Original Research Article

ANTIMICROBIAL ACTIVITY AND ANTIOXIDANT ACTIVITY OF CLOVE (S*yzygiumaromatium*) AND THYME (T*hymus vulgaris*) TRADITIONALLY USED AS FOOD PACKAGING AGAINST FOODBORNE PATHOGENS

ABSTRACT

This study investigated the antimicrobial and antioxidant activity of clove (*Syzygium aromaticum*) and thyme (*Thymus vulgaris*) essential oils, which are traditionally used as food packaging materials. The essential oils were extracted using hydro distillation and their antimicrobial activity was evaluated against common foodborne pathogens, including *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica*, and *Listeria monocytogenes*. The antioxidant activity was determined using the DPPH (2,2-dip). The study found that both clove and thyme essential oils exhibited antimicrobial activity against all four foodborne pathogens tested, with clove oil having the strongest activity. In addition, both essential oils showed antioxidant activity, with clove oil having the highest antioxidant capacity. Based on these results, clove and thyme essential oils have potential as natural food preservatives. This study provides further evidence of the beneficial properties of these two herbs and their potential use in food packaging. However, more research is needed to optimize the use of these essential oils and to ensure their safety and efficacy.

KEY WORDS: antimicrobial, antioxidant, clove, thyme, food-packaging, foodborne, pathogens

INTRODUCTION

Human infectious diseases and food consumption have long been linked; Hippocrates, who lived around 460 B.C., had already maintained that diet and human health were strongly related (Hutt, 1989). Food safety continues to rank among the top public health issues of our day. Foodborne pathogens continue to produce an unacceptably high number of foodborne illnesses outbreaks each year worldwide, despite efforts and advancements in hygienic precautions in food manufacturing procedures (Linscott, 2011). The primary cause of food-borne illnesses has been shown to be bacterial infections, whose management in the setting of food processing is thought to be extremely difficult. They pose a serious concern to food safety because they can proliferate, settle, and form biofilms on food surfaces (Hoveida*et al*., 2019). Furthermore, one of the main causes of foodborne illness outbreaks is the unsanitary conditions of surfaces, tools, and processing areas that come into touch with food (Khelissa*et al*., 2017). According to published research, eating food tainted with harmful bacteria like *Staphylococcus aureus* and *Escherichia coli* can be extremely harmful to one's health. A number of *E. coli* strains are harmful to people, resulting in cholecystitis, septicemia, and intestinal and urinary tract infections (Makvana*et al*., 2015).Ingestion of tainted food or water by excrement from infected humans or animals can

spread *E. coli* pathotypes, and cause foodborne illnesses. Animal products are frequently contaminated during the slaughter and processing of meat. The use of animal dung as fertilizer and tainted irrigation water are two sources of contamination for agricultural crops (García *et al*., 2010). Along with its ability to form biofilms under various conditions across the food production chain, *E. coli* is well-known for its acid resistance mechanisms, which enable the bacterium to survive harsh conditions found in food processing settings, including those associated with the use of disinfectants to combat food pathogens (Lajhar*et al*., 2017; Ejimofor*etal*., 2023). In addition to food poisonings (Doyleet*et al*., 2012), *S. aureus* is primarily responsible for toxic shock syndrome, endocarditis, and post-operative wound infections (Tong *et al*., 2015). Many contaminated foods, such as minced meat, pork sausage, minced turkey, salmon slices, oysters, shrimp, milk, and salads, are made up of *S. aureus*(Ejimofor*etal*., 2023; Bacon *et al*., 2005). The majority of staphylococcal food poisoning cases can be linked to food contamination during preparation as a result of poor personal hygiene, inadequate cooking or heating, or inadequate refrigeration. Although there are roughly ten staphylococcal enterotoxins known to exist, the majority of outbreaks are caused by types A and D (Schelin *et al*., 2017). Strategies for addressing the hygienic rise of foodborne illnesses are urgently needed. The world community currently places a high premium on food safety, and there is a heightened awareness of the need to enhance our knowledge and surveillance of foodborne illnesses and pathogens, as well as to apply systematic methods in food conservation plans. In recent decades, the food industry has made extensive use of synthetic chemical preservatives (Tian *et al*., 2014). Regarding the use of these synthetic chemical compounds to suppress foodborne germs, there has been disagreement. They have been linked to a number of unfavorable characteristics, such as acute toxicity, teratogenicity, and carcinogenicity (Dwivedi *et al*., 2017).Consumer preferences have recently shifted in favor of food products free of chemical additions as a result of increased worries about food safety (da Cruz *et al*., 2016). A search for efficient substitutes in the realm of natural goods has been spurred by the negative consequences linked to artificial food preservatives (Burt, 2004). There are now a number of substances under investigation that may be employed as safe and effective natural food preservatives. Because of their strong antibacterial and antifungal qualities, several plant extracts with broad antimicrobial activity are among the most potential substitutes (Awad*et al*., 2022).Aromatic oily liquids derived from plant material using various techniques, essential oils (EOs) may be a better option than some artificial chemical additives (Burt, 2004). Although EOs have been utilized for their preservation qualities since antiquity, there has recently been a resurgence of scholarly interest in their usage in food (Burt, 2004). They are a complex of bioactive molecules with a variety of intriguing biological properties, including antibacterial, antifungal, antioxidant, and antibiofilm (Tajkarimi*et al*., 2010; Donato *et al*., 2020). These properties enable their use in a variety of industries, including medicine, food, cosmetics, and pharmaceuticals. Herbs, spices, and other fragrant plants can all provide essential oils (EOs) (Tajkarimi*et al*., 2010). Clove (*Syzygium aromaticum* L.) and thyme (*Thymus vulgaris* L.) essential oils are two of the most valued essentialoils that are among the most appreciated essential oils, especially due to their significant antimicrobial properties.A blooming plant native to Southern Europe and found all over the world, *Thymusvulgaris*, also referred to as thyme, is a member of the Lamiaceae family and has significant pharmacological qualities (Benameur *et al*., 2018). According to Mohammed *et al*. (2020), thyme extracts, including essential oil extracted from plant aerial parts (flowers and leaves), has shown antibacterial properties against both Gram-positive and Gram-negative bacteria. Clove is the popular name for *Syzygium aromaticum* L., an aromatic plant that is a member of the Myrtaceae

