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The Antibacterial and Phytochemical Evaluation of Extracts of Grape *(Vitis Vinifera)* Seeds

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ABSTRACT: *The skins and seeds of grapes are known to be rich sources of phenolic compounds, both flavonoids and non-flavonoids. These compounds, when present in plants have been confirmed to have antibacterial property. The antibacterial and phytochemical evaluation of grape seeds was done in vitro on selected bacteria (Bacillus cereus, Escherichia coli, Proteus vulgaris, Staphylococcus aureus, Shigella dysenteriae and Salmonella typhi). The seeds were collected from a local farmer in Ijare, Ondo State, dried and extracted using 98% ethanol and methanol respectively after blending the seeds to powder. The qualitative phytochemical analysis of the seeds was done using standard methods. The results showed that the methanol extract was more effective than the ethanol extract and had the highest diameter of zone of inhibition of 16.90mm on Staphylococcus aureus. The methanol extract had an average minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 25mg/ml on the microorganisms. It however had both MIC and MBC of 12.5 mg/ml on S. aureus. The extracts of the seeds contained various phytochemicals such as alkaloids, phenolic compounds, flavonoids, saponins, glycosides, phytosterols, and tannin. However, both of the extracts showed significant levels of antibacterial activity. Methanol extract was the most active one with remarkable antibacterial activity on the various species tested. The findings of the present study indicated that the seeds of Vitis vinifera possess various secondary metabolites having the potential for developing pharmaceutical drugs, especially antimicrobial ones*.

KEY WORDS: antibacterial, phytochemicals, grape seeds, extracts.

INTRODUCTION

Fruits normally carry non-pathogenic epiphytic microflora. However, there are certain factors, which contribute to the microbiological contamination of these products with pathogens. Contamination can arise as a consequence of treating soil with organic fertilizers such as manure and sewage sludge and from irrigation water. Another aspect contributing to the microbial risk for

the consumer is the increasing consumption of new products (e.g sprouted seeds) or fruits (Afolabi *et al*., 2007).

The application of technologies such as cutting, slicing, skinning and shredding will remove the natural protective barriers of the intact plant and open the possibility for providing a suitable medium for the growth of contaminating microorganisms. Some pre harvest measures will reduce the microbial contamination of fruits. However, the relative contribution of these measures is not always equal in terms of efficacy or the level of safety achieved. Manure, bio-solids and irrigation water should be of a quality that does not introduce pathogens to the treated commodity (Edris, 2007)

Harvesting at the appropriate time and storing the harvested products under controlled conditions will help retard growth of post-harvested spoilage and pathogenic microorganisms (Afolabi *et al*., 2007). Humid and warm storage conditions encourage the growth of microbial contaminants. The use of additional post-harvest procedures could reduce the contamination level f fruits. Washing with water of potable quality can reduce the microbial load. Although a wide range of different agents is available for disinfecting or sanitizing fresh produce, their efficacy is variable and none are able to ensure elimination of pathogens. (Bennerman, 1993).

The skins and seeds of grapes are known to be rich sources of phenolic compounds, both flavonoids and non-flavonoids. There are two main types of grapes: European grapes (Vitis vinifera) and North American grapes (Vitis labrusca andVitis rotundifolia). The European grapes (Vitis vinifera) represent 95 percent of grapes produced. The European grapes have a thick skin and sweet flesh which adheres firmly to the skin. The most important North American grape is the Concorde grape (Afolabi *et al*., 2007). The grape vine is a woody climber with more or less twisted stems and large lobbed leaves. The grape vine attaches to other plants with its coiled climbing tendrils. The grape flowers are small and are formed on a light green panicle. In autumn, the grape vine forms blue, red or green colored grape berries (or simple called grapes) that contains between 5 and 10 seeds.

A lot of research has been done on grape juice, grape skin, the tree leaves, stem and roots etc. However, not much work has been done on the seeds, hence the objectives of this study are to evaluate the antibacterial property of ethanol and methanol extracts of grape seeds and determines the major phytochemicals present in these extracts of the seeds.

