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Full Length Research Paper

Bioethanol production from starchy part of tuberous plant (potato) using *Saccharomyces cerevisiae* MTCC-170

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Ethanol is one of the bio-energy sources with high efficiency and low environmental impact. Various raw materials have been used as carbon source for ethanol production. In the present study, one varieties of potato that is, Kufri Bahar (KB) flour was chosen as a carbon source. In order to obtain maximum conversion of starch into fermentable sugar, optimum parameters for the liquefaction were determined as 104 to 105°C, 0.15% v/w of α-amylase enzyme solution (300 U/ml) and 30 g dry-weight potato mash/100 ml distilled water, respectively with a 68.86% loss in dry weight during the process. For saccharification process, the optimum dose of amyloglucosidase was 0.35% w/v (300 U/ml) with 16.95% glucose production at pH 5.0 and temperature 60°C after 1 h. The maximum ethanol concentration 7.89% (v/v) was obtained with 10% inoculum size at pH 6.0 after 48 h. Furthermore, out of the three nitrogen (yeast extract, peptone and ammonium sulphate) sources tested for ethanol production, peptone at 1.5 g/l was found to be best (7.58%). In conclusion, this study demonstrates the potential utilization of potato powder for ethanol production.

Key words: Potato starch, bioethanol, liquefaction, saccharification, *Saccharomyces cerevisiae* MTCC-170.

INTRODUCTION

In the 21st century, the demand of energy for transportation, heating and industrial processing is increasing day by day. Environmental issues are a point of concern (Hahn-Hagerdal et al., 2006). Renewable energy sources receive attention not only to protect the environment but also to supply energy needs by reducing dependence on foreign oil. In recent years, bio-energy sources have become more important as a viable and economical alternative source. Ethanol is one of the bio-energy sources with high efficiency and low environmental impact. Worldwide production of ethanol is approximately 51,000 million litters. Fuel encompassed 73% of produced ethanol, while beverage and industrial ethanol constitute 17 and 10%, respectively (Sanchez and Cardona, 2008). As a fuel enhancer, ethanol has some advantages. Woodson and Jablonowskiy (2008) reported that "as an additive (ethanol), serves as a fuel volume extender, an oxygenate and an octane enhancer." Most of the countries have either ethanol blended gasoline or direct ethanol as fuel, such as: Brazil, USA, Canada, Colombia, Spain and France (Sanchez and Cardona, 2008). In 2005, 45.42 billion litters of ethanol were produced worldwide (Balat et al., 2008) with Brazil and the U.S as the two major producers of ethanol. Ethanol is an alcohol that is a product of microbial fermentation. Microorganisms meet their energy demand by converting carbon sources to by-products such as: carbon dioxide, lactic acid, ethanol, etc. *Saccharomyces cerevisiae*, *Zymomonas*

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mobilis, *Kluyveromyces* spp. and *Schizosaccharomyces pombe* are microorganisms able to convert sugars to ethanol.

Various feedstock and chemically defined media can be used for ethanol fermentation. The most commonly used types of feedstock for ethanol production are corn, sugar cane and wheat (Balat et al., 2008). Sugarcane, sugar beets and molasses are feasible for ethanol fermentation and have been used; however, these carbon sources are high value products as food sources (Nalley and Hudson, 2003; USDA, 2006). In order to meet the low cost requirement, lignocellulosic biomass is another option for ethanol fermentation. However, lignocellulosic biomass is complex and requires expensive pre-treatments. Currently, potatoes are an alternative feedstock for ethanol production. Minal and Deshpande (2010) stated that potatoes are the second most used food in the world. Potatoes are starchy crops which do not require complex pre-treatments. Although, it is also a high value crop, but 5 to 20% of crops that are waste potato by-products from potato cultivation could be utilized for bio-ethanol production (Limatainen et al., 2004; Adarsha et al., 2010). Moreover, during processing of potato, particularly in the potato chip industry, approximately 18% of the potatoes are generated as waste. Therefore, the waste from potato industry can also be utilized as growth media (economical carbon source) for the fermentation processes for the production of ethanol as it has high starch content. The wastes of potato industry are currently being utilized as animal feed (Yamada et al., 2009).

Starchy materials require a reaction of starch with water (hydrolysis) to break down the starch into fermentable sugars (saccharification). Hydrolysis is carried out at high temperature (90 to 110°C); however, at low temperatures, it is also possible and can contribute to energy savings (Sanchez et al., 2008). To convert starch into the fermentable sugars, either acid hydrolysis or enzymatic hydrolysis needs to be performed. Each has their own set of advantages and disadvantages for use. Enzyme hydrolysis is generally chosen even though high cost of enzymes and initial investment because of high conversion yield of glucose (Tasic et al., 2009). Amylases (α-amylase, β-amylase and glucoamylase) are employed for hydrolysis of starchy materials. Although, amylases are derived from plants, animals and microorganisms, microbial amylases are in use commonly (Kunamneni et al., 2005). α-Amylase hydrolyses the 1,4-α-D-glucosidic linkages in the linear amylase chain, randomly. However, amyloglucosidase cleaves the 1,6-α-linkages at the branching points of amylopectin as well as 1,4-α-linkages (Pandey et al., 2000). However, production of ethanol from waste potato still needs to be optimized because limited research has been conducted about the utilization of potato waste for ethanol production. Fadel (2000) and Liimatainen et al. (2004) showed that different wastes of potato industry can be a good carbon source for yeast during alcohol fermentation by studying waste from potato to chips industry (98.67% total carbohydrate) and different potato cultivations (starch content in a range of 11.2% to over 19.3%), respectively.

So, the aim of the present study was to use potato starch, a very cheap substrate for the production of ethanol and to optimize fermentation process.

MATERIALS AND METHODS

Raw materials

Potato tubers were procured from CCS HAU, Hisar and analyzed for different components by standard methods (AOAC, 1990). Thoroughly, washed peeled potato (1 kg) were dried overnight at 70°C and grounded to fine powder.

Enzyme for liquefaction and saccharification

Commercial α-amylase (specific activity 300 DUN U/ml) and amyloglucosidase (specific activity 400 GA U/ml) were obtained from SIGMA – ALDRICH PVT. LTD., India.

Preparation of potato flour slurry

Slurries of various concentrations (10, 15, 20, 25 and 30% w/v) of potato flour starch was prepared in water and treated with liquefying enzyme (0.15% v/w) at 104 to 105°C for 60 min in an autoclave. The slurry prepared by mixing 25 g flour in 100 ml water (1:4) being homogenous, loose, easy to handle was used for further experiments. Liquefaction of potato flour (100 ml slurry) was carried out at 104 to 105°C in an autoclave using varying concentration of alpha amylase (0.05 to 0.20% v/w) for different time intervals (10 to 240 min). The progress of liquefaction was monitored by employing starch-iodine (1 g of iodine and 2 g KI in 100 ml water) reaction. Saccharification of liquefied starch was carried out at 60°C for different time intervals using varying concentration (0.05 to 0.45% v/w) of amyloglucosidase. The reaction was monitored by the yield of total reducing sugars estimated by dinitrosalicylic acid method (Miller, 1959).

Yeast strain

A fast fermenting strain of *Saccharomyces cerevisiae* MTCC170 was obtained from Microbial Type Culture Collection, Chandigarh and maintained on yeast extract peptone dextrose (YEPD) agar medium containing yeast extract (1%), peptone (2%), dextrose (2%) and agar (2%). Dextrose inoculum medium (IM) used for inoculum preparation contained dextrose (6%), peptone (0.5%) and yeast extract (0.5%). Yeast cells pre-grown in inoculum medium for 18 h under shaking condition (120 rpm) was directly used as an inoculum at 10% (v/v).

Liquefaction

Potato slurry was liquefied with α- amylase at different enzyme dosages (0.05 to 0.20%v/w), incubation times (10 to 150 min) and temperatures (85 to 105°C). The initial pH of the slurry was 5.6. The extent of liquefaction was determined by disappearance of blue colour. The effects of enzyme dosage, incubation time and heating temperature on liquefaction ratio were evaluated.

Saccharification

Liquefied-mash of potato-powder obtained by employing the optimized conditions of liquefaction process were saccharified at 60°C for 1 h by using another enzyme that is, amyloglucosidase. This enzyme completes the process of breakdown of the starch into simple sugar that is, glucose. Saccharification process was determined by using dinitrosalicylic acid method (Miller, 1959).

Optimization of fermentation conditions

Effect of inoculum concentration

The hydrolysate was inoculated with different concentrations of inoculum that is 5, 10, 15 and 20% (v/v) and kept for fermentation at 35°C for 48 h.

Effect of temperature

The hydrolysate inoculated with the best combination of nutrients and fermentation was carried out at various temperatures namely 25, 30, 35 and 40°C. Ethanol content in fermented samples was estimated after 48 h of incubation.

Effect of pH

The pH of hydrolysate was adjusted to different levels and it was fermented after supplementation with the best combination of nutrients after inoculating with 10% inoculum (v/v). The fermentation was carried out at 35°C for 48 h.

Effect of nutrient concentration

To 100 ml hydrolysate, different nutrients like ammonium sulphate (0.3%), yeast extract (0.5%) and peptone (0.5%) was added in their single and double concentration. The flasks were inoculated with 10% yeast cells (v/v). The fermentation was carried out at 35°C for 48 h.

Analytical methods

Estimation of reducing sugars

The DNS method of Miller (1959) was used to estimate reducing sugars of the samples.

Ethanol determination

Ethanol concentration was determined by the method of Caputi et al. (1968).

Statistical analysis

All experiments were carried out in a completely randomized design and in triplicates. The results were subjected to analysis of variance (one-way ANOVA) and the treatment means were compared using the least significant difference (LSD) values at a significance level of $P < 0.05$. Simple ANOVA were evaluated using SPSS 16.0 software (SPSS, O.P. Sheoran Programmer, Computer Section, CCS HAU, Hisar).

RESULTS AND DISCUSSION

Potato flour contained 8.39% moisture, 73.25% starch and 4.86% proteins (Table 1).

Optimization of condition for liquefaction process

The optimum combination of temperature, dose of enzyme (α- amylase) and amount of potato flour slurry was determined as 104 to 105°C, 0.15% v/w of α-amylase enzyme solution (300 U/ml) and 30 g dry-weight potato mash/100 ml distilled water, respectively with a 68.86% loss in dry weight during the liquefaction process (Table 2).

Optimization of saccharification

For the saccharification process, dose of enzyme, temperature and saccharification time were also determined. The optimum dose of amyloglucosidase was 0.35% v/w (300 U/ml) with 16.82 g/100 ml glucose production after 1 h at 60°C for potato (KB) as shown in Table 3.

Optimization of fermentation conditions

Optimization of inoculum size for ethanol production

To determine the economic inoculum size of SSF of potato flour hydrolysate, different innoculum size that is 5, 10, 15 and 20% were used by keeping initial substrate concentration (100 g/l), initial pH (6.0), inoculum age (17 h old culture) and agitator speed (120 rpm) for 24, 36 and 48 h fermentation period as shown in Figure 1, there was significant difference among the inoculum size tested (5, 10, 15 and 20%) regarding kinetic parameters in ethanol production. The maximum ethanol concentration (7.89%) was produced by *S. cerevisiae* MTCC-170. Sugar utilization (94.83%) and ethanol yield that is, 91.39% was obtained with an initial inoculum of 10%, which is economic and environment friendly. It was observed that when the innoculum size was increased from 5 to 10%, ethanol production was also increased but above 10%, rate of alcohol production decreased after 48 h of incubation. Breisha (2010) reported that increasing the yeast inoculum volume from 3 to 6% showed positive effects on fermentation from 25% sucrose and reduced the fermentation time from 72 (3) to 48 h (6%). The fermentation time shorten along with the raise in inoculum size which was due to the fast cell growth within the reactor. Most of the substrate was immediately converted to ethanol. A maximum ethanol production of 88 from 200 g/l sucrose medium at 10% inoculum size in 16 to 18 h was obtained by Singh and Jain (1994).

According to the study of Fadel (2000), the maximum

Table 1. Composition of starchy raw materials.

Raw material	Source	Chemical composition % (w/w)					
		Starch					
		Acid hydrolysis	Enzymatic hydrolysis	Nitrogen contents	Protein contents	Phosphorus contents	Ash contents
Potato (KB)	CCS HAU, Hisar	72.13	73.25	0.81	4.86	0.61	4.40

Table 2. Summary of liquefaction.

Raw material	Slurry % (w/v)	Enzymes % (v/w)	pH	Temperature (°C)	Time (h)	Ca^{++} (mM)	K^{+} (mM)
Potato (KB)	25	0.10	6.2-7.0	104-105	1	0.36	0.30
	30	0.10	6.2-7.0	104-105	1	0.72	0.30

Table 3. Summary of saccharification.

Raw material	Slurry % (w/v)	Enzymes % (v/w)	pH	Temperature (°C)	Time (h)	Sugar production (Kufri Bahar) % (w/v)
Potato	25	0.10	5.0	60	1	15.34
	30	0.10	5.0	60	1	16.82

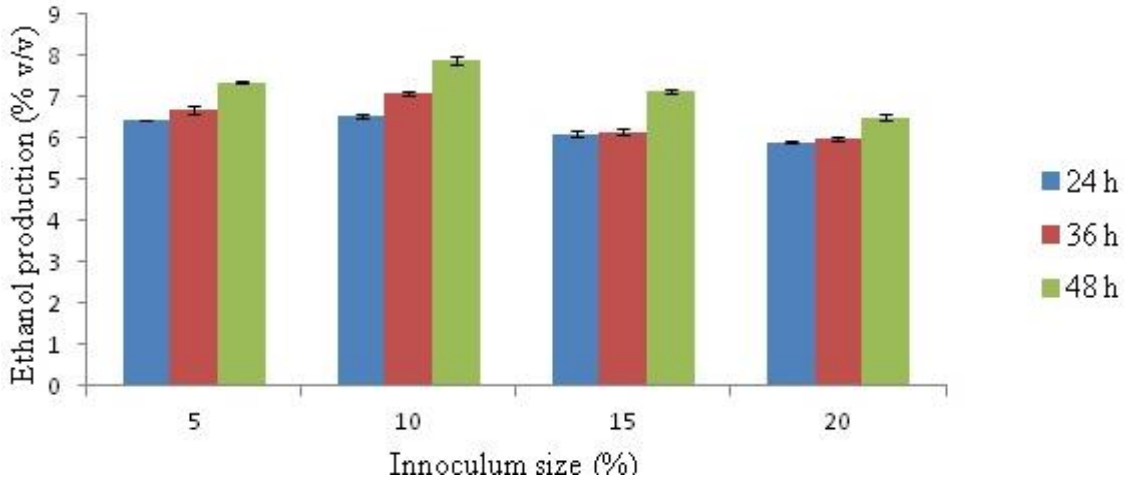

Figure 1. Effect of inoculum size on ethanol production from supplemented potato (Kufri Bahar) powder hydrolysate.

alcohol production (12.9%) was obtained when inoculated with 10% culture of *S. cerevisiae*.

Afifi et al. (2011) produced maximum ethanol from industrial solid potato wastes when inoculated with 10% (v/w) inoculum size of *S. cerevisiae.* Neelakandan and Usharan (2009) studied different inoculum size (2, 4, 6, 8 and 10% v/v) for a period of 24 h and observed that the maximum ethanol concentration that is, 8.8% was obtained at 10% inoculum size. In comparison of these results, Izmirlioglu and Demirci (2012) showed that 3% inoculum size was optimum for maximum ethanol concentration and production rate. Turhan et al. (2010) reported the ethanol production from carob extract by using *S. cerevisiae* and found that maximum ethanol concentration; ethanol productivity and ethanol yield were 42.90 g/L, 3.7 g/L/h and 45.0%, respectively, obtained

Table 4. Effect of temperature on ethanol production from supplemented[b] potato (Kufri Bahar) flour hydrolysate[a].

Yeast strain[c]		Ethanol % (v/v)[d]		
		35°C		
		Kufri Bahar		
	Temperature[e] (°C)	24 h	36 h	48 h
S. cerevisiae	30	5.90	6.28	7.50
	35	6.11	6.59	7.99
	40	4.07	2.02	4.30
	45	2.14	1.67	2.26

[a]Initial sugars 16 to 17% (w/v).
[b]Potato (KB) flour hydrolysate was supplemented with ammonium sulphate (0.2% w/v), peptone (0.25% w/v) and yeast extract (0.25% w/v).
[c]Inocula were grown in YEPD shake flask (210 rpm) at 35°C and used at 10% (v/v).
[d]Ethanol values are mean of three replications.
[e]Fermentation process was carried out at different temperatures.

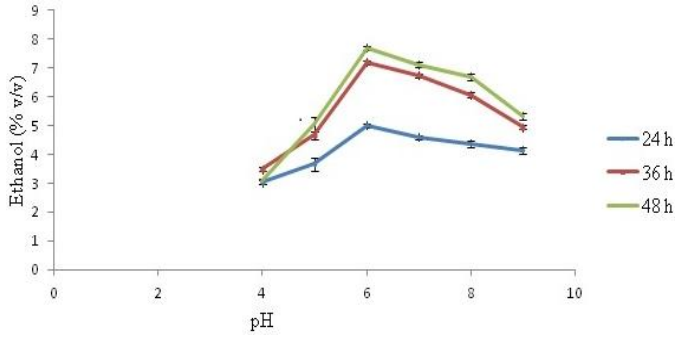

Figure 2. Effect of pH on ethanol production from potato (Kufri Bahar) powder hydrolysate.

with an initial inoculum of 3%.

Effect of temperature on ethanol production

Temperature is one of the major constraints that determine the ethanol production because temperature exerts a profound effect on growth, metabolism and survival of the fermenting organism. To know the optimum temperature for ethanol production, the fermentation media were kept at 25, 30, 35 and 40°C. The maximum ethanol concentration (7.99%) was obtained from *S. cerevisiae* MTCC-170 when culture was grown at 35°C. Above 35°C ethanol production was decreased to 4.30% (Table 4). Hashem and Darwish (2010) observed that production of ethanol by *S. cerevisiae* y-1646 was favoured at 35°C temperature and reached its maximum value (5.29 g/l) after 36 h. At 37°C, ethanol production was reduced to 4.38 g/l. Rani et al. (2010) observed that maximum ethanol content of 56.8 g/l was recorded after 48 h of fermentation at 30°C. However, at temperature 35, 37 and 40°C, the corresponding values were 53.6, 50.0 and 46.0 g/l, respectively showing a decline with increase in temperature of fermentation. Asli (2010) observed best ethanol production rate at 32°C temperature. Bio-ethanol production increases with increased in temperature and reaches its maximum value at 35°C.

Further, the increasing temperature reduced the percentage of ethanol production and it is mainly due to denaturation of the yeast cells (Periyasamy et al., 2009). Khan et al. (2012) studied the effects of temperature on bioethanol production and observed that maximum bioethanol was produced at 35°C as compared to bioethanol produced at 23 and 28°C, respectively.

