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EFFECT OF PHYTOL ON BACTERIAL ISOLATES FROM GRILLED MEAT

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ABSTRACT: *Effect of phytol on Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa and Enterococcus faecalis isolated from grilled meat was examined using a broth dilution method. Time kill effects of phytol concentration on the bacterial isolates after 4h and 24hrs incubation were determined and this showed that inhibitory effects of cell growth decreased at elevated concentrations. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of phytol against the bacterial isolates were found in the range of 31.25µg/ml to 250 µg/ml and 125 µg/ml to 250µg/ml respectively. Effect of phytol on the dehydrogenase activity of the bacterial isolates showed that gram negative Pseudomonas aeruginosa had higher dehydrogenase activity than the gram positive Bacillus cereus, Enterococcus faecalis and Staphylococcus aureus. Effect of phytol on protease activity of the bacterial isolates was assessed and it was observed that protease activity decreased with increase in phytol concentration. The effect of phytol on growth and protease activity was not statistically significant ($P<0.05$) but dehydrogenase activity varied significantly ($p>0.05$) among the bacterial isolates. This result indicates that phytol could be used as an antibacterial agent in food industry to inhibit the growth of certain foodborne pathogens.*

KEYWORDS: Phytol; dehydrogenase activity; protease activity; grilled meat.

INTRODUCTION

For years, food borne illness resulting from consumption of food contaminated with pathogenic bacteria and/or their toxins has been of vital concern to public health. Among food borne outbreaks in the United States during 1983-1987 in which the etiology was determined, bacterial pathogens caused the largest number of out breaks (66%) and cases (92%) (Tauxe, 2002). Thus, controlling pathogens could reduce food borne outbreaks and assure consumers a safe, wholesome and nutritious food supply.

Plants contain a great number of secondary metabolites, many of which display biological activity as “natural products” with a role in plant defense against bacteria, fungi and other microorganisms. There has been a growing interest in new and effective antibacterial substances from natural sources like plants to reduce cases of bacterial diseases. In many cases, antibacterial compounds from plants, herbs and spices in low concentrations have been used effectively in the food industry. Biologically active secondary metabolites of spices, herbs and medicinal plants, due to their diverse range of antibacterial activities, are becoming increasingly important in food industry as potential food preservatives to control food borne pathogenic bacteria (Aiyelagbe *et al.,* 2007).

Food borne pathogenic bacteria cause several food borne diseases (Tauxe, 2002). The indiscriminate and excessive use of a wide range of chemical bactericides has led to extended

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environmental pollution and the production of resistant pathogen populations. During the last few years, research on plant-based antibacterial agents has produced a diverse range of products with novel modes of action, which are expected to have a significant impact on the control of food borne diseases in coming decade. Therefore the demand for plant based antimicrobials as novel bactericide sources for controlling food borne pathogens is rapidly increasing (Ulubelen *et al*., 2000; Kubo *et al*., 2004; Giang *et al*., 2006).

Some synthetic chemicals have been used to control microbial growth and reduce the incidence of food borne illness. Although these synthetic preservatives are effective, they might be detrimental to human health. Consumers are concerned about the safety of food products that contain artificial preservatives. The ban of chemical additives by some consumers has driven the food industry and food research towards the search for natural antibacterial compounds from plant origin. Many natural compounds (e.g. terpenoids) found in dietary plants possess antibacterial activities against food borne pathogens (Deller *et al*., 1996; Singh and Singh 2003; Aiyelaagbe *et al*., 2007).

Phytol is an acyclic diterpene alcohol that can be used as a precursor for the manufacture of synthetic forms of vitamin E (Netscher, 2007) and vitamin K1 (Daines, 2003). Phytol is likely the most abundant acyclic isoprenoid compound present in the biosphere and its degradation products have been used as biogeochemical tracers in aquatic environments (Rontani and Volkman, 2003). The aim of this work is to determine the effect of phytol on the bacterial isolates from grilled meat.

MATERIALS AND METHODS

Materials

The materials used in this research work include; phytol from sigma chemical industry, USA, grilled meat (suya and pork),sterile bottles, Petri dishes, conical flask, test tubes, measuring cylinder, wire loop, glass rod, aluminum foil, cotton wool, pipettes, forceps, cork borer, autoclave, microscope, weighing balance, Bunsen burner ,incubator, spectrophotometer, rotary incubator. Reagents used include; dimethyisulphoxide (DMSO) obtained from chevron Philips chemical company USA, tannic acid from powder park chemical, India, casein-Universal buffer, amyl alcohol and triphenyl formazan were obtained from sigma chemical industry, USA. Trichloroacetic acid from hydrite chemical company, brook field,2,3,5-triphenyl tetrazolium chloride [TTC] from BDH England, methyl red from jigchem universal, mumbia and NaCL from cellmark , USA. All reagents were of analytical grade. The media used were nutrient agar, macConkey agar and nutrient broth-glucose obtained from becton Dickinson and company, USA respectively. Luria-bertani broth and yeast extract casein broth were obtained from sigma chemical industry, USA. Brain heart infusion broth and agar-solidified Mueller hinton broth were obtained from hardy diagnostic, USA.