MATERIALS AND METHODS

Materials

The materials used in this work include dried grape seeds, conical flask, Petri dishes, test tubes, bijou bottles, measuring cylinder, thermometer, sterile container for sample collection, foil paper, ethanol, methanol, distilled water, cotton wool, paper tape and slides.

Sample collection

The grape seed sample was obtained in a fresh form from a farmer in Ijare village, close to Akure and taken to the Microbiology Department of the Federal University of Technology, Akure, Ondo State. They were washed with sterile distilled water before drying them for further Laboratory analysis.

Sterilization of glass wares

All the glass ware used such as conical flask, Petri dishes, measuring cylinder, McCartney bottles and test tubes were properly cleaned and sterilized using oven at a temperature of 180^0C for 2 hours (Gnamani *et al*., 2003).

Preservation of culture

Nutrient agar powder was dissolved in distilled water according to the manufacturer specification to prepare a double strength agar. 10ml of the dissolved agar were dispensed into each bijou bottles and screwed tight for sterilization in an autoclave at 121^{o}C for 15 minutes, after sterilization the agar was allowed to set in a slanting position, with sterile inoculating loop, a loopful of the pure inoculum is streaked on the surface of the slant agar aseptically.

Antibiotic sensitivity test

The antibiotic sensitivity test was carried out in order to know the sensitivity of the microorganism to the different commercially available antibiotics. These antibiotics discs include: Augmentin, Amoxacillin, Ofloxacin, Gentamycin, Cotrimoxazole, Nitrofurantoin, Nalidixic acid and Tetracyclin. Disc diffusion method was to determine the effect of standard antibiotics on the bacterial isolates as described by Javasinghe *et al*.,(2008). Sterile Petri dishes were seeded aseptically with 18hours old pure cultures of the test organisms each while about 15ml of sterilized Muller- Hinton agar was poured aseptically on the seeded plates. The plate were swirled carefully for even distribution and allowed to gel. With the aid of sterile forceps the antibiotics disc were placed firmly on solidified plates and incubated for 24hours at 37^{o}C. After incubation, clear areas around the disc represent the zones of inhibition (McMahon *et al*, 2005) and the areas without clear zones were also observed. Seeded agar plates without antibiotics disc served as the control experiment. The zones of inhibition were measured in millimeter (mm). The experiment was carried out in triplicate.

Extraction from grape seeds
This was done using standard method. 100g of sample was soaked 400ml of solvent in ratio of 1:4 for 72hrs before filtering and drying the solvent using rotary evaporator.

Susceptibility of isolates to extracts
The ethanol and methanol extracts of grape seeds were prepared into 100 mg/ml of the extracts and tested *invitro* on the isolates according to the method of Sofowora (1993).

Phytochemical determination

Determination of Tannin
Zero-point-two millilitres (0 20ml) of the sample was weighed into test tubes and tannin was extracted in ten millilitres (10ml) 70% acetone. The test tubes were then placed in cold water bath for 10minutes to allow for complete extraction of tannin. Zero-point-two millilitres (0.2ml) was filtered into test tubes and made up to one millilitre (1ml) with distilled water. Two-point-five millilitres (2.5ml) of 20% Na_2CO_3 and zero-point-five millilitres (0.5ml) with distilled water were added and the content was mixed properly. The solution was incubated for 45min at room temperature to develop colour (blue colour). The absorbent of each samples were read at wavelength 700nm using a Coring colorimeter 253, Corning Ltd, Essex, England.

Determination of phenol
Phytate was determined by weighing four grams (4g) of the sample and soaking in one hundred millilitres (100ml) of 2% HCl for 3 hours and then filtered, twenty-five millilitres (25ml) of the filtrate was placed in a conical flask. Five millilitres (5ml) of 0.3% ammonium thiocyannate (NH_4SCN) solution was added as indicator and diluted with distilled water. This was titrated with standard $FeCl_3$ solution until a brownish yellow colour persisted for 5minutes.