Effect of pH on ethanol production

The initial pH is one of the important factors that affect the performance of SSF. The effect of pH on ethanol fermentation is studied by conducting batch experiments at different pH ranging from pH 4.0 to 7.0 for yeast strains namely *S. cerevisiae* MTCC- 170 by keeping initial substrate concentration (100 g/l), initial temperature (35°C), inoculum age (17 h old culture) and agitator speed (120 rpm) for 24, 36 and 48 h of fermentation period. As shown in Figure 2, the ethanol concentration was increased from pH 4.0 to 6.0 and then decreased marginally above this value. The maximum ethanol concentration 7.70% was obtained from *S. cerevisiae* MTCC- 170 culture grown at pH 6.0. Fadel (2000) reported that high ethanol production was obtained by using initial pH 5.0 to 6.0. It was also shown that no ethanol production exists lower than pH 4.0 (Graves et al., 2006). Turhan et al. (2008) reported that maximum ethanol yield, maximum growth rate and biomass concentration were obtained at pH 5.5 on carob as a medium for ethanol production. Osman et al. (2011) tested wide initial pH range and found that at pH 3.0 no growth was observed and no ethanol was produced, while pH 6.0 was the optimum for both biomass and ethanol production. Similar results were obtained by Kadambini (2006). Mohanty et al. (2009) reported that pH 6.0 was optimum for bioethanol production from mahula (*Madhuca latifolia* L.) flowers by

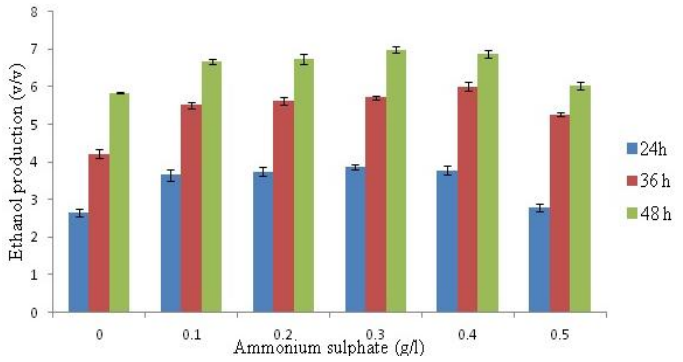

Figure 3. Effect of addition of ammonium sulphate on ethanol production from potato (Kufri Bahar) flour hydrolysate.

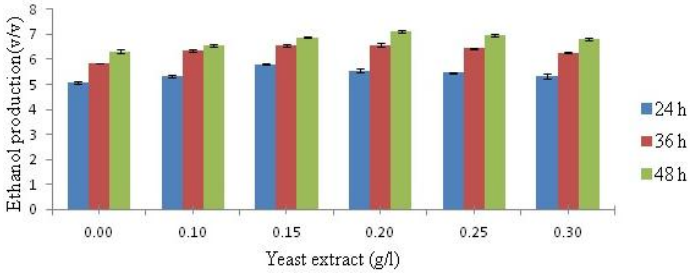

Figure 4. Effect of addition of yeast extract on ethanol production from potato (Kufri Bahar) flour hydrolysate.

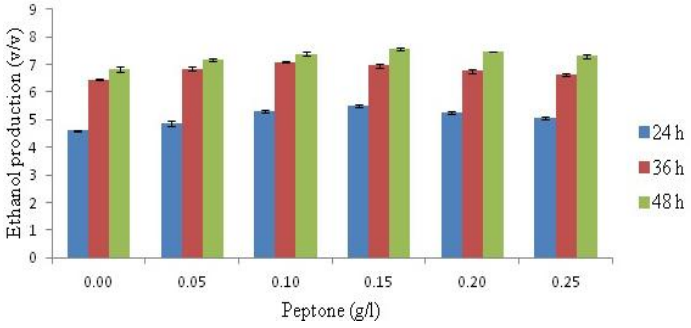

Figure 5. Effect of addition of peptone on alcohol production from potato (Kufri Bahar) flour hydrolysate.

production from mahula (*Madhuca latifolia* L.) flowers by solid-state fermentation.

Similar results were obtained by Togarepi et al. (2012) when *Ziziphus mauritiana* fruit pulp was used as a substrate (Akponah and Akpomie, 2011).

Effect of nutrients on ethanol production

Addition of nutrients such as ammonium sulphate, yeast extract and peptone play a vital role in boosting the ethanol production and its rate. Effect of ammonium sulphate as a nitrogen source was studied by varying its concentration between 1.0 to 5.0 g/l keeping rest of the parameters at their optimal conditions. Figure 3 shows that as the concentration of ammonium sulphate increased from 1.0 to 3.0 g/l, ethanol production also increased from to 5.84 to 6.98% for *S. cerevisiae*; above that concentration ethanol production was decreased when potato (Kufri Bahar) was used as substrates. Beltran et al. (2007) studied the effect of ammonium sulphate with different concentrations ranging from 0.01 to 0.09 g/l and observed that maximum production was obtained at 0.06 g/l concentration of ammonium sulphate. Amutha and Gunashekaran (2000) obtained higher ethanol yield of 44.2 and 54.9 g/l, respectively by supplementation of liquefied cassava starch with ammonium sulphate (1.0 g/l). Srichuwong et al. (2009) studied the SSF simultaneous saccharification and fermentation (SSF) of very high gravity (VHG) potato mash for the production of ethanol and results revealed that 2 to 2.5% ethanol concentration was increased with ammonium sulphate supplementation which corresponded to a decrease in residual glucose (0.25 to 3.3% w/v).

Anupama et al. (2010) obtained optimum ethanol yield of 5.6% with 3 g/l concentration of $(NH_4)_2SO_4$ as a nitrogen source.

Effect of yeast extract on ethanol production

Effect of yeast extract was studied by varying its concentration from 1.0 to 3.0 g/l keeping rest of the parameters at their optimal conditions. Figure 4 shows that as the concentration of yeast extract increased from 1.0 to 2.0 g/l, ethanol production was also increased from 6.55 to 7.11% for *S. cerevisiae* but above this concentration, ethanol production was decreased when potato (Kufri Bahar) was used as substrates. Nuanpeng et al. (2011) studied that sugar consumption, ethanol production and yeast cell viability during batch VHG fermentation of *S. cerevisiae* NP 01 from sweet sorghum juice supplemented with various yeast extract concentrations and observed that the highest ethanol concentration in the EP medium containing 9.0 g/l of yeast extract. Laopaiboon et al. (2009) observed that ethanol production efficiency was improved when 3.0 g/l of yeast extract (120.68 ± 0.54 g/l) was added to sweet sorghum juice under VHG conditions.

Effect of peptone on ethanol production

To examine the effect of peptone on ethanol production various concentrations that is, 0.5 to 2.5 g/l were used keeping rest of the parameters at their optimal conditions. Data in Figure 5 shows that as the concentration of peptone increased from 0.5 to 1.5 g/l, ethanol production increases from 6.83 to 7.58% for *S. cerevisiae*, above this concentration ethanol production was decreased when potato (Kufri Bahar) was used as substrate. Wang et al. (2007) observed that peptone was a critical factor

for ethanol production and 1.5% (w/v) peptone in the medium increased the final ethanol titre from 14.2 to 17% (v/v) in 48 h. Dake et al. (2010) observed that maximum ethanol was produced at 0.5% (w/v) of peptone concentration.

Conclusion

According to the results, it could be concluded that potato can be an attractive feedstock for the bioethanol production, especially in India where 5 to 20% waste potato by-products are obtained from potato cultivar and also due to poor storage facility (Adarsha et al., 2010). Since it provided the necessary nutrient element and the appropriate hydrogen balance for the fermentation, there was no need for supplementing these additionally or making any pH adjustment. Potato were dried overnight at 70°C and grounded to fine powder was used for ethanol fermentation by *S. cerevisiae* MTCC-170. Homogenised (1:4) slurry was obtained on treatment with α-amylase (300 U/ml) at 104 to 105°C for 60 min which was saccharified with glucoamylase at 60°C for 1 h. Optimum parameters for ethanol fermentation by this strain are pH 6.0, temperature at 35°C, initial sugar concentration of 16.82 g/100 ml, $(NH_4)_2SO_4$, yeast extract and peptone used as nitrogen source. The yeast concentration of 2.0 g/l of potato flour yielded the optimum ethanol concentration that is 7.11% v/v. Addition of peptone, ammonium sulphate, to the production medium also markedly influences the level of ethanol concentration. Evidently, treating the hydrolysate with nutrients after formation of hydrolysate enhanced the degree of ethanol production significantly ($p<0.05$). Holding the hydrolysate at 35°C for 48 h could increase in the ethanol production, consequently allowing greater growth of the yeast strain to act on the hydrolysate.

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Short Communication

Mycoplasma gallisepticum in free-range chicken from Northern Tocantins State, Brazil

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***Mycoplasma gallisepticum* is a major problem of the poultry industry worldwide. This infectious disease, which affects animals worldwide, is caused by bacteria. The aim of this study was to determine the prevalence of *M. gallisepticum* in free-range chickens raised in Araguaína, Tocantins, using rapid plate agglutination test (SAR). Blood samples of 175 free-range chickens from the city of Araguaína, Tocantins, Brazil, were evaluated through serological tests. Of the 175 blood samples from adult birds (*Gallus gallus domesticus*), 74 (42.29%) - [34.87 to 49.97%] had positive serology for *M. gallisepticum*. Positive results were significant and show the need for preventive measures in small farms in the region studied.**

Key words: Poultry, mycoplasmosis, biosafety.

INTRODUCTION

The avian mycoplasmosis was first known as enzootic pneumonia. It is regarded as one of the serious health problems of the poultry production chain (Yoder, 1991). This infectious disease, which affects animals worldwide, is caused by bacteria within the class Mollicutes (Latin mollis = soft, cutis = skin) distinguished by the absence of a cell wall. Thus, these agents may be present in many forms while spherical is the most frequent (Razin et al., 1998). The main species responsible for mycoplasmosis is *Mycoplasma gallissepticum* (MS) (Yoder, 1991; Nascimento, 2000).

The presence of *M. gallisepticum* results in severe direct and indirect losses to the poultry industry (Buim et al., 2009). The losses include decreased hatchability and egg production, poor quality chicks, reduced growth rate, in addition to increasing costs with disease eradication procedures, monitoring and control programs (Yilmaz et al., 2011).

The absence of a cell wall makes them naturally resistant to antibiotics, such as penicillin (Buim, 2007). However, since these micro-organisms do not survive outside the host, their survival is usually restricted to a few hours or days, under the usual circumstances of farms (Nascimento, 2001).

According to Mettifogo and Ferreira (2007), the most important sources of infection are either the sick birds or the ones carrying the pathogen. The mycoplasma can be eliminated through the eggs, or the agent can be present in the semen of roosters or the oviduct of hens. Transmission can occur through direct contact with infected birds and indirect contact via contaminated fomites, such as feces and contaminated feathers present in the water and food; and airborne transmission which involves aerosols and droplets. During winter, the respiratory disease is more frequent and more severe in young birds while in the adult bird population is directly related to decreased

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Table 1. Frequency of free-range chicken (*Gallus gallus domesticus*) seropositive for Mycoplasmosis in Araguaína, TO, Brazil, 2012.

Farm	Number of birds	Positive	Percentage (%)
1 (North region)	95	39	41.05
2 (North region)	32	23	71.87
3 (South region)	8	0	0.00
4 (Center)	40	12	30.00
Total	175	74	42.29

egg laying. Yoder (1991) reports that together with *Mycoplasma synoviae* (MG), *M. gallisepticum* (MS) is one of the most widespread agents in poultry.

According to Ito et al. (2002), the implementation of biosecurity is the main preventive measure that can be adopted to prevent the introduction of *M. gallisepticum* in poultry production. Based on this information, the aim of this study was to determine the prevalence of *M. gallisepticum* in free-range chickens raised in Araguaína, Tocantins, using rapid plate agglutination test (SAR).

MATERIALS AND METHODS

The blood samples were collected from four poultry farms of Araguaína, of which three were commercial and one small non-commercial farm where the chickens were raised for own consumption. Two are located in the northern region of the city; the small non-commercial farm, in the south; and, the last farm, in the urban area (center) of the city.

Blood samples of 175 birds were randomly collected in four small free-range chicken farms in Araguaína, Tocantins. Blood samples were drawn by humeral venipuncture, identified, placed under refrigeration and immediately sent for serology to the Laboratory of Hygiene and Public Health of the Escola de Medicina Veterinária e Zootecnia, Universidade Federal do Tocantins (UFT).

The blood samples collected from the chicken at the small farms were chilled and transported carefully in order to avoid hemolysis. The centrifugation to obtain the serum was performed in the laboratory of Hygiene and Public Health of the UFT. According to the manufacturer instructions, serology was performed at room temperature between 20 and 25°C and the samples were not frozen, since freezing favors nonspecific reactions. The positive reactions, which are characterized by the presence of lumps, were visible within a span of two minutes.

The SAR antigen used was the Myco-Galli Test®, which consists of the inactivated suspension of *M. gallisepticum* strain S-6. Firstly, the antigen, the serum to be tested and the positive and negative serum controls were removed from the refrigerator and kept at room temperature for 30 min. Subsequently, the serum sample to be tested was inactivated in a water bath at 56°C for 30 min. The test was performed according to the manufacturer's instructions. Confidence intervals of 95% were determined for the frequencies of seropositivity for *Mycoplasma gallisepticum*.

RESULTS AND DISCUSSION

Of the 175 blood samples from adult birds (*Gallus gallus domesticus*) 74 (42.29%) had positive serology for *M. gallisepticum* (Table 1). Table 1 also shows the results with respect to the four chicken farms studied.

Knowing the frequency of major infectious diseases in birds is critical to establish a poultry health program. The serological monitoring of large and smaller, more informal poultry farms is fundamental to the establishment of preventive measures (Buchala et al., 2006).

Determining the infection sources by detecting the presence of antibodies is a rapid and practical epidemiological tool. This information shows the contact of birds with the infectious agent, thus demonstrating the presence and circulation of the pathogen in poultry populations (Wray and Davies, 1994). Serological surveys are generally used to support mycoplasmosis control programs (Sato, 1996).

Birds infected with *Mycoplasma* sp can have their production indices severely affected causing significant losses. Respiratory diseases may condemn carcasses in the slaughtering process and reduce egg production by up to 10% while increasing mortality due to decreasing immunity (Cardoso et al., 2006). It is also important to know the number of birds positive for MG in the region because seropositivity is related to aerosaculitis that causes weight loss in broilers (Machado et al., 2012). This fact undermines the local economy, in addition to the health issues.

Farm 1 was more organized and had better health management because the aim was to sell the birds in the popular town market. The shaded, covered area was clean and equipped with adequate feeders and drinkers. The feed and the pickets were uniform since the chicks, young chickens, and birds of approximately four months (batch from which blood samples were obtained) were all separated. However, other species were also raised on the property, such as ducks, geese, turkeys and helmeted guinea fowl hens. In this property, 41% of samples were positive. The high number of seropositive samples may be due to the number and diversity of animals raised in the same location. Furthermore, the grouping and origin of the animals may also have influenced this result. The second farm, located in the northern part of the city also showed high number of positive samples (72%). The non-commercial farm, situated

in the southern region of the city, where the birds are only for domestic consumption eight blood samples were drawn. In this third farm, there was no sample positive for MG, which can be explained by the small number of birds surveyed. On the other hand, farm number 4 located in the downtown area had 30% positivity and a large total number of birds, which may have facilitated the spread of the disease.

The city Araguaína is located in northern Tocantins where control measures to ensure the health of birds are minimal. It is common to find backyard raised chicken near chicken farms, as well as birds of different age and growing stages mixed altogether. These factors contribute to the spread of the pathogen. The implementation of biosecurity measures is paramount in these cases as highlighted by Nascimento et al. (2000).

The high seropositivity for mycoplasmosis in all birds studied, points to the need for preventive measures in small commercial chicken farms.

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Full Length Research Paper

In vitro antibacterial activities of dietary medicinal ethanolic extracts against pathogenic reference strains of animal origin

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In vitro **antibacterial activities of five extracts from dietary medicinal plants were investigated by agar-well diffusion method (AWD), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against 13 foodborne pathogenic bacteria reference strains; four Gram positive bacteria including *Staphylococcus aureus* (NCINB 50080), *Bacillus cereus* (NCINB 50014), *Citrobacter freundi* (ATCC 8090) and *Listeria innocua* (ATCC 33090) as well as nine Gram negative bacterial reference strains including *Escherichia coli* (ATCC 11775), *E. coli* O157 (ATCC 700728), *Salmonella typhimurium* (ATCC 13311), *Shigella boydii* (ATCC 9207), *Shigella sonnei (*ATCC 25931), *Shigella flexneri* (ATCC 12022), *Pseudomonas aeruginosa* (NCINB 50067), *Klebsiella pneumoniae* (NCTC 9633) and *Proteus mirabilis* (ATCC 14153). Four ethanolic extracts underwent acetone wash then analyzed for their principal components using gas chromatography-mass spectrometry (GC-MS), Oleamide was the predominant compound in onion, garlic, wheat germ and *Nigella sativa* which have great antibacterial effect. The tested acetone extracts exhibit variable antibacterial activity against foodborne pathogens which differ according to the compounds clarified in the GC-MS analysis. Garlic extract showed the best antibacterial activities, GC-MS analysis showed the presence of five compounds including; tetrasulfide, monosilane, oleamide, stearoylamide and vitamin E. Testing for the presence of 91 pesticides in the tested extracts using GC-MS analysis proved complete absence of pesticides which indicate that the antibacterial activities showed was due to the active components in the tested extracts and not due to the pesticides contaminants. Antimicrobial activities of plant extracts revealed that garlic has greatest inhibitory effect against *S. aureus* NCINB 50080 followed by *S.* Typhimurium ATCC 13311 with zone of inhibition 28 mm, 30 mm for AWDT and and 2.61 µg/ml for MIC, respectively. The best hindrance abilities was shown with garlic extracts with mean zone of inhibition (23.46 mm) followed by onion (18.15 mm), wheat germ extract (17.38 mm), mint (17.15 mm) then *Nigella sativa* (15.69 mm). Results of MIC and MBC confirm the antibacterial activities of the tested extracts.**

Key words: Antibacterial activity, agar well diffusion test (AWDT) minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), seed, bulb, ethanolic extracts.

INTRODUCTION

Food poisoning is still a concern for both consumers and the food industry despite the use of various preservation methods. Food processors, food safety researchers and regulatory agencies are continuously concerned with the

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high and growing number of illness outbreaks caused by some pathogenic and spoilage microorganisms in foods. The increasing antibiotic resistance of some pathogens that are associated with foodborne illness is another concern (Perreten et al*.,* 1998; Stermitz et al*.,* 2000).

The emergence of antimicrobial resistance has its roots in the use of antimicrobials in animals and the subsequent transfer of resistance genes and bacteria among animals and animal products (McEwen and Fedorka-Cray, 2002). Consumers are also concerned about the safety of foods containing synthetic preservatives. Therefore, there has been increasing interest in the development of new types of effective and nontoxic antimicrobial compounds. There is growing interest in using natural antibacterial compounds, such as extracts of spices and herbs, for food preservation (Smid and Gorris, 1999).

Numerous studies have been published on the antimicrobial activities of plant extracts against different types of microbes, including foodborne pathogens (Beuchat, 1994; Lis-Balchin and Deans, 1997; Smith-Palmer et al*.,* 1998; Hara-Kudo et al*.,* 2004). However, the results reported for these different studies are difficult to compare directly, usually because of the low number of plant samples tested, different test methods and diverse bacterial strains and sources of antimicrobial samples used.

Garlic has been used worldwide for many centuries as a spice and herbal medicine and believed to treat and prevent various diseases. It is strong antibacterial against Gram positive and Gram negative bacteria including *Bacillus*, *Brucella*, *Klebsiella*, *Pseudomonas*, *Proteus*, *Shigella*, *Staphylococcus*, *Salmonella* as well as *Helicobacter pylori* (Cellini et al*.,* 1996, Chowdhury et al*.,* 1991). The main component of garlic responsible for its antibacterial activities is allicin (Lixin Xia and Ng, 2005).

Rahman et al. (2012) indicated that garlic has been a favorite additive in food for many years in various cultures as it possesses antimicrobial, antiprotozoal, antimutagenic, antiplatelet and antihyperlipidemic properties. Allicin, a thiosulfinate extract of garlic, has been presumed to be a very strong antioxidant. Garlic contains unique organo-sulfur compounds (Block, 1985), which provide its characteristic flavor and odor and most of its potent biological activity.