Methods

Source of Sample

A total of thirty samples of grilled meat (suya and pork) were collected from five different locations namely; world bank housing estate in new Owerri, Ihiagwa in Owerri suburb, Ama

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Awusa in Owerri town, Mbieri in Mbaitolu L.G.A and Akabo in Ikeduru L.G.A, all in Imo state.

Media Preparation

Nutrient agar and MacConkey agar were used for the isolation of the bacterial organisms and they were prepared according to the manufacturers' instructions.

Isolation of bacterial strains

Food-associated bacteria were isolated from grilled meat products, Suya and pork meat, using Nutrient agar and MacConkey agar. Ten grams of suya and pork meat were weighed aseptically into 90ml sterile distilled water, agitated for 5mins and serially diluted ten fold. 0.1ml of (10^{-8} dilution) was spread on Nutrient agar and MacConkey agar and the plates incubated at 37^0C for 24hrs and 48hrs respectively. After incubation, single morphologically well-formed colonies were picked and subcultured. The colony forming unit per gram (CFU/g) was calculated. Colonial morphology, microscopic characteristics, and biochemical and carbohydrate fermentation tests of the isolated organisms were used for characterization.

Purification of Isolates

Each of the sub-cultured isolates were further sub-cultured aseptically from the plates by streaking a loopful of the bacterial colony onto a freshly prepared Nutrient agar and MacConkey agar plates and incubated at 37^{o}C for 24hrs and 48hrs respectively. After incubation, discrete colonies were picked and Gram stained. Microscopy was carried out inorder to confirm their purity. Each pure colony was picked and inoculated onto sterile Nutrient agar slants in screw- capped bottles. These were incubated at 37^{o}C for 24hrs and stored in the refrigerator at 4^{o}C as stock culture for further tests.

Characterization of Bacterial Isolates

Colonial morphology, microscopic characteristics, and biochemical and carbohydrate fermentation tests of the isolated organisms were used for characterization.

Sugar Assimilation Test

This was carried out using a broth medium composed of 10g of peptone, 5g NaCl, 0.03g methyl red and 1litre deionised water. After shaking, 9ml of the above mixture were dispensed into test tubes. Durham tubes were then introduced into the tubes in an inverted position to ensure that each durham tube was filled with the medium. The media was sterilized at 121^{o}C for 15 mins.

A solution of each sugar (Glucose, Sucrose, Maltose, Lactose, Mannose and Xylose) was prepared by dissolving 1g of each sugar in 100ml of distilled water and sterilized at 121^{o}C for 8 minutes. After cooling, 1ml of each sugar solution was aseptically added into the 9ml of the basal medium. Each tube was inoculated with the bacterial isolate. This was incubated for 48hrs.The tubes were checked for gas production in the durham tubes and change in colour of the medium from red to yellow indicated acid production. The tube which was not inoculated with the bacterial isolate served as the control.

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Gram Stain

This is the most widely used procedure for staining bacteria. It is used in separating bacteria into two major groups: Gram-positive and Gram negative. The gram stain procedure involves four basic steps; the smear was first flooded with the primary stain, crystal violet. The smear was rinsed to remove excess crystal violet and then flooded with Gram's iodine. The stained smear was rinsed again and 95% alcohol was added. The red dye safranin was added and Gram-positive cells remained purple in colour while Gram- negative cells appeared red.

Determination of minimum inhibitory and bactericidal concentrations

The minimum inhibitory concentration (MIC) of the compound (phytol) was tested by a two-fold serial dilution method (Bajpai *et al*., 2009a). The test compound was first dissolved in dimethylsulphoxide (DMSO), incorporated into Luria-Bertani medium for bacterial pathogens to obtain a concentration of 2000µg/ml and serially diluted to achieve 1000, 500, 250, 125, 62.5, and 31.25µg/ml. The final concentration of DMSO in the culture medium was maintained at 0.5% (v/v). A 10μl standardized suspension of each tested organism (1.5×10^8 CFU/ml) was transferred to each tube. The control tubes containing only bacterial suspensions were incubated at 37ºC for 24h. The lowest concentration of the compound, which did not show any growth of test organisms after macroscopic evaluation was determined as MIC, and was expressed in µg/ml. Further, the concentrations showing complete inhibition of visual growth of bacterial pathogens were identified, and 50µl of each culture broth was transferred onto the agar plates and incubated at 37^0C for 24hrs. The complete absence of growth of bacterial colonies on agar surface in the lowest concentration sample was defined as the minimum bactericidal concentration (MBC). Each assay in this experiment was replicated three times.