Determination of phytosterol
One gram (1g) of the sample was placed into labelled plastic bottles followed by the addition of seventy-five millilitres (75ml) of H_2SO_4. The content was mixed properly and allowed to extract for one hour with constant agitation using a mechanical shaker. This was then filtered and twenty-five millilitres (25ml) of the filtrate was titrated with zero-point-one millilitres (0.1ml) of $KMnO_4$ while hot (80 – 90^oC) until a purple colour was observed. The titre value was then multiplied by 0.9004 to give the result expressed as mg/g.

Flavonoid determination
Ten grams (10g) of the sample was extracted repeatedly with 100ml of 80% aqueous methanol at room temperature. The whole solution was filtered through what man filter paper No 42 (125mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight (Doughari, 2012).

Alkaloid determination

Five grams of grape blended seeds was weighed into a 250ml beaker and two hundred millilitres (200ml) of 10% acetic acid in ethanol was added, covered and allowed to stand for four hours to allow it extract. This was filtered and filtrate was placed in a water bath to allow it concentrate to one quarter of the original volume. To the concentrated samples, ammonium hydroxide was added drop wisely until the precipitation was completed. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid which was dried and weighed.

Determination of glycoside in grape seeds

Four grams (4g) of blended grape seeds sample was soaked in a mixture of forty millilitres (40ml) of distilled water and two millilitres (2ml) of orthorphosphoric acid. The sample was thoroughly mixed and covered and left overnight at room temperature checking the absorbance (AOAC, 2005).

Statistical Analysis of Result

Result obtain will be subjected to descriptive one way analyses of variance, SPSS version 16 Microsoft windows 7 and Duncan multiple range test will be used as follow up test.

RESULTS

Extraction Results

The results of the extraction showed that after 72 hours of soaking the blended seeds in the both solvents, ethanol had the highest percentage yield of 14.5 %, while that of methanol extract was 11.0%.

***Invitro* antibacterial assay result**

The result of the invitro antibacterial assay showed that the methanol extract had a higher antibacterial effect than that of the ethanol extract. The highest diameter of zone of inhibition exerted on the bacteria by the methanol extract was on S. aureus, with a diameter of zone of 16.90 mm, while the lowest diameter of zone of inhibition exerted by the methanol extract was on S. typhi, with a zone diameter of 10.20 mm. The ethanol extract on the other hand had its highest inhibitory effect on both S. aureus and S. typhi with about 13.00 mm, while its lowest inhibitory effect was on Escherichia coli with a zone diameter of 8.20mm as shown in table 1.

Table 1: Diameter of zones of inhibition of ethanol and methanol extracts on test isolates.

S/N	Bacteria	Ethanol Extract	Methanol Extract
1	*Bacillus cereus*	8.75±1.15	12.54±1.02
2	*Escherichia coli*	8.20±2.05	14.10±1.10
3	*Proteus vulgaris*	10.00±0.00	13.65±0.55
4	*Staphylococcus aureus*	12.90±1.20	16.90±1.30
5	*Shigella dysenteriae*	10.25±1.58	12.30±1.57
6	*Salmonella typhi*	12.55±0.55	10.20±0.60

The minimum inhibitory concentration, (MIC) showed that S. aureus had the least inhibitory concentration of 12.5 mg/ml for both the ethanol and methanol extracts respectively. The same S. aureus had lowest minimum bactericidal concentration as shown in tables 2 and 3 respectively.

Table 2: Minimum inhibitory concentration (mg/ml) of the extracts on bacteria isolates

S/N	Bacteria	Ethanol Extract	Methanol Extract
1	*Bacillus cereus*	50.00	50.00
2	*Escherichia coli*	50.00	25.00
3	*Proteus vulgaris*	25.00	25.00
4	*Staphylococcus aureus*	12.50	12.50
5	*Shigella dysenteriae*	12.50	25.00
6	*Salmonella typhi*	25.00	25.00

Table 3: Minimum bactericidal concentration (mg/ml) of the extracts on bacteria isolates

S/N	Bacteria	Ethanol Extract	Methanol Extract
1	*Bacillus cereus*	50.00	100.00
2	*Escherichia coli*	50.00	50.00
3	*Proteus vulgaris*	100.00	25.00
4	*Staphylococcus aureus*	25.00	50.50
5	*Shigella dysenteriae*	50.00	50.00
6	*Salmonella typhi*	50.00	25.00

Comparative evaluation of the extracts and standard commercial antibiotics tested for showed that the extracts compete favourably with the antibiotics. Figure one (1) compares the inhibitory effect of the extracts and the two most effective or most inhibiting antibiotics on the bacterial isolates. Ofloxacin was the most inhibiting of all the antibiotics used, followed by nalidixic acid.The result of the phytochemical screening showed the presence of or detection of seven different phytochemicals. These are cyanogenic glycosides, phytosterol, alkaloids, saponin, phenol, tannin and flavonoid. This result is shown n table 5.