family. It is a great source of bioactive volatile chemicals and is primarily grown in tropical and subtropical regions. Because of its numerous uses in medicine and cosmetics, clove's essential oil—which is extracted from flower buds—has garnered a lot of scientific attention. Its antibacterial and antioxidant properties are well-established (Haro *et al*., 2021). In line with their potential use as natural preservatives, to create healthy food products, to increase shelf life, and to decrease harmful bacteria, several studies have shown the antibacterial properties of thyme and clove essential oil against a variety of food pathogenic isolates (Ginting *et al*., 2021). The emergence of multi-resistant bacteria, notably foodborne ones, has had severe health effects due to the inappropriate and overuse of conventional antimicrobial drugs. Essential oils may also be able to help address the developing issue of antimicrobial resistance because of their inherent antibacterial qualities (Hofer, 2019). Therefore, new and effective antimicrobial techniques must be introduced and employed in conjunction with established ones. Numerous pieces of data indicate that essential oils, especially when combined with or used as adjuvants of traditionalantimicrobials, could represent effective tools against resistant pathogens (Qian *et al.*, 2019). Essential oils can damage the cellular structure of microbes and have multi-target inhibitory effects, making them more vulnerable to other antimicrobial substances. Therefore, research on the antibacterial properties of essential oils, either alone or in combination, is very beneficial. In light of this, the purpose of our study is to examine the antibacterial qualities of clove (*Syzygium aromaticum*) and thyme (*Thymus vulgaris*) essential oils in vitro against foodborne pathogens, both separately and in combination. Determining the EO's antimicrobial profile against food isolates of *S. aureus* and *E. coli*, two significant and frequent food infection causing agents, was the specific goal. The bioactivity of volatile fractions has been evaluated by testing these essential oils in both the liquid and vapor phases. To explore combinatorial interactions and emphasize their synergistic antibacterial capabilities that can be used in food preservation, EOs were also tested in binary combination against the food isolates. This study is very important since the synergistic antibacterial activity of EO combinations, particularly because of volatile antimicrobial compounds, offers a great deal of untapped potential.

METHODOLOGY

Study Area

This work was conducted at Alpha laboratory Awka, Anambra State. Anambra State is located in the south-eastern part of Nigeria and situated between latitudes 6° 13' and 16' N and longitude 7° 4' and 7° 41' E and Altitude 160.8m respectively (Ezenwaji*et al*., 2014). The research is based on IN-vitro antimicrobial and antioxidant activity of clove and thyme extracts on preservation of moimoi.

Materials Used

The materials used for the study includedmoimoi (prepared in the laboratory), clove seed and thyme powder (gotten from eke Awka), Whitman's filter paper No 42, beakers, volumetric flasks, measuring cylinder, spatula, inoculating loop, bunsen burner, aluminum foil, cottonwool, scapel, microscope, sterile polythene bags, masking tape, petri dishes, blotting paper, sodium hypochlorite, test tubes and rack, micro pipette, funnels, slide, cover slip.

Sample preparation

The samples were ground into fine powder and stored in an air tight plastic container for extraction.

Extraction

Extraction was done with ethanol by cold maceration .250g of powder sample was weighed into a glass and extracting solvent (ethanol) was added until the medicinal plant residues were fully immersed. The vessel was closed with a tight-fitting glass cover and the contents in the vessel were shaken after every 4 h except at night, and left to stand for three days (72 h) but with subsequent agitation until this period was over. The contents of the flask were then strained through two clean pieces of cotton cloth placed on top of filter paper, both supported by a funnel, and the extracted solution (miscella) collected in a flask with a tight-fitting cover. The maximum yield of ethanol extract was obtained by squeezing the marc (solid residue) in the top clean dry piece of cloth, while drippings of liquid extract were allowed to pass through the second clean dry piece of cloth, to the contents of the flask through Whatman's filter paper. The volume of the yield was noted. Both the ethanol and water extracts were transferred to a hot-air oven for drying at temperatures between 50 °C and 70 °C, and later transferred into a desiccator for further drying.

Phytochemical screening

a. Preliminary Phytochemical Screening

The extracts will be subjected to preliminary chemical screening for their presence or absence of active phytochemical constituents by the following methods according to (AOAC, 2010).

Test for Alkaloids

The extracts were treated with dilute (10%) hydrochloric acid and filtered. The filtrates were treated with various alkaloidal reagents.

a. Mayer's test: The extracts were with Mayer's reagent (Potassium mercuric iodide). Appearance of cream colour indicates the presence of alkaloids in chloroform, methanolic and aqueous extracts.

b.Wagner's test : The extracts were treated with the Wagner's reagent (Iodine solution) the appearance of brown colour precipitate indicates the presence of alkaloids in chloroform, methanolic and aqueous extracts.

Phenolics

0.5 g of the powdered dried seeds of each sample was boiled with 10 ml of distilled water for 5 mins and filtered while hot. Then 1ml of ferric chloride solution was added. Formation of blue-black or brown colouration indicated the presence of phenol.

Test for terpenoids

5 ml of each extract was mixed in 2 ml of chloroform. 3 ml of concentrated H_2SO_4 was then added to form a layer. A reddish-brown precipitate colouration at the interface formed indicated the presence of terpenoids

Test for Cardiac Glycosides

a. **Keller-Killanitest :** When a pinch of the extracts were dissolved in the Glacial acetic acid and few drops of ferric chloride solution was added, followed by the addition of concentrated Sulphuric acid, formation of red ring at the junction of two liquids indicates the presence of glycosides in methanolic and aqueous extracts.

Test for Flavonoids

a. Shinoda's test: The extracts were dissolved in alcohol, to that one piece of magnesium followed by conc. hydrochloric acid was added drop wise and heated. Appearance of magenta color shows the presence of flavonoids in methanolic and aqueous extracts.

b. Ferric Chloride test: To the extracts, few drops of neutral ferric chloride were added. Blackish red colour was observed in methanolic and aqueous extracts.

