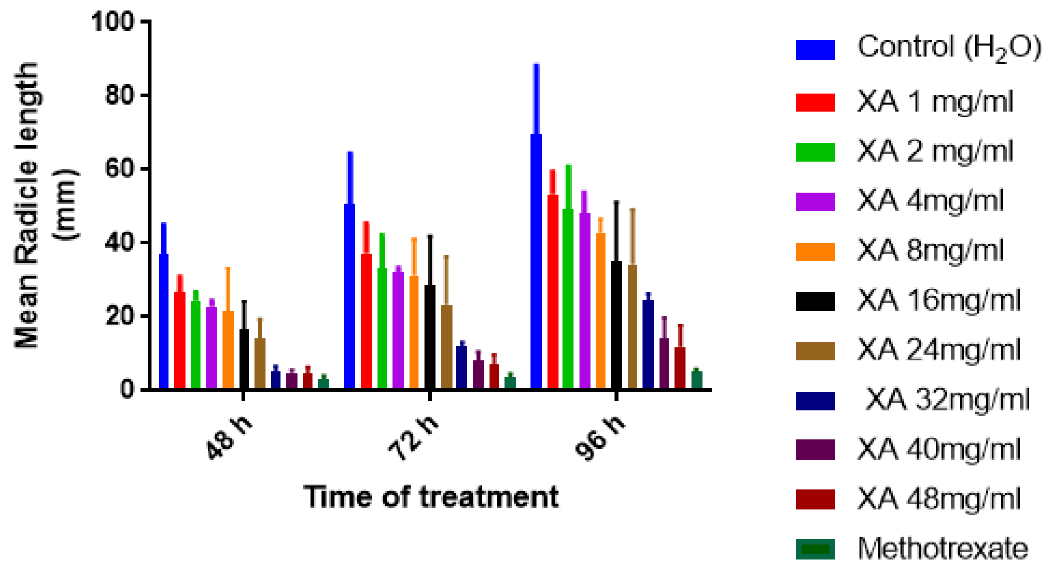

Figure 2. Inhibitory effects of *Ximenia americana leaf* aqueous extract (XA) on the growth of *Sorghum bicolor* seed radical.

Table 1. Mean radical length, percentage inhibition and percentage growth for *Sorghum bicolor* seeds treated with XA.

Treatment	Mean radicle length			% Inhibition*			% Growth†		
	48 h.	72 h.	96 h.	48 h.	72 h.	96 h.	48 h.	72 h.	96 h.
Control (H_2O)	37.28±1.66	50.48±3.00	69.5±4.06	0	0	0	100	100	100
methotrexate	3.28±0.17	3.73±0.18	5.03±0.21	91.20	92.61	92.76	8.80	7.39	7.24
XA (1 mg/ml)	26.68±0.92	37.38±1.75	53.35±1.34	28.43	25.95	23.24	71.57	74.05	76.76
XA (2 mg/ml)	24.38±0.48	33.03±1.98	49.30±2.48	34.60	34.57	29.06	65.40	65.43	70.94
XA (4 mg/ml)	22.90±0.34	32.00±0.31	48.18±1.21	38.57	36.61	30.68	61.43	63.39	69.32
XA (8 mg/ml)	21.58±2.55	31.45±2.13	42.90±0.84	42.11	37.70	38.27	57.89	62.30	61.73
XA (16 mg/ml)	16.53±1.70	28.00±2.86	35.05±3.53	55.66	44.53	49.57	44.34	55.47	50.43
XA (24 mg/ml)	14.03±1.18	23.35±2.82	34.48±3.20	62.37	53.74	50.39	37.63	46.26	49.61
XA (32 mg/ml)	5.03±0.36	12.25±0.22	24.60±0.38	86.51	75.73	64.60	13.49	24.27	35.40
XA (40 mg/ml)	4.88±0.17	8.30±0.49	14.13±1.23	86.91	83.56	79.67	13.09	16.44	20.33
XA (48 mg/ml)	4.95±0.40	7.25±0.57	12.50±1.32	86.72	85.64	82.01	13.73	14.36	17.99

*Percentage Inhibition = [(mean radicle length of control - mean radicle length of treated) / mean radicle length of control] X 100. †Percentage growth = 100 - percentage inhibition, n = 20. $p<0.0001$.

Therefore, the inhibitory effect of XA was concentration-dependent. Inhibitory effects of *Ximenia americana* aqueous extract on the growth of *Sorghum bicolor* seed radicle was determined for different concentrations: 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml, 16 mg/ml, 24 mg/ml, 32 mg/ml, 40 mg/ml and 48 mg/ml. Radicle lengths (mm) were measured at 48, 72 and 96 h. Distilled water without *Ximenia americana extract* was used as negative control, methotrexate 0.167 mg/ml was used as positive control. Mean radicle length ± standard error of mean, percentage growth inhibition and percentage growth at 48, 72 and 92 h for seeds treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml, 16 mg/ml, 24 mg/ml, 32 mg/ml, 40 mg/ml, 48 mg/ml aqueous extract of *X. americana* as well as the negative control, and methotrexate 0.167 mg/ml used as positive control are as shown in Table 1.

DISCUSSION

As a preliminary and preparatory assay to antiproliferative test on a cancer cell line system, the radicle lengths of fast growing seeds such as *Sorghum bicolor* have been utilized as a parameter for the testing of suspected anticancer agents. Generally, cancer cells have a characteristic of fast proliferation, and this is also the case with meristematic cells of *S. bicolor* seeds when exposed to favourable conditions [18]. Hence, the use of the method for this study.

