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Trials for Replacing Antibiotics Used in Production of Tissue Culture Vaccines by Natural Antibacterial and Antifungal Extracts

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Abstract

The continuous use of antibiotics for tissue culture adapted vaccines production has led to the increase in the bacterial resistance to these antibiotics. This study aims to evaluate the antimicrobial potential of thyme (*Thymus vulgaris*) and clove (*Syzygium aromaticum*) on bacterial and fungal contamination, in the production of tissue culture vaccines. The active agents in each plant were extracted by the conventional extraction technique using ethanol and water as solvents followed by concentration (steam distillation and boiling). The antimicrobial activities of different solvent extracts were determined in well agar diffusion technique using *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Candida albicans* as model for gram positive, gram negative and fungal contamination, respectively. The cytotoxic effects of the different solvent extracts were tested on VERO and MDBK cell culture. The obtained results indicated that water and ethanolic extracts from thyme and clove plants showed significant antimicrobial activities ($P < 0.05$) as they could inhibit the growth of *E. coli*, *S. aureus* and *Candida albicans*. Ethanolic extract of thyme had the maximum zone of inhibition against *E. coli* (2.40±0.20) and *Candida albicans* (3.07±0.3), and the lowest inhibition zone against *S. aureus* (1.53±0.23), whereas the thyme water extract didn't show any antimicrobial activity. The ethanolic extract of clove showed the greatest zone of inhibition against *Candida albicans* (2.63±0.2), *E. coli* (2.63±0.2), while the lowest was against *S. aureus* (1.87±0.3). Water extract of clove showed the greatest zone of inhibition against *E. coli* and *S. aureus* (1.93±0.4, and 2.47±0.1), respectively and 0.97±0.1 against *Candida albicans*. The ethanolic extracts of thyme and clove showed changes in the cell wall until concentration 1 mg/ml for clove and 10 µg/ml for thyme on VERO cells, while the cytotoxic effect on MDBK cells was observed till the concentration of 100 µg/ml for clove and thyme water extracts. In conclusion, the antimicrobial potential of clove water extract on bacterial and fungal contaminant could replace antibiotics in the production of tissue culture vaccines at a concentration of 10 µg/ml.

KEYWORDS
Thyme, Clove, Antimicrobial, Tissue culture, Cytotoxic effect.

INTRODUCTION

The main objectives of veterinary vaccinations are to enhance the health and welfare of companion animals, increase livestock output while minimizing costs, and prevent the spread of diseases from domestic and wild animals to humans (Meeusen *et al*., 2007).

The manufacture of viral vaccines has benefited greatly from the advent of animal cell culture technologies. Use of antibiotics while cultivating cells is one of the most common precautionary measures employed during *In vitro* investigations because bacterial and fungal contamination of cell lines occurs as a result of impure procedures and source material. The American Type Culture Collection (ATCC) lists standard cell culture techniques that specifically call for the use of antibiotics as a media supplement, including gentamicin and penicillin-streptomycin (Ann *et al*., 2017).

The inappropriate use of antibiotics in human and veterinary medicine has increased the frequency with which some dangerous microbes, including *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus* spp., *Salmonella* spp., and *Campylobacter* spp., are resistant to conventional antibiotics. In order to combat the emergence of resistance, it is also crucial to consider the potential synergistic effects of several natural compounds and conventional antibiotics (Maja *et al*., 2019).

Historically, both conventional and folk medicine has used medicinal herbs. Various bioactivities of natural compounds, including antibacterial activity, are confirmed by numerous research. Natural antibacterial substances, such isolated chemicals, and essential oils, are now a significant source for the food and pharmaceutical industries. They exhibit high antibacterial activity as well as "green consumerism" requirements. Additionally, they are the richest and cheapest source of pharmaceutical intermediates, food additives, food preservatives, and chemical entities for synthesized pharmaceuticals (Maliehe *et al*., 2015).

