# Accessing the Antimicrobial Potency of *Piper guineense (ODUSA)* Seed Extracts on Bread Spoilage Organisms

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***Abstract***

*The increasing demand of consumers for more natural foods and food safety has drawn the attention of Food Scientists and Technologists to the development of natural antimicrobials to preserve food instead of the chemical preservatives. The aim of this present work was to evaluate the antimicrobial potency of Piper guineense seed on bread spoilage organisms using ethanolic and aqueous solution. Room temperature dried seed of Piper guineense were screened for it phytochemical constituents and antimicrobial activity using aqueous and ethanolic extracts. The antimicrobial activity was determined based on the presence and absence of zones of inhibition. The seed extract of Piper guineense contain secondary metabolites. The ethanolic extract exhibited highest zones of inhibition that ranged from 7-25mm and aqueous extract imbibition ranged from 7-22mm with concentration of 500mg/ml - 100mg/ml. The result of this research suggests that Piper guineense seed is a promising preservative agent because of it valuable phytochemicals known for microbial inhibition.*

***Key Words:*** *Piper guineense, seed, phytochemicals, Zones of inhibition, Antimicrobial constituent.*

## Introduction

The existence of food borne illnesses, food spoilage, food waste and the resulting negative economic impact of these issues compelled with consumer interest have all pushed the food industry to find alternative, safe and natural antimicrobials to use in foods and beverages. In recent times, consumers are even more concerned of the processed foods they eat not only because of the high risk of contaminations but also because of the added synthetic preservatives which may be hazardous to health. The increasing demand for food with longer shelf-life, food with little or no chemical preservatives, coupled with the concern about toxic effects of some preservatives has also resulted in increased pressure to find alternatives to better health care (Negi, 2022).

*Piper guineense* (Odusa in Ibibio) is a flowering vine in the family piperaceae, cultivated for its leaf and fruit. The fruit is usually dried and used as spices for seasoning. It is commonly utilized in Nigeria due to its potent and effective medicinal properties. It has been used to treat a range of illnesses such as dysentery and bronchitis (Cowan, 2019). Bactericidal and bacteriostatic properties have been reported in this plant, which are traced back to the high concentrations of powerful metabolites in the leaves and seeds (Omodanioro and Evalone,

2013), The secondary metabolites that are most important in these extracts are alkaloids, reducing sugars, tannins and saponins (Konning *et al*., 2014). Ethanolic and methanolic extractions of this crude plant extract have better antimicrobial activity when compared to the aqueous plant extract, due to the solvents abilities to extract more phytochemicals from the plant matter (Nwinyi *et al*., 2019; Negi, 2021).

Bread is a food product that is universally accepted as a very convenient form of food that has desirability to all population both rich and poor, rural and urban. Its origin dates back to the Neolithic era and is still one of the most consumed and acceptable stables in all parts of the world. Bread is subjected to various spoilage problems, ranging from physical, chemical and microbial where the latter is the most serious one (Saranraji *et al.,* 2012)

The growing concern about food safety due to the use of artificial or chemical preservatives has recently led to the development of natural antimicrobials to control food borne pathogens and spoilage bacteria (Vazirian *et al.,* 2015; Hogue *et al.,* 2018). There has been constant increase in search of alternative and efficient compounds for preservation aimed at a partial or total replacement of chemical preservatives in bread production. Since chemical preservatives could be toxic to human, therefore, plants materials with antimicrobial properties has a possible application in bread production hence, the research.

Major antimicrobial compounds in *Piper guineense* (Odusa) have been reported to inhibit growth of both gram positive and gram-negative microorganisms. Therefore the purpose of this study was to investigate the antimicrobial potency of *Piper guineense* seed as a preservative recipe in the production of bread.

**Statement of the Problems**

In recent years, there has been pressure from both legislation and consumers to reduce the quantity of preservatives added to food products. However, a reduction in the dose of preservatives used to control mold spoilage in baking product may lead, under certain conditions to a reduction in the shelf-life of the product as spoilage in bread is a serious economic concern [Magan and Aldred, 2014]. Secondly, the need to fill the gap associated with the economy and the high cost of artificial preservatives necessitated this research.