The objectives of this study were: (1) to evaluate and compare the *in vitro* antibacterial activity of five plant extracts against 13 highly pathogenic reference strains responsible of food poisoning form food of animal origin (2) to establish the relationship between bacterial inhibition and total ethanolic extract content to confirm whether the ethanolic constituents are responsible for antibacterial activity (3) to ensure that the bactericidal activities are due to the compounds shown by gas chromatography-mass spectrometry (GC-MS) - gas chromatography mass selective detector (GC-MSD) analysis and to ensue the absence of pesticides in the extracts.

MATERIALS AND METHODS

Preparation of ethanolic extracts (Nanasombat and Lohasupthawee, 2005)

Five plant seeds or bulbs Table 1 were cut into small pieces; 20 g of each, were soaked in 100 ml of 95% ethanol, and shaken at 150 rpm for four days at ambient temperature. The mixtures were then filtered. The filtrates were evaporated using vacuum rotary evaporator (BÜCHI Rotavapor R-200/205, Model R205V800), and frozen at -80°C before freeze drying (Labconco, Model Lyph. Lock 6). Stock solutions of crude ethanolic extracts were prepared by diluting the dried extracts with 10% acetone solution to obtain a final concentration of 400 mg/ml.

Gas chromatography mass selective detector (GC-MSD) of acetone extract

GC-MSD operating parameters (Chin-Kai and Bruce, 2005 and Wylie, 2006)

The following parameters were used; gas chromatography (GC): Agilent Technologies 6890N, mass selective detector (MSD): 5975. EPC Split/ Splitless with inlet temperature: 250°C, 1 µl injection. Agilent Technologies column: HP5MS, p/n 19091S-433, stationary phase 5% phenyl methyl siloxane. Dimension 30 m × 0.25 µm ID × 0.25 mm film thickness, UHP Helium gas with 1.3 ml/min flow.

Oven programme: 90ºC (2 min), ramp 20ºC / min, 150 (0 min), ramp 6ºC /min, 270°C (10 min); total run time is 35 min. The used MSD temperature was 290ºC, quad temperature was 150ºC, and ion source temperature was 230ºC. The MSD mode with synchronous scan/SIM (selected ion monitoring); 3 selected ions for each compound. MS library: Wiley7/NIST5 and RTL pesticides mass spectral libraries. Four calibration levels were prepared including 0.01, 0.05, 0.10, and 0.50 µg/ml to construct the multi-level calibration curve. Aldrin was added to each level as internal standard (ISTD) with suggested concentration value of 0.1 µg/ml.

Gas chromatography mass spectrometer detector (GC-MSD) *of ethanolic extract (Chin-Kai and Bruce, 2005 and Wylie, 2006)*

Acetone wash was carried out for the tested ethanolic extract before introduction to Mass Spectrometer Detector. Optimized analytical method, that employing single quadrupole gas chromatograph equipped with mass spectrometer detector (GC-MSD) instrument has been developed for the simultaneous screening of 91 residues of different pesticide types including organophosphorus, organochlorine, pyrethroids and others as shown in Table 2 were monitored in Acetone wash was carried out for the tested ethanolic extracts of garlic, *Nigella sativa*, onion and wheat germ respectively.

Antimicrobial assay

Preparation of bacterial suspensions (NCCLS, 2003)

Antibacterial activities were carried out against thirteen highly pathogenic foodborne pathogenic strains of animal origin including four Gram positive bacterial reference strains; *Staph.aureus* (NCINB 50080), *Bacillus cereus* (NCINB 50014), *Citrobacter freundi* (ATCC 8090) and *Listeria innocua* (ATCC 33090) and nine Gram negative bacterial reference strains including *E. coli* (ATCC 11775), *E. coli* O157 (ATCC 700728), *Salmonella* Typhimurium (ATCC

Table 1. Botanical name of plant extracts and their edible parts.

Common name	Botanical name	The used plant parts in the experiment
Onion	*Allium cepa*	Bulbs
Garlic	*Allium sativum*	Bulbs
Mint	*Mentha canadensis*	Leaves
Wheat germ	*Triticum vulgare*	cereal grain
Black cumin	*Nigella sativa*	Seed

13311), *Shigella bodyii* (ATCC 9207), *Shigella sonnei* (ATCC 25931), *Shigella flexeneri* (ATCC 12022), *Pseudomonas aeruginosa* (NCINB 50067), *Klebsiella pneumoniae* (NCTC 9633) and *Proteus mirabilis* (ATCC 14153). Agar well diffusion test (qualitative method) and minimum inhibitory concentration (MIC) as well as minimum bactericidal concentration (MBC) (quantitative method) were used in this study. Wherein a suspension of bacterial strains were freshly prepared by inoculating fresh stock culture from each strain into separate broth tubes, each containing 7 ml of Muller Hinton Broth. The inoculated tubes were incubated at 37°C for 24 h. Serial dilutions were carried out for each strain, dilution matching with 0.5 Mc-Farland scale standard was selected for screening of antimicrobial activities. Ciprofloxacin 100 µg/ml was used as reference drugs.

Agar well diffusion method

The antimicrobial activity of 5 ethanolic extracts; onion, garlic, mint, wheat germ and *Nigella sativa* against bacterial strains were evaluated by using agar-well diffusion test (Katirciolu and Mercan, 2006). Hundred µl of cell culture suspension matching with 0.5 McFarland of target strains were spread onto the plates. For the investigation of the antibacterial activity, 100 µl of extracts (400 mg/ml), ciprofloxacin (100 µg/ml) as control positive and DMSO as control negative were added into wells of agar plates directly. Plates were left for 1 h at 25°C to allow a period of pre-incubation diffusion in order to minimize the effects of variation in time between the applications of different solutions. The plates were re-incubated at 37°C for 24 h. After incubation, plates were observed for antimicrobial activities by determining the diameters of the zones of inhibition for each of the strains. For an accurate analysis, tests were run in triplicate for each strain to avoid any error.

Determination of the minimum inhibitory concentration using micro broth dilution test (Jorgensen et al., 1999)

The dilution test was performed to determine minimum inhibitory concentrations (MICs). One hundred microliters of Mueller-Hinton broth (MHB) were added in each well of the 96-well sterile micro-titer plate. The 100-µl aliquot of stock solution of crude ethanolic extract (400 mg/ml) was added in the first well, and subsequently two- fold serially diluted with MHB. The inoculum suspension (20 µl) of each bacterial reference strains (0.5 McFarland, ~1 × 10^8 cfu/ml) were then added in each well containing crude ethanolic extract and MHB. The final concentrations of the extract were 166.7, 83.3, 41.7, 20.8, 10.4, 5.2, and 2.6 mg/ml. The negative and positive controls were also performed using DMSO and Ciprofloxacin, respectively. Duplicate wells were run for each concentration of herbal extracts. The plates were incubated at 37°C for 24 h. The lowest concentration that inhibited visible growth of the tested organisms was recorded as the MIC.

Determination of minimum bactericidal concentration (MBC) (Alade and Irobi, 1995)

After culturing the test organisms separately in nutrient broth containing various concentrations of the active ingredients, the broth was inoculated onto freshly prepared agar plates to assay for the bactericidal effect. The culture was incubated at 37°C for 24 h. The lowest concentration of extracts that does not yield any colony growth on the solid medium after the incubation period was regarded as minimum bactericidal concentration.

RESULTS

Gas chromatography mass spectrometer detector (GC-MSD) analysis

GC-MSD was carried out for four out of the five tested extracts; *Nigella sativa*, garlic, onion and wheat germ. Mint extract was not included as it contains heavy matrix which affect the column and interfere with a chromatographic analysis. Results reveal that the tested extracts vary in their compounds. The extract active compounds showed different antibacterial effects. 9-octadecenamide (oleamide) was found in the four tested extracts. Palmitic acid was found in *Nigella sativa*, Onion and Wheat germ extracts. Compounds found in each extracts play an important role in their antibacterial activities. Results revealed that eleven compounds were analyzed in Onion extract, nine compounds in *Nigella sativa* extract, eight compounds in Wheat germ extract and five compounds in garlic extract as shown in Table 3.

Results clearly indicate that no residues of the 91 tested pesticides were found in any of these four tested extracts Table 2. Results indicate that the pesticide residues have no roles in the antibacterial activities of the studied extract and results confirm that the active ingredients found in the extracts have bactericidal activities against the tested strains.

Antimicrobial studies

Results of agar well diffusion test (AWDT) Table 4, Figures 1, 2 and 3 reveal that garlic extract showed the highest antibacterial activities with mean zone of inhibition equals 23.46 mm. On the contrary, *Nigella sativa*

Table 2. List of pesticides tested in the portion of the extracted materials.

No.	Pesticide	No.	Pesticide	No.	Pesticide
1	Alachlor	32	Deltamethrin	63	Imazalil
2	Amitraz	33	Dichlobenil	64	Iprodione
3	Atraton	34	Dicloran	65	Isophenphos
4	Bifenthrin	35	Dicofol	66	Isophenphos-methyl
5	Biphenyl	36	Dieldrin	67	Methoxychlor
6	Bromophos-ethyl	37	Diniconazole	68	Metribuzin
7	Bromophos-methyl	38	Diphenylamine (DPA)	69	Mirex
8	Bromopropylate	39	Ditalimfos	70	Orthophenylphenol (OPP)
9	Cadusafos	40	Endosulfan-alpha	71	Oxadiaxyl
10	Captafol	41	Endosulfan-beta	72	Oxadiazon
11	Captan	42	Endosulfan-sulfate	73	Oxyfluorfen
12	Chlordane-cis	43	Endrin	74	Pentachloroanisole (PCA)
13	Chlordane-trans	44	Ethoxyquin	75	Pentachlorobenzene
14	Chlorfenapyr	45	Etofenprox	76	Permethrin
15	Chlorobenzilate	46	Etridiazole	77	Procymidone
16	Chlorpyrifos	47	Fenarimol	78	Profluralin
17	Chlorothalonil	48	Fenazaquin	79	Propiconazol
18	Chlorpropham	49	Fenitrothion	80	Prothiofos
19	Chlorthal-dimethyl	50	Fenpropathrin	81	Quintozene
20	Chlozolinate	51	Fenvalerate	82	Spiromesifen
21	Cinmethylin	52	Flucythrinate	83	Sulfur
22	Cyanophos	53	Fludioxonil	84	Tecnazene
23	Cyfluthrin	54	Folpet	85	Tefluthrin
24	Cyhalothrin-lambda	55	HCH-alpha	86	Tetradifon
25	Cypermethrin	56	HCH-beta	87	Thiometon
26	Dazomet (Basomid)	57	HCH-delta	88	Triadimefon
27	DDD o.p`-	58	HCH-gamma (Lindane)	89	Triadimenol
28	DDD p,p`-	59	Heptachlor	90	Trifluralin
29	DDE p.p`-	60	Heptachlor-endo-Epoxide	91	Vinclozolin
30	DDT o,p`-	61	Heptachlor-exo-Epoxide		
31	DDT p,p`-	62	Hexachlorobenzene (HCB)		

Table 3. The GC-MSD analysis of the tested extracts.

Onion extract	Garlic extract	Wheat germ extract	*Nigella sativa* extract
Bisabolol oxide A	Tetrasulfide	Tetradecanoic acid (**Myristic acid)**	Tetradecanoic acid (**Myristic acid)**
n-difluoro phosphine dimethyl hydroxyl amide	**Silane** (**monosilane**)	Palmitic Acid -methyl ester	**Palmitic Acid**
3-Eicosene	9-Octadecenamide (**Oleamide)**	**Palmitic Acid**	Ethyl palmitate
Palmitic Acid	Stearoylamide	9,12-Octadecadienoic acid	9,12-Octadecadienoic acid
9,12-Octadecadienoic acid	Vitamine E	9-Octadecenamide **(Oleamide)**	Ethyl linoleate
9,17-Octadecadienal		1,2-Benzenediccaroxylic acid	Ethyl Oleate
Linoleic acid, ethyl ester		Beta-Tecopherol	Stearic acid ethyl ester
Palmitic acid amide		Campesterol	9-Octadecenamide (**Oleamide)**
9-Octadecenamide **(Oleamide)**			Ethyl nonadecanoate
Tetradecanamide			
Cholesteryl alcohol			

Table 4. Antibacterial activities of plant extracts against bacterial reference strains using Agar well diffusion method, results given in (mm).

Bacterial Ref. strain	Extract						
	Onion	Garlic	Mint	Wheat germ	*Nigella sativa*	Mean	CIP
S. aureus NCINB 50080	11	30	10	12	18	16.20	40
B. cereus NCINB 50014	20	23	14	12	10	15.80	34
C. freundi ATCC 8090	12	24	18	18	16	17.60	50
L. innocua ATCC 33090	22	25	20	20	18	21.00	40
E. coli ATCC 11775	12	20	12	18	12	14.80	40
E. coli O157 ATCC 700728	12	26	16	10	14	15.60	40
S. Typhimurium ATCC 13311	20	28	10	30	24	22.40	40
Shigella bodyii ATCC 9207	28	26	30	20	14	23.60	36
Shigella sonnei ATCC 25931	28	20	20	14	22	20.80	40
Shigella flexeneri ATCC 12022	25	22	24	30	20	24.20	30
Ps. aeruginosa NCINB 50067	14	12	20	20	10	15.20	42
K. pneumoniae NCTC 9633	18	25	14	12	14	16.60	26
Proteus mirabilis ATCC 14153	14	24	15	10	12	15.00	30
Mean	18.15	23.46	17.15	17.38	15.69	---	37.54

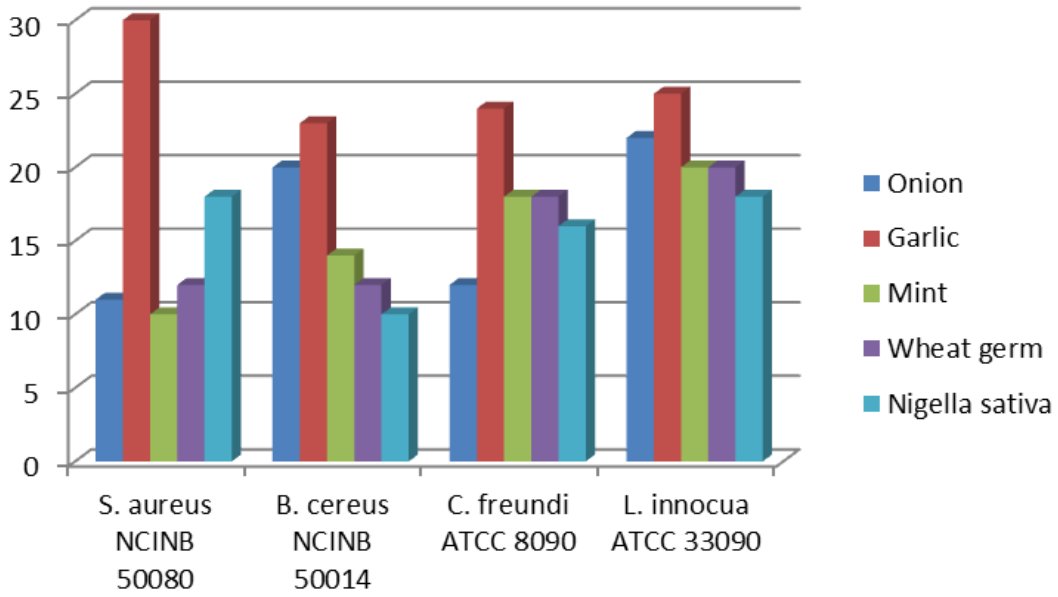

Figure 1. The Agar Well Diffusion Test of the tested extracts against Gram positive reference strains.

showed the least zone inhibition (15.69mm). Among the tested strains, the best results was shown against *Shigella flexeneri* ATCC 12022 (24.2mm), then *Shigella bodyii* ATCC 9207 (23.6mm) followed by *S.* Typhimurium ATCC 13311 (22..4mm) then *L. innocua* ATCC 33090 (21mm).On the other hand *E. coli* ATCC 11775showed the least hindrance of abilities with zone of inhibition 14.8mm then *Proteus mirabilis* ATCC 14153 (15mm) then *Ps. aeruginosa* NCINB 50067 (15.20mm) then *E. coli* O157 ATCC 700728 (15.60mm). Results were confirmed by the results of MIC shown in Table 5 and Figure 4 the best MIC was given against *Shigella flexeneri* ATCC 12022 (2.61 to 10.42 µg/ml). On the other hand, garlic showed the best MIC results range from (2.61 to 5.21 µg/ml).

Results of MIC Table 5 confirm the previous finding of AWDT showing the efficiency of garlic for hindrance of the tested strains with the highest dilution 2.61µg/ml for hindrance of *S. aureus* NCINB 50080 and *S.* Typhimurium ATCC 13311. MIC was 5.21 µg/ml against *B. cereus* NCINB 50014, *C. freundi* ATCC 8090, *L. innocua* ATCC 33090, *E. coli* O157 ATCC 700728,

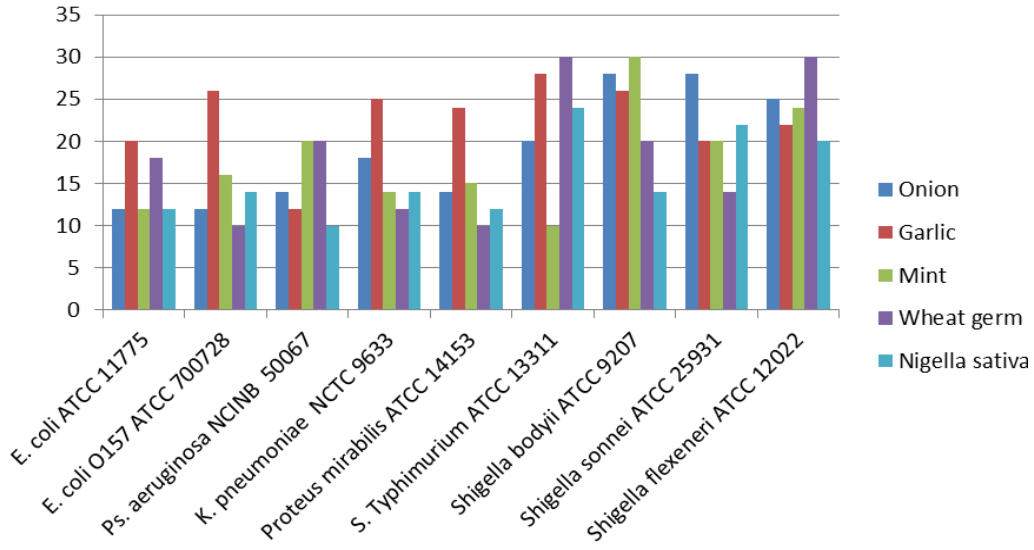

Figure 2. The Agar Well Diffusion Test of the tested extracts against Gram negative reference strains.

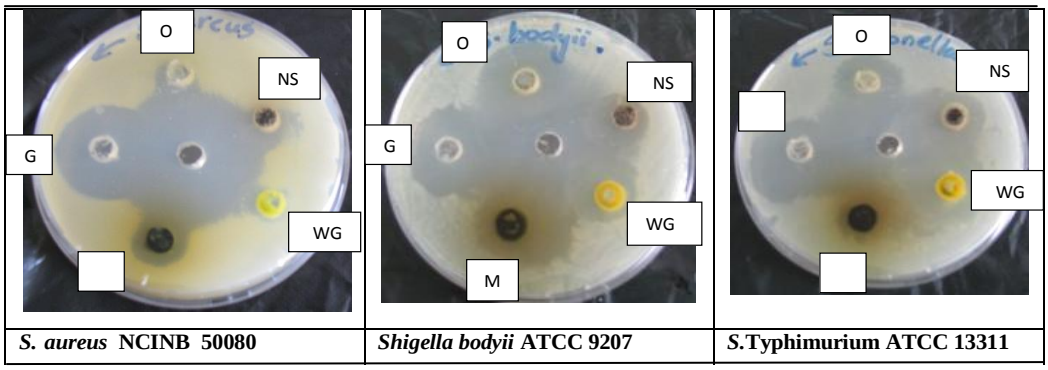

Figure 3. Showing the antimicrobial activities of some of the tested extracts against some of the tested reference strains. O=onion, G=garlic, M=mint, WG=wheat germ, NS=*Nigella sativa, CIP at the center.*

Shigella bodyii ATCC 9207, *Shigella flexeneri* ATCC 12022, *K. pneumoniae* NCTC 9633, *Proteus mirabilis* ATCC 14153.