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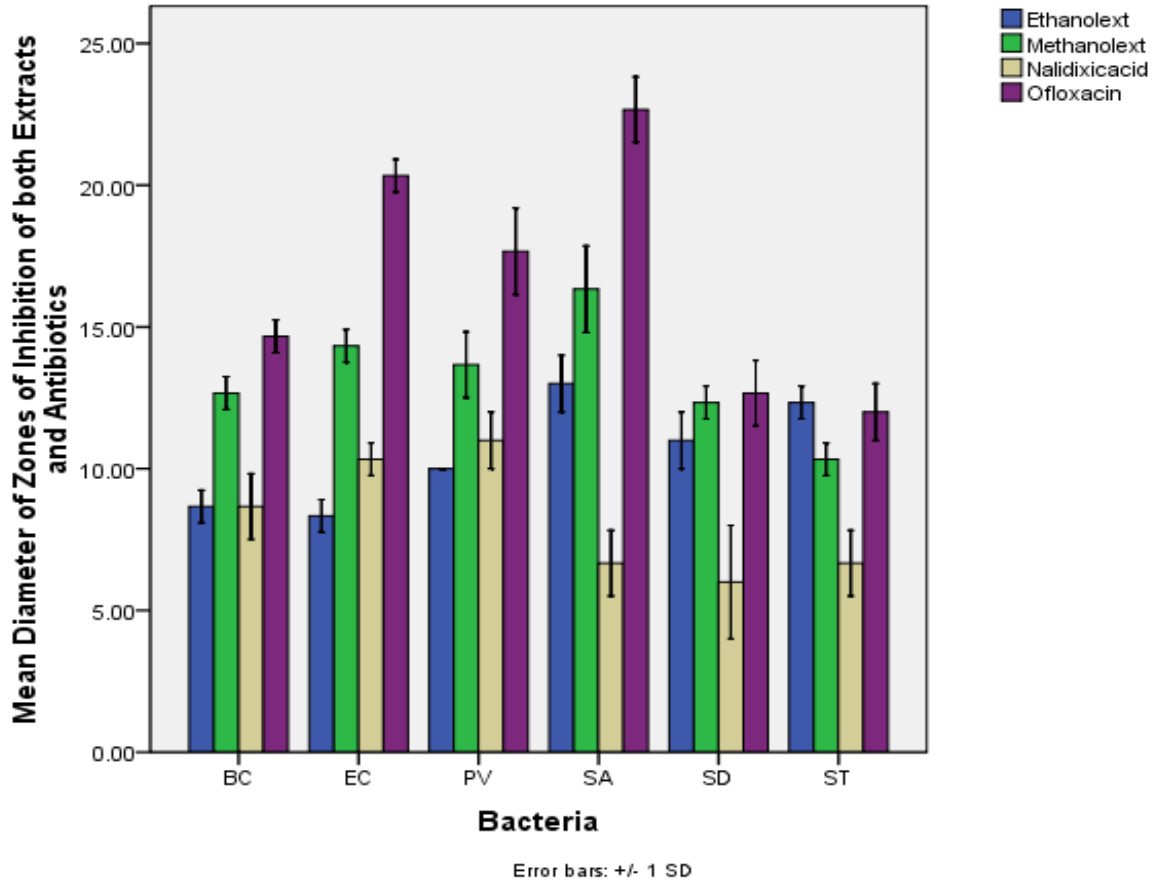

Figure 1: Comparative inhibitory effect of both extracts and the two most inhibiting antibiotics.