Determination of antibacterial activity using the bds method (broth dilution with shaking)

The method as described by Arai *et al*. (1996), was adopted. The compound to be tested was added at the indicated concentrations, to 10ml aliquots of brain heart infusion (BHI) broth in test tubes without any solubilizing agent or surfactant. An aliquot of an overnight culture of bacterial isolates was added to each sample to give approximately 10^5 CFU of the different bacterial isolates per ml. Each culture was incubated, with shaking at 40rpm, for 24hrs at 37ºC. The inhibitory activity of the compound being tested was monitored turbidimetrically. The optical density at 660nm was determined with a biophotorecorder.

Time kill assay

Cells from an overnight culture of the bacterial isolates were washed twice with phosphate-buffered saline (PBS; pH 7.0). The cell pellet was re-suspended in 1ml of PBS. An aliquot of 100µl of this suspension was added to 10ml of buffer that contained phytol at the indicated concentrations,(0-2000 µg/ml) and the mixture was incubated at 37ºC. The buffer did not contain any solubilizer. Samples of the above mixture were removed for determination of viable cell counts at 4hrs and 24hrs. Serial ten-fold dilution of the samples of bacterial suspensions (10^{-1} to 10^{-4}) was prepared in PBS. Aliquots of 50µl of each serially diluted sample were placed on Mueller Hinton agar. The plates were incubated at 37ºC for 24hrs, and then colonies were counted. Killing curves were constructed by plotting numbers of viable cells against time. All assays were performed in triplicate.

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Effect of phytol concentration on dehydrogenase activity of bacterial isolates.

Dehydrogenase assay method as described by Nweke *et al*. (2007) was employed.

Dehydrogenase activity was determined using 2, 3, 5-triphenyltetrazolium chloride (TTC) as the artificial electron acceptor, which is reduced to the red-coloured triphenylformazan (TPF). The dehydrogenase activity of the bacterial isolates was determined in 3.4ml volume of nutrient broth-glucose-TTC medium supplemented with varying concentrations of phytol (0 - 2000µg/ml) in separate screw-capped test tubes. A 0.3ml of suspension of the bacterial isolate was inoculated into triplicate 20ml screw-capped tubes containing 3.4ml of phosphate buffered (pH 7.2) nutrient broth-glucose medium amended with toxicant (phytol 0-2000 µg/ml) and preincubated on a rotary incubator (150rpm) at room temperature (28± 2°C) for 24 hrs.

The TPF produced was extracted in 4ml of amyl alcohol and determined spectrophotometrically.

The amount of formazan produced was determined from a standard dose-response curve (0-50mg/l TPF in amyl alcohol) at a reasonable R^2 value. Dehydrogenase activity was expressed as micrograms of TPF formed per mg dry weight of cell biomass per hour.

Protease Assay

Ten grams of suya and pork meat were weighed aseptically into 100ml of sterile distilled water, agitated for 45mins on a shaker and 0.1ml was spread on Nutrient agar and MacConkey. This was incubated at 37^{o}C for 24hrs and 48hrs respectively. A total of 8 bacterial isolates from grilled meat (suya and pork) was plated over Nutrient agar medium containing 0.4% gelatin. After 24hrs of incubation, plates were flooded with 1% tannic acid. Isolates having higher ratio of clearing zone to colony size were grown in liquid broth (peptone water) and the amount of protease production was determined from culture filtrate. The isolate which showed higher protease activity was selected and maintained on Nutrient agar slants and sub-cultured after every fifteenth day.

Selected isolate was identified based on morphological & biochemical characteristics.

Enzyme Production Medium

Yeast extract casein medium was used as production medium. It contained (g/L) glucose 10, casein 5, yeast extract 5, KH_2PO_4, 10; Na_2Co_3, 10. Hundred milliliter of yeast extract casein medium in a 250ml conical flask was sterilized in autoclave at 121^{O}C for 15mins.After cooling the flask was inoculated with 2% over night grown bacterial culture and incubated at 37^{o}C in a shaker for 24hrs. At the end of the fermentation period, the culture medium was centrifuged at 5000 xg at 4^{o}C for 15mins to obtain the crude extract which served as enzyme source.

Protease Assay Activity

Three hundred microliters of each supernatant, which contains the crude enzyme, was added to the same volume of the casein-universal buffer at the different phytol concentrations (0 - 2000µg/ml).The enzyme-substrate mixture for each phytol concentration was incubated at 37^{o}C for 30 mins. The reaction was stopped by adding 60µl of trichloroacetic acid. The mixture was allowed to stay at room temp for 15mins, then centrifuged at 1000rpm for 10mins (Biofuge 15-Heraeus sepatach). The absorbance of each sample was determined spectrophotometrically

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at 280nm (Perkin elmer-uv/vis spectrometer lambda).Their tryrosine content was derived from the tyrosine standard curve and each enzyme activity was determined as unit per ml.

Data analysis

The results obtained from the data were subjected to Analysis of variance (ANOVA) and values of $P \leq 0.05$ were considered statistically significant according to Onuh and Igwemma (2000).