On the other hand, Onion showed great hindrance capability against *Shigella bodyii* ATCC 9207 and *Shigella sonnei* ATCC 25931 with concentration 2.61 µg/ml followed by *Shigella flexeneri* ATCC 12022 and *L. innocua* ATCC 33090 with concentration 5.21µg/ml. Wheat germ and *Nigella sativa* were effective against *S.* Typhimurium ATCC 13311 with concentration 2.61 and 5.21 µg/ml, respectively. On the other hand wheat germ and mint were effective against *Shigella flexeneri* ATCC 12022 with concentration 2.61 and 5.21 µg/ml.

Results of AWDT and MIC were confirmed by MBC shown in Table 6 and Figures 5; results reveal that garlic extract showed bactericidal effect using high dilution among the five tested extracts, with MBC 5.21µg/ml against *S.aureus* NCINB 50080 and *S.* Typhimurium ATCC 13311. MBC was 10.42 µg/ml against *B. cereus* NCINB 50014, *C. freundi* ATCC 8090, *L. innocua* ATCC 33090, *E. coli* O157 ATCC 700728, *Shigella bodyii* ATCC 9207, *Shigella flexeneri* ATCC 12022, *K. pneumoniae* NCTC 9633, *P. mirabilis* ATCC 14153.

On the other hand, Onion and Mint showed the highest bactericidal activities against *Shigella bodyii* ATCC 9207. Onion was effective against and *Shigella sonnei* ATCC 25931 and Wheat germ showed bactericidal effect against *S.* Typhimurium ATCC 13311 and *Shigella flexeneri* ATCC 12022 with concentration 5.21 µg/ml.

DISCUSSION

Screening of the antibacterial activities of the tested

Table 5. Minimum Inhibitory Conc. of plant extracts against bacterial reference strains compared with Ciprofloxacin.

Strain	Extract					
	Onion	Garlic	Mint	Wheat germ	Nigella sativa	CIP
S. aureus NCINB 50080	166.7	2.61	166.7	166.7	20.84	3.125
B. cereus NCINB 50014	10.42	5.21	83.35	166.7	166.7	1.56
C. freundi ATCC 8090	166.7	5.21	20.84	20.84	41.68	1.56
L. innocua ATCC 33090	5.21	5.21	10.42	10.42	20.84	3.125
E. coli ATCC 11775	166.7	10.42	83.35	20.84	166.7	3.125
E. coli O157 ATCC 700728	166.7	5.21	41.68	166.7	83.35	3.125
S. Typhimurium ATCC 13311	10.42	2.61	166.7	2.61	5.21	3.125
Shigella bodyii ATCC 9207	2.61	5.21	2.61	10.42	83.35	1.56
Shigella sonnei ATCC25931	2.61	10.42	10.42	83.35	5.21	3.125
Shigella flexeneri ATCC 12022	5.21	5.21	5.21	2.61	10.42	3.125
Ps. aeruginosa NCINB 50067	83.35	166.7	10.42	10.42	166.7	3.125
K. pneumoniae NCTC 9633	20.84	5.21	83.35	166.7	83.35	6.25
P. mirabilis ATCC 14153	83.35	5.21	83.35	166.7	166.7	3.125

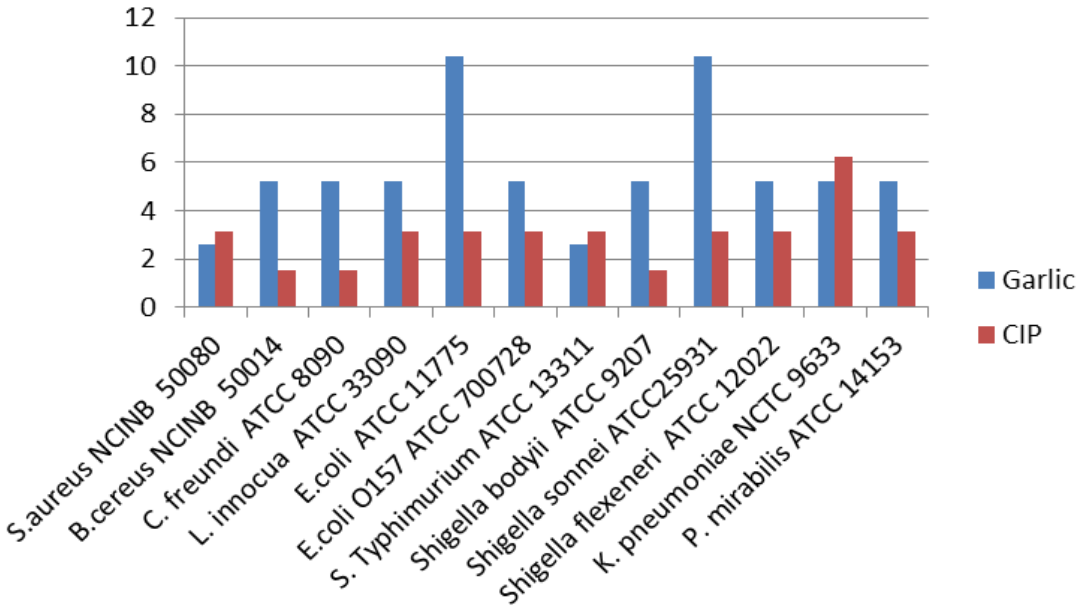

Figure 4. The Minimum Inhibitory Concentration of the garlic extract and Ciprofloxacin against reference strains.

extracts using AWDT, MIC and MBC revealed that garlic extract showed the highest hindrance capability AWDT Table 4, Figures 1, 2 and 3 with mean zone of inhibition equals 23.46 mm. On the contrary, *Nigella sativa* showed the least zone inhibition (15.69 mm). Among the tested strains, the best results was shown against *Shigella flexeneri* ATCC 12022 (24.2 mm), *Shigella bodyii* ATCC 9207 (23.6 mm), *S.* Typhimurium ATCC 13311 (22.4 mm) then *L. innocua* ATCC 33090 (21 mm). On the other hand, *E. coli* ATCC 11775, *Proteus mirabilis* ATCC 14153, *Ps. aeruginosa* NCINB 50067 and *E. coli* O157 ATCC 700728 showed the least hindrance abilities. Results agree with Suree and Pana (2005) who indicated that crude ethanolic herbal extracts showed different degrees of growth inhibition, depending on the tested strains. They added that *E. aerogenes* and *E. coli* were resistant to most of the ethanolic extracts. The present study showed that *E. coli* was one of the most resistant strains among the tested once.

MIC results Table 5 and Figure 4 and MBC (Table 6) and Figures 5 confirmed AWDT results; the best MIC was shown against *Shigella flexeneri* ATCC 12022 with

Table 6. Determination of Minimum Bactericidal Concentration (MBC) of extracts against bacterial reference strains compared with reference drugs.

Strain	Extract					
	Onion	Garlic	Mint	Wheat germ	*Nigella sativa*	CIP
S. aureus NCINB 50080	166.70	5.21	166.70	166.70	41.68	6.25
B. cereus NCINB 50014	20.84	10.42	166.70	166.70	166.70	3.125
C. freundi ATCC 8090	166.70	10.42	41.68	41.68	83.35	3.125
L. innocua ATCC 33090	10.42	10.42	20.84	20.84	41.68	6.25
E.coli ATCC 11775	166.70	20.84	166.70	41.68	166.70	6.25
E.coli O157 ATCC 700728	166.70	10.42	83.35	166.70	166.70	6.25
S. Typhimurium ATCC 13311	20.84	5.21	166.70	5.21	10.42	6.25
S. bodyii ATCC 9207	5.21	10.42	5.21	20.84	166.70	3.125
S. sonnei ATCC25931	5.21	20.84	20.84	166.70	10.42	6.25
S. flexeneri ATCC 12022	10.42	10.42	10.42	5.21	20.84	6.25
Ps. aeruginosa NCINB 50067	166.70	166.70	20.84	20.84	166.70	6.25
K. pneumoniae NCTC 9633	41.68	10.42	166.70	166.70	166.70	12.5
P. mirabilis ATCC 14153	166.70	10.42	166.70	166.70	166.70	6.25

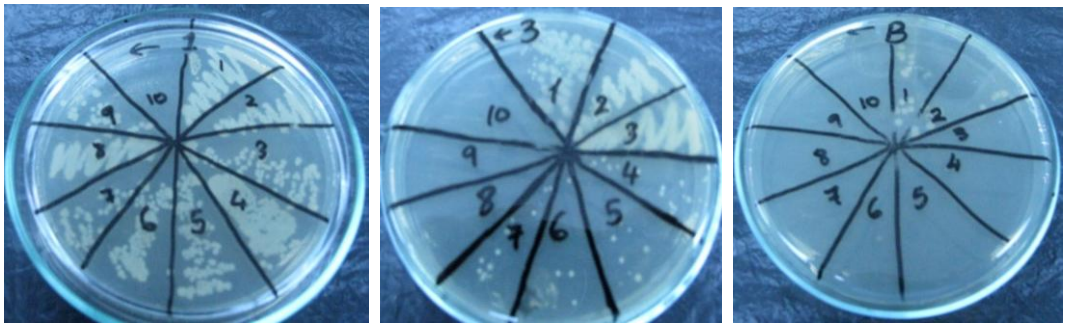

Figure 5. Minimum Bactericidal concentration using onion (a), *Nigella sativa* (b) and garlic (c), respectively against *S. aureus* NCINB 50080.

concentration range from 2.61-10.42 µg/ml. Garlic showed the best MIC results range from 2.61-5.21 µg/ml among the tested strains with exception of *Ps. aeruginosa* NCINB 50067 which was the most resistant strain with MIC 166.70 µg/ml. *E. coli* ATCC 11775 and *S. sonnei* ATCC 25931 were highly resistant to garlic with MIC 20.84 µg/ml. *E. coli* ATCC 11775 was resistant to onion and mint with conc. 166.70 µg/ml. Results agree with Ziarlarimi et al. (2011) who indicated that MIC of the garlic aqueous extract was 5%, but *E. coli* was resistant to the aqueous extracts of onion and mint. Results agree with Bin Shan et al. (2005) who found that *E. coli* was the most resistant strain to the 46 tested extracts.

Suree and Pana (2005) proved that the MIC values of garlic and ginger oils varied depending on the bacterial strains. Garlic extracts have been found to possess antibacterial property against several bacteria including *S. Typhimurium, S. Typhi, E. coli, Bacillus cereus*, *S. epidermidis*, and *S. aureus* (Arora and Kaur, 1999; Johnson and Vaughn, 1969; Saleem and Al-Delaimy, 1982).

The present study shows that highest hindrance abilities of the tested extracts using MIC was shown using concentration 2.61 µg/ml, given with garlic extract against *S. aureus* NCINB 50080 and *S. Typhimurium* ATCC 13311, onion extract against *Shigella bodyii* ATCC 9207 and *Shigella sonnei* ATCC 25931 and wheat germ extract against *S. Typhimurium* ATCC 13311 and *Shigella flexeneri* ATCC 12022. Results match with the findings of Suree and Pana (2005) who indicated that MIC results showed that *S. Typhimurium* was the most susceptible strain to most of the ethanolic extracts. Also, results agree with Bin Shan et al., 2005 who found that *S. aureus* was the most sensitive to the 46 extracts. The highest sensitivity of *S. aureus* may be due to its cell wall structure and outer membrane (Zaika, 1988).

GC-MS analyzed indicated that the number of compounds analyzed from each extract have no role in the antibacterial activities as garlic contains only five compounds but it was the most effective extract against the tested strains. While onion contains eleven compounds, *Nigella sativa* (nine compounds) and wheat germ extract (eight compounds) showed lower bactericidal activities. The present work shows that antibacterial activity is closely related to the type of ethanolic extracts. Other researchers have reported that compounds from different plant sources could inhibit various foodborne pathogens (Nychas, 1995; Prashanth et al*.,* 2001; Kim et al*.,* 2005). Results clearly indicated that no residues of the 91 tested pesticides were found in any of these 4 tested extracts (Table 2). Thus the pesticide residues have no roles in the antibacterial activities of the studied extract which confirm that the active ingredients found in the extracts have bactericidal activities against the tested strains. Sheikh et al*.* (2013) showed that onion samples were contaminated with profenofos, and enosulfan. Recent study showed that mint and onion were contaminated with pesticide residues with an incidence reach 100 and 33.3%, respectively. The pesticides detected in all samples were chlorpyrifos, chlorpyrifos-methyl, malathion, cypermethrin, l-cyhalothrin and sulfur Farag et al*.* (2011).

Results of AWDT and MIC were confirmed by MBC shown in Table 6 and Figures 5. The highest bactericidal effect with concentration 5.21 µg/ml was given with garlic against *S. aureus* NCINB 50080 and *S. Typhimurium* ATCC 13311, onion against *Shigella bodyii* ATCC 9207 and *Shigella sonnei* ATCC 25931, wheat germ against *S. Typhimurium* ATCC 13311 and *Shigella flexeneri* ATCC 12022 and mint against *Shigella bodyii* ATCC 9207. GC-MS revealed that Oleamide is the predominant compound found in the four tested extracts, which must have an important role in bacterial inhibition activities. Palmitic acid found in *Nigella sativa*, onion and wheat germ extracts may play an important role in the antibacterial activities of the tested extracts. Crude ethanolic herbal extracts showed different degrees of growth inhibition, depending on the tested strains. The mechanisms of action of each compound against various bacteria are very complicated. Results agree with Ultee et al*.* (1999) and Lambert et al*.* (2001) who proved that the antimicrobial activities of compounds may involve multiple modes of action as degrading the cell wall, interacting with the composition and disrupting cytoplasmic membrane. Raccach (1984) found that compounds may cause damaging of membrane protein, interfering with membrane integrated enzymes, causing leakage of cellular components, coagulating cytoplasm, depleting the proton motive force, changing fatty acid and phospholipid constituents, impairing enzymatic mechanisms for energy production and metabolism, altering nutrient uptake and electron transport (Taniguchi et al*.*, 1988). All of these mechanisms are not separate targets; some are affected as a consequence of another mechanism being targeted. The mode of action of antimicrobial agents depends on the type of microorganisms and is mainly related to their cell wall structure and the outer membrane arrangement. Plants including spices and herbs contain complex compounds. The mechanisms of action of each compound against various bacteria are very complicated (Kalemba and Kunicka, 2003; Burt, 2004). The main component of garlic responsible for its antibacterial activities is allicin. The enzyme allicinase converts allicin into these volatile compounds, once garlic is damaged by crushing or cutting, most antimicrobial agents are able to modify bacterial cell membranes and this leads to leakage and autolysis, thereby preventing growth and causing cell death (Lixin Xia and Ng, 2005). Garlic has been found to have a morphological effect on various bacterial cells, resulting in changes to the outer surfaces, internal properties as well as behavior of the cells (Ankri and Mirelman, 1999).

Conclusions

The degree of antibacterial property of tested ethanolic extracts can be put in the following order: garlic > wheat germ > mint > onion > *Nigella sativa*. These spices may be selected for use as potentially useful anti-bacterial agents in fermented meat products and other foods, depending upon the desired flavor of the products. *S. flexeneri* ATCC 12022, *S. bodyii* ATCC 9207, *S. Typhimurium* ATCC 13311 and *L. innocua* ATCC 33090 are the most vulnerable to crude ethanolic extracts, while *E. coli* ATCC 11775, *Pr. mirabilis* ATCC 14153 and *Ps. aeruginosa* NCINB 50067 were the most resistant. This study reported a highly positive relationship between antibacterial activity and total ethanolic content in a large number of herb extracts. This suggested that the ethanolic compounds might significantly contribute to their antibacterial activity. The present study also demonstrated that garlic among many of the ethanolic extracts tested possessed strong antibacterial activity. They could be a potential source for inhibitory substances against some foodborne pathogens. Garlic, wheat germ and mint extracts showing high antibacterial activity may be subjected to future studies of synergism, compatibility and activity in food-processing systems against specific pathogens of animal origin.

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Detection of acute toxoplasmosis in rural women in Sudan using different diagnostic tests

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Blood samples from 254 childbearing age women from two villages (EL Nuba and EL Massoudia) were collected after seeking their consent; their written consent form was obtained. Detection of IgM for acute toxoplasmosis was done using two different enzyme-linked immunosorbent assay (ELISA) techniques. Prevalence of IgM was 18.9 and 20.3%; this was obtained by using ELISA IgM in Khartoum for all samples and ELISA IgM in Prague for selected samples, respectively. Diagnostic test was used to confirm acute toxoplasmosis. This consists of detecting IgA and IgE using ELISA technique. The result was the same in both techniques (6.8%). Confirmation tests were used for different purposes. IgG avidity was used to determine the exact time the selected samples had the infection. Six cases had recent infection; five cases had old infection and two cases were in between. Western blot (WB) was used to confirm the antibodies detected by screening and diagnostic tests. Western blot confirmed that 61.5% of samples had antibodies against P30 gene.

Key words: Acute toxoplasmosis, ELISA- IgG avidity, Western blot, rural women, Sudan.

INTRODUCTION

Toxoplasmosis is one of the more common parasitic zoonoses world-wide and one of the most common human infections. Infection is acquired by ingestion of viable tissue cysts in meat or oocysts excreted by cats that contaminate food or water (Jones et al., 2009). Diagnosis of toxoplasmosis in humans is performed using different techniques. A few examples of these techniques are mouse inoculation, detection of anti-*Toxoplasma* antibodies, histological demonstration of tachyzoites in tissue sections or smears of body fluid, and detection of *Toxoplasma gondii* DNA by molecular methods (Montoya, 2002; Lappalainen and Hedman, 2004; Remington et al., 2004). Many serological methods for the diagnosis of toxoplasmosis have been established over the years. Most have been developed for detection of *Toxoplasma* infection in human and are commercially available in kit form (Buxton and Brebner,1998). The diagnosis of infection caused by *T. oxoplasmagondii* is carried out by the detection of specific anti-*Toxoplasma* immunoglobulin (IgM and IgG); and to discriminate chronic from reactivated infection IgG avidity is also determined with VIDAS instrument (bioMerieux, France) (Calderaro et al., 2009). Detection of anti-*Toxoplasma* antibodies indicates that a person

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has been infected with *T. gondii* some time in the past. There are many different serological techniques available; for example, the dye test, complement fixation test, indirect immunofluorescent test, latex agglutination test, enzyme linked immunosorbent assay, and immunosorbent agglutination test (Hill and Dubey, 2002; Montoya, 2002).

In asymptomatic infections, the only indication of a primary infection is seroconversion indicated by anti-*Toxoplasma* IgM or IgG antibodies. IgM antibodies usually become detectable within days after infection, while IgG antibodies become detectable after 1-2 weeks and may have lifelong persistence. The presence of IgM antibodies indicates a recent infection, but the tendency of IgM to remain detectable for a long time has been demonstrated (Lappalainen and Hedman, 2004; Petersen et al., 2005). Detection of anti-*Toxoplasma* IgM and IgG is essential for the diagnosis of *Toxoplasma* infection in pregnant women (Panah et al., 2013). Measure of IgA or IgE antibodies is also helpful in the diagnosis of acute infections (Ronday et al., 1995).