Table 4: Diameter of zones of inhibition of commercial antibiotics used on test isolates.

Bacteria	Aug	Amp	Ofl	Gen	Cot	Nit	Nal	Tet
B.cereus	8.20±0.40	0.00±0.00	14.80±1.40	0.00±0.00	5.00±0.00	0.00±0.00	8.00±0.00	0.00±0.00
E.coli	4.12±0.20	2.00±0.00	20.20±0.10	0.00±0.00	4.50±0.10	0.00±0.00	10.20±0.60	0.00±0.00
P.vulgaris	4.00±0.00	6.00±0.00	18.00±0.00	2.00±0.00	0.00±0.00	0.00±0.00	10.00±0.00	5.13±0.17
S.aureus	2.00±0.00	0.00±0.00	22.00±0.00	0.00±0.00	0.00±0.00	4.00±0.00	6.03±0.33	0.00±0.00
S.dysenteriae	6.00±0.00	5.00±0.00	12.00±0.00	0.00±0.00	3.00±0.00	4.20±0.50	4.10±0.20	9.05±0.55
S.typhi	2.00±0.00	3.00±0.00	11.00±0.00	0.00±0.00	6.50±0.20	0.00±0.00	4.50±0.05	5.15±0.25

Key: Aug=Augmentin, Amp=Ampicillin, Ofl=Ofloxacin, Gen=Gentamycin, Cot=Cotrimoxazole, Nit=Nitrofurantoin, Nal=Nalidixic acid, Tet=Tetracyclin

Table 5: Phytochemical Results

S/N	Phytochemical	Present
1	Cyanogenic glycosides	+
2	Phytosterol	+
3	Saponin	+
4	Alkaloid	+
5	Phenol	+
6	Flavonoid	+
	Tannin	+

DISCUSSION

The result of the extraction showed that ethanol is a good solvent of extraction for higher yield of the active ingredients of these grape seeds. This is because the percentage yield obtained from the extraction using ethanol was more than the percentage yield obtained from the use of methanol as solvent. This result is in agreement with the result obtained by Sahoo et al., (2012), who used six different solvents to extract from a particular plant leaf and root and got the highest yield from ethanol solvent.

The result of the antibacterial effect of the extracts however showed that the methanol extract of the grape seed exert a better inhibitory effect than that of the ethanol extract. This probably might be due to the ability of the methanol to extract the active ingredients of the seeds with less impurity. According to Kar, (2007), Jothinel and Paul, (2008) and Maizura et al., (2011), most of the extractions with methanol have proven to have more antibacterial effect than ethanol extract. Kumar, (2011), said that most of the extractions done using methanols, especially methanol above 90% concentration have had little impurities during purification via chromatography. This deduction however, was strictly in comparison with ethanol and water as solvents.

The fact that the highest zone diameter of inhibition was on Staphylococcus aureus for both extract means that the grape juice could be effective as well against the bacteria. According to previous work on the antibacterial property of grape fruit juice, Forbes *et al*., (2007) got grape juice to have the highest antibacterial effect on Gram positive bacteria. Cabello *et al*., (2009), carried out an investigation on the factor inbuilt in microorganisms that help them resist antimicrobial agents. They concluded that the cinnamon-derived Michael acceptor cinnamic aldehyde which impairs melanoma cell proliferation wasresponsible in S. aureus. Therefore, any substance that will inhibit the bacteria must have high content of phenols which are escharotics in nature and has the ability to reduce invasiveness of bacterial cell.

Phenols and flavonoids were detected in the grape seed extract and therefore could have contributed to the antibacterial property of the extracts. The result of the phytochemical analysis is agreement with the result obtained by Doughari *et al*, (2012), who got similar result when he analyzed different species of citrus plants. The result obtained in this work has shown that grape seed extracts have antibacterial property and that the biologically active components could be extracted for the development of new antibacterial drugs or compounds.

This project work has shown that both the ethanol and methanol extracts of grape seeds have antibacterial property and inhibited the growth of the test bacteria used in this work. It has also shown that the methanol extract of grape seed was more effective since it had higher diameter of zones of inhibition on the test bacteria.

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