Test for Saponins

a. Foam test: The extracts were diluted to 20 ml with distilled water and shaken well in a graduated cylinder for 15 minutes. The formation of foam in the upper part of the test tube indicates the presence of saponins in each extract.

b. **Demonstration of emulsifying properties:** 2 drops of olive oil was added to the solution obtained from diluting 2.5 ml filtrate to 10 ml with distilled water (above), shaken vigorously for a few minutes, formation of a fairly stable emulsion indicated the presence of saponins.

Test for Steroids

a. **Salkowski reaction:** To 2 ml of extract, added 2ml chloroform and 2 ml conc. H_2SO_4. Shaked well. Chloroform layer showed red color and acid layer showed greenish yellow fluorescence.

b. **Liebermann-Burchard test:** When the extracts were treated with concentrated sulphuric acid, few drops of glacial acetic acid, followed by the addition of acetic anhydride, absence of green colour indicates the absence of steroids in all extracts.

Test for Tannins

a. **Lead acetate solution**: When the extracts were treated with 10% lead acetate solution, appearance of white precipitate indicates the presence of tannins in methanolic and aqueous extracts.

b. Ferric Chloride Solution: When the extracts were treated with ferric chloride solution, NaOH, &AgBr Solution appearance of green colour precipitate indicates the presence of tannins in methanolic and aqueous extracts.

Total phenolic content

Total phenolic content of the EO was determined using a Folin-Ciocalteu reagent and gallic acid (Sigma–Aldrich Chemie, Steinheim, Germany) as a standard phenolic compound. Briefly, 0.1 ml of the solution containing the EO was mixed with 46 ml distilled water. Then, 1 ml Folin-Ciocalteu reagent was added to the solution and the mixture was shaken vigorously. After 3 min, 3 ml sodium carbonate solution (2%; Na_2CO_3) was added and the mixture was shaken, gently for 2 h. The absorbance of the solution was measured at 760 nm. The same procedures were carried out with gallic acid (as a standard) and a calibration curve was obtained. The total phenolic content was presented as mg of gallic acid equivalent per g of the EO.

Flavonoids content

The flavonoid contents were measured according to the aluminum chloride colorimetric method. Rutin was used to obtain the calibration curve. Various concentrations of the EO were prepared for this test. Each diluted EO of lemon peel (500 µl) was mixed with 500 µl aluminum chloride methanolic solution (2%). Each prepared mixtures were incubated at room temperature for 15 min, then the absorbance of the reaction mixture was measured at 430 nm with a UV–Vis spectrophotometer (Unico Inc, Shanghai, China). Using the same procedure, the calibration curve was obtained for rutin (in the range of 5 to 60 mg/ml). Finally, flavonoids content was expressed in mg of rutin equivalent/g of the EO.

Determination of vitamin E

This will be determined by the futter-mayor colometric met. 1g of the sample will be mixed with 10ml of ethanoic sulphuric acid and boiled gently under reflux for 5minutes. It will be transferred to a separating funnel and treated with 3 x 30ml diethyl ether and recovering ether layer each time, the ether extract will be transferred to a Dessicator and dried for 30 minutes and later evaporated to dryness at room temperature. The dried extract will be dissolved in 10ml of pure ethanol. 1ml of the dissolved extract and equal volume of standard vitamin E will be transferred to separate tubes. After continuous addition of 5ml of absolute alcohol and 1ml of concentrated nitric acid solution, the mixture will be allowed to stand for five minutes and the respective absorbance measured in a spectrophotometer at 410nm with blank reagent at Zero.

Vitamin D

Weigh 10g of sample and Add 5 ml of water, 20 ml of methanol, 1 ml of sodium-sulphate solution and 3 ml of a freshly prepared 50 per cent m/m solution of potassium hydroxide . Heat under a reflux condenser on a water-bath for 30 min. Cool rapidly under running water. Transfer the liquid to a separating funnel to separate. Shake vigorously for 30s. Allow to stand until the two layers are clear. Transfer the lower aqueous alcoholic. Take the extract and read the absorbance with a spectrophotometer at wavelength 432nm.

DPPH radical scavenging assay

The free radical scavenging activity was determined using DPPH as previously described (Shimada *etal*., 2012). DPPH was first dissolved in EtOH to a concentration of 0.1 mM and a solution of DPPH (1 mL) was added to an EtOH solution (3 mL) of the tested samples at different concentrations (200, 150, 100, 50, and 25 µg/mL). An equal volume of EtOH was added in the control test. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance at 517 nm was measured with a UV–Vis

spectrophotometer. The **percentage** of inhibition (I%) of DPPH radical was calculated as follows:

$$I\% = \left[\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}\right] \times 100,$$

where A_{blank} is the absorbance value of the control reaction (containing all reagents except the sample) and A_{sample} is the absorbance value of the test sample. The sample concentration providing 50% inhibition (IC_{50} value) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC_{50} values were reported as mean ± SD of triplicates.

FRAP assay

The total reducing capacity was determined using FRAP assay (Benzie & Strain, 1996). The stock solutions included 300 mM acetate buffer pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ, and 2.5 mL $FeCl_3 \cdot 6H_2O$. The temperature of the solution was raised to 37 °C prior to use. The essential oil (150 µL) was allowed to react with 2850 µL of the FRAP solution for 30 min in the dark condition. After incubation, the absorbance was read at 593 nm using a UV–vis spectrophotometer. The results were calculated by standard curves prepared with known concentrations of ascorbic acid (AA) and were expressed as mg AA/g.

Determination of antioxidant property of essential oil by TBARS assay

According to Dorman and colleagues (2016), the oxidation of egg yolk lipids was measured in order to assess the antioxidant property. The egg yolk (10%, v/v) solution used in this test was made in 1.15%, w/v KCl. Before being used, it was held at 4 °C after being homogenized for 30 seconds and ultrasonically sonicated for 5 minutes. An 8.1% (w/v) sodium dodecyl sulphate (SDS) solution was used to manufacture the EO, vitamin E, and BHT at 0.01% (w/v) concentrations. 1.1% (w/v) SDS solution was mixed with 0.8% (w/v) thiobarbituric acid (TBA) to create a solution. Tubes holding 0.5 mL of egg yolk homogenate were filled with test solutions (0.1 mL). Following the addition of 1.5 mL of 20%, v/v acetic acid, 1 Mol NaOH was used to bring the pH down to 3.5.