RESULT AND DISCUSSION

Characterization of Bacterial Isolates

Out of a total of 30 samples of grilled meat analyzed, 8 pathogenic organisms were isolated. The most common isolates were *B. cereus*, *S. aureus*, *E. faecalis* and *P. aeruginosa* as shown in Table 1 and 2.

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Table 1 Colonial morphology and microscopic characteristics of bacteria isolated from grilled meat.

Sample code	Colony code	Colony count	Cell morphology	Colonial morphology	Motility	Gram stain	Spore	Flagellum	Capsule	Probable organism
Suya And Pork Meat	X1	$1.6x10^{10}$	Spherical cells in pairs and short chains	Small smooth and shiny round colonies	-	+S	-	-	-	*Enterococcus sp*
	X2	$5.0x10^{9}$	Straight rods in short chains with oval central spores	Dull and dry irregular flat cream colonies	+	+R	+	+	-	*Bacillus sp*
	X3	$3.0x10^{9}$	Straight or slightly curved rods with tapered or clubbed end	Circular umbonate cream colonies	-	+R	-	-	-	*Corynebacterium sp*
	X4	$2.0x10^{9}$	Slightly curved rods in singles	Bluish-green colonies spreading on plates	+	-R	-	+	-	*Pseudomonas sp*
	X5	$1.6x10^{10}$	Spherical cells in irregular clusters. Few in pairs.	Golden yellow smooth and circular colonies	-	+S	-	-	-	*Staphylococcus sp*
	X6	$5.0x10^{9}$	Cocci in pairs, tetrads.	Small round yellow colonies	-	+S	-	-	-	*Micrococcus sp*
	X7	$3.0x10^{9}$	Straight rods with oval and central spores	Mucoid and slimy irregular raised colonies	+	+R	+	+	-	*Bacillus sp*
	X8	**2.0x10**	**Cocci predominantly in tetrads. Few in pairs and clusters**	**Small round convexed orange colonies**	-	**+S**	-	-	-	*Micrococcus sp*

R – Rods

S – Spherical cocci

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Determination of minimum inhibitory and bacteriocidal concentrations

The response of these bacterial isolates to phytol showed that phytol has potent inhibitory effect in terms of MIC and MBC values against all the tested bacterial pathogens. As shown in Table .3, the MIC and MBC values of phytol against the tested bacterial pathogens were found in the range of 31.25µg/ml to 62.5µg/ml and 125µg/ml to 250 µg/ml, respectively. *Pseudomonas aeruginosa* and *Staphylococcus aureus* were found highly susceptible bacterial pathogens to the phytol having MIC values of 62.5µg/ml and 31.25µg/ml respectively and MBC values of 125µg/ml respectively, for each bacterial pathogen. Phytol also had potential antibacterial effect against *B.cereus* and *E. faecalis.*

Similarly, Mathabe *et al*.(2008), reported the antibacterial activity of plant based terpenoid compounds against a panel of food borne pathogenic bacteria,showing their potential antibacterial effect

Table 3: Determination of MIC and MBC of phytol against bacterial pathogens isolated from grilled meat.

Bacterial pathogen	Gram reaction	PHYTOL	
		MIC(µg/ml)	MBC(µg/ml)
Bacillus cereus	+	31.25	250
Pseudomonas aeruginosa	-	62.5	125
Staphylococcus aureus	+	31.25	125
Enterococcus faecalis	+	31.25	250

MIC) Minimum inhibitory concentration (value in µg/ml)

MBC) Minimum bactericidal concentration (value in µg/ml)

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Table 2: Biochemical and carbohydrate fermentation test of bacteria isolated from samples.

Colony	Cat	Oxi	Coag	In	MR	VP	Cit	H_2S	$N0_3$	Urease	TSI	Carbohydrate fermentation						**Identity of isolates**
												Glu	Suc	Mal	Lac	Mann	Xyl	
X1	-	-	-	-	+	-	+	nd	-	+	Nd	+	+	-	+	+	nd	***E. faecalis***
X2	+	-	-	-	-	+	+	nd	+	-	Nd	+	-	-	+^{S}	+	nd	***B. subtilis***
X3	+	-	-	-	-	+	+	nd	+	-	Nd	+	-	+	+^{S}	-	nd	***Corynebacterium sp***
X4	+	+	-	-	+	-	+	nd	+	+	Nd	+	-	-	-	+	nd	***P.aeruginosa***
X5	+	-	+	-	-	+	-	nd	+	+	Nd	+	+	+	+	+	nd	***S. aureus***
X6	+	-	-	-	+	-	+	nd	-	+	Nd	-	-	-	-	-	nd	***M.cuteus***
X7	+	-	-	-	-	+	+	nd	+	-	Nd	+	-	-	+^{S}	-	nd	***B.cereus***
X8	+	-	-	-	+	-	+	nd	+	-	Nd	+	+	-	-	-	**nd**	***M.roseus***

