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Effect of Heat Treatment on Antioxidant and Antimicrobial Activity of Croton gratissimus and Xylopiaaethiopica Spices

Afraa Awadallah Ahamed
Yassir Salah Ibrahim Adam
Amna Mokhtar Hussien
Faculty of agricultural Technology & fish science || Alneelain University || Sudan
Mohammed Elamin Hassan
Sudan Academy of Banking and Financial Sciences || Sudan

Abstract: Background and objective Croton gratissimus and Xylopiaaethiopicaare tropical African shrubs or small tree with corky bark. It is traditionally used as a febrifuge, styptic, cathartic and is medic for dropsy. This study was designed to determine antioxidant, antimicrobial and toxicity of local spices Crotongratissimus (A), Xylopiaaethiopica (B) and measure effect of heat process on their characteristics of antioxidant, antimicrobial activity, and toxicity. Materials and methods Samples were purchased from JubelAulia and Klakla Al Lafa Markets, Sudan and prepared samples for analysis in three replicates. All samples were extracted by ethanol (80%) A Crotongratissimus and B Xylopiaaethiopica and extract treated by heat treatment (80°C) C Crotongratissimus and D Xylopiaaethiopica. Antioxidant activity was determined by using (DPPH) detector. Results: There were found the extracts with and without heat treatment (A, B, C and D) were grading from highest to lowest, respectively. Antimicrobial activity of (Croton gratissimus, Xylopiaaethiopica) extracts were determined against Bacillssubtilis, Staphylococcus aureus, Escherichia coli, Pseudomonasaeruginosa, and Candida albicans. Toxicity of extracts (Croton gratissimus, Xylopiaaethiopica) was determined for edible matter by using (MTT) assay method, and there were no toxic in both of them. Conclusion: African spices (Croton gratissimus, Xylopiaaethiopica) exhibited highest antimicrobial, antioxidant activities without any toxicity even in cooked food products.

Keywords: spices, Croton gratissimus, Xylopiaaethiopica, antioxidant, antimicrobial, activity, toxicity.

تأثير الحرارة على نشاط مضادات الأكسدة والنمو الميكروبي في بهارات الكومبا وأم غليلة

عفراء عوض الله أحمد
ياسر صلاح إبراهيم آدم
آمنه مختار حسين
كلية التقانة الزراعية وعلوم الأسماك || جامعة النيلين || السودان
محمد الأمين حسن
أكاديمية السودان للعلوم المصرفية والمالية || السودان

الملخص: الخلفية والأهداف: بهار أم غليله وبهار الكومبا عبارة عن شجيرات استوائية تنمو عشوائياً بأفريقيا. تستخدم تقليدياً كخافض للحمى ومانعة للنزيف ومسهل وكدواء لداء الاستسقاء. صممت هذه الدراسة لتقييم النشاط المضاد للأكسدة وللنمو الميكروبي وكذلك

السمية لتلك البهارات المحلية أم غليله(أ) والكومبا (ب)، وقياس تأثير أثر المعاملة الحرارية 80مº على خصائص النشاط المضاد للأكسدة و للنمو الميكروبي والسمية. الطرق والوسائل: تم شراء العينات من أسواق جبل أولياء والكلالة اللفة - السودان وتجهيزها للتحليل، حيث تم استخلاص جميع العينات بواسطة الايثانول 80% (أ) و(ب) والمستخلص المعامل بالحرارة 80مº (ج) و(د) كما تم تحديد نشاط المضاد للأكسدة بواسطة كاشف (DPPH). النتائج: قد تبين من خلال النتائج أن المستخلصات المعاملة بالحرارة والغير معاملة (ا، ب، ج، ود) تتدرج في نشاطها من الأعلى إلى الأقل على التوالي. تم تقييم النشاط المضاد للنمو الميكروبي لهذه المستخلصات ضد الميكروبات. Bacillssubtilis, Staphylococcus aureus, Escherichia coli, Pseudomonasaeruginosa, and Candida albicans. أما بالنسبة لسمية مستخلصات أم غليله والكومبا فتم تقييمها باستعمال طريقة assay(MTT)، ولم يستدل على أي سمية في كل منهما. الخلاصة: أظهرت البهارات الإفريقية أم غليلة والكومبا نشاطا مضادا للأكسدة وكمضادات للميكروبات متميزا بالإضافة إلى خلوها من أي سمية حتى في منتجات الغذاء المطبوخة.