The final volume was then adjusted to 4 mL with deionized water after 1.5 mL of 0.8% TBA was added. After being vortexed, the samples spent 60 minutes in a water bath at 95°C. The absorbance of the butanol layer was measured at λ 532 nm (UV-160, Shimadsu, Japan) against an n-butanol blank when they had cooled, after which 5 mL of n-butanol was added, vortexed, and centrifuged. For the control, 0.1 mL of 8.1% (w/v) SDS was used in place of the test solution, and the same steps were taken as before. As positive controls, BHT and vitamin E were both used. The experiment was conducted twice, in duplicate each time.

Antioxidant index percentage (AI %) was calculated using the following formula:

$$\text{AI \%} = ((1 - T/C)) \times 100$$

where, T = the absorbance of the test sample C = the absorbance of the fully oxidized control

Microbial analysis of sample

Preparation of Culture Media

The major media use for the isolation and characterization of bacteria isolates include: Nutrient agar (NA), MacConkey agar (MA), potato dextrose agar (PDA) and Peptone water. Appropriate grams of the agar were measured and poured into a conical flask, dispensed into the volume of water according to the manufacturer's instruction. The mixture was heat in the autoclave for 15 minutes at 121°C.

Sterilization of materials

All the glassware to be use for the research including Conical flask, Petri dishes, test tubes were washed with detergent and rinse with clean water and were assemble in the autoclave with the more fragile equipment wrap in aluminium foil and were sterilized at 121°C for 15 minutes.

PROCEDURE

Total Bacterial Count

One gram of each sample was homogenized using vortex mixer (VM-300, Taiwan) with 9 ml sterile peptone water to obtain first dilution. 1ml from the first test tube was pipette into the second test tube already containing 9ml of peptone water, this continued following the same procedure till the last dilution (ie the last test tube) using the pour plate method 1ml each of each sample unit from the test tubes was pipetted into the sterile Petri dishes containing already prepared Nutrient agar media. The plates were incubated at 37°C for the 24hr. After incubation the representative colonies on the plates was subcultured on fresh nutrients agar to obtain pure cultures of the isolates. The pure cultures were then transferred into nutrient agar slants for biochemical identification (Abbott, *et al.*, 2008).

Total fungi count

The serial diluted sample was used in the total fungi analysis. Using the streak method 1ml each of each sample unit from the test tubes was collected with wire loop and streak into the already prepared PDA agar Petri dishes. The plates were incubated at 37°C for the 72hr. After incubation the representative colonies on the plates was subcultured on fresh PDA agar to obtain pure cultures of the isolates. The pure cultures were then used biochemical identification (Abbott, *et al.*, 2008).

The number of colonies were counted on all the agar agar and calculated using the formula below;

$$\text{CfU (ml)} = \frac{N}{VxD}$$

Where

Cfu = Colony forming unit
N = Mean number of colonies
V = Volume of innoculum
D = Dilution factor.

Identification and Characterization of Bacterial Isolates

Purification of isolates:

Single colonies of bacteria were randomly selected from different media plates based on their morphology. These bacterial cultures were subsequently isolated in pure forms by subculturing on nutrient agar plates incubated for 24hrs and used for microscopic characterization and biochemical analysis.

Identification of Microorganisms

a) Morphological identification: The isolated bacteria were identified on the basis of motility and Gram's-staining.

Gram's staining

The pure bacterial isolates were stained according to Gram's techniques. A thin smear was prepared on clean glass slide, air dried, and heat fixed by placing the slide gently over the flame of the spirit lamp. The smear was stained with crystal violet for 1 minute, and then rinsed with tap water. The smear was then covered with Lugol's iodine for 60 seconds and washed off under gentle running tap water. The slide was then decolourized using 70% ethanol after which it was washed under tap water and then counterstained with safranin for 30 seconds. It was again rinsed with tap water and the slide blotted dry with a piece of filter paper. The stained cells were examined with the oil immersion objective lens of the light microscope. The gram positive organism is characterized by a purple colour while a gram negative organism takes on a pink colour as well as the shape of the cells were also examined.

Motility test

The stabbing technique was used to carry out this test. Test-tubes containing sterilized Sim Agar were prepared. Sterilized inoculating needle was used to pick up isolates from their pure cultures. Each test-tube was stabbed with the needle rubbed with each isolate in the middle. The test-tubes were then incubated at 37^0C for 24hours. After 24 hours, the tubes were observed for the motility of the isolates. A motile isolate usually grows away from the point where the medium was stabbed.

Urease Test

This test was used to demonstrate the ability of the isolates to produce the enzyme urease which splits urea forming ammonia. The test is usually used to differentiate organisms like proteus from other non urease positive organisms. A loop full of the isolates was used to inoculate a tube of urea-agar. The tubes were incubated at 37^oC. a change in colour from yellow to red confirmed the presence of urease.

Catalase Test

This test was used to demonstrate which of the isolates could produce the enzyme catalase that release oxygen from hydrogen peroxide.

A loopful of the pure colony was transferred into a plane, clean glass slide. The sample was then mixed with a drop of 3% v/v hydrogen peroxide. The reaction was observed immediately Gas production indicated by the production of gas bubbles confirmed the presence of catalase.

Indole Test

This test was used to determine which of the isolates has the ability to split indole from tryptophan present in buffered peptone water. The test is usually used as an aid in the differentiation of Gram negative, *Bacilli* especially those of the *Enterobacteriaceae* (Baker, 1976). Tubes of peptone water were inoculated with young culture of the isolates. The tubes were incubated at 37°C for 48hrs. About 4 drops of Kovac reagent were added into 1ml of each of the culture tubes. Positive test was indicated by a red colour that occurs immediately at upper part of the test tube.

Citrate Utilization Test

This test was used to identify which of the isolates can utilize citrate as the sole source of carbon for metabolism. The- test is usually used as an aid in the differentiation of organisms in the *Enterobacteriaceae* and most other genera. The medium used for this test was the Simon's citrate agar. Slant tubes of Simon's citrate agar were inoculated with young culture of the isolates. The inoculation was done by stabbing the medium on the tubes using sterile straight inoculating wire containing the culture. The tubes were then incubated at 37°C for about 24hours. Change in colour from green to blue after about 24hours of incubation indicated positive result.