Cat, Catalase; Oxi, Oxidase; Coag, coagulase; In, indole; MR, methyl red; VP, Voges Proskaeur; Cit, citrate; H2S, Hydrogen sulphide utilization; NO3, Nitrate reduction; Glu, glucose; Suc, sucrose; Mal, maltose; Lac, lactose; Mann, mannose; Xyl, xylose, +s, slow reaction

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Determination of inhibitory effect of phytolon the growth of the bacterial isolates

Broth dilution with shaking (BDS) method was used to investigate the inhibitory effect of phytol on the growth of *S. aureus*, *B. cereus*, *P. aeruginosa* and *E. faecalis*. Fig 1 shows that in comparison to control absorbance; phytol was stimulatory to the growth of *B.cereus*; *P.aeruginosa* and *E. faecalis* at all concentrations but inhibitory to *S.aureus* at concentrations between 250µg/ml and 2000µg/ml.

The time kill assay demonstrated that the growth of the different bacterial isolates decreased with increase in concentration except in *S.aureus* and *P.aeruginosa*. Inhibitory effects of cell growth decreased at elevated concentrations even though a certain fraction of the bacteria in each bacterial suspension was killed. Thus, surviving bacteria grew faster than did cells under control conditions. It appears that phytol has both growth-inhibitory and growth accelerating activities. The net effects depended on the final concentration, with inhibitory activity predominating at lower concentration.

Effect of phytol on dehydrogenase activity of the bacterial isolates

Results obtained from the control samples showed that these organisms were able to reduce TTC to red formazan at variable rates and extent. The gram-negative *P. aeruginosa* had higher rates of dehydrogenase activity than the gram + positive *B.cereus, E.fecalis* and *S.aureus*. This variation may be due to differences in cell wall components or dehydrogenase systems, since different micro-organisms have been reported to have different dehydrogenase systems (Praveen-kumer, 2003). This result is also in consonance with the work of Nweke *et al*., (2006).

The effects of the different concentrations of phytol on the bacterial isolates with respect to the dehydrogenase activity and its inhibition are shown in Fig. 4. The responses of the bacterial dehydrogenase activities to phytol are concentration – dependent and vary between the organisms. The dehydrogenase activity inhibition observed in this study is consistent with those observed by other workers using plant extracts (Alisi *et al*., 2008; Nwaogu *et al*., 2008; Nwaogu *et al*; 2007). Result presented in Fig. 5 showed that *P.aeruginosa* had higher percentage inhibition than the other bacterial isolates at all concentrations of phytol. This implies that *P.aeruginosa* was more sensitive to the deleterious effect of phytol than the other bacterial isolates.

The effect of phytol on the protease activity of the bacterial isolates are shown in and Fig .6. In comparison to the control culture, protease activity decreased with increase in phytol concentration in all the bacterial isolates.

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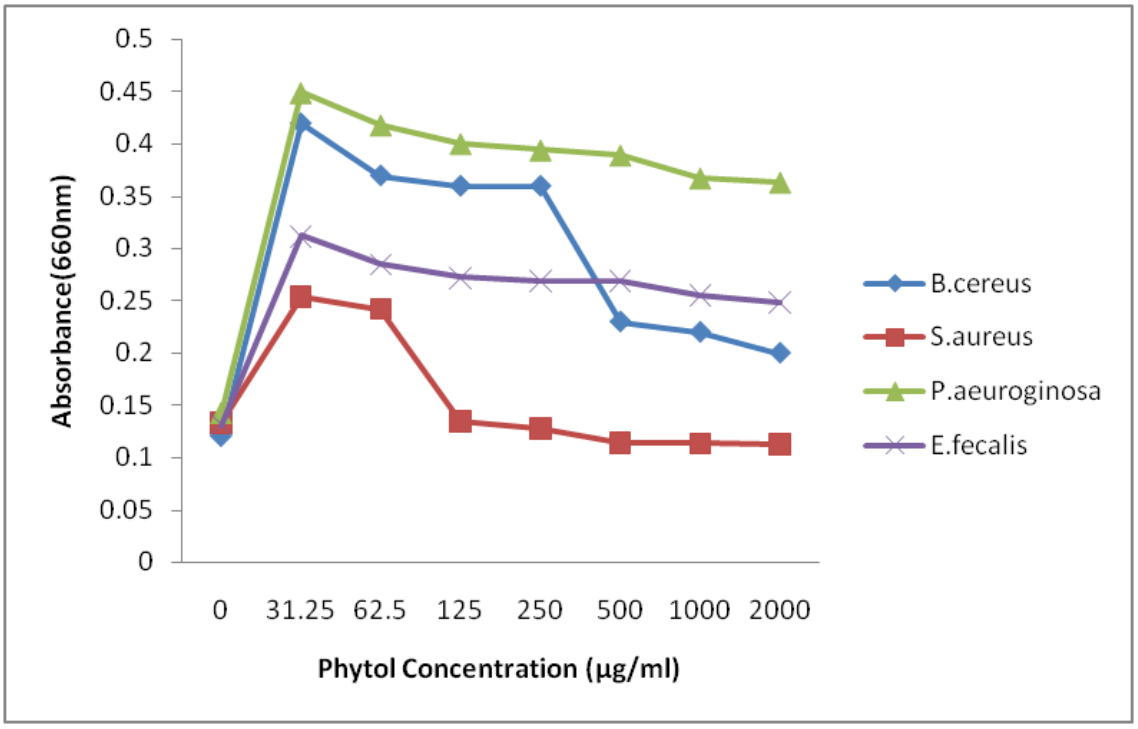