The phytochemical analysis of XA showed the presence of tannins, saponins, flavonoids, and carbohydrates. The HPLC spectrum of XA revealed the presence of rutin and ferulic acid at retention times of 6.886 and 7.796 minutes, respectively (Fig. 1). Flavonoids have been reported to have relatively low toxicity compared to other metabolites like alkaloids. Flavonoids have also been referred to as 'natural biological response modifiers' because of the strong experimental evidence of their ability to modify the body's reactions to allergens, viruses and carcinogens. Flavonoids have been reported to possess anti-allergic, anti-inflammatory, antimicrobial and anticancer activities. Saponins have been reported to exhibit antioxidant, anticancer and anti-inflammatory activities and tannins to have antibacterial, antiviral and anti-tumor activities. Generally, the pharmacological properties of medicinal plants depend on their secondary metabolites constitution [19].

Ximenia americana had been reported to possess anticancer properties. Physicochemical characterization showed that the active antineoplastic components of the plant material were proteins with galactose affinity [20]. Cytotoxic and antiproliferative activities of *Ximenia americana* against six cancerous cells lines had been reported. The study revealed the presence of flavonoids (13%), gallotannins (5%), phenolic acids (0.7%), ellagic acid (0.3%) and an abundance of condensed tannins (81%) [21]. This finding is in agreement with the results obtained with the Nigerian material (Fig. 1) reported herein, where rutin and ferullic acid were identified as the bioactive constituents of *Ximenia americana* leaf.

In a review of the chemistry, pharmacology and biological properties of *Ximenia americana*, the chemical constituents and anticancer properties were reported [22]. Antioxidant activity study and total phenolic determination of leaf extracts of *Ximenia americana*, an anti-tumor plant used traditionally in Mali, revealed the presence of phenolic compounds with potential anticancer properties [23]. Flavonoids have been reported to possess anticancer potential [24]. Rutin isolated from *Triticum aestivum* showed anticancer activity [25]. Currently it has been observed that cinnamic acid and its analogs such as ferulic acid and isoferulic acid display various pharmacological activities including anticancer [26].

CONCLUSION

The hot aqueous extract of *Ximenia americana* leaf (XA) exhibited growth inhibitory effects on fast proliferating cells of *S. bicolor* seed radicle. Hence, by extension it can inhibit cancerous cells. This study provided preliminary evidence that supports the ethno medicinal use of *X. americana* leaf growing in Nigeria for the treatment of breast cancer. The observed growth inhibitory properties may be attributable to the flavonoid rutin and ferulic acid content.

AUTHORS' CONTRIBUTION

ACN wrote the initial draft of the manuscript; SEO designed the study and did the statistical analysis; UEB proof read, and edited the word. All authors were involved in the execution of the research plan. The final manuscript was read and approved by all authors

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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RESEARCH ARTICLE

Aloe vera and garlic ameliorate thermoxidized palm oil-induced haemostatic derangement in albino Wistar rats

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ABSTRACT
This study examined the effects of *Aloe vera* and garlic on haemostatic status of rats fed thermoxidized palm oil diet (TPO). 35 male Wistar rats (140-170 grams) used in this study were randomly assigned five groups (n=7): Control, TPO, TPO + garlic juice (TPO+G), TPO + Aloe gel (TPO+A) and TPO + garlic/Aloe gel (TPO+G+A). TPO diet was prepared by mixing 85 g of rat chow with 15 g of thermoxidized oil. The juice and gel were orally administered at doses of 2 ml/kg and 6 ml/kg respectively. After three months, the rats were sacrificed and blood collected through cardiac puncture for analysis. Plasma fibrinogen was significantly (P<0.001) reduced in TPO group compared to Control, whereas, fibrinogen increased in all treated groups. Platelet count was significantly (P<0.001) decreased in TPO compared with control. Platelet count was significantly decreased (P<0.001) in TPO+G compared to TPO and control, but significantly (P<0.001) increased in TPO+A, and TPO+G+A compared to TPO. Prothrombin and clotting times were significantly increased (P<0.001) in TPO and TPO+G compared to control. Bleeding time was significantly increased (P<0.001) in TPO compared to control, but significantly reduced in TPO+A compared to TPO. Chronic consumption of TPO has deleterious haemostatic by reducing plasma fibrinogen, platelet count and causing an increase in prothrombin, clotting and bleeding times. However, these debilitating effects were seen to be markedly ameliorated following separate and combined administrations of *Aloe vera* gel and garlic juice. Additionally, *Aloe vera* gel seemed to have a more significant effect compared with garlic juice.

Keywords: Garlic; *Aloe vera*; Thermoxidized palm oil; Haemostatic status; Platelet; Fibrinogen.

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Ethical considerations: permission was granted by the ethical committee of the Faculty of Basic Medical Sciences, University of Calabar, Calabar, Nigeria (approval number 18/17).

INTRODUCTION

Thermoxidized palm oil, derived from fresh palm oil of *Elaeis guineensis* fruit is widely used as cooking oil in Nigeria and other parts of the world. The oil is thermoxidized when its fresh form is subjected to several rounds of heating at high temperatures [1]. Chronic consumption of TPO diet has been implicated in several ailments in the body such as cardiovascular, haematological diseases, reproductive dysfunction, thrombosis,

fatty livers, essential fatty acids deficiency and micronutrient malnutrition leading to deactivation of key metabolic enzymes [2-9].

Thermoxidation negatively affects most vegetable oils, thus, inducing alterations in their chemical properties when compared to the nutritionally healthy fresh form [10]. Thermoxidation of palm oil has been reported to be the root cause of most deleterious consequences associated with consumption of palm oil diets [5]. This results from formation of peroxides and increased free radical generation known to be highly reactive, cytotoxic and destructive to tissues [2, 3].