Coagulase Test

1. A very homogenous suspension of the inoculum was mixed on a drop of normal saline in a grease free slide.

2. Loopful undiluted rabbit plasma was added to the suspension and mixed thoroughly for 5 seconds.

3. A control was set up in the same manner without blood plasma.

4. Coagulase positive staphylococci showed clumping or agglutination within 5-15 seconds while negative suspension showed no clumping.

OXIDASE TEST

This was carried out to identify bacterial species that will produce the cytochromeoxidase enzyme.

A piece of filer paper was placed in a clean Petri dish and 2-3 drops of fresh or nascent oxidase reagent was added. A colony of test organism was collected using a glass rod and

smeared on the filter paper and observed. Blue-purple color within few a seconds showed a positive test.

Identification and Characterization of FUNGAL Isolates

The isolates were identified using cultural characteristics and morphology with reference to De Hoog *et al*. (2020) and Jay (2012).

Cultural Characteristics

The growth pattern, pigmentation and size of colonies were recorded at the incubation period to aid identification of the organisms.

Colony Morphology

A drop of lactophenol (LP) was placed on a clean microscopic slide. A small portion of the isolate was placed in the drop of lactophenol (LP) and suspended. A clean cover glass was placed over the suspension and observed microscopically.

Spore Staining

The staining procedure for identification of spore was carried out by placing heat-fixed slide (containing the smear of the isolate) over a steaming water bath and placing of blotting papers over the area of the smear without sticking out past the edges of the slide. The blotting paper was then saturated with 5.6% solution of malachite green and steamed for 5 min. Following this, the slide was cooled to room temperature and then rinsed thoroughly with tap water. Safari was then applied for one minute and rinsed briefly but thoroughly before blotting dry. After which the slide was examined microscopically.

Motility Test

Fungal motility was determined by transferring a small drop of live isolates to the centre of a slip of a depression slide using petroleum jelly or 2-3 drops of peptone water with growth of the organism replaced on a clean slide with wire loop. Then cover slip was placed over the slide, the slide was left for some time and then examined microscopically with the high-power objective. Motile organisms were seen swimming around.

Biochemical Test

Carbohydrate Assimilation Test: Filtered and sterilized carbohydrates were added to the medium at concentration of 1% while the pH was adjusted to 5.4 by addition of NaOH or HCl. 2 ml of the media were dispensed into 10 ml test tube. The tubes were also inoculated with isolates and carbohydrates. All tubes were incubated at 20^{o}C for 14 days. A change in the color of the medium of orange and yellow were taken as positive result. A change to pink or purple was considered negative result.

Amino-acid Assimilation Test:

Medium preparation and indication were as described for the carbohydrate assimilation test. 10 mm test tubes containing 2 ml of the media were inoculated with the isolate and control tubes for

each fungus and amino acid. Also, tubes were incubated at 20o C for 14 days. A change to pink or purple was considered positive result while a change in color of the medium to orange was taken as negative result.

Hydrolysis Test:

The basal medium was similar to that of amino acid assimilation test with addition of 0.05 mg milk and 1.2 mg agar. After autoclaving at 110^{o}C for 30 min, the medium was poured into petri dish. Isolates were inoculated at the centre of the plate and incubated at 20^{o}C for 14 days. The appearance of a clear zone around the fungal colony was taken as a positive result.

Lipase Activity Test:

The medium of 0.5% peptin, 0.3% yeast extract and 1.0% agar were autoclaved at 121^{o}C for 10 min. It was filtered and dispensed into sterilized test tubes. Isolates were inoculated into the surface of the medium and incubated at 20^{o}C for 7 days. The occurrence of clearance in the medium column was taken as a positive result.

Pathogenicity of isolated fungi

Pathogenicity or decay test was carried out in order to know if the isolated fungi were really responsible for the spoilage of citrus and banana fruits. Healthy fruits were surface sterilized with ethanol. Cylindrical plug tissues were cut out from the fruits using a sterilized 2mm sized cork borer. Agar plate containing a week-old fungal culture were aseptically placed in these holes, then covered and sealed off by means of petroleum jelly. The procedure was repeated separately across each of the fungal isolates. The inoculated samples and the control were placed in sterile polythene bags and incubated in an oven for 5days. The point of inoculation of each type of fungus was examined and recorded. The diameter of the rotten portion of the watermelon fruits was measured. The fungi were later re–isolated from the inoculated fruits and compared with the initial isolates.

Antibacterial activity

The 100 µl of adjusted bacterial suspension was pipetted using a micropipette and applied on the surface of Mueller Hinton agar and was swabbed at 60^{o} rotation to uniformly distribute bacteria throughout media surface using a cotton swab. The swabbed Mueller Hinton agar stood for 15 min to provide time for the attachment of bacteria on the media. After that, the sterilised cork borer of 6 mm diameter was perforated with the swabbed media to create 6 mm diameter wells. At the time of punching media for different test bacteria, the cork borer was sterilised by immersing in alcohol and burning with Bunsen burner flames (Umer *et al*., 2013). The created wells were filled with 50 µl extracts at a concentration of 400, 200 and 100 mg/ml, and negative control, but the positive control disc (gentamicin) was placed on the media surface. After all the wells on the Petri dishes were filled, and the positive control was placed on Petri dishes, then the Petri dishes were placed in the refrigerator at 4°C for 2 h to facilitate diffusion of extracts or fractions in the media. Subsequently, Petri dishes were incubated at 37°C for 24 h in the incubator (BioTechnics India). The inhibition zone diameter after 24 h incubation was measured by a ruler in millimetre and recorded. The experiment was done in triplicate.

Antifungal activity

The 100 µl adjusted fungi suspension was pipetted using a micropipette and applied on the surface of sabouraud dextrose agar and swabbed at 60^{o} rotation to uniformly distribute yeast throughout the media surface using a cotton swab. The swabbed sabouraud dextrose agar stood for 15 min to provide time for the attachment of fungi on the media. After that, the sterilised 6 mm diameter cork borer was used to perforate the swabbed media to create a 6 mm diameter of wells. The concentration of extracts for the experiment was determined based on a previous study on the plant. The created wells were filled with the 50 µl extracts at 400, 200 and 100 mg/ml, negative, and positive control. The inoculated Petri dishes were placed in the refrigerator at 4°C for 2 h to facilitate diffusion of extracts or fractions in the media. Next to that, Petri dishes were incubated at 37°C for 24 h in the incubator. The inhibition zone diameter after 24 h incubation was measured by a ruler in millimetre and recorded. The experiment was done in triplicate.