الكلمات المفتاحية: بهارات، أم غليله، كومبا، نشاط، مضاد أكسدة، نمو ميكروبي، سم.

Introduction

Spices can be defined as vegetable products used for flavoring, seasoning and imparting aroma in food (FAO, 2005). Spices and herbs come from the following parts of aromatic plants: fruits (fennel, fenugreek, mustard), rhizomes, roots (ginger, turmeric), leaves (bay, marjoram, parsley, sage, and thyme), barks (cinnamon and cassia), floral parts (saffron, cloves), or bulbs (onion, garlic) andstems (coriander, cinnamon) (Ref??). Flavor is the primary purpose of spices and herbs, however some have been shown to have antimicrobial properties (Tiwari et al., 2009; Tajkarimi et al., 2010).

Similarly, many spices and extracts contained compounds that have been shown to have antioxidant activity (Sasse et al., 2009).

Spices had active compounds in class of naturally occurring food preservatives and have their inclusion in foods allowed by food production regulator (Raghavan, 2007).

Spices and herbs have been added to food since ancient times, and used for many centuries by various cultures to enhance flavor and aroma of our foods as our ancestors have recognized the usage of spices in food preservation and in treatment of clinical ailments and there are several reports on development of antibiotic resistance in diverse bacterial pathogens (Javed, 2011). Not only as flavoring agents, but also as folk medicine and food preservatives (Susheela, 2000). Furthermore, certain spices and herbs prolonged the storage life of foods by preventing rancidity through their antioxidants activity or through bacteriostatic or bactericidal activity, also to food-borne pathogenic bacteria (Susheela, 2000).

Much studies indicated that lipid oxidation and microbial growth in meat products can be controlled or minimized by using either synthetic or natural food additives (Mielniket al., 2003). Natural agents possessed antioxidant and antimicrobial properties have the advantage of being readily accepted by consumers, as they are considered natural (Sallama et al., 2004). The same author mentioned some spices and herbs used today are valued for their antimicrobial activities and medicinal effects in addition to their flavor and fragrance qualities. The extracts of many plant species have become popular in recent

years and the attempts to characterize their bioactive principles have gained momentum for varied pharmaceutical and food processing applications (Shan et al., 2007).

Spices are used widely in the food industry as flavors and fragrances. Besides, they also exhibited useful antimicrobial properties (Roller, 2003) and were used in food industry for shelf-life extension and wholesomeness. Therefore, there has been increasing interest to replace synthetic preservatives with natural, effective and non-toxic compounds (Roller, 2003). Those are, in the first place, extracts and essential oils (EOs) of spices and herbs (Javed 2011). As natural food stuffs, spices and herbs appeal to all who question safety of synthetic food additives and demand high-quality products that at the same time are safe and stable (Nwinuka et al., 2005), used and considered indispensable in many types of meat products for its colouring, flavouring, antioxidative and antimicrobial properties (Honikel, 2008). The key technological measures needed during storage is the preservation of the meat from microbial spoilage and contamination/proliferation of pathogenic microorganisms (Jang &Lee, 2005; Brightwelet al., 2009; Pennacchia et al., 2011). The using about 30 types of spices in meat products, but it is only in recent years that modern science has started paying much attention to the exploitation of desirable properties of spices (Aberle et al., 2001). The same author mentioned as technologies improved, processing and storage conditions reduced bacterial growth and meat processors were able to lessen the amount of salt added to products. This allowed greater diversity of flavors and subtle use of spices because salt did not overpower the other flavors (Aberle et al., 2001).