## Materials and Methods

### Source of Materials

The seed of *Piper guineense* used in this study was procured from Itam market in Itu Local government Area of Akwa Ibom State, Nigeria. It was authenticated in the Department of Pharmacognosy and Natural Medicine, University of Uyo, Nigeria.

### Processing of Materials

#### Preparation of Plant Sample

The seed of *Piper guineense* was thoroughly cleaned and dried at room temperature for two weeks. The dried seed was then grounded to fine powder using Molino Victoria traditional grain mill (high hopper Ret 600009 columbia) and stored in air tight bags. A 850g of the pulverized seed of *Piper guineense* was obtained the *Piper guineense* seed.

#### Ethanolic Extract Preparation

A 425g of the pulverized plant material was weighed using an electronic scale and soaked separately in 70% ethanol in conical flask and allowed to stand at room temperature for 72

hours with occasional stirring twice in every 24hours. The extracts were filtered using cottonwool, beaker and funnel for up to three times. The resulting solution was concentrated using water bath at 45°C until a semi solid extract was obtained. It was then transferred to a small beaker and weighed, and thereafter stored in the refrigerator at 4°C until required for use.

**Aqueous Extract Preparation**

A 425g of the Pulverized Plant Material was weighed using an electronic scale and soaked in an aqueous solution. After 24hours the preparation was filtered using cotton wool, beaker and funnel for up to three times. The resulting solution was concentrated using water bath at 45°C until a semi solid extract was obtained. It was then transferred into a small beaker and stored in the refrigerator at 4°C until required for use.

**The flow chart for the preparation of *Piper guineense* seed extract is shown in fig. 1**

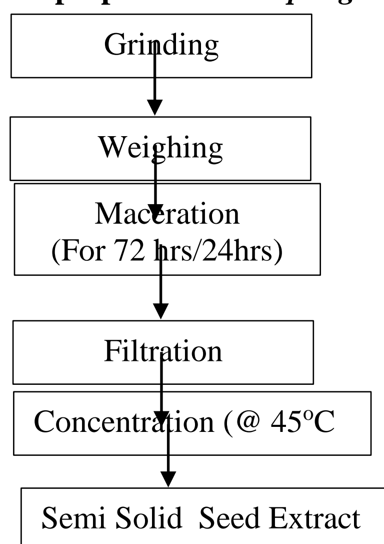


**Figure 1:** Flow chart for *Piper guineense* seed extract preparation

(Negi, P.S. 2022)

**Qualitative and Quantitative Phytochemical Screening of *Piper guineense* Seed Extract**

The qualitative phytochemical screening was carried out using the methods described below:

**Test for Phenols and Tannins:** 2ml of 2% solution of $FeCl_3$ was mixed with the crude extract. Black or blue green colour indicated the presence of tannins and phenols.

**Test for Flavonoids: Shinoda Test:** Pieces of magnesium ribbon and concentrated Hcl was mixed with the crude seed extract after few minutes, the appearance of pink colour scalet indicated the presence of flavonoids.

**Test for cardiac glycosides:** 5ml of extract was treated with 2ml of glacial acetic acid containing one drop of ferric choloride solution. This was underplayed with 1ml of concentrated sulphric acid a brown ring at the interface indicated the deoxysugar characteristics of cardenolides.

**Test for saponins:** 5ml of distilled water was added to crude plant extract in a test tube and it was shaken vigorously. The foam formation indicated the presence of saponins.

Liebermann's test 2ml of acetic acid and 2ml of chloroform mixed with seed entire crude extract. The mixture was then cooled and concentrated. $H_2SO_4$ was added, a green colour indicated the entity of aglycone steroidal part of glycosides.

**Test for terpenoids:** 2ml of chloroform was mixed with the plant seed extract and evaporated on the water bath, then boiled with 2ml of concentrated $H_2SO_4$. A gray colour produced indicated the entity of terpenoids.

**Test for Anthraquinone family of quinine:** Bontrager's test to a portion of the extract in a dry test tube, 5ml of chloroform was added and shaken for at least 5minutes. This was filtered and shaken with equal volume of 10% ammonia solution, there was no colour change observed which indicated the absence of anthraquinone.

**Test for Alkaloids:** About 2ml of 10% aqueous hydrochloric acid was stirred with 2ml of plant seed extract. 1ml was treated with a few drops of Dragendorff's reagent.