Since they have antimutagenic, antioxidant, anti-inflammatory, and antimicrobial properties as well as being used as a food preservative, clove (*Syzygium aromatic*) and thyme (*Thymus vulgaris*) are two of the most used plant extracts in the world (Ajobiewe *et al*., 2022). Clove's essential oil extracts' ability to kill numerous Gram's positive and negative species, including some fungi, led to the discovery of clove's antibacterial potential (Gis-

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lene *et al*., 2000).

In order to replace antibiotics used in the development of tissue culture vaccines, this study aimed to assess the antibacterial and antifungal activity of ethanolic and aqueous extracts of clove (*Syzygium aromaticum*) and thyme (*Thymus vulgaris*) on specific microorganisms.

MATERIALS AND METHODS

Plant extraction

Thymus vulgaris (Thyme) and *Syzygium aromaticum* (clove) were used as medicinal plants for replacing antibiotics. They were purchased from local market in Cairo in a dried form. In this study the conventional extraction method was applied by using water and ethanol as dissolving solutions to evaluate the efficacy of their extraction yields.

Water extraction preparation

Twenty grams of each plant material was heated for 30 minutes at 90ºC in a round bottom flask with 180 ml of distilled water, then incubated overnight at 37ºC.

Preparation for ethanol extraction

Round bottom flasks containing 10 g of each studied plant material were separately mixed with 9:1 ethanol before being incubated overnight at 37°C and 150 rpm. The resultant liquid extracts were then concentrated using a rotary evaporator after being separated from the solid residue by filtration using filter paper. The distilled extracts were packaged with labels and kept in the refrigerator until needed (Gonelimali *et al*., 2018). The following equation has been used to determine the extraction yield of a few different plants (Felhi *et al*., 2017):

Yield (%) = X_1*100/ X_0

Where X_0 is the dry weight of the plant powder prior to extraction and X_1 is the weight of the extract after the solvent has evaporated.

Preparing the inoculum of standard microorganisms

The standard microorganisms used to test the antimicrobial effects of plant extracts included *Candida albicans* (ATCC10231) as a pathogenic fungus, *Escherichia coli* (ATCC 25922) as a Gram-negative bacteria, and *Staphylococcus aureus* (ATCC 25923) as a model for Gram-positive bacteria. These microorganisms were pre-cultured in brain heart infusion broth (BHI) for an overnight period at 37°C, and each strain was adjusted to a concentration of 108 cells/ml for the 48-hour incubation of the fungus.

The antimicrobial potential screening of plant extracts

The minimum inhibitory concentrations (MIC) of various solvent plant extracts against the last mentioned standard microorganisms have been determined using the Agar well diffusion method. Agar plates were inoculated with 1 ml of standardized inoculum of each bacterium (in triplicates), spread, and then 12 ml of Muller Hinton agar were added. The plates were then placed in the refrigerator for 30 minutes to solidify. 100 µl of plant extracts were added to each of the five 6 mm-diameter wells created using sterile borer tools into the agar plates containing the bacterial inoculum. The negative control was distilled water that had been sterilized.

For ten minutes, the plates were left at room temperature to allow the extracts to diffuse into the agar. After a 24-hour incubation period. The plates were examined at 37°C. An inhibitory zone surrounding the well containing the plant extract served as a visual cue that antibacterial activity was present on the plates. The Diameter of Inhibition Zone (DIZ) was quantified in cm and expressed as such. According to the procedure (CLSI, 2020), the mean values of the diameter of the inhibition zones were computed.

Detection the cytotoxicity effect of plant extract

On development of a monolayer of Madin-Darby bovine kidney cell culture (MDBK) and an African green monkey kidney cell culture (VERO), seven serial tenfold dilutions of the plant extracts in Hank's balance salt solution (under test) were introduced as 100 µl of each dilution in five wells of tissue culture. As a control, a 96-well plate with normal cell culture was used. After that, incubate the plate at 37°C for up to 7 days while doing a daily microscopic examination for the detection of any cell abnormalities (Kiki *et al*., 2023).