The detection of *T. gondii* specific IgM antibodies is the most common method used to determine the infection (Tekkesin, 2012). Positive IgM results are not sufficient as evidence of recent infection, as these antibodies are often present for many months (Kaul et al., 2004; Ali-Heydari et al., 2013). Repeat testing of IgM is not sufficient to confirm acute infection (Bobic et al., 1991; Gorgievski-Hrisoho et al., 1996; Meek et al., 2001). There is need to detect specific IgG, IgM, and IgA antibodies in order to increase diagnostic sensitivity (Partanen et al., 1984; Wilson et al., 1987; Wilson and Auley 1991; Roos et al., 1993). As is true of IgM antibodies to the parasite, IgA antibodies may persist for many months or more than a year. For this reason, they are of little additional assistance for diagnosis of acute infection in the adult. In contrast, the increased sensitivity of IgA assays over IgM assays for diagnosis of congenital toxoplasmosis is advancement in the diagnosis of the infection in foetus and newborns with congenital toxoplasmosis and negative IgM antibodies. Serologic diagnosis has been established by the presence of IgA and IgG antibodies (Stepick-Biek et al., 1990).

The persistence of specific IgE several months after seroconversion is suggestive of active toxoplasmosis and should be taken into account. Specific IgE is almost present in symptomatic acquired toxoplasmosis and can thus contribute to the etiologic diagnosis of lymphadenopathy (Foudrinier et al., 2003). Detection of IgA has been introduced as supplementary method to detect the acute phase of the disease (Jenum and Stray-Pedersen, 1998).

Measurement of *T. gondii* IgG avidity is a powerful tool for distinguishing recent from past infection. *T. gondii* IgG avidity measurement is particularly valuable for approximating the time of infection in pregnant women (Prince and Wilson, 2001; Sadraie et al., 2013). IgG avidity measurement is based on the differential elution of antigen bound IgG using urea-containing wash buffer; low avidity IgG dissociates from antigen in the presence of urea, whereas high avidity IgG remains bound to antigen (Hedman et al., 1989). Results for avidity assays are generally expressed as an avidity index (AI).

Since the original description of immunoblotting for diagnosis of congenital toxoplasmosis was done (Remington et al., 1985), almost 10 years elapsed before additional reports for its use appeared in the literature (Remington et al., 2004). Western blot is a sensitive and specific assay for diagnosis of congenital toxoplasmosis (Gallego-Marin et al., 2005). It is utilized as confirmation test, particularly in mothers and newborns. Immunoblotting technique is easy to perform and might be useful as additional serological assay for routine diagnosis of *T. gondii* infection. However, it can not replace other serological test system but can serve as additional method to improve diagnosis (Gross et al., 1992).

The specific aim of this study was to differentiate between acute toxoplasmosis and chronic toxoplasmosis in Sudanese women using immuno techniques.

MATERIALS AND METHODS

Study area and population

The study was performed in two villages: EL Massoudia and EL Nuba in the North of EL Geizera State (middle Sudan) located near Blue Nile. These villages belong to EL Kamleen Province about 50 km South of the Capital, Khartoum. Most of the population in these villages belongs to the same ethnic group. People in these areas have low socio-economic status. They are farmers, animal breeders or workers in a big industry, newly established near this area. Women live simple life; although most of them do not work, they lend a hand to improve the economic situation by bringing water from river, wood for cooking or taking care of animals; they also help animals to give birth or participate in farms and agricultural process.

Study design

The study was a cross-sectional study. The sample size was 254; a prevalence of 20% was obtained from first 10 samples collected (d = 0.05 at a confidence level of 95%). A total of 5% of the sample population was added to the sample size. One sample was missed due to lack of plasma. From the total samples size, 254 samples were detected for IgM in Khartoum and 59 samples were examined by using ELISA IgM, IgA, and IgE in Prague. About 13 samples which were highly positive by CFT, ELISA IgG, and ELISA IgM, IgA, IgE were chosen to confirm the acute and recent infection.

Data collection

Consent form and questionnaire were filled for each individual. Consent form was signed and fingerprinted by each one after agreeing to participate in the study. Data were collected after convenient interview.

Table 1. Detection of Acute Toxoplasmosis in EL Nuba and EL Massoudia Villages Using Different Investigation Tests.

Village	ELISA IgM Done in Khartoum		Number examined	ELISA IgM Done in Prague		Number examined	ELISA IgA		Number examined	ELISA IgE		Number examined
	Positive	Negative		Positive	Negative		Positive	Negative		Positive	Negative	
EL Nuba	24 (17.7%)	112 (82.6%)	136	6 (24.0%)	19 (76.0%)	25	1 (4.0%)	24 (96.0%)	25	1 (4.0%)	24 (96.0%)	25
EL Mass	24 (20.3%)	94 (79.7%)	118	6 (17.7%)	28 (82.4%)	34	3 (8.8%)	31 (91.2%)	34	3 (8.8%)	31 (91.2%)	34
Total	48 (18.9%)	206 (81.9%)	254	12 (20.3%)	47 (79.7%)	59	4 (6.8%)	55 (93.2%)	59	4 (6.8%)	55 (93.2%)	59

Samples collection

The blood samples were collected under direct medical supervision by medial venipuncture using 5 ml syringe in heparinized tubes. Plasma was obtained by centrifugation of the blood at 5000 rpm for 10 min. Plasma was kept in different labeled cryo tubes in -20°C until it was used. Plasma samples were sent in dry ice to National Reference Laboratory for Toxoplasmosis, Prague, Czech Republic where more investigations were done.

Detection of *T. gondii* antibodies using enzyme link immunosorbent Assay IgM (In Khartoum)

The ELISA IgM (Toxo IgM®, Human Germany) is planned for the detection of immunoglobulin M (IgM) class antibodies to *T. gondii*.

Enzyme link immunosorbent assay IgM, IgA and IgE (In Prague)

ELISA IgM, IgA, and IgE (Test-line®) demonstration of specific IgA and IgE antibodies is an indication of current or recent infection. ELISA IgM, IgA, and IgE were used as diagnostic and differential tests to see the recent and acute infection of *T. gondii* in selected patients.

Indirect immunofluorescent test

The IIFT (Test-line®) test is simple, quantitative and reproducible, and the titers can be related to clinical events. However, visual assessment of fluorescence of the whole-cell antigen is subjective (Gordon et al., 1981).

Western blot IgM

The test (Test-line®) serves for a detailed determination of specific IgM antibodies to the respective *T. gondii* antigens. The diagnosis of *T. gondii* infection using western blotting is based on the detection of specific antibodies to a number of high-specific antigens that may characterize their molecular weights (Remington et al., 1985).

Enzyme link immunosorbent assay IgG Avidity

The avidity (Test-line®) of an antibody expresses the strength of the bond between an antigen and an antibody. The methods of avidity determination are usually based on splitting the antigen-antibody bond using avidity solution (Hedman et al., 1989); and the very important and usefulness of using this test is determination of the time of infection.

Data analysis

Statistical evaluation was done by entering the data obtained into the personal computer (PC) using two different programs: (1) Statistical package for social science (SPSS) version 13.0 (SPSS Inc. Chicago, IL. USA) and (2) Statistical analysis was performed by statistical software Stata, version 9.2 (Stata Corp LP, College Station, TX).

RESULTS

ELISA IgM done in Khartoum

A total of 254 plasma samples were detected by ELISA IgM after collecting them directly. Prevalence of acute toxoplasmosis was 48 (18.9%). The numbers of cases were equal in each village; in EL Nuba village, the prevalence was 24(17.7%), while the prevalence was 24(20.3%) in EL Massoudia as shown in Table 1.

ELISA IgM done in Prague

About 59 samples were selected according to the results of screening tests done before; they were examined in the reference laboratory of toxoplasmosis in Prague including ELISA IgM of positive samples done in Khartoum. The prevalence of acute toxoplasmosis was 12 (20.3%). The prevalence was 6 (24.0%) in EL Nuba village while the prevalence was 6 (17.7%) in EL Massoudia as shown in Table 1. Significant difference was found between the result done in Khartoum and result obtained in Prague (p-value<0.05).

Using ELISA IgA

The same samples selected in ELISA IgM in Prague were detected using ELISA IgA to diagnose the acute toxoplasmosis. The samples which were positive were 4 out of 59 samples. The prevalence of IgA as indicator of acute toxoplasmosis was 6.8% from the two villages. In EL Nuba village, the prevalence was 1.7% and the prevalence was 5.1% in EL Massoudia village as shown

Table 2. Detection of Acute Toxoplasmosis in 13 samples using Diagnostic and confirmation tests in both villages.

Sample Number	Location	Age	ELISA (OD)				IIFT dilution	WB	IgG Avidity
			IgM (K)	IgM (P)	IgA	IgE			
27n	EL Nuba	30	1.632	2.063	0.841	0.799	2048	+	14
103n	EL Nuba	20	0.378	0.602	0.071	0.196	512	-	25
108n	EL Nuba	16	0.616	0.851	0.226	0.228	1024	+	28
110n	EL Nuba	35	1.439	0.472	0.129	0.095	256	+	35
124n	EL Nuba	40	0.449	0.475	0.067	0.111	512	+	24
137n	EL Nuba	35	0.518	0.692	0.071	0.080	256	-	30
1o	EL Mass.	50	0.102	0.524	0.081	0.933	256	-	35
12o	EL Mass.	40	0.472	0.140	0.288	0.171	2048	-	49
65o	EL Mass.	26	1.685	1.529	0.432	0.535	4096	+	13
66o	EL Mass.	34	0.370	0.282	0.252	0.225	1024	+	39
80o	EL Mass.	33	0.432	0.754	0.187	0.220	512	+	68
100o	EL Mass.	38	1.051	0.236	1.020	0.851	8192	-	54
118o	EL Mass.	33	0.844	0.664	0.197	0.131	512	+	42

n, code used for samples collected from EL Nuba; o, code used for samples collected from EL Massoudia; K, code for ELISA IgM done at lab. of TMRI in Khartoum; P, code for ELISA IgM done in toxoplasmosis reference lab. in Prague; + = Positive by WB; - = Negative by WB

in Table 1.

Using ELISA IgE

Samples selected were test with ELISA IgA to diagnose the acute toxoplasmosis via detection of IgE. The result is shown in Table 1. The prevalence of IgE specific antibodies for *T. gondii* was 6.8%. The prevalence was 1.7% in EL Nuba village and 5.1% in EL Massoudia village. The significant difference between ELISA IgA and ELISA IgE was high (p-value=0.001); also, there was high significant difference between ELISA IgA and ELISA IgM (p-value=0.001). There was significant difference between ELISA IgE and ELISA IgM (p-value=0.02).

Confirmation of acute toxoplasmosis

A total of 13 cases were suspected to be in acute phase of toxoplasmosis from both villages. For confirmation of these cases, several confirmation tests were done as shown in Table 2. Two cases were positive in all diagnostic and confirmation tests with low avidity.

Indirect immunofluorescent test (IIFT)

This test was done for the 13 cases with high dilutions to confirm the presence of antibodies against *T. gondii*. The results shown in Table 2 confirmed the positivity of all cases and some cases were positive in higher dilutions as shown in Table 2.

Western blot IgM test

Western blot IgM test was done on the suspected acute cases. The positive cases were 8/13; 4 cases from each village (Table 2). The prevalence of acute toxoplasmosis was 61.5%. The results show the reaction between antibodies found in the plasma and the target gene of *T. gondii* P30.

Avidity IgG Test

This test was done to determine the exact time of infection. The results confirmed six cases were in low avidity; five of them in EL Nuba village and five cases in high avidity; all of them in EL Massoudia village (Table 2).

DISCUSSION

The prevalence of acute toxoplasmosis using ELISA IgM and different kits in this study ranges between 18.9 and 20.3%. In some countries, the prevalence of IgM has been reported to be as high as 2.4% (Svobodova and Literak, 1998). In the United States, the incidence of acute *Toxoplasma* infection during pregnancy has been estimated to be between 0.2-1.0% (Wong and Remington, 1994). In Iran, prevalence of IgM in women was 11.7% (Saeedi et al., 2007) and in Mexico none of the women studied had IgM anti-*T. gondii* antibodies (Alvarado-Esquivel et al., 2006); also in pregnant women in Turkey (Ertug et al., 2005). In Saudi Arabia, the prevalence of IgM in pregnant women was 5.6% (Al-Harthi et al., 2006). Similar results were obtained in differ-

ent target groups in Khartoum State (Adnan, 1994; Khalil 2004). IgM test is still used by most laboratories to determine if a patient has been infected recently or long ago; and because of the hurdles posed in interpreting a positive IgM test result, confirmatory testing should always be performed (Montoya, 2002). Detection of IgM antibodies was performed only in samples with high titration by CFT and IgG positive, because the presence of IgM antibodies alone is rarely seen. Anti-*T. gondii* IgG antibodies appear very early after infection (Pfrepper et al., 2005); therefore, the period between the appearance of IgM and the appearance of IgG is extremely short and the probability to find an IgM positive/IgG negative infected subject seems to be quite low (Alvarado-Esquivel et al., 2007). In addition, seropositivity to IgM alone is not considered an acceptable diagnostic criterion for acute infection. Anti-*T. gondii* specific IgM antibodies are detectable early after infection and can persist for prolonged times after infection (Liesenfeld et al., 1997; Montoya and Liesenfeld, 2004).

IgM antibodies are the first to be produced between 7 and 15 days; IgA antibodies are produced at the end of the first month (Bessieres et al., 1992). IgM and IgA antibodies increase in parallel. Several reports have indicated the role that specific IgA plays in the acute infection process (Decoster et al., 1988; Huskinson et al., 1990; Stepick-Biek et al., 1990; Decoster et al., 1992; Gross et al., 1992; Saathoff and Seitz 1992; Gross et al., 1993). During the course of toxoplasmosis infection, the kinetics of IgA antibodies are similar to those displayed by IgM antibodies (Decoster et al., 1988; Wong and Remington, 1994; Remington et al., 1995); therefore, joint detection of IgA and IgM antibodies may be useful regardless of whether it may be discriminated at the expense of which antibody of reaction proved positive.

IgE antibodies are detectable by ELISA in sera of acutely infected adults, congenitally infected infants, and children with congenital toxoplasmic chorioretinitis (Pinon et al., 1990; Wong et al., 1993). Their demonstration does not appear to be particularly useful for diagnosis of *T. gondii* infection in foetus or newborn when compared with IgA tests. The duration of IgE seropositivity is briefer than that with IgM or IgA antibodies and hence appears useful for identifying recently acquired infection (Wong et al., 1993; Montoya and Remington, 1995).

Confirmatory testing should be done for all IgM positive cases (Liesenfeld et al., 1996; Wilson et al., 1997; Liesenfeld et al., 2001a). Accordingly, Western blot is performed because it detects antibodies against *T. gondii* P30. P30 is the major surface antigen of *T. gondii*. It is a very abundant protein (Kasper et al., 1983), conserved in most strains (Ware and Kasper, 1987), and is present both in the vesicular network of the parasitophorous vacuole and on the surface of the parasite (Sibley et al., 1986). It is very immunogenic, eliciting high titers of antibodies in infected individuals (Potasman et al., 1986). The gene coding for P30 has been cloned and completely sequenced (Burg et al., 1988). Western blot proved more sensitive than ELISA (Remington et al., 2004). Western blot was performed after PCR was done. The results obtained by PCR were negative using P30 gene primer; therefore, WB was used to confirm the antibodies against which antigen to interpret the result obtained by PCR. The WB confirmed that 61.5% of samples that were negative by PCR had specific antibodies against P30 surface gene of *T. gondii*.

Recently, a number of tests for avidity of *T. gondii* IgG antibodies have been introduced to help discriminate between recently acquired and distant infection (Hedman et al., 1989; Liesenfeld et al., 2001b). It has been observed that the functional affinity of specific IgG antibodies is initially low after primary antigenic challenge and that it increases during subsequent weeks and months by antigen-driven B cell selection. Protein-denaturing reagents including urea are used to dissociate the antibody-antigen complex. The avidity result is determined using the ratios of antibody titration curves of urea treated and untreated samples (Montoya, 2002). Therefore, IgM positive results are not sufficient as evidence of recent infection, as these antibodies are often present for many months (Kaul et al., 2004). The results we obtained show that there is high prevalence in EL Massoudia village, but the recent infection and new cases are found in EL Nuba village.

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Full Length Research Paper

Optimization of phytase production in solid state fermentation by different fungi

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The present study was conducted to explore the potential fungal isolates for maximum production of phytase by optimizing solid substrate preference and growth parameters (pH, temperature, fermentation time) of solid state fermentation (SSF). It has been inferred from the results that all the tested fungi (*Aspergillus niger, Aspergillus flavus, Aspergillus versicolor, Aspergillus nidulans, Cladosporium cladosporioides, Trichoderma reesei,* and *Trichoderma viride*) showed inducible expression of phytase. Fungal isolates showed different preference of solid substrate out of wheat bran, lentil, oat, corn and bagasse for maximum production of phytase. Lentil and bagasse were not used preferably by any tested fungi. Among the tested fungi, maximum phytase production was observed in *A. flavus* (80 U/g of solid substrate) using wheat bran as solid substrate at pH 6 after 7 days of fermentation period at 30°C. It was established that solid substrates with high phytate and low inorganic phosphate (P*i*) contents are substrate of choice for phytase production by SSF.

Key words: Phytase, solid state fermentation, optimization.

INTRODUCTION

Cereals, legumes and oilseed crops are cultivated globally over approximately 90% of agricultural land and serve as major source of nutrients for animal kingdom (Reddy et al., 1982). In these plants, more than 80% of the total phosphorous is stored in the form of phytate (myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate, IP_6) molecules (Spier et al., 2008). Phytate (phytic acid) has highly stable structure and differs from other organo-phosphates because of its high phosphate content (Kerovuo et al., 2000).

Monogastric animals poorly digest phytate and excrete most of it in fecal waste. While passing through the digestive track, the indigestible phytate exerts strong anti-nutritive effects such as chelation of essential minerals like iron, calcium, zinc and magnesium resulting in insoluble phytate mineral complexes. These complexes limit the bioavailability of essential minerals (Lopez et al., 2002). It is also reported that binding of phytic acid with dietary proteins and amino acids reduces their digestibility and the functionality of digestive enzymes like trypsin, and amylase (El-Batal and Karim, 2001; Li et al., 2008).

In animal feeding industries, feed is usually supplemented with inorganic phosphate in order to meet the phosphorous need for proper growth and development of animals. However, anti-nutritional effects of phytate remained unaffected (Sandberg and Andlid, 2002). Excretion of indigestible phytate having large amount of phosphorous in manure leads to redistribution of phosphorous in soil (Turner and Leytem, 2004). It may leach in waterways and execute eutrophication that generates water quality issues. Hence, elevated level of phosphorous in water and soil also creates several environmental problems.

To avoid the phytate related issues, there is need to introduce the methods for degradation of phytate. The

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physical and chemical methods are expensive and reduce the nutritional value of feed as well (Khetarpaul and Chauhan, 1990). Therefore, enzymatic degradation of phytate appears to be of great interest. Phytase (myo-inositol hexakisphosphate phosphohydrolase; EC 3.1.38 and EC 3.1.3.26) hydrolyzes phytate stepwise into lower inositol phosphates, myo-inositol and inorganic phosphate (Kim and Lei, 2005).

The use of phytase in animal industry is not only to enhance the utilization of phosphorous, but it also eliminates the anti-nutritive effects of phytate which ensure the bioavailability of minerals and phosphorous to promote bone strength (Lei et al., 2011). It also reduces the fecal phosphorous contents up to 50% (Konietzny and Greiner, 2004). Therefore, phytases are considered as potential candidate for removing some of the negative environmental impacts exerted due to livestock production (Hegeman and Graban, 2001). The phytase is also gaining attention of animal industries for the degradation of phytate present in animal feeds (Li et al., 2008).

Phytases are present naturally in plants, animals and microbes. The activity of animal phytases is minimal in comparison to the plant and microbial phytases (Weremko et al., 1997). The intrinsic phytases of some plants, such as wheat and barley, have high activity but their pH spectrum (4-7.5) is narrow. These enzymes are also more heat labile (Greiner and Konielzny, 2006).