Plants derived natural substances; some of which are antioxidants have captured great interest due to their numerous therapeutic importances like scavenging free radicals. Proximate analysis of *Aloe vera* and garlic has revealed the presence of several bioactive compounds with different medicinal efficacies. Allicin, a thiosulfinate extract of garlic has been reported to possess strong antioxidant properties [11]. Treatment with *Aloe vera* gel has been presumed to reduce lipid peroxidation levels, increases the levels of some antioxidant enzymes (SOD, CAT and GPx) including glutathione [12]. Several health benefits of *Aloe vera* and garlic exist in literatures [13-17].

In view of the increasing incidence of complications associated with TPO consumption, research on remedies which are cheap and potent in preventing these ailments is desirable and expedient. Due to their therapeutic effects, this study was therefore aimed at investigating if both garlic and *Aloe vera* which are readily available and affordable could help prevent or ameliorate the devastating effects attributable to thermoxidized palm oil diet consumption.

MATERIALS AND METHODS

Plant materials and extraction

Garlic cloves were purchased from Watt Market while *Aloe vera* was collected from a domestic garden located in Uwanse street, all in Calabar, Cross River State, Nigeria. The authentication of the plant materials was done by a botanist in the Department of Botany, University of Calabar, Nigeria.

Garlic cloves were washed, thinly sliced and blended for 25 minutes using electric blender to extract the natural juice. The blended mixture was then left to stand for 10 minutes in order to allow a total enzymatic reaction of alliin with allinase [18]. The mixture was first filtered using filter paper and filtered again with a syringe filter to ensure that no garlic particles would obstruct the column. *Aloe vera* leaves were thoroughly washed with clean tepid water to remove dirt. The base of the leaves was cut off with surgical blades and these were sliced open along the margin to reveal the transparent mucilage. The transparent mucilage was carefully scooped into a beaker with the aid of a spatula. The gel was then homogenized using an electric blender for 10 minutes. This was allowed for 20 minutes and afterwards sieved using Whatman filter paper to obtain a particulate-free gel [19]. Garlic and Aloe extracts were preserved in the refrigerator (at temperatures between 4-6^{0}C) for three days in the research laboratory of the Department of Physiology, University of Calabar, Calabar, after each day's use.

Preparation of thermoxidized palm oil diet

Fresh palm oil was purchased from Mbukpa Market, Calabar, Cross River State, Nigeria. The fresh oil was thermally oxidized by heating the oil at 150°C in a stainless steel pot intermittently for five times at about 20 minutes for each round of heating [2]. After each round, the thermoxidized oil was allowed to cool for five hours. TPO diet was prepared by mixing 85 g of rat chow with 15 g of cooled thermoxidized palm oil as used in previous studies [1, 20, 21].

Experimental animals and protocol

Thirty five male Wistar rats weighing 140-170 grams were purchased from the Department of Physiology, University of Calabar, Calabar. They were randomly assigned into five groups (n = 7), namely; control, TPO – fed group, TPO+garlic juice group, TPO+A group and TPO+G+A group. This was done after permission was granted by the ethical committee of the Faculty of Basic Medical Sciences, University of Calabar, Calabar, Nigeria on the use of experimental animals for scientific purposes with approval number 18/17. Garlic juice and *Aloe vera* gel were orally administered at doses of 2 ml/kg and 6 ml/kg, respectively (the safe dose taken as 1/3 of the LD_{50}) from the result for lethality studies as reported by Ime et al. [22].

The animals were kept in clean cages in the animal house of the Department of Physiology for one week, for acclimatization, before feeding and administration for three months. The control and TPO groups were also given oral administration of normal saline at 6 ml/kg body weight, thus exposing all the groups to the same physiological condition. All animals had access to feed and water *ad libitum* while the juice and gel were given via oral route administration.

After three months of administration and feeding, the rats were allowed an overnight fast before sacrifice. In line with the guidelines of the European Convention for the Protection of Vertebrate animals used for scientific purposes, they were anaesthetized with chloroform and afterwards their thorax were opened and blood collected via cardiac puncture with sterile syringe into EDTA sample bottles to obtain serum for analysis.

Determination of plasma fibrinogen

Plasma fibrinogen concentration was determined as defined by the clot weight method, though with modifications to accommodate the procedure for use of thrombin time (TT) test kit as given by the manufacturers of the kit [23]. Blood was collected with the aid of plastic disposable syringes into sample vials containing 3.2% sodium citrate in the ratio 1:9 with blood. Blood plasma was obtained by centrifuging blood in a stopped vial at 1000 g for 10 min. 0.2 ml of the test plasma was put into a test tube and incubated in a water bath for 3 min at 37°C. 0.2 ml of thrombin time-reagent was added to test plasma, mixed and the clot formed harvested with a wooden applicator stick. The resulting clot was transferred into a tube containing acetone to dry and harden for about 10 min; the acetone was decanted and the clot placed on a filter paper for the remaining acetone to evaporate. The clot was then recovered and weighed. The process of fibrinogen concentration determination was completed within 3 h of blood collection. Thus, fibrinogen concentration of citrated plasma in mg/dl equals clot weight (mg) divided by plasma volume (dl).

Determination of bleeding time

The time elapsed the moment of escape of blood outside the vessel and the moment of cessation of the flow is known as bleeding time. Bleeding time was determined by Duke's method [24].

The tail of albino Wistar rat was cleaned with methylated spirit and allowed to dry. A quick deep prick with a lancet was made in the tail resulting in blood flowing out and the time noted. With a filter paper, the blood was dabbed every half a minute using a fresh part of the filter paper for each touch. It was noted that the blood spots on the filter paper gets smaller till it disappears when bleeding stops and the blood spots counted and the number divided by two giving the bleeding time in minutes.