Fig. 1: Effect of Phytol concentration on the growth of the bacterial isolates

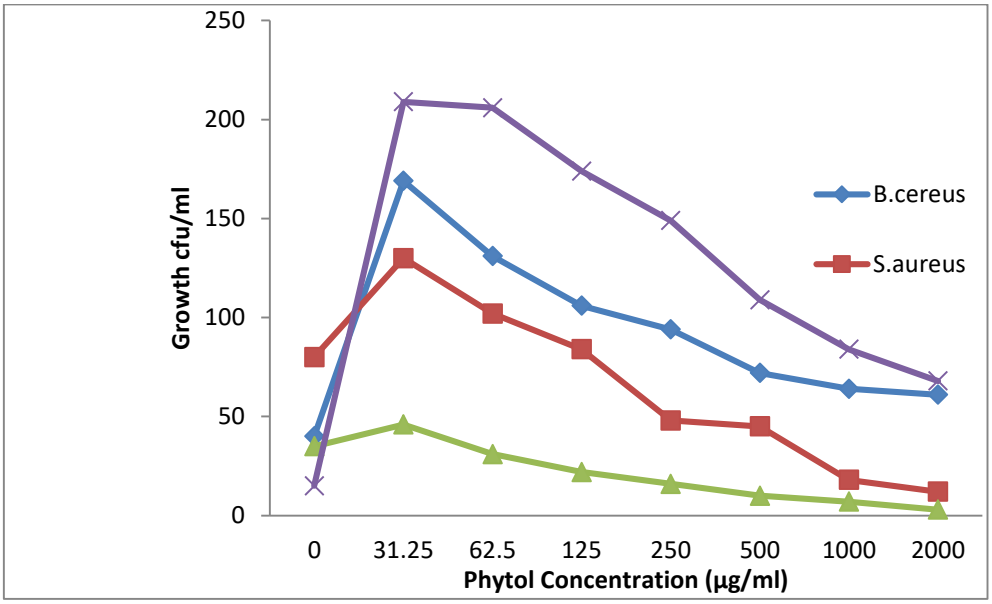

Fig. 2: Effect of phytol concentration on the bacterial isolates at 4hrs incubation.

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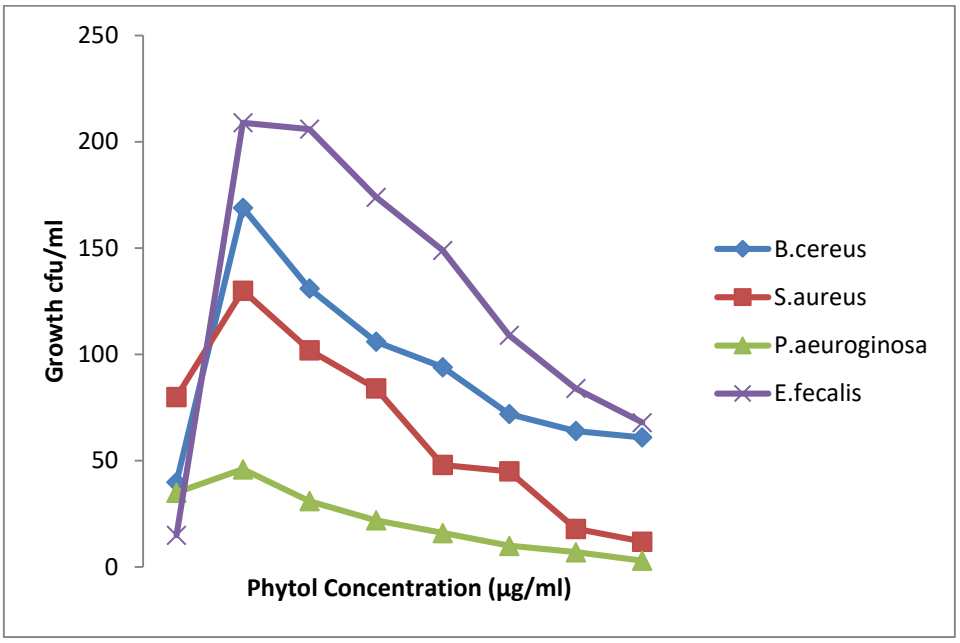

Figure 3: Time Kill Assays of the Effect of Phytol on Bacterial Isolates at 24hr Incubation