Determination of minimum inhibitory concentration for pathogenic bacteria

Minimum inhibitory concentration is the minimum concentration of extracts or fractions which have inhibited the growth of microorganisms. The minimum inhibitory concentrations were determined using the broth microdilution technique for extracts or solvent fractions as their inhibition zones equal to or greater than 7 mm in agar well diffusion techniques. The serial double dilution technique was employed for extracts in broth filled wells. The serial double dilution was performed as 100 µl extracts or fractions were added to the first well and thoroughly mixed for five times by rinsing using micropipette and 100 µl of the mixture was transferred to the second well using a new micropipette tip and thoroughly mixed as above. A 100 µl of the second well mixture was pipetted using a new micropipette tip and transferred to the third well, and then thoroughly mixed as above. The process was continued until the tenth well and 100 µl mixture of the tenth well was pipetted and discarded to have an equal volume of fluid in wells (CLSI, 2015). The twofold serially diluted concentrations of extracts for the experiment were determined from a previous study on the plant. The serially diluted concentrations used in the experiment were 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 and 0.3906 mg/ml (Abew*et al.*, 2016). The 100 µl broth filled 11th and 20th wells were used as growth and sterility control, respectively. The 10 µl diluted bacterial suspension (10% of 100 µl well volume) was pipetted to wells from eleventh to first wells to reduce contamination to sterility control and attained a final concentration of 5×10^5 CFU/ml bacteria in each well, but 10 µl broth was pipetted to the 12th well. Finally, microtitre plates were sealed using parafilm and incubated at 37°C for 24 h (CLSI, 2015). The incubated microtitre plate wells were filled with 0.01% resazurin sodium salt indicator from 12th to 1st well and incubated for 2 h at 37°C. The resazurin sodium salt reaction with actively growing microorganisms produces colour changes which are important to determine the MIC of extracts or fractions based on colour changes. The blue or purple colour appears if the growth of microorganisms is inhibited, while pink or colourless change is observed for those actively growing cells which reduced resazurin sodium salt to resorufin. The experiment was performed in triplicate.

Determination of minimum inhibitory concentration for pathogenic fungi

The serial double dilution technique was employed for extracts in broth filled wells commenced from the first to tenth wells. The serial double dilution was performed as 100 µl extracts or fractions were added to the first well and thoroughly mixed five times by rinsing using a

micropipette and 100 µl of the mixture was transferred to the second well using a new micropipette tip and thoroughly mixed as above. A 100 µl of the second well mixture was pipetted using a new micropipette tip and transferred to the third well and thoroughly mixed as above. The process was continued until the tenth well and 100 µl mixture of the tenth well was pipetted and discarded to have an equal volume of fluid in the wells (EUCAST, 2003). The twofold serially diluted concentrations of extracts for the experiment were determined from a previous study on the plant. The serial double dilution concentrations used in the experiment were 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 and 0.3906 mg/ml. The 100 µlbroth□filled 11th and 20th wells were used as growth and sterility control, respectively. The 10 µl diluted yeast suspension (10% of 100 µl broth volume) was pipetted to wells from the eleventh to first wells to reduce contamination on sterility control and the attained final concentration of yeast suspension (2.5×10^4 CFU/ml) in each well, but 10 µl broth was pipetted to the 12th well. The incubated microtitre plate wells were filled with 0.01% resazurin sodium salt indicator from the 12th to the 1st well and incubated for 2 h at 37°C. The MIC of extracts and fractions were determined as blue or purple resazurin colour changed to pink or colourless (Blazic *et al.*, 2019; Ohikhena*et al.*, 2017). The experiment was done in triplicate.

Determination of minimum bactericidal concentration (MBC)

The minimum bactericidal concentration was determined through sub□culturing of 10 µl content of microtitre plate well which is greater or equal to the lowest minimum inhibitory concentration on the Mueller Hinton agar and incubated for 24 h. After 24 h incubation, the Petri dish was assessed for the presence of growth, and the minimum concentration of extracts or fractions with no visible growth was taken as a minimum bactericidal concentration (Akinduti*et al.*, 2019). The experiment was done in triplicate.

Determination of minimum fungicidal concentration (MFC)

The minimum fungicidal concentration was determined through sub□culturing of 10 µl content of microtitre plate well which is greater or equal to the lowest minimum inhibitory concentration on the sabouraud dextrose agar and incubated for 24 h. After 24 h incubation, the Petri dish was assessed for the presence of growth, and the minimum concentration of extracts or fractions with no visible growth was taken as minimum fungicidal concentration (Akinduti*et al*., 2019). The experiment was done in triplicate.

Data analysis

The data were entered into an excel spreadsheet for statistical analysis using Statistical Package for Social Science (SPSS) version 20. The descriptive statistics, one□way ANOVA, Tukey's post hoc test and linear regression R^2 (Coefficient of determination) were utilised for statistical analysis and inference. The descriptive statistics were employed for calculation of group mean of inhibition zone diameter as mean ± SEM. The one□way ANOVA was performed to determine the significant difference among group means. Whereas, Tukey's post hoc test followed one□way ANOVA to determine the significant difference between each group mean. The linear regression R^2 was calculated to determine the concentration dependence of extracts and fractions on antimicrobial activities against test microorganisms. Statistically significant differences were declared at a p value of less than 0.05.

RESULTS

Table 1: Extraction yield (%)

Clove	Thyme
7.19%	**5.44%**

TABLE 2: Qualitative phytochemical composition of ethanolic extract

PHYTOCHEMICALS	Clove	Thyme
SAPONIN	++	++
FLAVONOID	-	-
TANNIN	+++	-
STEROIDS	++	+
TERPENIODS	-	-
GLYCOSIDES	++	-
PHENOL	+++	++

Key

+++ = Present in high concentration

++= Present in moderate concentration

+ = Slightly or sparingly present

- = Absent.