Determination of clotting time by Wright's capillary method

This was determined by Wright Capillary method [25]. The tail of albino Wistar rat was cleaned with methylated spirit and allowed to dry. A quick deep prick with a lancet was made in the tail resulting in blood flowing out. This was drained immediately into the capillary tube by placing one end of the capillary tube on the drop of the blood. The capillary tube was tilted downwards so that the blood can easily flow into the tube.

When the capillary tube (10 cm nearly) was more or less filled, it was removed. After about two minutes, small lengths of the tube were snapped off at 30 seconds interval. Initially, the blood column broke easily and cleanly. At the end point, a thick strand or coagulated blood column was seen stretching between the broken ends and the time noted. It is important to break the tube gently without jerking the ends apart or else the strand will be snapped by the movement before it is observed. Normal clotting time by this method is about three to eight minutes.

Estimation of prothrombin time

0.1 ml of plasma was delivered into the bottom of a 75×10 mm tube in a water bath at 37^0C and 0.1 ml of thromboplastin added to it. After a delay of about one minute, 0.1 ml of warmed 0.025 M calcium chloride was added and the contents of the tube were carefully mixed. A stop watch was started and the tube was held with its lower end submerged. The tube was continuously but gently inclined from the vertical to just short off the horizontal so that its contents can be observed for the first signs of clotting. A fibrin clot developing within a second marked the end point. The test was repeated thrice and the average reading was taken [26].

Statistical analysis

The data obtained was analyzed by one way ANOVA of the SPSS statistical program and post hoc tests (LSD) between groups using Microsoft (MS) excel program and the result presented as mean ± SEM. P value of less than 0.05 was accepted as statistically significant.

RESULTS

Comparison of platelet count in the different experimental groups

Platelet count was significantly reduced (P<0.001) in TPO when compared to control. More so, TPO+G induced a marked significant reduction (P<0.001, P<0.05, P<0.001 and P<0.001) in Platelet count compared to control, TPO, TPO+A, and TPO+G+A respectively. However, TPO+A, and TPO+G+A caused a progressive significant increase (P<0.001) in platelet count when compared to TPO and TPO+garlic (Table 1).

Comparison of plasma fibrinogen in the different experimental groups

Plasma fibrinogen level was significantly reduced (P<0.001) in TPO compared to control. Though Fibrinogen was also reduced in the three treated group, but there was progressively increase in all treated groups when compared to TPO. Meanwhile, TPO+A induced a tremendous significant increase (P<0.001) in plasma fibrinogen level when compared to TPO+G and TPO+G+A (Table 1).

Comparison of prothrombin time in the different experimental groups

Prothrombin time was significantly increased (P<0.001) in all four groups when compared to control. Meanwhile, there was a progressive significant decrease (P<0.05, P<0.001 and P<0.001) in prothrombin time of TPO+G, TPO+A and TPO+G+A when compared to TPO respectively (Table 1).

Comparison of clotting time in the different experimental groups

There was a significant increase (P<0.001) of clotting time in all four experimental groups compared to control. However, clotting time was significantly decreased (P<0.001) in TPO+A when compared to TPO, TPO+G and TPO+G+A (Table 1)

Comparison of bleeding time in the different experimental groups

A significant increase (P<0.001) was observed in bleeding time of TPO, TPO+G and TPO+G+A compared to control. However, TPO+A significantly decreased (P<0.001) bleeding time when compared to TPO, TPO+G and TPO+G+A (Table 1).

Table 1. Comparison of haemostatic parameters in the different experimental groups

	Groups				
Variables	**Control**	**TPO**	**TPO+G**	**TPO+A**	**TPO+G+A**
Platelet count ($\times 10^9$/l)	606.7±22.1	433.7±8.3***	393.6±7.8$^{***, a}$	549.9±10.7$^{**, c, f}$	501.1±11.6$^{***, c, f, y}$
Fibrinogen level (g/dl)	213.7±4.7	102.1±8.7***	114.3±9.0$^{***, a, z}$	176.3±10.7$^{***, c}$	153.6±12.8$^{***, c, z}$
Prothrombin time (seconds)	9.4±0.4	15.7±0.2***	14.7±0.3$^{***, a}$	12.7±0.2$^{***, c, f}$	11.9±0.3^{***,c,f,x}
Clotting time (seconds)	3.7±0.1	9.0±0.2$^{***, z}$	8.5±0.3$^{***, z}$	5.1±0.2***	6.7±0.3$^{***, c, f, z,}$
Bleeding time (seconds)	8.7±0.3	13.3±0.3***	11.9±0.4$^{***, b}$	8.7±0.3$^{c, f}$	10.2±0.3$^{***, c, f, z}$

Values are mean ± Standard deviation
*p<0.05 vs Control; **p<0.01 vs Control; ***p<0.001 vs Control
a = p<0.05 vs TPO; b = p<0.01 vs TPO; c = p<0.001 vs TPO
d = p<0.05 vs TPO+Garlic; e = p<0.01 vs TPO+Garlic; f = p<0.001 vs TPO+Garlic
x = p<0.05 vs TPO+Aloe; z = y<0.01 vs TPO+Aloe; z = p<0.001 vs TPO+Aloe.

DISCUSSION

The platelet count was significantly reduced in TPO group compared to control. It was however found that garlic treated group caused a greater reduction in platelet count compared to TPO diet fed group. Interestingly, platelet count was significantly increased in TPO+*Aloe vera* group compared to TPO group although it was significantly less than control. Platelet count was significantly increased in TPO+garlic+aloe group compared to TPO group although less than TPO+*Aloe vera* group. Several reports have established decrease platelet count induced by TPO [1, 20, 27].