The present study is designed to optimize the nutritional and physical parameters of solid state fermentation for the maximal production of extracellular phytase from various fungi.

MATERIALS AND METHODS

Preparation of inoculum

All the fungi (*A. niger, A. flavus, A. versicolor, A. nidulans, Cladosporium cladosporioides, T. reesei,* and *T. viride*) used in the present study were obtained from the Fungal Bank of Institute of Agricultural sciences, University of the Punjab, Lahore, Pakistan. The cultures were grown and maintained on malt extract agar slants composed of 2.5% malt extract, 0.5% yeast extract, 0.5% glucose and 2% agar. After sterilization, the slants were inoculated with respective fungus and incubated at 30°C for growth for seven days. After growth, the slants were stored at 4°C until further use.

Seven days old fully sporulated slants were used for the preparation of inoculum by suspending the fungal spores in 10 ml of 0.1% Tween-80 separately. The spore suspension was adjusted to $1x\ 10^7$ spores per ml and 100 µl of spore suspension was used to inoculate the media for respective fungi unless otherwise mentioned.

Qualitative screening of fungal strains for phytase production

The fungal strains were qualitatively screened for extracellular phytase production using phytase screening medium (PSM) containing glucose (2%), tryptone (1%), NaCl (0.5%), KCl (0.1%), potassium phytate (0.2%) and agar (2%). The PSM plates were inoculated and incubated at 30°C for 3 days. The plates were observed for zone of clearance around fungal growth. The zone forming fungi were selected for quantitative screening.

Quantitative screening

The qualitatively positive fungal cultures were checked for the expression of phytase enzyme whether it is constitutive or inducible. Each fungal strain was grown on phytase screening broth and in the medium including K_2HPO_4 (0.5%) instead of potassium phytate. The media was inoculated with fungal strains in 250 ml Erlenmeyer flasks and incubated at 30°C for 5 days. The mycelial mat was removed by passing the suspension through two layers of muslin cloth. The filtrate was centrifuged at 9447 g for 20 minutes at 4°C. The cell free supernatant was used for phytase assay. In this assay, the expression of phytase enzyme was observed inducible in all the tested fungi.

Substrates

The substrates used for phytase production by solid substrate fermentation (SSF) includes: wheat bran, lentil, oat, corn and bagasse. The substrates were purchased from the local market of metropolitan city of Lahore, Pakistan. The phytic acid contents of these substrates were used as inducer for phytase expression.

Optimization of phytase production by solid substrate fermentation (SSF)

The nutritional and physical parameters of SSF were optimized by changing one parameter at a time to get maximal production of phytase from each fungus. The physical parameters included pH (4-9), temperature (20-40°C) and incubation time (24-192 h).

The medium for SSF was prepared by adding 2.5 g of finely ground substrate (180 µm particle size) in 250 ml Erlenmeyer flask supplemented with 5 ml of nutrient solution (0.2% glucose, 1% tryptone, 0.5% NaCl, 0.1% KCl) and sterilized at 121°C under 15 psi for 20 min. After cooling, fermentation medium was inoculated and initially incubated at 30°C for 5 days. Phytase production was evaluated by phytase assay.

Preparation of enzyme extract

In order to prepare the enzyme extract, 10 ml of 0.2 M potassium acetate buffer was added in each fermented flask and incubated at 4°C for 24 h in orbital shaker at 200 rpm. The mycelial mat was removed by passing the suspension through two layers of muslin cloth. The filtrate was centrifuged at 9447 *g* for 20 min at 4°C. The clear supernatant was used as crude enzyme extract for phytase assay.

Phytase assay

Phytase activity was determined spectrophotometrically by measuring the inorganic phosphorous released from the substrate as described by Harland and Harland (1980).

One unit of phytase activity is defined as ''the amount of phytase required to liberate 1 µmole of inorganic phosphorous per unit time under assay conditions.''

RESULTS AND DISCUSSION

The phytase is gaining importance at industrial level and has become an object of extensive research because it is involved in the breakdown of phytate which is associated with several problems of the animal industry and environ-

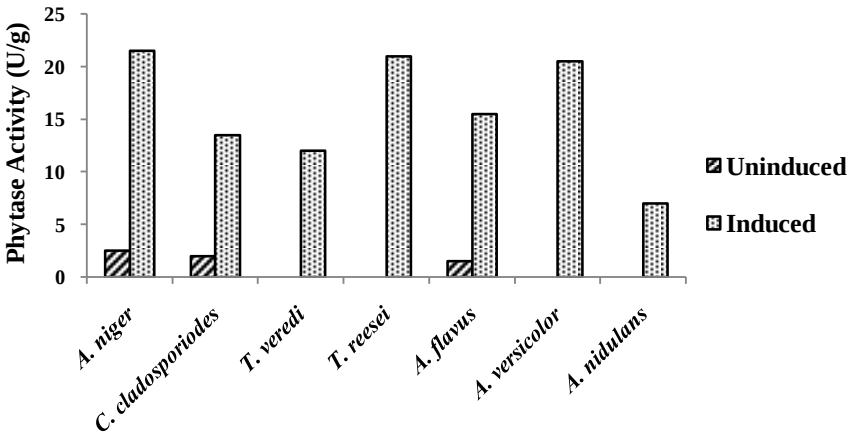

Figure 1. Phytase production under uninduced and induced conditions using KH_2PO_4 as inducer.

mental pollution. In order to ensure the maximal enzyme yield at low cost, the selection of microorganism, culture conditions, substrate selection and physical conditions are critical which play key role in enhancing the enzyme production (Vats and Banerjee, 2004; Sasirekha et al., 2012).

In the present study, a correlation between solid substrate, physical parameters of SSF and production of phytase from fungi has been studied.

Inducible expression of phytase

The fungi showing zone of clearance around its growth circle were selected for qualitative screening. In qualitative screening, the selected fungi were assessed for phytase production under induced and uninduced conditions. In uninduced condition, KH_2PO_4, while in case of induction conditions potassium phytate, were used as sole sources of phosphorous. The results show that all tested fungi had inducible expression of phytase (Figure 1). The results are similar to the work of Sasirekha et al. (2012) who have reported expression of phytase only in media including phytate as source of phosphorous and negligible expression, when inorganic phosphate is added in media.

Optimization of SSF parameters

For the production of phytase and other metabolites from filamentous fungi, SSF is preferred over submerged fermentation because substrate provides physical support and energy for fungal growth (Pandey et al., 2001). In the present study, five solid substrates including wheat bran, corn, oat lentil and bagasse were evaluated for phytase production. These substrates served both as source of energy and as inducer for phytase production due to their phytic acid contents. During optimization of substrates, the pH was adjusted to 7 and fermentation was performed at 30°C for 5 days. The substrate preference of tested fungi for maximum phytase production is given in Figure 2. The results indicate that lentil and bagasse are not preferably used in SSF by all fungi to produce phytase. It was interesting to note that tested fungi showed different preferences for solid substrate for maximal production of phytase. These results do not corroborate with many other reports which supports only wheat bran as potential substrate for phytase production (Salmon et al., 2012; Sreedevi and Reddy, 2012; Pandey et al., 1999) whereas, the studies of Roopesh et al. (2006); Bogar et al. (2003), Spier et al. (2008) and Selvamohan et al. (2012) support our finding that other solid substrates may also be considered as good candidate for phytase production. However, this observation is dependent on the type of microorganism used for SSF. It has been reported that phytate contents (% of dry weight) of lentil (0.27%) and bagasse (0.038%) are low in comparison to the wheat bran (3.29%), oat (1.37%) and corn (1.05%) (Coulibaly et al., 2011; Mittal et al., 2012). In the present study, it was observed that substrates with high phytate and low inorganic phosphate (P*i*) content are preferred over the substrates with low phytate and high inorganic phosphate (P*i*) contents for production of phytase. Hence, it is suggested that the substrates with high phytate and low P*i* are more preferable for phytase production by SSF, which is also supported by the studies of Vats and Banerjee (2004).

Physical parameters significantly affect the growth of microorganism as well as the growth associated production of desirable metabolite in SSF. In the present study, physical parameters (pH, temperature and incubation time) were optimized using standardized solid substrate specific for each fungus to get the maximum phytase production.

In order to optimize pH for phytase production from

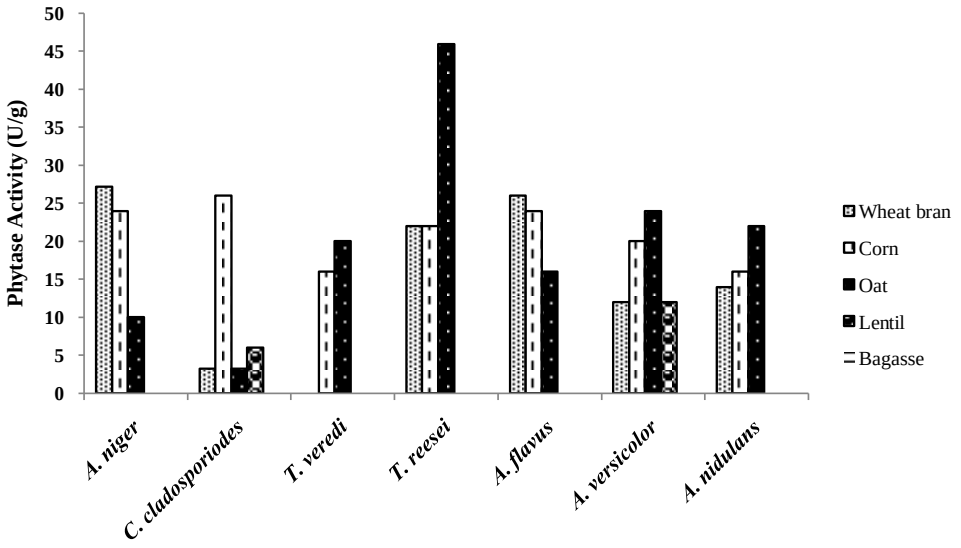

Figure 2. Optimization of solid substrates for phytase production.

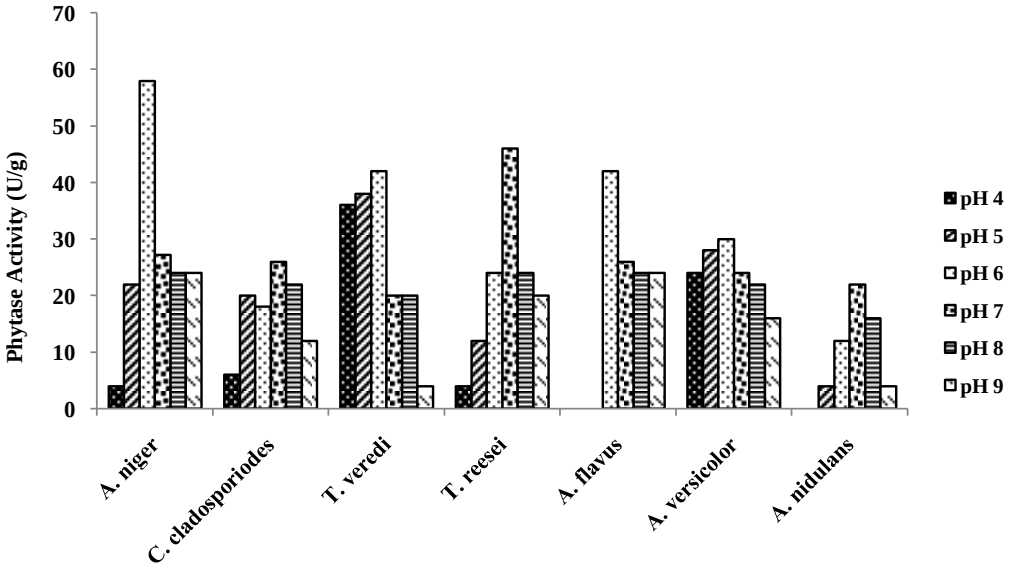

Figure 3. Optimization of pH for phytase production from tested fungi using SSF on specific substrates: Wheat bran for *Aspergillus niger* and *Aspergillus flavus*; Oat for *Trichoderma reesei*, *Trichoderma viride*, *Aspergillus versicolor* and *Aspergillus nidulans*; Corn for *Cladosporium cladosporioides*.

tested fungi, the pH of media was varied from 4 to 9 and SSF was conducted for 5 days at 30°C. The results show that the optimum pH of all tested fungi falls in pH ranging from 6 to 7. *A. niger* (58 U/g), *A. flavus* (42 U/g), *T. viride* (42 U/g) and *A. versicolor* (30 U/g) had the optimum pH 6 while *T. reesei* (46 U/g), *C. cladosporioides* (26 U/g) and *A. nidulans* (22 U/g) have pH 7 (Figure 3). These findings corroborate several investigations that report that acidic to neutral pH of fermentation medium favors the optimal phytase production (Howson and Davis, 1983; Shimizu, 1993; Sano et al., 1999; Andlid et al., 2004; Gulati et al., 2007; Singh and Satyanarayana, 2012; Selvamonan et al., 2012).

Using optimized substrate type and pH, SSF was performed at different temperatures for 5 days to standardize optimum temperature of all tested fungi for phytase production. It is observed that all tested fungi show maximum phytase production at their optimal growth

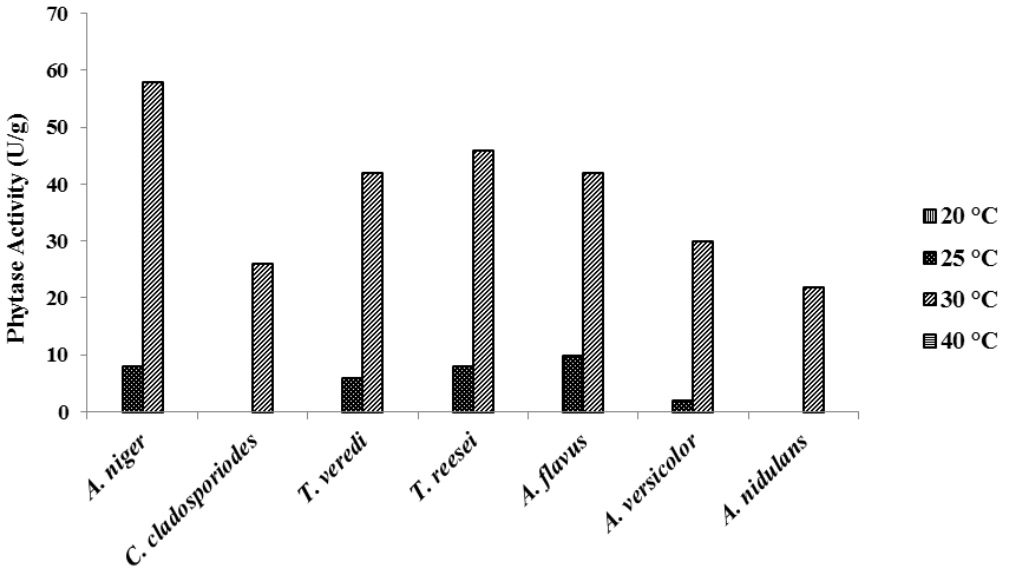

Figure 4. Optimization of temperature for phytase production by SSF.

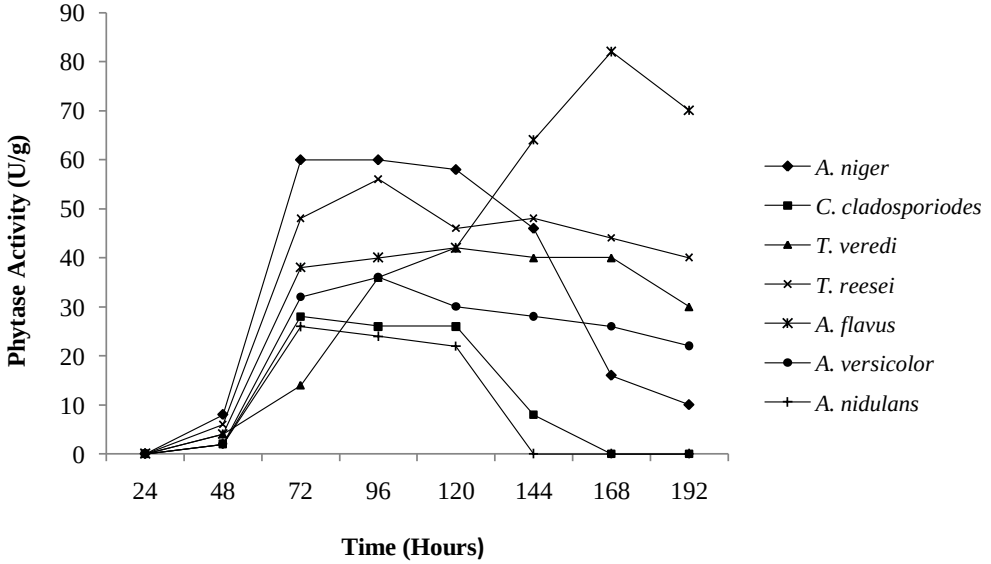

Figure 5. Optimization of fermentation time for phytase production from tested fungi using SSF.

temperature (30°C) (Figure 4), which corroborates other studies (Al-Asheh and Duvajak, 1995). Fungi produce phytase maximally at their optimal growth temperature. The results of our study clearly established the fact that phytase production is growth associated as well and fermentation performed above or below the optimum growth temperature significantly affects the enzyme yield.

In order to standardize the time interval for optimal phytase production, SSF was performed for different time intervals using standardized substrate, pH and temperature for each fungus. It was observed that each tested fungus showed optimal phytase production after specific time intervals of fermentation. The maximum phytase production was observed in *A. flavus* (80 U/g) after 7 days of SSF (Figure 5).

Finally, it was concluded that *A. flavus* is a potential candidate for phytase production using SSF. The culture conditions significantly affect the enzyme production. SSF performed using standardized nutritional and physical parameters, which are specific for each fungus improves the enzyme yield. The fungal cultures showed differential preference for solid substrates and the solid substrate with high phytate: P*i* ratio is more preferable for phytase production.

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Full Length Research Paper

Thermoanaerobacter spp. recovered from hot produced water from the Thar Jath oil-field in South Sudan

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Two bacterial isolates, designated S1.1 and S3.1, were recovered from oil-well produced water extracted from a deep and hot oil-well in the Thar Jath oil-field in South Sudan, and characterized. Based on their 16S rRNA gene sequences and phenotypic properties, the isolates were identified as members of the genus *Thermoanaerobacter*, possibly representing novel species. Both strains are strict anaerobes, grow optimally at 65 – 70°C at neutral pH and with ~0.3% NaCl, and can use various carbohydrates, proteinaceous compounds and organic acids as growth substrates. Strain S1.1 differs from strain S3.1 in its ability to grow on xylan and D-ribose, and S3.1 from S1.1 in its capacity to grow on acetate, arabinose, cellulose and lactate. Both produced acetate, ethanol, carbon dioxide and hydrogen as fermentation by-products from glucose, and growth was stimulated by thiosulphate. Strain S1.1 actively reduced Fe(III) as revealed by the formation of a dark paramagnetic precipitate and increased growth in cultures supplemented with Fe_2O_3. Cultures of both strains survived autoclaving at 121°C for 40 min, suggesting the formation of extremely heat-resistant endospores.

Key words: *Thermoanaerobacter*, oil-well, produced water, deep biosphere, petroleum microbiology, thermophiles, Thar Jath.