Croton gratissimus and Xylopiaaethiopica are tropical African shrubs or small tree with corky bark. It is traditionally used as a febrifuge, styptic, cathartic and is medic for dropsy. The spices occurred in Ethiopian, South Sudan, Ghana and part of South Africa. Croton gratissimus Burch (leaves with reddish scales below) C. gratissimus is used to treat coughs, fever, abdominal disorders, respiratory disorders, skin inflammation, earache, malarial and chest complaints (Van Vuuren, &Viljoen, 2008 ;Langat et al., 2011; Ashwell et al., 2013).

Xylopiaaethiopica or Ethiopian pepper as it is usually called, is an angiosperm belonging to the family "Annonaceae" and is among the species that thrive in the evergreen rain forests of tropical and subtropical Africa (Woode et al., 2011). This plant possesses great nutritional and medicinal values in African traditional medicine for several centuries owing to its wide array of therapeutic indications in the treatment of cough, bronchitis, malaria among other diseases. Almost all parts of Xylopiaaethiopica are very useful medicinally, but the fruits are most commonly used for therapeutic purposes (Ayodele et al., 2019). The objectives of the study to determine Antioxidant and antimicrobial activity for Croton gratissimus and Xylopiaaethiopica and Toxicity for edible, beside the effect of heat processing on Croton gratissimus and Xylopia aethiopica.

MATERIALS AND METHOD

Sample collection:

The spices samples collected from Sudanese local market (JabelAulia and Lafa). Two types of different spices purchased for use in three replicate.

Preparation of samples:

The samples were grounded to homogenous mass in a grinder, and then used for extraction.

Preparation of extract:

Extract of sample A Croton gratissimus and B Xylopiaaethiopica were treated by 80 °C for C Croton gratissimus and D Xylopiaaethiopica method describe by (Sukhdev et al., 2008). Weight 50 g of each plant sample was grounded using mortar and pestle and extracted by soaking in 80 % ethanol for about seventy two hours with daily filtration and evaporation. Solvent was evaporated under reduced pressure to dryness using rotary evaporator apparatus (Panchun Scientific Co., Kaohsiung, Taiwan) and the extracts were combined together. Each sample of plant were extract by the same methods and evaporated solvent by using Soxhlet apparatus at 80 °C to determined their activity for each one cooked.

Measurements of antioxidant content:

The free radical scavenging activity of the fractions was measured in vitro by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was determined according to the method of (Shimadaet al., 1992). with some modification. The test samples were to react with (DPPH) for half a hour at 37°C. The concentration of DPPH was kept as (300 µM). The test sample were dissolved in di methyl sulphoxide (DMSO) while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517nm using multiplate reader spectrophotometer (Varian Cary 50, Melbourne, Australia). Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. All tests and analysis were run in triplicate. Propyl gallate (PG) was used as standard.

Antimicrobial activity of spices extract:

The extracts were reconstituted to concentration of 100% in di methyl sulphoxide (DMSO). Antimicrobial activity was assessed by the agar – well diffusion method (NCCLS, 2002). The inoculum size of each tested bacterium was adjusted to a suspension of 10^6 cells. The inoculum suspension was spread over a Mueller Hinton Agar (MHA) plate, to achieve confluent growth, and allowed to dry. 10 mm diameter wells were bored in the agar using a sterile cork borer and the agar dices were removed. A 100 µl aliquot of the reconstituted extract was placed into a well with pipette and the plate was held for 1h at

room temperature for diffusion of extract into the agar. Subsequently, the plate was incubated for 18 h at 37 °C. After incubation, the diameters of the zones of inhibition were measured to the nearest mm.

Cytotoxicity screening:

Micro-culture-tetrazolium MTT-assay was utilized to evaluate the cytotoxicity of plants. This colorimetric assay is based on the capacity of mitochondria-succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) into an insoluble, blue colored-formazan, a product measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells (Patel et al., 2009**)**.

Cell Line and Culture Medium:

L20B (Normal cell line) cells were cultured in a culturing flask containing a complete medium consisting of 10% fetal bovine serum and 90% minimal essential medium (MEM) and then incubated at 37^{o}C. The cells were sub cultured twice a week (Stone et al., 2009).