Statistical analysis

With SPSS version 20.0 (Statistical Package for the Social Sciences, Inc., Chicago, IL, United States), the results were subjected to multiway analysis of variance, and mean comparisons were carried out using Tukey's multiple range test.

RESULTS AND DISCUSSION

Since ancient times, people have used plants as food, spices, and medicines. Plants were an integral element of many communities' traditional medicine, which was utilized to cure a variety of infectious ailments, long before germs were discovered to exist. Thyme and clove essential oils have been shown in recent years to exhibit a variety of bioactivities, including antibacterial, antifungal, and mycotoxin formation inhibition.

The basic operations of cells are impacted by the hazardous substances in the culture media. The cytotoxicity effect might cause the cells to die or change how they function. Oil extracts of thyme and clove are known to have some antibacterial properties. Additionally, Nzeako *et al*. (2006) observed that thyme oil extract could reduce the growth of *Candida albicans* and Pseudomonas aeruginosa. Doddanna *et al*. (2013) evaluated the effect of several plant extracts on the growth of *Candida albicans*.

The obtained results in Table 1 showed the yield percentage of ethanol and water extraction for thyme and clove, the yield percentage of water extract of thyme (10.75%) is more than the ethanolic extraction (3%). It has been reported previously that the water extract of different plants usually yields significantly higher amounts compared to the ethanolic extracts of same plants

Table 1. Plant extracts used in this study and their extraction yield percentage by ethanol and water extraction.

Plant extract	Yield % by Ethanol extraction	Yield % by water extraction
Thyme (*Thymus vulgaris*)	3.00%	10.75%
Clove (*Syzygium aromaticum*)	17.50%	11.00%

(Caleja *et al*., 2016). While the water extract (11%) for clove is lower than the ethanolic extraction (17.5%).

As stated in Table 2, ethanolic extract of thyme had the maximum zone of inhibition against *E. coli* (2.40±0.20) and *Candida albicans* (3.07±0.3) with a significant antibacterial activity (P < 0.05) and the lowest inhibition zone against *S. aureus* (1.53±0.23) whereas the thyme water extract didn't show any antimicrobial activity, whereas the ethanolic extract of clove showed significant antimicrobial activity (P < 0.05) with greatest zone of inhibition against *Candida albicans* (2.63±0.2), while the lowest was against *E. coli* (2.00±0.2). Water extract of clove showed significant antibacterial activity (P < 0.05) against all tested bacterial strains, the greatest zone of inhibition was against *E. coli* and *S. aureus* (1.93±0.4 and 2.47±0.1), respectively and 0.97±0.1 against *Candida albicans*. Likely, Gonelimali *et al*. (2018) demonstrated that water and ethanolic extracts from thyme and clove plants showed antimicrobial activity as they could inhibit the growth of *E. coli*, *S. aureus* and *Candida albicans*.

From the presented results in Table 2, we excluded the thyme water extract from our study on tissue culture. In addition, the selected extracts were tested on VERO and MDBK cell culture to study the cytotoxic effect of such extracts. As recorded in Table 3 and 4, the ethanolic extracts of thyme and clove showed changes in the cell wall until concentrations of 1 µg/ml and 1 mg/ml, respectively on VERO cells while the cytotoxic effect on MDBK cells was observed until concentration of 100 µg/ml for thyme and clove water extracts.

Clove water extract showed no effect on VERO and MDBK cells as in Tables 3 and 4 at concentrations of 100 µg ml and 10 µg/ml, respectively. These results agreed with De Faria *et al*.

Table 2. Antimicrobial activity of plant extracts against 3 microorganisms.

Microorganism	Inhibition zone (cm)[a]			
	Thyme (*Thymus vulgaris*)		Clove (*Syzygium aromaticum*)	
	Ethanol extraction	water extraction	Ethanol extraction	water extraction
E coli	2.40±0.20	N	2.00±0.20	1.93±0.40
S. aureus	1.53±0.23	N	1.87± 0.30	2.47±0.10
Candida albicans	3.07±0.31	N	2.63±0.20	0.97±0.10

Values are means of triplicate determination (n = 3)±standard deviations. N: no zone of inhibition was found.