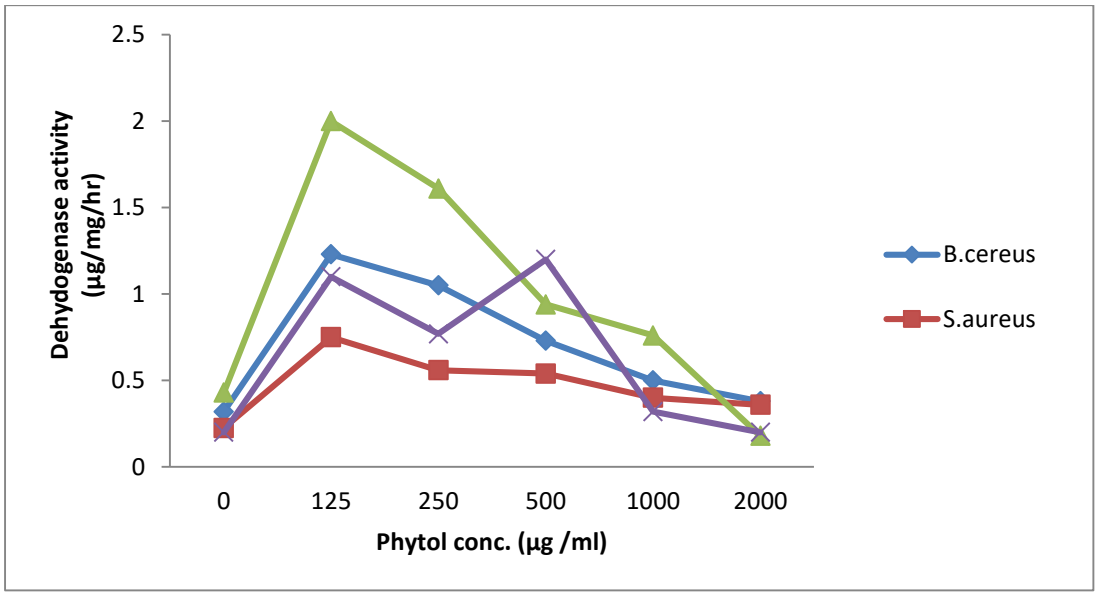

Fig .4: Effect of phytol concentration on dehydrogenase activity of bacterial isolates.

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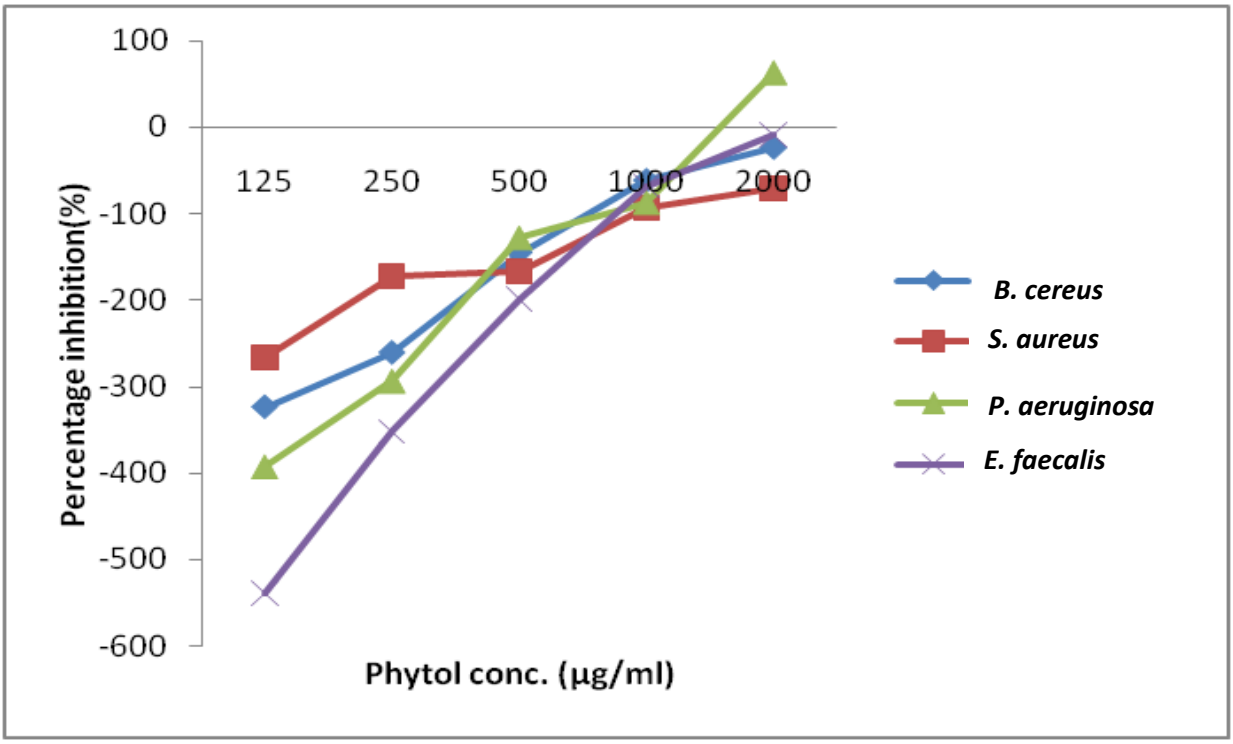

Fig 5: Inhibition of Dehydrogenase Activities of the Bacterial isolates by phytol.