**Quantitative Phytochemical Screening**

**Determination of Alkaloids:** Alkaloid determination was performed by the method reported by Ezeonu and Ejikeme (2016). 200cm$^3$ of 10% acetic acid in ethanol was added to powdered sample (2.5g) in a 250cm beaker and allowed to stand for 4 hours. The extract was concentrated on a water bath to one quarter of the original volume followed by addition of 15 drops of concentrated ammonium hydroxide dropwise, to the extract until the precipitration was complete immediately after filtration. After 4 hours of mixture sedimentation, the supernatant was discarded and the precipitates were washed with 20cm$^3$ of 0.1m of amoninum hydroxide and then filtered using filter paper.

**Determination of Flavonoid**

About 50cm$^3$ beaker, covered and allowed to stand for 24hours at room temperature. After discarding the supernatant, the residue was re-extracted (three times) with the same volume of ethanol. Filter paper was used to filter whole solution of each sample. The filtrate was later transferred into a crucible and evaporated to dryness over a water bath. The content of the crucible was cooled in a desciator and weighed until constant weight was obtained.

**Determination of saponin**

About 100cm$^3$ of 20% aqueous and ethanol solution was added to 5grams of powdered sample in a 250cm$^3$ conical flask. The mixture was heated over a hot water bath for 4 hours with continuous stirring at a temperature of 55$^o$C. The residue of the mixture was re-extracted with another 100cm3 of 20% aqueous and ethanol after filtration and heated for 4 hours at a constant temperature of 55$^o$C with constant stirring. The combined extract was evaporated to 40cm$^3$ over water bath at 90$^o$C. 20cm$^3$ of diethyl ether was added to the concentrate in a 250cm$^3$

seperator funnel and vigorously agitated from which the aqueous layer was recovered while the ether layer was discarded. This purification process was repeated twice. $60cm^3$ of n-butanol was added and extracted twice with $10cm^3$ of 5% sodium chloride. After discarding the sodium chloride layer the remaining solution was heated in a water bath for 30 minutes after which the solution was transferred into a crucible and was dried in an oven to a constant weight.

**Isolation of Fungal species from Bread Samples**

The spread plate method was used to isolate fungi from bread sample. This was carried out by sprinkling a little quantity of water on the bread and stored for five days at room temperature.

**Preparation of medium for inoculation of fungal isolates:**

Sabouraud Dextrose Agar used for the experiment was prepared according to manufacturer's instruction.

**The spread plate method**

The spread plate method was used for the culture of isolates from the bread sample. Precisely, 1g of bread sample was accurately measured and placed on sterile Sabouraud Dextrose Agar plates (Prescott 2004).

**Incubation of Fungal plates**

Fungal plates were incubated at room temperature for 5-7days.

**Purification and Maintenance of Microbial Isolates**

Discrete colonies from culture plates were picked for characterization. Fungal colonies were sub-cultured into sterile SDA plates and incubated at $28^oC$ for 5days to allow for sporulation and thereafter maintained on agar slants as stock and preserved in the refrigerator for further used.

**Preparation of test organisms used for the Experiment.**

Test organisms were serially diluted using 10-fold dilution to $10^3$. Thereafter, the last dilutions were compared with McFarland turbidity standard.

**Determination of antimicrobial assay of plants extracts: (Zones of Inhibition)**

Antimicrobial activity of the extracts were evaluated using the Agar well diffusion technique (Okeke *et al.,* 2019). Test organisms were diluted using Malt extract broth for the isolates. The last dilution for each isolate was compared to McFarland Turbidity standard. 0.1ml of each diluted test organisms were aseptically transferred and spread on the surface of the Muller Hinton agar (MHA), sterile swab sticks were used to spread the inoculum on the surface of the medium and allowed them to dry on the bench.

A sterile cork-borer of 5mm was used to bore holes on the surface of the medium that were seeded with the test organisms. In each of the well bored on the seeded plates with test

organisms, 0.2ml of the two plant extracts dilution of different concentrations were introduced into the wells.

Control experiment was set up alongside with the extracts using commercial antifungal drug (Nystatin) 10mg/ml.

All plates were left on the bench for 1hr before incubating at 28ºC for 3-5days. After incubation, antifungal activities were determined by measuring the Inhibition Zone Diameter (IZD) in all the plates containing the plant extracts.