Cell counting: (Stone et al., 2009).

Cells were counted using the improved Neubauer chamber. The cover slip and chamber were cleaned with detergent, rinsed thoroughly with distilled water and swapped with 70% ethanol, then dried. An aliquot of cell suspension was mixed with equal volume of 0.4% trypan blue in a small tube. The chamber was charged with cell suspension. After cells had settled, the chamber was placed under light microscope. Using 40 X objective, cells in the 4 large corner squares (each containing 16 small squares) were counted. The following formula was used for calculating the cells:

Number of cells counted X Dilution factor* X 10^{4}

*Dilution factor is usually 2 (1:1 dilution with trypan blue), but may need to further dilute (or concentrate) cell suspensions (Stone et al., 2009)..

MTT assay: (Patel et al., 2009)

Serial dilutions of extract were prepared in a 96 well flat bottomed plate (NalgeNunc, Inter.). The outer walls of the plate were filled with 250 µl of in-complete culture medium except the last row 6 middle wells (B - G), which were used for the negative control receiving 50 µl of culture medium and 2µl of sterile 0.5% Triton X. 50 µl/wells complete culture medium (CCM) were added and 30 µl more were added to second column wells (B – G) that were used as first extract dilution wells. To the first dilution wells in the row, 500 µg of c suspension extract were added to the 80 µl. extract were then serially diluted by two-fold dilution from well B3 till B11 by transferring 250 µl to the next well after proper mixing. From the last dilution wells (B-11), 50 µl were discarded. Each compound was tested in triplicate.

Cell suspension in a complete culture medium containing 2.5 X 10^5 /ml was properly mixed, and 150 µl of it were transferred into each well of the plate. The plate was covered and placed in 5% CO_2 incubator at 37 °C for three-five days (72 hours-120 hours). On the third/fifth day, the supernatant was removed from each well without detaching cells. MTT ((3- (4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole) stock (5 mg/ml) was prepared earlier in 100 ml PBS (phosphate buffer saline). MTT suspension was vortexed and kept on magnetic stirrer until all MTT dissolved. The clear suspension was filter sterilized with 0.2 µ Millipore filter and stored at 4 °C or – 20 until use. MTT was diluted (1:3.5) in a culture medium and brought to room temperature. To each well of the 96 well plates, 50 µl of diluted MTT were added. The plate was incubated further at 37° C for 2 to 3 hours in CO_2 incubator. MTT was removed carefully without detaching cells, and 200 µl of DMSO were added to each well. The plate was agitated at room temperature for 15 minutes then read at 540 nm using micro plate reader**.** The percentage growth inhibition was calculated using the formula below:

% cell inhibition = 100-{(Ac-At)/Ac} × 100

Where, **At**=Absorbance value of test compound;

Ac=Absorbance value of control.(Patel et al., 2009)

Statistical analysis:

All determinations were carried out at least in triplicates. One way ANOVA was used to find statistical difference between the means of the values reported. The means were separated by using new Duncan multiple range technique with (SPSS.v. 20, 2011).

Results and Discussion:

Antioxidant activity:

The results of this study for antioxidant activity for extract of Croton gratissimus and Xylopiaaethiopica with and without heat treatment shows in Table 1 and there were found high percent of antioxidant activity for C, and D which were treated by 80 °C, than A and B (without heat treatment), on the other hand Croton gratissimus extracts antioxidant activity a higher percent than Xylopia aethiopica Extract. Adam et al.(2016) mentioned the sample of cloves (92%) had a higher value compared with the other samples and lower value for Cumin (0.3%). The samples Thyme (29.5%) (AnetaWojdyło et al., 2007), cardamom (24.2%), ginger (23.5%), fennel (30.3%) and cumin (37.4%) (Iris hinneburget al., 2006) this values were not agreement with the samples cardamom (22 %), ginger (77%), fennel (44%) and cumin (0.3%). The Antioxidant activity was significantly different among spices samples it might be refer to type, and solvent, or individual (Ashwell et al., 2013). However, natural agents possessing antioxidant and antimicrobial properties have the advantage of being readily accepted by consumers, as they are considered natural (Sallama et al., 2004).