Platelets are synthesized in the bone marrow by fragmentation of the cytoplasm of a group of cells called megakaryocytes which arise by a process of cell differentiation from the haemopoietic stem cell [28]. From the result, it is likely that there exist constituent(s) in *Aloe vera* that help ameliorate the devastating effect caused by TPO on blood. *Aloe vera* is known to be highly rich in antioxidants that could help mob up the free radicals associated with TPO. From the result, garlic is seen to worsen the effect caused by TPO on platelet. It is therefore likely that garlic may either be harmful to haemopoietic stem cells or committed platelet progenitors that give rise to platelets or on mature platelets themselves although the exact mechanism was not studied in this research. Garlic has been reported to inhibit platelet aggregation which could affect haemostasis [29].

Bleeding, clotting and prothrombin time were all markedly increased in TPO fed rats and garlic treated group compared to control. However, these parameters witnessed a great progressive decrease in *Aloe vera* treated group. Bleeding, clotting and prothrombin time are markers of haemostatic status in which clotting factors play important role. While clotting time is the measure of intrinsic pathway, prothrombin time measures the extrinsic pathway [29]. Therefore, clotting time measures the functions of factors: I, II, V, VIII, IX, X, XI and XII whereas prothrombin time indicates the functions of factors: III, V, VII and X. These factors are mostly

synthesized in the liver. It is therefore possible that TPO has adverse effect on the function of the liver either through free radical damage to liver cells or mechanisms that are yet to be established. The increase in bleeding time could be attributed to antiplatelet effect of garlic as seen from the result of this study and its reported interaction with warfarin [28].

The mechanism through which *Aloe vera* reduces clotting and bleeding time is yet unknown but from the result of this research, it could be attributable to its marked increase in fibrinogen although this is in contrast with a research work [30] which reported a dose dependent decrease in prothrombin, clotting and fibrinogen following administration of *Aloe vera* gel on alternative days for 14 days. This variation may have resulted from the difference in duration of administration.

Plasma fibrinogen level was greatly reduced in TPO fed rats. However, *Aloe vera* and garlic proved to be potent in reversing this effect. Many plasma proteins including fibrinogen are synthesized by the liver. Amino acids, which are the building blocks for synthesis of proteins, are necessary to maintain adequate supply of these substrates for such liver synthetic function. From this study, it is likely that garlic and *Aloe vera* improves liver integrity by supplying substrates for its synthetic function or improves upon the integrity of gastrointestinal tract for absorption of nutrients as reported [31, 32].

The results of this study have shown that chronic consumption of TPO has deleterious effects on haemostatic status of Wistar rats by reducing plasma fibrinogen, platelet count and causing an increase in prothrombin, clotting and bleeding times. However, these debilitating effects were seen to be markedly ameliorated following garlic juice and *Aloe vera* gel administration. If these results are to be applicable to humans, then the chronic consumption of thermoxidized palm oil diet should be seriously discouraged.

AUTHORS CONTRIBUTION

This research was carried out by four authors. EJA designed the study. This research and laboratory work was done by AUI with the assistance of CEO. Data analysis was carried out by VUN while AUI interpreted and wrote the article. EJA read and corrected the initial draft before the manuscript was submitted. The final manuscript was read and approved by all authors.

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SHORT COMMUNICATION

RNA isolation from paraffinated material of histological´s prepared slides

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ABSTRACT

The parafinization of solid biopsies allows a better preservation and preparation of microscopy slides used for histological evaluations. Techniques of RNA extraction are suitable to be applied in microtomized paraffinated material, but the extraction of paraffinic material already disposed in a slide has not yet been described and can be useful for gene expression studies. We adapted the RNA isolation technique of paraffinated material for slide-laid paraffinited material, using the Rneasy® FFPE Kit. Total RNA so isolated is suitable to gene expression analysis by RT-qPCR.

Keywords: Paraffinated material; Slides; RNA isolation.

Advances in molecular biology and its techniques, such as RT-qPCR and genomic and proteomic sequencing, provide the elucidation of the functioning of our genetic material, its molecular mechanisms and its relation to the phenotypic characteristics of each individual of the most varied species, both from the physiological as well as pathological point of view.

Recently, gene expression studies have generated a lot of fundamental information for a better understanding of the adaptive and pathological changes related to diseases such as cancer, besides characterizing biological markers that facilitate the diagnosis and the prognostic monitoring of several disorders. Among the techniques most used in this type of study, RT-qPCR - which consists of quantifying the expression of genes of interest by means of the amplification of cDNA fragments in real time, synthesized from the RNA isolated from several types of biological material - stands out. One of the major limiting factors for adequate reaction efficiency is the quality of the biological material. It is known that RNA is a very instable molecule and the sample condition, such as the RNA extraction technique, may partially damage or completely degrade the molecule, if certain concerns are overlooked [1].

Often, solid biopsy may be fixed in formalin-fixed paraffin-embedded (FFPE) and blocked for performing arranges in slides, consisting on the most adequate way for retrospective histological studies, in addition to allowing the storage and preservation of genetic material for a long time. Despite RNA isolation

from FFPE tissues leads to a highly degraded total RNA [1], these FFPE block samples may be used for nucleic acids extraction aiming to perform genome and gene expression studies [2, 3]. RNA extraction techniques for gene expression studies from FFPE tissue after microtomy are already well described and kits with this purpose are commercialized [4, 5]; however, this procedure in paraffin-embedded material fixed in a slide has not yet been described and there are no protocols and kits for this purpose.

This study aims to describe an adaptation in the technique of RNA isolation from slide-laid FFPE tissues, allowing these samples to provide the quality and concentration suitable for the realization of RT-qPCR using a known commercial kit.