Table 3 :Quantitative phytochemical composition

PARAMETERS	CLOVE	THYME
ALKALOIDS(mg/100g)	3.75	11.10

FLAVONOIDS (mg/100g)	16.75	12.70
PHENOL(mg/100g)	1.80	1.30
SAPONIN(Mg/100g)	6.86	5.33
TANNIN(mg/100g)	9.76	13.77
STEROIDS (mg/100g)	0.85	0.43
TERPENIODS (mg/100g)	1.68	1.53
CARDIAC GLYCOSIDE (mg/100g)	**7.75**	**8.62**

***Values are mean scores of three (3) replicates**

Table 4 :ANTIOXIDANT PROPERTIES

PARAMETERS	**CLOVE**	**THYME**
TOTAL PHENOLIC CONTENT (mg/100ml)	1.86	1.36
TOTAL FLAVONOID CONTENT (mg/100ml)	16.69	11.70
B-CAROTENE (mg/ml)	0.65	1.86
VITAMIN E (mg/ml)	21.75	31.70
DPPH (µg/mL)	3.66	13.70
FRAP (mg AA/g)	113.70	216.00
TBARS (%)	7.42	4.17

Table 5 :ANTIBACTERIAL ACTIVITIES OF THE EXTRACTS

ISOLATE	EXTRACT	ZONE OF INHIBITION(mm)	RESULT
Staphylococcus spp	**Clove Ethanol extract**	24.00± 0.28	S
	Thyme Ethanol extract	19.50± 0.28	S
	Gentamycin	32.00± 0.00	S
Bacillus cereus	**Clove Ethanol extract**	12.80±0 .00	I
	Thyme Ethanol extract	10.50± 0.00	R
	Gentamycin	18.00± 1.36	S
Pseudomonas aeruginosa	**Clove Ethanol extract**	7.33± 0.28	R
	Thyme Ethanol extract	21.50± 0.28	S
	Gentamycin	25.00± 0.00	S
Salmonella spp	**Clove Ethanol extract**	13.50±1 .00	I
	Thyme Ethanol extract	5.00± 0.02	R
	Gentamycin	32.27± 1.00	S
Escherichia coli	**Clove Ethanol extract**	20.00±1 .00	S
	Thyme Ethanol extract	13.00± 0.02	I
	Gentamycin	30.00± 1.30	S

***Values are mean scores ± Standard deviation of three (3) replicates**
N/B:
***R** = Resistant,*
***I** = Intermediate,*
***S** = Susceptible;*

Table 6 :ANTIFUNGAL ACTIVITIES OF THE EXTRACTS

ISOLATE	EXTRACT	ZONE OF INHIBITION(mm)	RESULT
Rhizopus stolonifer	**Clove Ethanol extract**	8.13± 0.28	R
	Thyme Ethanol extract	6.10± 0.28	R
	Fluconazole	12.00± 0.00	S
Aspergillus spp	**Clove Ethanol extract**	22.00±0 .00	S
	Thyme Ethanol extract	15.00± 0.00	S
	Fluconazole	32.27± 1.36	S
Penicillium spp	**Clove Ethanol extract**	17.30± 0.28	S
	Thyme Ethanol extract	21.00± 0.28	S
	Fluconazole	18.00± 0.00	S

***Values are mean scores ± Standard deviation of three (3) replicates**

N/B:

***R** = Resistant,*

***I** = Intermediate,*

***S** = Susceptible;*

Table 7 :MIC and MBC of extracts against spoilage bacteria

EXTRACT	Activities	*Staphylococcus* spp	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Salmonella* spp	*Escherichia coli*
Clove Ethanol extract	**MIC**	12.5	12.50	250.00	100.00	25.00
Thyme Ethanol extract	**MIC**	175	5.00	500.00	100.00	125.00
Clove Ethanol extract	**MBC**	100.00	100.00	ND	N D	200.00
Thyme Ethanol extract	**MBC**	200.00	200.00	100.00	ND	50.00

Table 8 :MIC and MFC of extracts against spoilage fungi

EXTRACT	Activities	*Rhizopus stolonifer*	*Aspergillus* spp	*Penicillium* spp
Clove Ethanol extract	**MIC**	66.67	16.67	6.25
Thyme Ethanol extract	**MIC**	ND	3.125	8.33
Clove Ethanol extract	**MFC**	100.00	200.00	100.00
Thyme Ethanol extract	**MFC**	200.00	50.00	200.00

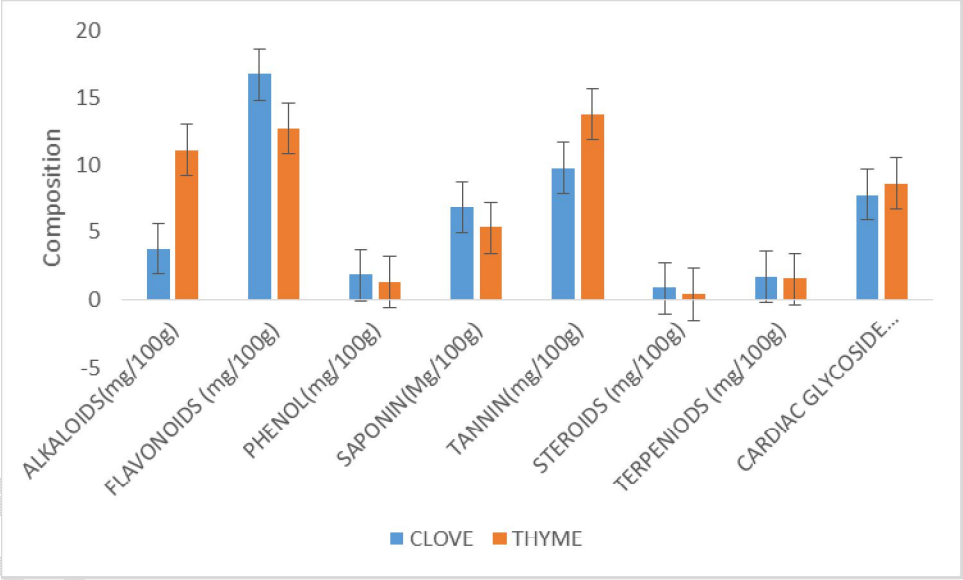

Fig .1 Bar graph showing antimicrobial activity and antioxidant activity of clove and thyme