**Determination of Minimum Inhibitory Concentration (MIC), Fungi static and Fungicidal concentrations of the plant extracts on fungal isolates**

The minimum inhibitory concentrations (MIC) of the extract were determined using the tube dilution method. To obtain this, 1ml from the last concentration of the extract that had activity for any organism was further diluted to obtain lower concentrations of the extract. A loop-full of each diluted test organism i.e fungal isolates that were susceptible to the plant extracts were inoculated into the different concentration of the broth and incubated for 48hrs. The concentration that inhibits the growth of any test organisms was noted as MIC for that extract on the test organism, Ekong *et al*., (2004).

The bacteriostatic concentration were determined by inoculating the broth from the concentration that showed no turbidity on the MIC tubes on sterile Sabouraud Dextrose agar plates and incubated at 28oC for 48hrs. Concentrations that did not grow at 48hrs on the Sabouraud Dextrose agar plates but grew at 72hrs were recorded as Fungi-static concentration, but concentrations that did not grow at all even after 72hrs were recorded as Fungicidal concentrations.

**RESULT**

Yield from Aqueous and Ethanol Extractions
The powder form of the seed extract was extracted with Aqueous solution and ethanol solution respectively. The highest yield was achieved with ethanol (66g) followed by the Aqueous solution extract to be 53.3g.

Determination of minimum inhibitory concentrates (MCC), Fungi static and fungicidal concentrations of plant extracts on fugal isolates.

**Phytochemical Screening**

**Table 4.2 Phytochemical Screening (qualitative) screening of Piper guineense extracts**

| S/N | Test | Ethanol Extract | Aqueous Extract |
|---|---|---|---|
| 1 | Alkaloid | + | + |
| 2 | Saponins | + | + |
| 3 | Tannins | + | + |
| 4 | Anthraquinone | + | + |
| 5 | Cardiac glycosides | + | + |
| 6 | Flavonoids | - | - |
| 7 | Phenols | + | + |
| 8 | Terpenes | + | + |

**KEY:** + = Present; - = Absent

Table 4.2 shows the phytochemical (qualitative) screening of the extract (Piper guineense) in both ethanolic and aqueous forms. From the result (Table 4.2) it was observed that alkaloids, saponins, tannins, anthraquinone, cardiac glycosides, phenols and terpenes were present in both extracts. Flavonoids was however absent from both forms of extracts.

**Table 4.3 Phytochemical (quantitative) screening of Piper guineense extracts**

| S/N | Test | Ethanol Extract | Aqueous Extract |
|---|---|---|---|
| 1. | Alkaloids | 2.083 ± 0.64 | 1.803 ± 0.096 |
| 2. | Saponins | 0.028 ± 0.004 | 0.009 ± 0.002 |
| 3. | Tannis | 0.178 ± 0.011 | 0.101 ± 0.004 |
| 4. | Flavonoids | 0.013 ± 0.013 | 0.097 ± 0.002 |
| 7. | Phenols | 0.208 ± 0.013 | 0.179 ± 0.004 |

Table 4.3 shows the phytochemical (quantitative) screening of the seed extract of *Piper guineense* in both ethanolic and aqueous forms. From the result in table 4.3 alkaloids and tannins were observed to have higher values while flavonoids and saponins were observe to have low values.

**Table 4.4 Macroscopic and microscopic Characteristics of Fungal Isolates from Bread (control) samples**

| Colony colour | Types of soma | Nature of hyphae | Vegetation structure | Asexual spore | Special reproductive structure | Conical head | Vesicle shape | Probable organisms |
|---|---|---|---|---|---|---|---|---|
| Brownish colony becoming dark with age | Filamentous | Septate | Footcell | Globose conidia | Short conidiophores | Long columnar | hemispherical | *Aspergillus terreus* |
| Smoky or gray green colony | Filamentous | Septate | Footcell | Globose conidia | Short conidiophores | Typically columnar | Dome shaped broadly clavate | *Aspergillus fumigatus* |
| Compact white or yellow basal black colony | Filamentous | Septate | Footcell | Globose conidia | Smoothen walled erect conidiophores | Globose | Globose | *Aspergillus niger* |
| Grayish green colony | Filamentous | Septate | Footcell | Sub globose conidia | Hyaline conidiosphores | Radiate | Subglobose | *Aspergillus glaueus* |
| White becoming grayish brown | Filamentous | Coenocytic | Stolons rhizoids | Ovoid sporangiophores | Sporangiosphores in groups, black brown sporangia | - | - | *Rhizopus stolonifer* |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Creamish yellow colony | Filamentous | Coenocytic | - | Sporangiospore | Sympodially branched sporangiosphore | - | - | *Mucor sp* |