Table 3. Cytotoxicity testing of plant extract on VERO cells

Tested dilution of plant extract	Tested concentration plant extract	*In vitro* assay Cytotoxicity in VERO cells			
		Thyme		Clove	
		Ethanol extraction	Water extraction	Ethanol extraction	water extraction
Original concentration	1 g/ml	+ve	+ve	+ve	+ve
1:10	100 mg/ ml	+ve	+ve	+ve	+ve
0.11	10 mg/ml	+ve	+ve	+ve	+ve
0.74	1 mg / ml	+ve	+ve	+ve	+ve
1:10000	100 ug / ml	+ve	+ve	+ve	-ve
1:100000	10 ug / ml	+ve	+ve	+ve	-ve
1:1000000	1 ug / ml	-ve	-ve	-ve	-ve

Inoculation of different concentrations of plant extracts in tissue culture VERO (starting from the master concentricity 1 g / ml up to 1 ug / ml) showed abnormal cell growth and cellular changes. A regular growth rate from conc 100 ug/ml in clove extracted by water. Clove and thyme ethanol extraction showed cytotoxic effect until 10 ug/ml.

Table 4. Cytotoxicity testing of plant extract on MDBK cells.

Tested dilution of plant extract	Tested concentration plant extract	*In vitro* assay Cytotoxicity in MDBK cells			
		Thyme		Clove	
		Ethanol extraction	Water extraction	Ethanol extraction	water extraction
Original concentration	1 g/ml	+ve	+ve	+ve	+ve
1:10	100 mg/ ml	+ve	+ve	+ve	+ve
0.11	10 mg/ml	+ve	+ve	+ve	+ve
0.74	1 mg / ml	+ve	+ve	+ve	+ve
1:10000	100 ug / ml	+ve	+ve	+ve	+ve
1:100000	10 ug / ml	+ve	-ve	+ve	-ve
1:1000000	1 ug / ml	+ve	-ve	+ve	-ve

Inoculation of different concentrations of plant extracts in tissue culture MDBK (starting from the master concentricity 1 g / ml up to 1 ug / ml) showed abnormal cell growth and cellular changes. A regular growth rate from conc 10 ug/ml in clove and thyme extracted by water.

Table 5. Antimicrobial activity of clove (water extract) at concentration 100 µg/ml against 3 microorganisms.

Microorganism	Inhibition zone (cm)[a] of Clove (*Syzygium aromaticum*) water extraction
E. coli	0.90±0.08
S. aureus	0.90±0.10
Candida albicans	1.40±0.05

Values are means of triplicate determination±Standard Deviations.

(2013) who studied the cytotoxic effects on Vero and MDBK cells *In vitro*. Their study demonstrated that clove extract had a concentration-dependent cytotoxic effect on both types of cells. They concluded that caution should be taken when using high concentrations of clove, as it might have cytotoxic effects on cells.

Table 5 showed the antimicrobial activity of clove (water extract) against 3 microorganisms after inoculation of 100 µg/ml, the greater inhibition zone was recorded against *Candida albicans* as 1.4 cm, then 0.9 cm against *E. coli* and *S. aureus*, respectively.

The active ingredient in clove extract is eugenol, which acts as an antimicrobial agent; eugenol has been shown to be effective against a wide range of microorganisms, including bacteria, and fungi. It works by disrupting the cell membrane and interfering with the metabolic and enzymatic processes of the microorganism, ultimately leading to its death (Marchese *et al*., 2017). Our study agreed with Ajobiewe *et al*. (2022) who showed that the active ingredient in the clove extract is eugenol, which has been found to be effective against bacteria, and fungi, and it can be used as a natural alternative to synthetic antimicrobial agents.

CONCLUSION

Based on the results, the antimicrobial potential of clove (*Syzygium aromaticum*) water extract on bacterial and fungal contaminants has been assumed to replace antibiotics in the production of tissue culture vaccines but caution should be taken when using high concentrations of the clove extract.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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