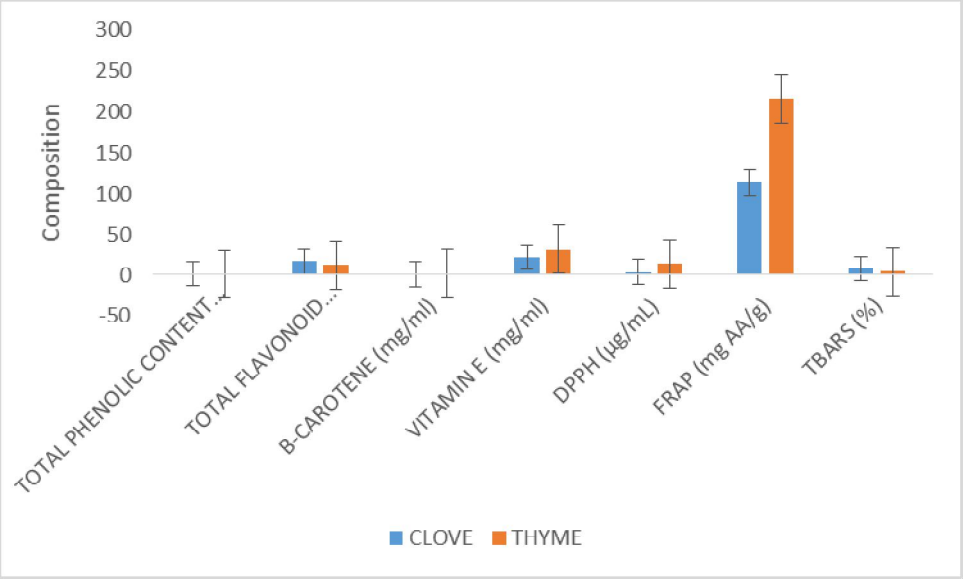

Fig .2 :Bar graph showingantioxidant properties

SHELF LIFE STUDY ON MOI MOI

STORAGE	EXTRACT CONC.(mg/ml)		**Clove leaf extract**(cfu/ml)	**Thyme extract**(cfu/ml)
DAY 1				
	5.00%	TBC	No Growth	No Growth
		TFC	No Growth	No Growth
	3.00%	TBC	No Growth	No Growth
		TFC	No growth	No Growth
	1.0%	TBC	No Growth	No Growth
		TFC	No Growth	No Growth
	CONTROL	TBC	$1.17x10^2$	
	TBC		$1.73x10^3$	
DAY 5	5.00%	TBC	No Growth	No Growth
		TFC	No Growth	No Growth
	3.00%	TBC	$2.07x10^2$	$4.17.00x10^2$
		TFC	$1.83x10^3$	$3.06x10^2$

	1.0%	TBC	$4.33.x10^3$	$6.85x10^3$
		TFC	$5.13x10^3$	$6.15x10^3$
	CONTROL	TBC	$4.80x10^4$	
	TBC		$3.88.00x10^6$	
DAY 10				
	5.00%	TBC	$6.62x10^3$	$7.15x10^3$
		TFC	$5.30x10^4$	$8.03x10^3$
	3.00%	TBC	$5.17x10^3$	$7.27x10^3$
		TFC	$5.80x10^3$	$7.13x10^3$
	1.0%	TBC	$8.79x10^3$	$9.70x10^3$
		TFC	$9.43x10^3$	$1.15x10^4$
	CONTROL	TBC	$4.80x10^6$	
	TBC		$3.88.00x10^6$	
DAY 15				
	5.00 %	TBC	$3.23x10^4$	$7.25x10^4$
		TFC	$5.75x10^4$	$8.38x10^4$
	3.00%	TBC	$8.11x10^4$	$3.52x10^5$
		TFC	$7.00x10^4$	$2.60x10^5$
	1.0%	TBC	$2.02x10^6$	$5.00x10^5$
		TFC	$2.92x10^6$	$5.09x10^8$
	CONTROL	TBC	TNTC	
	TBC		TNTC	
DAY 20				
	5.00%	TBC	$6.11x10^6$	TNTC
		TFC	$8.16x10^6$	TNTC
	3.00%	TBC	$7.00x10^6$	TNTC
		TFC	$8.87x10^6$	TNTC
	1.0%	TBC	TNTC	TNTC
		TFC	TNTC	TNTC

	CONTROL TBC TBC	TNTC TNTC	

FAO/SON STANDARD FOR MICROBIAL COUNT IN FOOD
No growth = Safe for consumption
Less than or equal to 10^4cfu/ml = Significant
Above 10^4 cfu/ml = highly significant and unsafe for consumption

DISCUSSION

The result of this study demonstrates the potential of clove and thyme as natural antimicrobial and antioxidant agents in food packaging. The extracts of the both plants exhibited significant antimicrobial activity against foodborne pathogens, including *E. coli*, *Salmonella*, and *Staphylococcus aureus*.

The antimicrobial of clove can be attributed its high content of eugenol, a compound known for its bactericidal properties (kim*et al*., 2016). Thyme, on the other hand, contains thymol, which has been shown to disrupt the cell membrane of microorganisms, leading to cell death (Sokovic*et al.*, 2007). The antioxidant activity of clove and thyme can be attributed to their high content of phenolic compounds, which is known to scavenge free radicals and prevent oxidative stress (Kumar *et al.*, 2017). The antioxidant activity of this plants can help prevent lipid oxidation and spoilage in food. The synergistic effects of combing clove and thyme extracts observed in this study are consistent with previous findings (Perez-Rosales *et al*., 2014). This combination may provide a more effective antimicrobial and antioxidant activity than using individual extracts. The use of clove and thyme as natural preservatives in food packaging has several advantages, including reduced toxicity and environmental impact compared to synthetic preservatives (Gould, 2016). Additionally, these plants are readily available and inexpensive.

This study had some limitations which is the extraction methods used may not have optimized the antimicrobial an antioxidant activity of the plants. Further studies are needed to optimize extraction methods and evaluate the stability of these extracts in food packaging.

CONCLUSION

In conclusion, this study demonstrates the potential of clove and thyme as natural antimicrobial and antioxidant agents in food packaging. The results suggests that these plants can be used to develop sustainable and effective food preservation system.

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