**Table 4.5 Antimicrobial activity of *Piper guineense* (aqueous extracts) on fungal isolate**

| Test organisms | Zones of inhibition diameter in (mm) | | | | | Control drugs (mg/ml) |
|---|---|---|---|---|---|---|
| | 500 mg/ml | 400 mg/ml | 300 mg/ml | 200 mg/ml | 100 mg/ml | Nystatin (10 mg/ml) |
| *Aspergillus fumigatus* | 18 | 13 | 10 | 7 | NA | 25 mm |
| *Aspergillus terreus* | 20 | 16 | 11 | 8 | NA | 27 mm |
| *Aspergillus niger* | 21 | 17 | 14 | 10 | NA | 30 mm |
| *Rhizopus stolonifer* | 16 | 12 | 8 | NA | NA | 33 mm |
| *Mucor sp* | 19 | 15 | 11 | 8 | NA | 39 mm |
| *Aspergillus glaueus* | 20 | 16 | 11 | 8 | NA | 28 mm |

**KEY:** NA = No Activity

**Table 4.5 Antimicrobial activity of *Piper guineense* (ethanol extracts) on fungal isolate**

| Test organisms | Zones of inhibition diameter in (mm) | | | | | Control drugs (mg/ml) |
|---|---|---|---|---|---|---|
| | 500 mg/ml | 400 mg/ml | 300 mg/ml | 200 mg/ml | 100 mg/ml | Nystatin (10 mg/ml) |
| *Aspergillus fumigatus* | 18 | 13 | 10 | 7 | NA | 25 mm |
| *Aspergillus terreus* | 20 | 16 | 11 | 8 | NA | 27 mm |
| *Aspergillus niger* | 18 | 15 | 11 | 8 | NA | 30 mm |
| *Rhizopus stolonifer* | 20 | 14 | 9 | NA | NA | 33 mm |
| *Mucor sp* | 25 | 21 | 15 | 10 | 7 | 39 mm |
| *Aspergillus glaueus* | 21 | 13 | 8 | NA | NA | 28 mm |

**KEY:** NA = No Activity

**Table 4.6 Minimum Inhibitory Concentration (MIC), Fungi-static Concentration (FSC), Fungi-cidal Concentration (FCC)**

| Isolates | MIC. Aqueous Extract (mg/mL) | MIC. Ethanol Extract (mg/mL) | Fungi-static conc. Aqueous Extract (mg/mL) | Fungi-static conc. Ethanol Extract (mg/mL) | Fungicidal conc. Aqueous Extract (mg/mL) | Fungicidal conc. Ethanol Extract (mg/mL) |
|---|---|---|---|---|---|---|
| *Aspergillus fumigatus* | 200 | 200 | 250 | 200 | ND | **ND** |
| *Aspergillus terreus* | 200 | 100 | 200 | 150 | ND | **ND** |
| *Aspergillus niger* | 200 | 200 | 250 | 200 | ND | **ND** |
| *Verticillium sp* | ND | ND | ND | ND | ND | **ND** |
| *Rhizopus stolonifer* | 250 | 300 | 250 | 400 | 500 | **ND** |
| *Mucor sp* | 200 | 100 | 300 | 150 | ND | **400** |
| *Aspergillus glaueus* | 200 | 300 | 250 | 400 | ND | **ND** |

**Key:** ND = Not Determined

Table 4.6 shows the Minimum Inhibitory Concentration (MIC), Fungi-static Concentration (FSC) and Fungi-cidal Concentration (FCC) of Piper guineense against the fungal isolates from the bread sample. From the result in Table 4.6, the lowest MIC was observed from the ethanolic extract of Piper guineense on Mucor sp. (100 mg/mL) and the highest MIC was observed from the ethanolic extract on Rhizopus stolonifer and Aspergillus glaueus respectively at 300 mg/mL. The lowest FSC was observed from ethanolic extract on Aspergillus terreus and Mucor sp. Respectively at 150 mg/mL while the highest FSC was observed from ethanolic extract on Rhizopus stolonifer and Aspergillus glaueus respectively at 400 mg/mL. The lowest FCC was however observed from ethanolic extract on Mucor sp. at 400 mg/mL, while the highest FCC was from aqueous extract on Rhizopus stolonifer at 500 mg/mL.