INTRODUCTION

Deep Oil-field reservoirs represent extreme anaerobic environments with high temperature and pressure, toxic compounds, and limited nutrient availability. Nevertheless, a number of anaerobic bacteria and archaeal isolates believed to be indigenous to these environments have been recovered and described, including fermentative organisms, methanogens, metal reducers, acetogens, and sulphate and nitrate reducers (Birkeland, 2004; Magot et al., 2000). Samples from oil-field reservoirs are usually taken from the well-head or at subsequent points in the production pipeline and the possible sources of sample contamination are therefore numerous (Magot et al., 2000; McInerney and Sublette, 1997). Contaminants can also be introduced into the production systems and oil-wells through the drilling process, well operations and from water re-injection of the wells. The frequent recovery of certain thermophiles, including members of the order Thermotogales, the fermentative genera *Thermoanaerobacter* and *Thermoanaerobacterium* and gram-positive sulphate-reducing *Desulfotomaculum* species from geographically widely separated high-temperature reservoirs, is taken as evidence for an indigenous microbial community in these habitats. This is also supported by the fact that members of the genera *Geotoga*, *Petrotoga* and *Thermovirga* have only been obtained from oil-well produced waters (Dahle and Birkeland, 2006; Davey et al., 1993; Lien et al., 1998; Miranda-Tello et al., 2007; Miranda-Tello et al., 2004). Subsurface microbial communities could represent a major part of the biosphere and

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possibly serve as analogous models for extraterrestrial life (Fredrickson and Balkwill, 2006). The main primary energy source for this microbial community is still uncertain, and information on the biogeochemical cycling of carbon and other nutrients is very limited. In reservoirs with temperatures below 80°C, the hydrocarbons tend to have been partially biodegraded over geological time scales (Head et al., 2003), but up to now, microorganisms capable of degrading hydrocarbons anaerobically under *in situ* conditions have not been identified. A number of microbial diversity analyses of high-temperature oil reservoirs using cultivation-independent methods have been carried out (Dahle et al., 2008; de Oliveira et al., 2008; Kaster et al., 2009; Kotlar et al., 2011; Li et al., 2007a; Li et al., 2006; Li et al., 2007b; Orphan et al., 2000; Sette et al., 2007), that basically confirm the presence of a diverse community including extremophilic populations unique to oil-bearing strata. Here we describe two thermophilic Gram-positive anaerobes belonging to the genus *Thermoanaerobacter* isolated from hot produced water from the Thar Jath oil-field in South Sudan. Members of this genus are common in oilfields worldwide and thus represent interesting organisms for analysis of bio-geographical structuring and special adaptations to this extreme deep biosphere habitat.

MATERIALS AND METHODS

Enrichment and isolation

Produced water from the Thar Jath oil-field (Block 5A) in the Unity State, South Sudan, was sampled from a sedimentation tank in December 2006. The water was collected in 100 ml sterile and anaerobic serum bottles and transported to Bergen, where they were kept at ~5°C. The reservoir, with an *in situ* temperature of ~70°C, had not been injected with any chemicals or reinjection water and had been operating for 6 months prior to sampling. Enrichment and growth was performed using an anaerobically prepared basal mineral salts medium (MMF) containing the following components (l^{-1} distilled water): 3 g NaCl, 0.7 g $MgSO_4$, 0.37 g KCl, 0.16 g NH_4Cl, 0.16 g $CaCl_2$, KH_2PO_4, 1 ml trace element solution SL-10 (Widdel et al., 1983), and 0.5 ml resazurin (0.02%). Following autoclaving in a dispenser (Lien and Beeder, 1997) and cooling to ~60°C under continuous flushing with dinitrogen, the medium was reduced by addition of 4 ml 0.5 M Na_2S (Lien et al., 1998). 10 ml of a vitamin solution (Balch et al., 1979) was added and the pH was adjusted to 6.8 with 1 M HCl. The medium was dispensed into 50 ml serum bottles. Substrates that are peptone, yeast extract and dextrin were added from stock solutions to give final concentrations of 0.3% (w/v) each prior to inoculation with 10% produced water. Pure cultures were obtained by dilution series using the shake tube culture method (Widdel and Pfennig, 1984) with anoxic Gelrite gellan gum (0.3%; Merck) as gelling agent.

Microscopy

Cells were observed with an Eclipse E400 (Nikon) phase microscope to determine purity, morphology and Gram staining.

Growth and metabolism

Growth was determined by monitoring increase in OD_{600} and total cell counts. For testing of substrate utilization, a fresh overnight culture that had been passed for at least five times in MMF containing 0.05% yeast extract (Sigma), was used as inoculum (10%). Growth experiments were performed in triplicates in MMF using 0.5% (w/v, final concentration) of the substrates as listed in Table 1, except for the following: yeast extract was used at 0.25% final concentration, lactate, sodium acetate and pyruvate at 40 mM. The polysaccharides used as substrates were maltodextrin from maize (Dextrin 20, Fluka), microcrystalline α-cellulose (Sigma) and xylan from birchwood (Sigma). Organic acids and alcohols, and hydrogen and carbon dioxide were determined using an HP 5890A and HP 6890 GC, respectively (Dahle and Birkeland, 2006). Growth rates at 45, 55, 65, 70, 75 and 80°C, and optimal pH and salinity, were determined in triplicates in MMF medium containing 0.5% glucose. Iron reduction was tested in medium supplemented with amorphic Fe(III) oxide (Slobodkin and Wiegel, 1997).

16S rRNA gene sequence determination and analysis

Genomic DNA was isolated using the cetyltrimethylammonium bromide method as modified by Lien et al. (1998). The 16S rRNA sequence was amplified using PCR with the universal primers, 5'-AGA GTT TGA TCC TGG CTC AG-3' (*Escherichia coli* 16S rRNA positions 8 to 27) and 5'-AAG GAG GTG ATC CAG CCG CA-3' (*E. coli* 16S rRNA positions 1541 to 1522) (Loffler et al., 2000). The PCR was performed with an initial denaturation at 96°C for 3 min, followed by 30 cycles of denaturation at 96°C for 45 s, annealing at 55°C for 30 s, extension at 72°C for 2.5 min and, finally, an extension at 72°C for 10 min. Purification and sequencing of the PCR product were performed as described by Dahle and Birkeland (2006).

The 16S rRNA gene sequences were compared with other sequences in the GenBank database using BLAST (Altschul et al., 1997). Alignments were made using CLUSTAL X (Thompson et al., 1997). The phylogenetic tree was constructed using the neighbor-joining algorithm as implemented in MEGA5 (Tamura et al., 2011).

RESULTS

Enrichment and isolation

Following overnight incubation at 70°C with a mixture of peptone, yeast extract and dextrin as substrates, the primary enrichment cultures became strongly turbid, with a dominance of rod- shaped bacteria. Enrichments incubated at 45°C in the same medium did not yield any visible growth. Dilution series prepared in Gelrite yielded single colonies in tubes diluted up to 10^8-fold after 3 – 5 days of incubation. Ten colonies were picked and successfully regrown in liquid medium. They were all pure cultures as revealed with phase contrast microscopy and sequencing of the 16S rRNA genes.

Phylogenetic analyses

The isolates were grouped into 2 OTUs sharing 95.5% sequence identity. Sequence identities within the groups were 100%, demonstrating that each group represented a distinct species. One isolate from each group, termed S1.1 and S3.1 (NCBI accession numbers KC994642 and KC994643, respectively), were chosen for further

Table 1. Substrate utilization range for strains S1.1 and S3.1 in comparison to their closest phylogenetic relatives shown in Figure 1.

Substrate	S1.1	*T. b. b*	*T. b. f*	*T. b. l*	*T. e*	*T. p*	S3.1	*T. i*	*T. m. m*	*T. m. a.*	*T. t.*
Acetate	-	N	N	N	N	N	+	N	N	N	N
Arabinose	-	-	-	-	N	N	+	+	+	+	+
Casamino acids	+	N	N	N	N	N	+	N	N	N	N
Cellobiose	+	N	N	+	+	+	+	+	+	+	+
Cellulose	-	-	-	-	-	-	+	-	-	-	+
D-Fructose	+	N	N	+	+	N	+	+	+	+	+
Galactose	+	+	+	+	+	N	+	+	-	+	+
D-Glucose	+	+	N	+	+	+	+	+	+	+	+
M-Inositol	+	N	N	N	-	N	+	N	-	N	N
Lactate	-	N	N	N	N	N	+	N	N	N	N
Lactose	+	N	N	+	+	N	+	+	+	N	+
Maltodextrin	+	N	N	N	N	N	+	N	N	N	N
Maltose	+	N	N	+	N	+	+	+	N	N	+
Mannitol	+	N	+	+	-	N	+	N	+	-	-
Mannose	+	-	+	+	+	N	+	N	+	N	+
Melezitose	+	N	N	N	-	N	+	+	+	-	-
Melibiose	+	N	N	-	-	N	+	+	+	-	-
Peptone	+	N	N	N	+	N	+	N	N	N	N
Pyruvate	+	N	N	+	+	N	+	N	N	N	N
L-Rhamnose	+	N	N	-	-	N	+	N	-	N	+
D-Ribose	+	N	N	+	+	N	-	N	+	+	-
Salicin	+	N	N	N	N	N	+	N	-	N	+
Sorbitol	+	N	N	N	-	N	+	N	-	N	+
Sucrose	+	N	N	+	+	+	+	+	+	+	-
Xylan	+	+	N	N	N	N	-	N	+	N	+
D-Xylose	+	-	N	+	+	+	+	N	+	N	N
Yeast extract	+	-	N	+	-	N	+	N	N	-	N

*N, not determined; +, growth; – , no growth; *T.m.m., Thermoanaerobacter mathranii* subsp. *mathranii* (Larsen et al., 1997); *T.m.a., Thermoanaerobacter mathranii* subsp. *Alimentarius* (Carlier et al., 2006); *T.t.*, *Thermoanaerobacter thermocopriae* (Collins et al., 1994); *T.i., Thermoanaerobacter italicus* (Kozianowski et al., 1997); *T.e, Thermoanaerobacter ethanolicus* (Wiegel and Ljungdahl, 1981); *T.p., Thermoanaerobacter pseudethanolicus* (Onyenwoke et al., 2007); *T.b.b., Thermoanaerobacter brockii* subsp. *brockii* (Cayol et al., 1995); *T.b.f., Thermoanaerobacter brockii* subsp. *finii* (Cayol et al., 1995); *T.b.l, Thermoanaerobacter brockii* subsp. *lactiethylicus* (Cayol et al., 1995). References are given in brackets. The dotted vertical line separates strain S1.1 and S3.1 and their respective closest relatives. The substrate utilization data for previously described species and subspecies are provided in the above citations.

analysis. They shared 99.3% 16S rRNA gene sequence identity with *Thermoanaerobacter ethanolicus* and *Thermoanaerobacter pseudoethanolicus*, and 99.1% with *Thermoanaerobacter mathranii*, respectively, as closest hits in Blast searches. Construction of a phylogenetic tree encompassing all the validly described species of the *Thermoanaereobacter* genus, placed strain S3.1 in a distinct lineage branching between *T. mathranii* and *Thermoanaerobacter italicus*, with a significant bootstrap value (Figure 1). Strain S1.1 branched within the *Thermoanaerobacter brockiil T. ethanolicus* clade, but with a low bootstrap value.

Morphology

Phase contrast microscopy of both cultures in exponential growth revealed rod-shaped cells, sometimes in chains (Figure 2). The cell size varied from 0.3 - 0.5 µm in width to 1 – 20 µm in length.

Motility was observed for both strains. Strain S3.1 formed highly refractive intra- and extracellular particles that could represent sulphur granules and/or endospores (Figure 2). Strain S1.1 formed terminal spore-like structures. Cultures of both strains survived autoclaving at 121°C for 40 min, indicating endospore-forming capa-

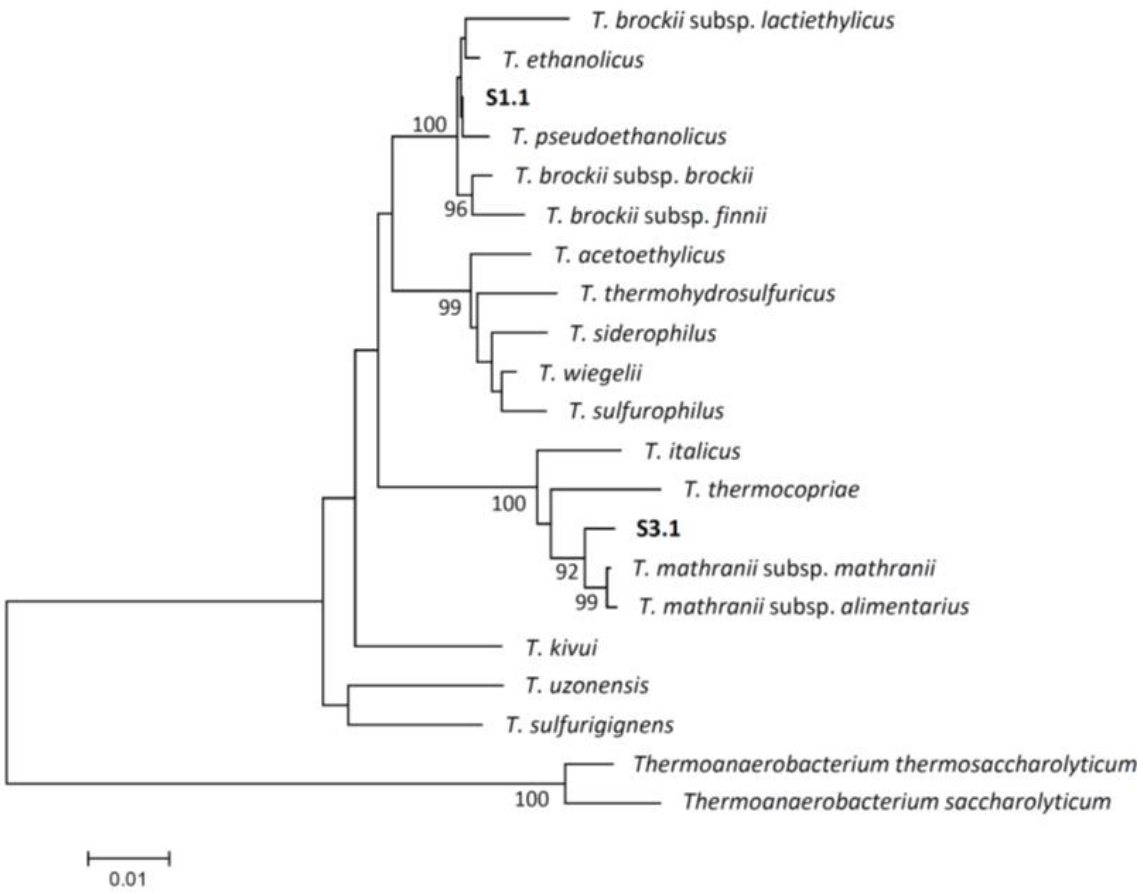

Figure 1. Phylogenetic dendrogram based on 16S rRNA gene sequences indicating the position of strains S1.1 and S3.1 within the *Thermoanaerobacter* genus. Only type strains of validly described species are included. Accession numbers for the sequences used in the analysis are as follows: Strain S1.1, Strain S3.1, *T. acetoethylicus,* L09163*; T. brockii subsp. brockii,* L09165*; T. brockii subsp. finnii,* CP002466; *T. brockii subsp. lactiethylicus,* U14330; *T. ethanolicus,* L09162; *T. italicus,* AJ250846; *T. kivui,* L09160; *T. mathranii subsp. alimentarius,* AY701758; *T. mathranii subsp. mathranii,* Y11279; *T. pseudoethanolicus,* CP000924; *T. siderophilus,* F120479; *T. sulfurigignens,* AF234164; *T. sulfurophilus,* Y16940; *T. thermocopriae,* L09167; *T. thermohydrosulfuricus,* L09161; *T. uzonensis,* EF530067; *T. wiegelii,* X92513. The 16S rRNA gene sequences of *Thermoanaerobacterium saccharolyticum* and *Thermoanaerobacterium thermosaccharolyticum* were used as outgroup. Bootstrap values ≥92% are indicated at nodes. The bar indicates the number of base substitutions per site.

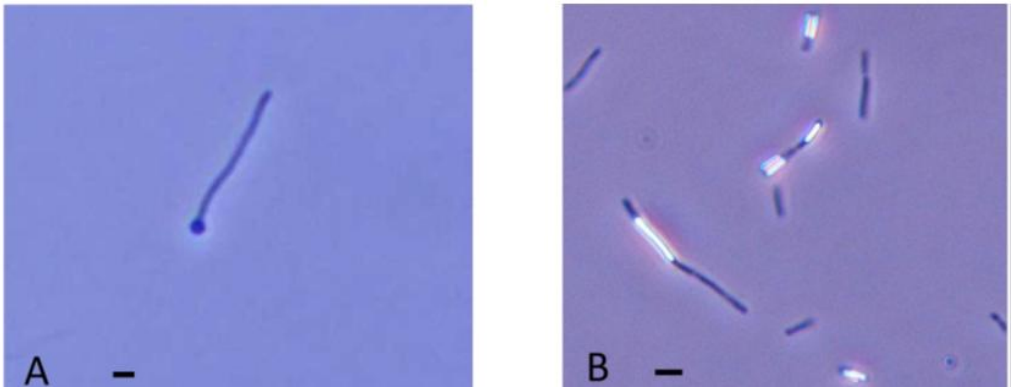

Figure 2. Phase contrast microscopy of (A) strain S1.1 indicating the presence of a terminal spore-like structure, and (B) strain S3.1 indicating the presence of sulphur granules. The size bar indicates 1 µm.

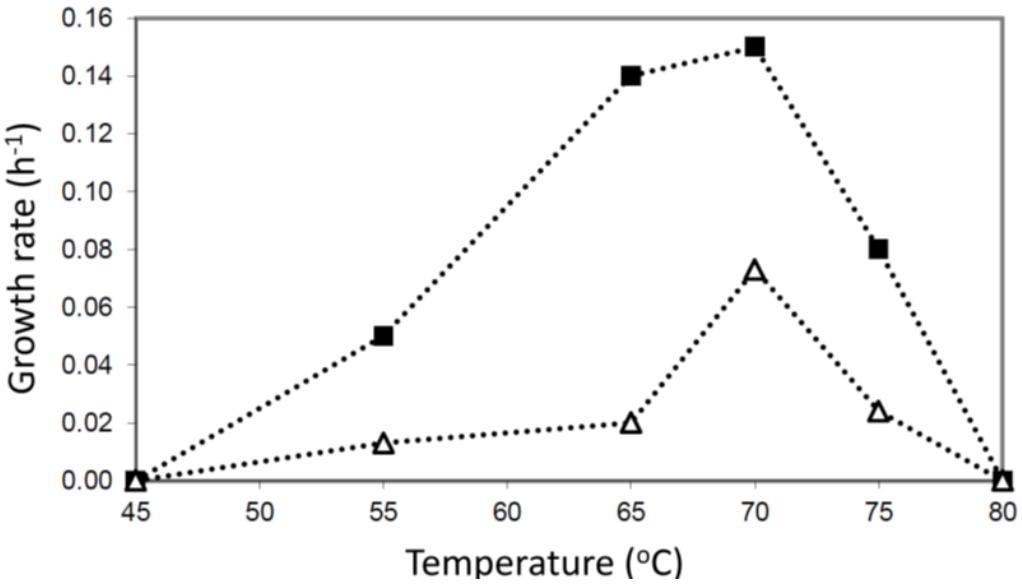

Figure 3. Growth rate of strain S1.1 (Δ) and S3.1 (▪) as a function of temperature in the 45 – 80°C temperature range.

bility. Both strains stained Gram-positive in early exponential growth phase while Gram-variable in late exponential phase and yielded a negative result for the KOH-test (Gregersen, 1978), indicating a Gram-positive cell wall structure.