Table (1) Antioxidant activity of spices extracts measured by DPPH inhibition %.

NO	Sample Code	% RSA±SD (DPPH)
1	A	86±0.02
2	C	88±0.03
3	B	87±0.09
4	D	90± 0.08
Standard	Propyl Gallate	92±0.01

Antimicrobial activity of Croton gratissimus and Xylopiaaethiopica extracts were examined against to Escherichia coli, Pseudomonas, Staphylococcus aureus, Bacillus subtilisand, candida presented in Table2. The highest antimicrobial activity against E.coli for hot extract of Xylopiaaethiopica and lowest one for Croton gratissimus extract according to method (NCCLS 2002). The extract of Croton gratissimus and Xylopiaaethiopica with and without heat treatment there were a highest activity against E.coli. Also, Pseudomonas, Staphylococcus aureus, Bacillus subtilis and Candida albicans were sensitive to Xylopiaaethiopica and Croton gratissimus extract with and without heat treatment

Table (2) Sensitivity of E.coli, Bacillus subtili, Pseudomonas, Staphylococcus areus, Candida albicans in 100 concentration of Spices.

	A	B	D	C
E.coli	17.5±0.2	18.5±0.8	23.5±0.2	20±0.00
Pseudomonas	20±0.0	19.5±0.2	15±0.00	19.5±0.2
Staphylococcus	16.5±0.2	18.5±0.8	20 ± 0.00	17.5±0.2
Bacillus	16±0.00	19±0.00	16±0.00	14.5±0.2
Candida	16±0.00	13.5±0.8	18.5±0.8	19±0.5

Toxicity of extract:-

There were no toxicity for Crotongratissimus and Xylopiaaethiopica extracts with and without heat treatment in three different concentration (125, 250and 500 µg/ml).When IC_{50} < 30 µg/ml: high toxic, >100 µg/ml: no toxic *Control= Triton –x100 was used as the control positive at 0.2 µg/ml.

Table (3) Cytotoxicity screening MTT assay of Croton gratissimus and Xylopiaaethiopica

Name of extract	Concentration(µg/ml)			IC50 (µg/ml)	IC50
	500	250	125		
A	79.71±0.03	72.86±0.04	49.43±0.09	121.51	>100
C	80±0.02	76.86±0.04	51.71±0.08	112.75	>100
B	78.57±0.03	67.71±0.05	51.71±0.03	110.56	>100
D	71.14±.0.02	68.86±0.01	46.57±0.09	142.32	>100
Control		96.28±0.01			>30

The results agreement with Ayodele et al.(2019), Xylopiaaethiopica fruit ethanol extract (XAFEE) exerted its oral acute toxicity at the concentration higher than 3000 mg/kg. However had no effect at the 1000, 2000 and 3000 mg/kg. The median lethality dose of Xylopiaaethiopica fruit ethanol extract (XAFEE) suggested that the fruit may not be completely safe for consumption at a dose higher than 3000 mg/kg. As though no report yet on the median lethality (Ayodele et al. 2019).

Conclusion:

This study was concluded that Croton gratissimus and Xylopiaaethiopica exhibited a high antioxidant activity and increased by heat process, beside antimicrobial activity against E.coli, Staph. areues, Bacillus subtili, Pseudomonas, candida albicans.

All samples of Croton gratissimus and Xylopiaaethiopicawithout toxicity for edible matter. From results of this study. It could be recommended to use Croton gratissimus and Xylopiaaethiopicain food industry especial meat products for prolong shelf-life, whereas it added in the traditional cooked and improved taste.

Significant statement

The study significant to discovered African spices which had excellent preservative proprieties Croton gratissimus and Xylopiaaethiopica in food processing without side effect, but must be treated carefully for the flavor and food taste. Some mixing of selected spices should be replacement the chemical preservation in food industry. However, these spices had good power to enhance or prolong the food shelf-life which had a higher antibacterial and antioxidant contents.

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