## References

Adu, A. A. Adennola, O. J., Mekuleyi, G. O. and Uyi, O. J. (2020). Assessment of heavy metals and phytochemical composition of *Piper guineense* leaves collected from three markets in Lagos, Nigeria. *International Journal of Advanced Academic Research,* 6 : 2488.

Benjamin, T. Oguntimehin, B. O., Inya-Agha, S. I. (2013). Phytochemical and antibacterial *Studies on the essential oil of piper guineennse* Plant Pathol, 5 : 536 – 538.

Centers for Disease Control and Prevention (2015). Surveilance for food borne disease outbreaks. United States, 2012, annual report. Atlanta, Georgia: US Department of Health and Human Service.

Cowan, M. M. (2019). Plant Products as antimicrobial agents chemical microbiology Review 12(4), 564 – 582.

Ebi, G. C. (2017). Antimicrobial activities of Alchomea cordifolia. Fitoterapia, 72(1), 69-72.

Edeoga, H. Okwu, D. E. and Mbaebie, B. O. (2015). Phytochemical constituents of some Nigerian Medicinal Plants. *African Journal of Biotechnology*, 4(7), 685-688.

Ezeonu, C. S. and Ejikeme, c. M. (2016). Qualitative and quantitative determination of phytochemical contents of indigenous Nigeria softwoods, *New Journal of Science,* 1 – 9.

Hogue, M. M., Bari, M. L., Junja, V. K., and Kawamoto, S. (2018). Antimicrobial activities of plant extract against food borne pathogens and spoilage bacteria. *Natural Food Research Institution*, 72 : 9-21.

Konning, G. H., Agyara, C., and Ennison, B. (2014). Antimicrobial activity of some medicinal plants from Ghana. *Fitoterapia*, 75(1), 65 – 67.

Magan, N. and Aldred, D. (2014). Managing Microbial spoilage in Cereal and bakery Productions, 194 – 212. In: C. W. Blackburn (ed.) Food Spoilage Micro-organism woodhead publications, Cambridge.

Mgbeahuruike, E. E., Fyhrquist, P., Vuorela, H., Julkunen-Tilto, R. Holm, Y. (2018). Alkaloids – rich crude extracts, fractions and piperamide alkaloids of *Piper guineense* posses promising antibacterial effects. Antibiot, 7:98.

Negi, P. S. (2022). Plant extracts for the control of bacterial growth: Efficacy, Stability and Safety Issues for Food Application, *International Journal of Food Microbiology,* 156(1), 7 – 17.

Nwinyi, O. C., Chinedu, N. S. and Ajani, O. O. (2019). Evaluation of antibacterial activity of *Pisidium guajava* and *Gongronema latifolium*. *Journal of Medicinal Plant Research*, 2:189–192.

Okeke, I. N., Ogundaini, A. O., Ogungbamila, F. O. and Lamikanra, A. (2019). Antimicrobial spectrum of alchornea Cordifolia Leaf extract. *Phytotheraphy Research*, 13(1), 67-69.

Saranray, P. Geetha, M. (2012). Microbial Spoilage of bakery products and its control of preservatives. *International Journal o Pharmaceutical and Biological Achieves*, 3 : 38 – 48.

Sharma, S. (2015). Food preservatives and their harmful effects. *International Journal of Scientific and Research Publication,* 5(4) : 1 – 2.

Sujafa, S. I. Vshal, R. P., Talib, M. I., Dandi, B. N. (2017). Shelf life extension of bread using anti-microbial activity of spices. IJCRY pp. 335 – 340.

Vazirian, M. Alehabib, S., Jamalifar, H, Fazeli, M. R., Toosi, A. N., Knamavi, M. (2015). Anticrobial effect of Chinagron bare essential oil in cream-filled caves and pastires. *Research Journal of Pharmacy*, 2(4): 11 – 16.