Total RNA isolation were performed in 30 formalin-fixed paraffin-embedded biopsies of oral squamous cell carcinoma (SCC) that were microtomized and disposed in slides for histological studies. Before biopsies, all patients have signed an Informed Consent Term in compliance with the Helsinki Convention and approved by FMABC Ethics Committee (protocol number 384/2007). The material of the slides was scraped off completely and transferred to a 2 ml microtube with the aid of a scalpel slide, requiring at least three slides for each sample to obtain an adequate amount of material to perform the extraction. After scraping of slides, the isolation of total RNA was performed with the RNeasy® FFPE Kit (Qiagen, Valencia, CA, USA) and xylene was used as deparaffinization agent. The steps outlined in the kit protocol were followed closely.

The cDNA of the samples was synthesized using the SuperScript® III Reverse Transcriptase kit (Invitrogen, cat no 11752050, following the manufacturer's protocol), using random primers, and subjected to RT-qPCR in the Applied Biosystems® 7500 Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) for the amplification of a 148 bp fragment of the endogenous gene GAPDH. The RT-qPCR was prepared to a final volume of 15 µl containing: 1X SYBR Green mix (Quantitec SYBR Green PCR kit, QIAGEN cat. no. 204054), 10 pmol of each specific primer (F-GACCACAGTCCATGCCAT and R-CAGCTCAGGGATGACCTT) and 2 µl cDNA. The primer was designed with the help of the software Primer3 Input 0.4.0 software, available at http://frodo.wi.mit.edu/primer3. The sequence was checked for specificity by the Primer-BLAST program, available at http://www.ncbi.nlm.nih.gov/tools/primer-blast. The MCF7 cells cDNA amplification was used as reaction standard for comparison of amplification pattern and melt curve. The cyclic parameters were an initial hot start step at 95°C for 10 min, followed by 40 repetitions of 95°C for 15 sec and 60°C for 25 sec.

The concentration and quality of RNA obtained were measured in NanoVue®. The mean concentration of RNA extracted was 0.95 µg/µl, ranging from 0.025 µg/µl to 4.9 µg/µl, depending on the amount of biological material available. A260/A280 absorbance ratio obtained with these samples averaged 1.46, ranging from 0.98 to 1.84. The average A260/A280 ratio for FFPE tissue is 1.47, close to the average value obtained in this study [6].

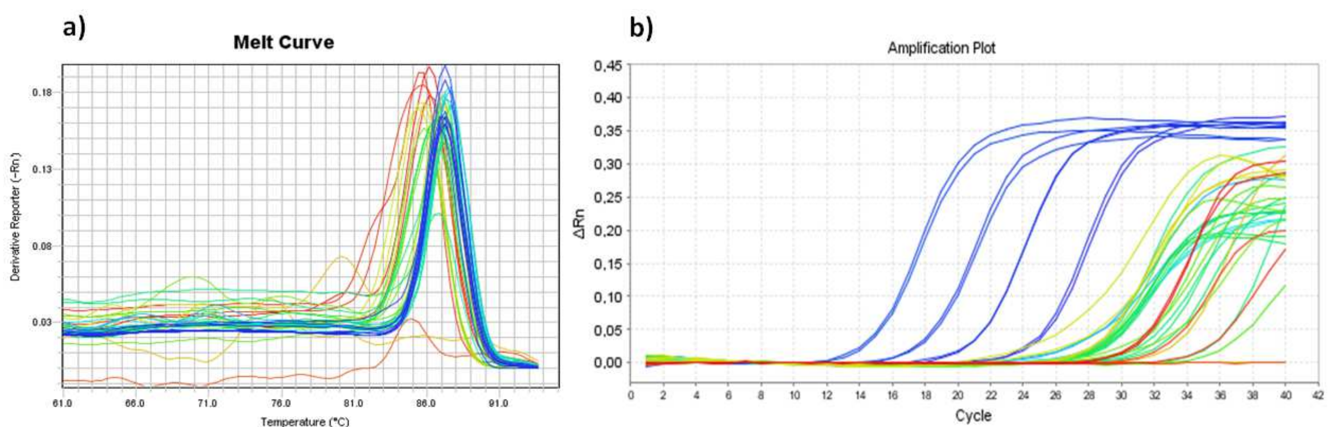

Figure 1. a) Melting curve of GAPDH amplification in all CEC samples. b) Amplification Plot of GAPDH gene. Curve of expression in blue belongs to standard curve (MCF7). All of the others belong to CEC samples.

Because RNA from FFPE samples does not have the same quality as RNA from fresh samples, it is possible that the poly-A tail of the transcript is not intact [7]. For this reason, reverse transcription was performed with random primers as an alternative to oligo-dT. The melt amplification and curve graphs of the samples and the MCF7 (in blue) are shown in Figure 1. The melt temperatures (around 87°C) are similar between all samples and standard (MCF7 cells), showing that the amplified product is the same.

Although RNA extracted from both paraffin embedded and slide-laid material does not have the same A260/A230 absorbance ratio of fresh samples, amplification of the endogenous GAPDH gene was satisfactory for all samples. For this reason, the adaptation of the protocol of RNA extraction from FFPE tissues laid on histological slides proved to be efficient to obtain adequate material for gene expression studies.

AUTHORS CONTRIBUTION

MMP: designed the study and performed all experiments; ALAF and FP: provided all CEC biological samples and designed the study; BB, FLAF and BCAA: analyzed the results and prepared the manuscript. The final manuscript was read and approved by all authors.