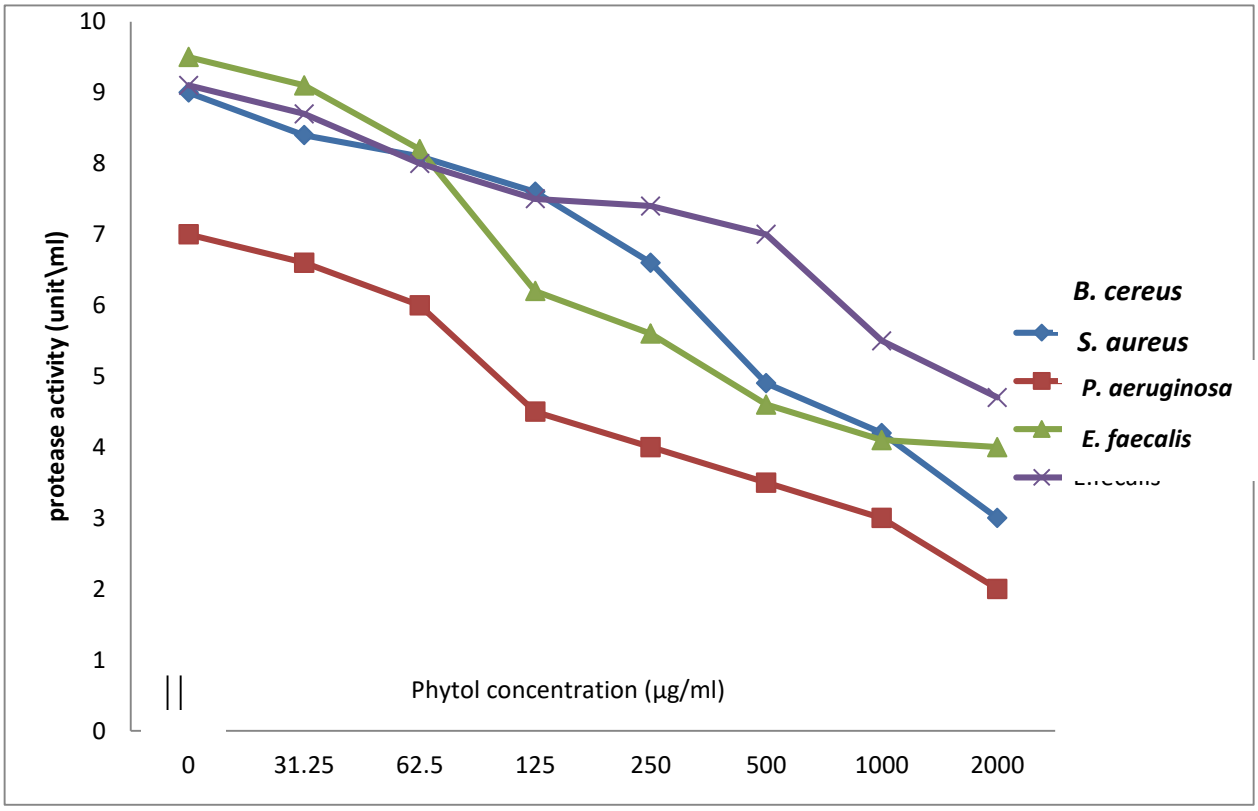

Fig .6: Effect Of Phytol Concentration On Protease Activity of the bacterial isolates

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CONCLUSION

Food safety is a fundamental concern of both consumers and food industry, especially as the number of reported cases of food – associated infections continues to increase and is rapidly changing (Alzoreky and Nakahara, 2003). Recurring out breaks of food borne illness caused by food borne pathogenic bacteria have sustained the demand for preservation systems that limit the proliferation of food borne pathogens in refrigerated, minimally processed and ready to eat foods. In this regard, plant – based antimicrobials could be potential alternatives in the food industry to control food borne pathogens.

Utilization of bioactive secondary metabolites as natural antibacterial agents may offer many new applications for food industry. Natural food preservatives targeted at food and food products that are easily contaminated by bacteria are highly desired.

The availability of natural compounds developed by exploring more chemistry, activation of plants natural resistance mechanisms and natural products will contribute to sustainable food and food products.

The results described in this study clearly indicate that phytol, a diterpenoid possesses the potential to control food borne pathogens, and these finding are in strong agreement with previous reports (Singh and Singh 2003; Murthy *et al*., 2005).

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