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RESEARCH ARTICLE

Status of iron during radon-sulfide balneotherapy in osteoarthritis patients

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ABSTRACT

Osteoarthritis and other arthropathies are still one of the most debilitating musculoskeletal disorders among the elderly population. Pathogenesis of these diseases is multifactorial and mainly connected with increasing of oxidative stress. Pharmacotherapy, physico- and balneotherapy are the most commonly used methods of treatment. The mechanism of action balneotherapy in arthropathies is not fully understood. Probably it is a combined effect of used forms of biological interactions which influence different metabolic pathways. One of the potential factor connected with decreasing oxidative stress status during balneotherapy can be iron bioavailability and changes its concentration during this therapy. The aim of this study was assessing change of iron blood status during the routine 21 days' radon-sulfide balneotherapy course in patients with osteoarthritis. The study group consisted of 35 osteoarthritis patients without impediment to comprehensive treatment at spa. The age of patients ranged 32-67 years (average 53.5). The blood samples were collected before the therapy and after 21 days of treatment at a spa. The levels of iron, transferrin, total iron binding capacity and transferrin saturation percent were assessed. Within statistical comparison before and after spa course reveled decreasing trend for all iron status parameters after 21 days' spa treatment, but the observed changes were significant only for TIBC. In the course of spa therapy Osteoarthritis patients, the level of the systemic iron components is not affected and changes in blood oxidative status during this therapy probably are not influenced by iron status and vice versa.

Keywords: Balneotherapy; Iron; Osteoarthritis; Oxidative stress; Radon-sulfide water.

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INTRODUCTION

Osteoarthritis and other arthropathies are still one of the most debilitating musculoskeletal disorders among the elderly population. Joint damages characteristic for those diseases mainly occur because of an imbalance between the processes of degeneration and regeneration of articular cartilage structures. Until now many risk factors favoring the development of degenerative joint disease have been identified, such as age, weight, previously sustained traumas to joints, sports, sex and genetic predisposition [1, 2].

The latest scientific reports confirm that the pathogenesis of changes in osteoarthritic joints is complex, occurs on many levels and strictly connected with increase of oxidative stress [3, 4]. In a face of constantly lengthening life expectance, treatment and care for patients with these progressive and irreversible diseases generate large social and economic costs [5].

It is important to introduce effective pharmacological and biological forms of therapy. In some countries, especially in Europe spa therapies and 21-days' curative courses are very popular and have a long tradition in treatment of many rheumatic diseases. One form of adjuvant therapy for patients with osteoarthritis in Poland is a routine 21-days balneotherapy e.g. in Przerzeczyn Zdrój spa resort with using unique, on a European scale, natural curative radon-sulfide waters [6].

Balneotherapy is an important component of arthropathy treatment called also as spa medicine or medical spa therapy. It refers to the therapies that utilize of spa waters, peloid, physiotherapy, kinesiotherapy, therapeutic massage and different forms of hydrotherapy to reduce pain and improving functional mobility.

Biomedical mechanism of action of balneotherapy and physiotherapy in spa is not entirely clear, because the health resort treatment may affect many factors. In spa treatment, there are used different kinds of energies, as well as natural healing materials such as: peat, gas or medicinal water treatment. This type of therapy brings not only local, but general results, and changes the reactivity of the immune system by activating the adaptive responses. Probably all elements of the balneotherapy influence by mechanical, thermal and chemical effect [7] and can activate the changes of different metabolic pathways in human organism [8-10]. It is suggested that all these forms of medicinal spa treatments have also an antioxidant effect proven in *in vitro* and clinical studies [7].

It is believed that the ferric iron status changes are related with oxidative stress in various diseases (metabolic, inflammatory, infection, cancer) as well as disorders of musculoskeletal system. Iron is an important trace element involved in many biochemical reactions essential for maintaining homeostasis and simultaneously takes part in generating of free radicals due to their high volatility redox potential. Free iron particles are sources of reactive oxygen species mainly via Fenton reaction. Both indispensability and their possible toxic effects cause that the concentration and availability of iron ions remains under strict biochemical control [11].

Among other factors, increasing of reactive oxygen species concentration and cellular oxidative stress products is connected with onset and progression of arthropathies [12, 13]. New studies confirm the antioxidant effect of balneotherapy in osteoarthritis patients [14, 15]. However, the mechanisms by which spa therapy influence on oxidative status are still not fully understood. One of potential factor linking these two aspects can be iron bioavailability and changes of its concentration during balneotherapy course.

The aim of this study was assessing changes of iron blood status during the routine 21 days' radon-sulfide balneotherapy course in patients with osteoarthritis.

MATERIALS AND METHODS

The project was conducted in a health resort Przerzeczyn Ltd. in Przerzeczyn Zdrój, Poland and was approved by the Medical University of Wrocław Ethics Committee (KB-401/2008). The study group consisted of 35 patients (28 women and 7 men) with pain in joints and spine, caused by osteoarthritis and spinal or degenerative disc disease without impediment to comprehensive treatment at a spa. The age of patients ranged

32-67 year (average 53.5). Patients were qualified for treatment by a referring physician and balneologist working as a medical consultant in the spa in accordance with applicable standards. All patients declared voluntary participation in the study in writing; they were also informed about the purpose and protocol of the research. In addition, the study participants declared that during the study they will restrain from taking any vitamin supplements, alcohol consumption and smoking tobacco.

Patients undergoing spa treatment within the 21-days' health resort stays were on the normal diet or highly digestible diet, which was dominated by dishes cooked with low fat content. Both diets were norm calories diets. The main element of therapy accounted for treatments using medicinal radon-sulfide baths. Patients were also prescribed a series of 10 treatments of various types of therapy depending on the needs and type of disease. A sample set of patient treatments used at spa resort included radon-sulfide baths, peat wraps, therapeutic individual and group exercises, laser biostimulation, interference currents.

Table 1 presents the content of the individual components characteristic for Przerzeczyn-Zdrój curative waters.

Table 1. The results of physicochemical studies performed on 24.04.2008 at source of medical waters and at mineral spring spa (MSP).

No	Drilling place location	Water temp. in^o C	pH	The content of the ingredient in 1 dm^3 of water			
				H_2S Mg	HCO_3 Mg	Rn Nci	Rn Bq
1.	Borehole No. II	12.0	7.62	1.96	263.2	2.21	81.8
2.	Borehole No. IX	12.0	7.72	1.70	289.6	1.71	63.3
3.	MSP tank	16.0	7.65	1.87	277.9	2.20	81.4

The blood samples were collected from patients by venipuncture before the therapy and after 21 days of treatment at the spa resort. The level of iron was measured by Feren S method, transferrin by immunoprecipitation method, total iron binding capacity (TIBC) by the Unsaturated Iron Binding Capacity (UIBC) method and transferrin saturation percent were calculated. All reagents were supplied from DiaSys, Holzheim, Germany and laboratory tests were determined using Konelab 20i biochemical analyser (ThermoScientific, Vantaa, Finland). The results were analyzed statistically, by the Wilcoxon test for related data using STATISTICA12.PL. The values of $p < 0.05$ were regarded as significant.

RESULTS

Results of iron status parameters before (start point) and after 21 days' spa therapy (end point) are given as median, Q_1-Q_3 range and min.-max. values in Table 2.

The study showed that medians and range of Q_1-Q_3quartiles nearly all iron status parameters, except of transferrin concentration, were in the reference value ranges in both estimated points. However, the minimum and maximum values of the iron, transferrin concentration and transferrin saturation were located both above and below references cut points. Only for TIBC we found all statistical descriptors inside references values bounds. Statistical comparison before and after spa course reveled decreasing trend for all iron status parameters after 21 days' spa treatment. The iron, TIBC and transferrin concentration declined by 7.7 %, 4.9 %, 9.1% respectively, and transferrin saturation by 3.9 %. Observed changes were significant only for TIBC ($p<0.05$).

Table 2. Descriptive statistics of the exanimated parameters before and after balneotherapy course.

Parameters	N	Start point median (Q_1-Q_3) (min.-max.)	End point median (Q_1-Q_3) (min.-max.)	p value
Iron [µmol/l] Reference value: 10.6-28.3	35	18.2 (13.6-24.0) (6.7-38.0)	16.8 (12.1-21.0) (6.6-32.0)	0.317
TIBC [µmol/l] Reference value: 44.8-73.4	35	54.4 (51.0-60.1) (46.7-69.3)	52.1 (49.2-57.4) (50.0-66.9)	**0.014**
Transferrin saturation [%] Reference value: 20-50	35	33.5 (26.7-39.9) (14.4-54.9)	32.2 (24.6-36.6) (13.7-47.9)	0.650
Transferrin [g/l] Reference value: 2.0-3.6	35	3.3 (2.5-4.1) (1.0-5.7)	3.0 (2.3-3.9) (1.1-5.4)	0.342

DISCUSSION

In the scientific literature, there are few reports about the using of natural medicinal resources like water and muds as a form of therapy. Their use is more popular in countries that have large resources and long tradition of use spa therapy. Such forms of therapy are of great interest and readily used in many old spa resorts located in different areas of Poland. Balneotherapy and physiotherapy are established adjuvant methods used in osterarthritis treatment [16]. Spa therapy, by influence on systemic changes, seems to be important factor in management of these diseases. We revealed in our previous investigation, that spa therapy and outpatient treatment reduce the level of pain in patients with degenerative joints and disc disease. What interesting, the reduction of pain level was more effective in case of therapy conducted in the spa [6]. Moreover, our unpublished study has shown significant increase in blood antioxidant status of patients' underling 21-days balneotherapy in Przerzeczyn Zdrój resort.

In presented study, we investigated iron status parameters before and after routine 21 days' radon-sulfide spa treatment because of importance of iron in the oxidant/antioxidant balance.

Among examined parameters we observed significant decrease only in Total Iron Binding Capacity. The remaining parameters of iron status showed only decreasing trend. Despite the fact significant shift for TIBC before and after balneotherapy, in our opinion all this difference due to the small degree of values change, seems to be not important in clinical practice. In available literature, we found only single studies in similar area to our research. Jokic et al. in 2010 have showed that sulphur baths and mud packs application during 21-days' spa treatment influenced hematological blood parameters and caused an increase in: number of circulating red blood cells, mean corpuscular hemoglobin concentration (MCHC) and decrease in lipid peroxidation in plasma [15]. But in their study iron status parameters during spa therapy were not investigated. Findings, for iron metabolism, similar for observed in our study, were published only by Olah et al. [17]. In overweight and hypertensive participants, they observed changes transferrin, but not iron and C-reactive protein concentration after spa therapy. They did not also find changes in antioxidant status [17]. Based on obtained by us results, we can conclude that in the course of routine 21-day spa therapy in patients with disorders of the musculoskeletal system the level of the systemic iron components is not affected. Probably changes in blood oxidative status during this kind of therapy are not influenced by iron status and vice versa. It is worth to notice there are only a few publications about influence of balneotherapy on antioxidative/oxidative balance and the mechanisms regulating antioxidant capacity, especially about iron status and its contribution to this state [15, 18, 19].

Taking to the account only preliminary character of our study and limited number of participants it is appropriate to continue research in this area for examination exact ways of influence of radon-sulfide balneotherapy and role of iron in the network of metabolic disturbances in osteoarthritis patients.

AUTHORS CONTRIBUTION

JKL conceived the idea for and designed the study, were involved in data collection and performed the research. SP, AP, LPS analyzed the data, conducted the literature search, study selection and prepared manuscript. SP, LPS, IK, AP revised the manuscript for final submission. The final manuscript was read and approved by all authors.

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