Physiology

The isolates only grew under strict anaerobic conditions, with optimal growth between 65 - 70°C (Figure 3). Generation times were determined as 2 and 6 h for strain S3.1 and S1.1, respectively, at optimal temperature with glucose as carbon source. The strains were tested for growth on a large variety of carbon sources and were found to be extremely versatile. They could utilize a large number of carbohydrates, proteinaceous compounds and organic acids as substrates (Table 1). Strain S1.1 differed from strain S3.1 in its ability to grow on xylan and D-ribose, and S3.1 from S1.1 in capability to grow on acetate, arabinose, cellulose and lactate. Both strains displayed a fermentative metabolism, producing acetate, ethanol, carbon dioxide and hydrogen as fermentation products when grown on glucose. Growth of strain S3.1 was weakly stimulated by the addition of thiosulphate, which led to formation of suphur granules (Figure 2B). When strain S1.1 was grown on pyruvate, addition of Fe_2O_3 stimulated growth significantly, and the cultures yielded a strongly paramagnetic precipitate (Figure 4), indicating the capability of an iron-reducing anaerobic respiration. Growth of strain S3.1 was only slightly stimulated by Fe_2O_3 addition, and the development of paramagnetism was marginal.

DISCUSSION

The two *Thermoanaerobacter* spp. strains, designated S1.1 and S3.1, isolated from produced water from the deep and hot That Jath oil-field in South Sudan showed distinct but highly diverse substrate utilization patterns (Table 1). Out of 27 substrates tested, growth was observed on 21 substrates for both strains. Six substrates: acetate, arabinose, cellulose, lactate, ribose and xylan, were differentially utilized. A phylogenetic analysis placed strain S3.1 in a branch most closely related to *T. mathranii*, while the phylogenetic affiliation of strain S1.1 was less clear except for a close relationship to *T. ethanolicus* and *T. pseudoethanolicus* (Figure 1). S3.1 differs from *T. mathranii* subsp. *mathranii* and *T. mathranii* subsp. *alimentarius* by its ability to utilize cellulose, while many of the other substrates have not been tested for the *T. mathranii* subspecies, and a firm differentiation between *T. mathranii* and S3.1 based on substrate utilization thus cannot be made. Although strain S1.1 is phylogenetically closely related to *T. ethanolicus*, it differs significantly from the latter in substrate utilization pattern. S1.1, in contrast to *T. ethanolicus*, can utilize M-inositol, mannitol, melezitose, melibiose, L-rhamnose, sorbitol and yeast extract for growth. However with regards to substrate utilization, *T. pseudoethanolicus* has not been adequately characterized to be properly compared with strain S1.1. The results of phylogenetic and physiological analyses of S1.1 and S3.1 indicate that these isolates represent novel species, although a more thorough comparison with their closest relatives, using techniques such as genomic DNA: DNA hybridization

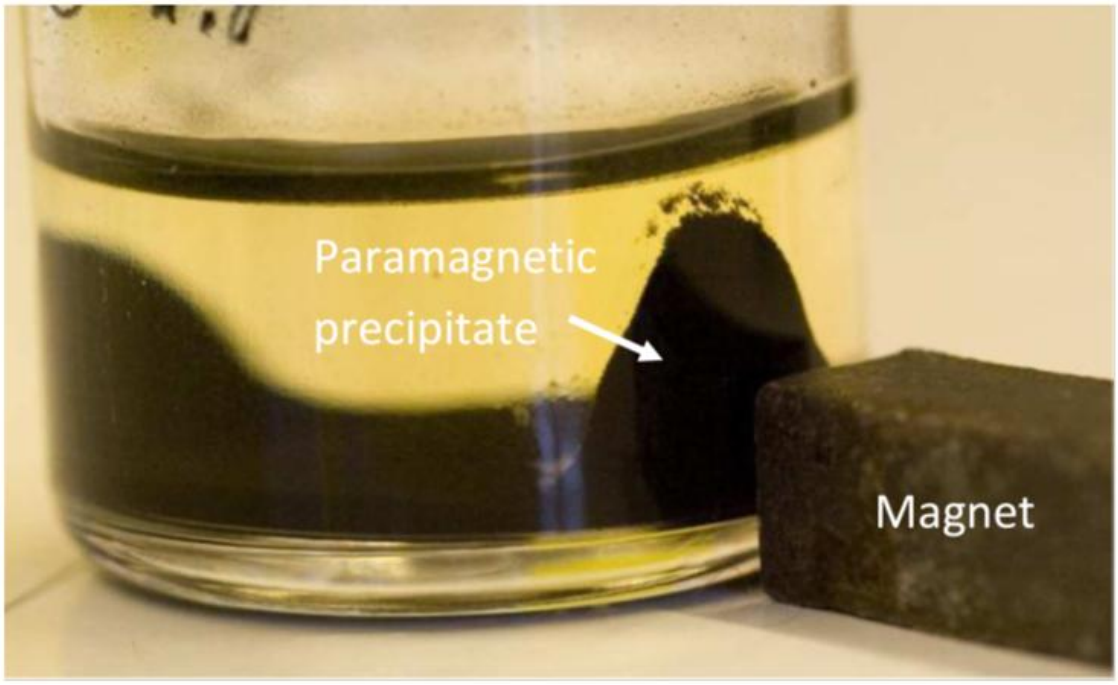

Figure 4. Demonstration of paramagnetic precipitate in a culture of strain S1.1 supplemented by Fe_2O_3.

and further substrate analyses are needed to provide further evidence. *Thermoanaerobacter* spp. growing at *in situ* temperatures have been recovered from oil wells around the world (Cayol et al., 1995; Fardeau et al., 2000; Grassia et al., 1996; Lan et al., 2012; Slobodkin et al., 1999) and is thus believed to represent one of the indigenous members of this subsurface community. Due to their physiological properties and potential capability for biological activities *in situ*, a mixed anaerobic culture dominated by *Thermoanaerobacter* spp. has been successfully used for microbially enhanced oil recovery (MEOR) of heavy oil fractions in an experimental simulated reservoir condition setting (Castorena-Cortes et al., 2012a; Castorena-Cortes et al., 2012b). Production of surface-active agents, solvents and gases, and degradation of heavy hydrocarbons by this mixed culture represent highly useful feature (Castorena-Cortes et al., 2012a). Thermoanaerobacters have also been noted for their anti- corrosion behaviour through their ability to reduce thiosulphate to elemental sulphur without sulphide formation (Lan et al., 2012). The above features underpin the importance of further analysis of the diversity of this microbial group due to its potential roles and applications in petroleum-related biotechnology. Moreover, magnetite biogenically formed by *Thermoanaerobacter* spp., a property confirmed for strain S1.1, also has potential as a biosignature for thermophilic iron-reducing bacteria in the deep hot biosphere as well as for the emergence of iron respiration on the early Precambrian earth (Li, 2012).

Conclusion

As part of an investigation of the bacterial diversity of the Thar Jaht oil-field in South Sudan, heterotrophic anoxic enrichments on a rich organic medium using produced water as inoculum, were made at reservoir *in situ* temperature of ~70°C. Two fermentative thermophilic anaerobes phylogenetically related to the genus *Thermoanaerobacter* were isolated and characterized. They utilize a variety of carbohydrates, proteinaceous compounds and organic acids, and may represent novel species. Reduction of thiosulphate to elemental sulphur by one of the isolates is a feature of interest for biological control of corrosion. One of the isolates efficiently reduced Fe(III) oxide. Further analysis is required to establish the exact taxonomic status and potential applications of the isolates.

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Full Length Research Paper

In vitro antimicrobial activity of *Ruta chalepensis* methanol extracts against the cariogenic *Streptococcus mutans*

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Medicinal plants have been used for centuries and have become part of complementary medicine worldwide because of their potential health benefits. Since dental caries is one of the most common oral diseases and is considered a major public health problem, the present study evaluated *in vitro* antimicrobial potential of methanol extracts of *Ruta chalepensis* against the major etiologic agent of dental caries, *Streptococcus mutans*. The antimicrobial effect of *R. chalepensis* was evaluated in liquid medium by the dimethylthiazol-diphenyltetrazolium bromide (MTT) reduction colorimetric assay and in solid medium by the determination of colony forming units (CFU). We found that the minimum inhibitory concentration (MIC) was 250 µg/mL (p <0.05) in liquid medium and 3.9 µg/mL (p <0.05) in solid medium.

Key words: Antimicrobial agents, plant extracts, *Ruta chalepensis*, dental caries, *Streptococcus mutans.*

INTRODUCTION

Infectious diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide. The importance of plants to modern medicine is recognized; for a long time, natural remedies and medicinal plants were the main or even the only resource for the physicians. For all cultures and in all times, medicinal plants have been used as the basis of their own medicine (Nuñez, 1982). Among the many diseases afflicting the world's population, infections, both bacterial and fungal diseases with inflammatory processes, which in some cases incapacitate the sufferer, represent a major group (Drusano, 2004). There are several plants of the Mexican medicinal plants that exhibit antimicrobial activities and are used in treating various human diseases such as burns, diabetes, anti-inflamatory, skin diseases and hypertension (Pushpam, 2004).

Oral diseases are still a major health problem worldwide (Petersen et al., 2005). Problems such as oropharyngeal cancer and soft tissue injuries are considered as oral health problems, however, dental caries and periodontal disease are considered the most important ones (Petersen, 2003). Dental caries is a transmissible infectious disease that remains as a major public health problem in many developing countries and disadvantaged populations of developed countries (Mattos et al., 1998).

Streptococcus mutans is considered one of the main

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etiological agents of dental caries. The World Health Organization (WHO) estimates that five billion people worldwide suffer from tooth decay, which affects 60 to 90% of the school population and the vast majority of adults in developed countries. In Mexico it is estimated that 44% of the population have cavities or oral diseases that affect their health (Petersen, 2003).

In the present study, leaf methanol extracts of *Ruta chalepensis*, a plant of the Rutaceae family, was tested on *St. mutans* growth, the major etiologic agent of dental caries, which might be important for the development of alternative treatments in dental health.

MATERIALS AND METHODS

Preparation of *Ruta chalepensis* leaf methanol extract

R. chalepensis leaves were collected in the city of Aramberri in the State of Nuevo Leon, Mexico (Latitude: 24° 06´ 05´´ N, Longitude: 99° 51´19´´ W), they were rinsed to remove traces of dust and insects material, and allowed to dry at 37°C for five days; then the plant material was pulverized and stored in 50-mL Falcon tubes. Five grams of pulverized material were transferred to a 10 x 10 cm gauze and a pouch was formed, securing it with a wire to the rim of a beaker where 80 ml of methanol were placed, which were in contact with the pulverized leaves in the gauze. The methanol extraction was facilitated by stirring with a magnetic bar in a magnetic stirrer (Laboratory Stirrer PC-410, Corning, NY), and allowed to mix for 24 h at room temperature. One milliliter of the extract was distributed in Eppendorf tubes, previously weighed, and then they were dried in a vacuum concentrator (CentriVap Desiccator Labconco) for 4 h. Extracts were dissolved in culture medium and adjusted to experimental concentrations.

Effect of *R. chalepensis* methanol extract on *S. mutans* growth

Fifty microliters of 1 x 10^3 *S. mutans* bacteria/ml suspensions were plated in brain heart infusion broth (BHI) medium (Remel, Lenexa, KS), in flat-bottomed 96-well plates (Corning Incorporated, Corning, NY), in the presence or absence of serial dilutions (1:2) of 50 µl of *R. chalepensis* methanol extract), antibiotic control (1 µg/ml tetracycline), plant extract free-methanol vehicle control and culture medium (vehicle control was similarly processed as with plant methanol extractions, but without plant material). Plates were then incubated for 6 h at 37°C, after which the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO) was added to all wells at a final concentration of 0.5 mg/ml in saline solution, and plates were incubated for 4 additional hours. At the end of the incubation period, 50 µl of extraction buffer [this buffer was prepared by dissolving 20% (wt/vol) sodium dodecyl sulfate (SDS) at 37°C in a solution of 50% each N,N-dimethylformamide (DMF) and demineralized water, and the pH was adjusted to 4.7 (SDS and DMF were purchased from Sigma-Aldrich)] were added to all wells and plates were incubated for 16 h at 37°C (Yamato IC600 incubator); optical densities resulting from dissolved formazan crystals were then read in a microplate reader (Beckman Coulter, Inc., Fullerton, CA) at 570 nm (Gomez-Flores et al., 1995). In regard to CFU determination, 50 µl of 1 x 10^3 *S. mutans* bacteria/ml suspensions were plated in BHI broth medium, in flat-bottomed 96-well plates (Corning Incorporated), in the presence or absence of serial dilutions (1:2) of the *R. chalepensis* methanol extract (50 µl), antibiotic control (3 mg/ml tetracycline), and vehicle controls (methanol and culture medium); the vehicle controls were similarly processed as with plant methanol extractions, but without plant material, similarly as mentioned above. Then, 1:10,000 dilutions were prepared from the wells and 100 µl were plated on BHI agar plates (Becton Dickinson, Mexico, D.F.) using sterile bent glass rods. Agar plates were then incubated at 37°C for 24 h and colonies were counted in a colony counter (ULB-100, Lightbox 37864-2000, Scienceware BEL-ART products, Pequannock, NJ) (Kansal et al., 1997).

RESULTS AND DISCUSSION

Inhibition of *Streptococcus mutans* growth by *Ruta chalepensis* methanol extracts

R. chalepensis methanol extract showed MICs of 250 µg/ml and 3.9 µg/ml, and induced a maximum of 63 and 94% growth inhibition against *S. mutans*, as measured by the MTT reduction (Figure 1a) and CFU methods, respectively (Figure 1b), whereas the vehicle control and medium alone (both free from plant extract) did not alter bacterial growth (data not shown). Medicinal plants have become part of alternative medicine worldwide because of their potential health benefits. These plants can be consumed or directly applied to treat infections (Rojas et al., 2006). Compounds synthesized by plants have a wide therapeutic potential due to their chemical constituents, for which the evaluation of their biological activity is important to develop new and alternative products with pharmacological potential and to validate treatments traditionally used by the Mexican population and other people from developing countries (Rodriguez-Fragoso, 2008).

Because of the increasing resistance of many pathogens to common therapeutic agents used today, such as antibiotics and antiviral agents, there is a renewed interest in the discovery of new compounds to treat systemic and oral diseases (Chinedum, 2005; Moreillon, 2000; Russell, 2000). Plant antimicrobials are not commonly used in a health program because of their low activity, unless their MICs are in the range of 0.1 to 1 mg/ml (Drusano, 2004); thus, the results of the present study may be an indication of an important antibiotic activity of *R. chalepensis* extracts.

Dental caries, periodontal disease, and tooth loss affect most of the population and can alter overall health. The value of medicinal plants to treat cariogenic bacteria is well known (Ramakrishna et al., 2011) and antimicrobial phytochemicals capable to treat oral diseases have been reported by many. In this regard, allicin from garlic has been shown to have antimicrobial activity against oral bacteria such as *Streptococcus mutans, Streptococcus sobrinus, Actinomyces oris, Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* (Bachrach et al., 2011). In addition, compounds such as oleanolic acid, oleanolic aldehyde, linoleic acid, linolenic acid, betulin, betulinic acid, 5-(hydroxymethyl)-2-furfural, rutin, beta-sitosterol, and beta-sitosterol glucoside from raisins were shown to suppress *in vitro* adherence of *S. mutans* biofilm (Wu, 2009). Furthermore, *Dryopteris*

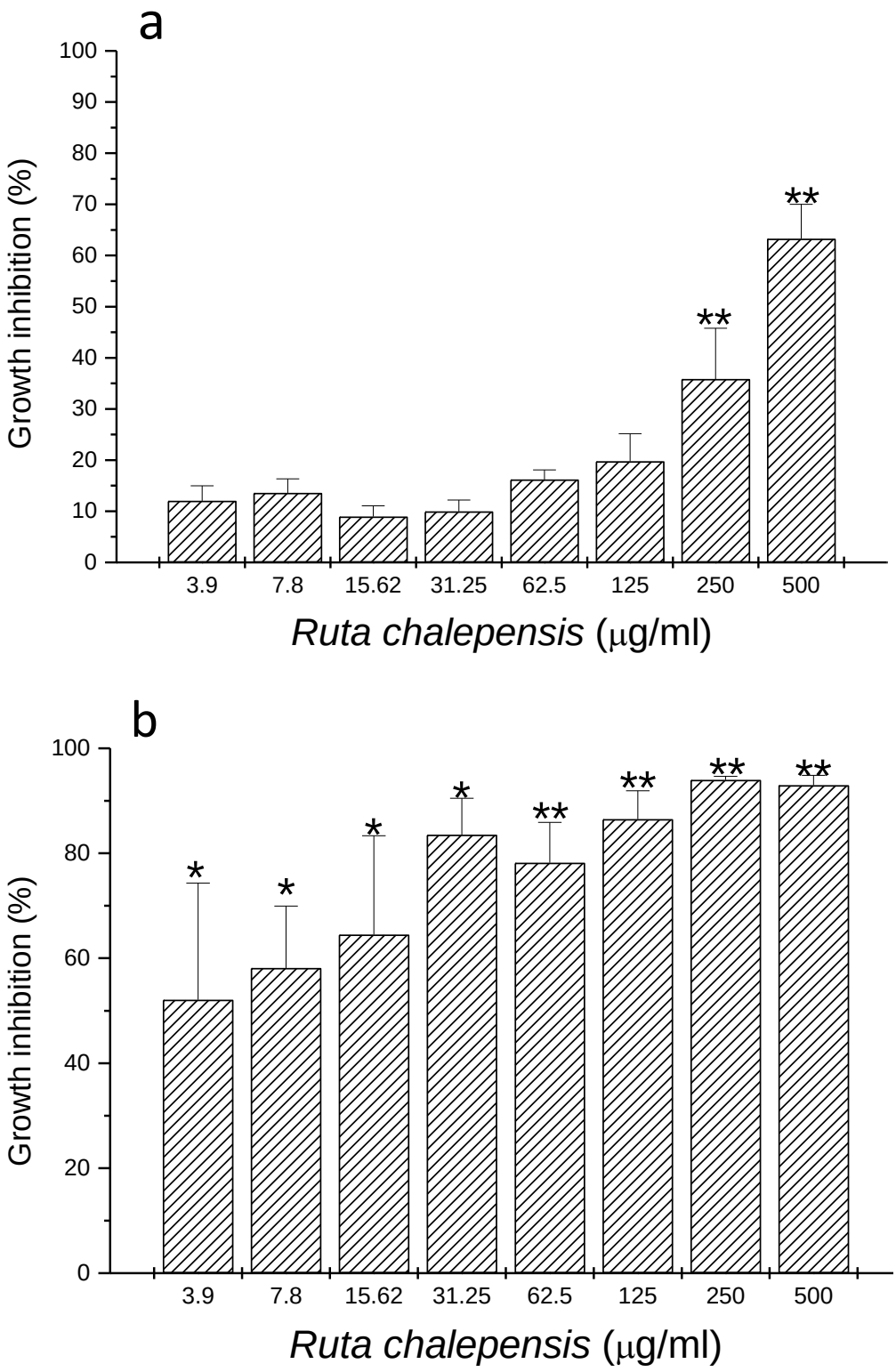

Figure 1. Antimicrobial effect of *R. chalepensis* leaves methanol extract on *S. mutans* (ATCC UA130 serotype *c*) growth. *S. mutans* culture suspensions ($1x10^3$ bacteria/ml) were incubated in the presence or absence of various concentrations of *R. chalepensis* methanol extract, after which growth was measured by the MTT reduction (*a*) and CFU (*b*) methods. Data represent means ± SEM of triplicate determinations from three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ when compared with *R. chalepensis* extract-untreated control. Optical density at 570 nm for untreated cells was 0.59 ± 0.05 for the MTT reduction technique, whereas CFU control value for untreated cells was $5.8 \times 10^8 \pm 68 \times 10^6$.

crassirhizoma and *Aloe vera* extracts were reported to have bactericidal and bacteriostatic activity against *S. mutans* (Ban et al., 2012; Fani and Kohanteb, 2012),

To our knowledge, this is the first report showing that *R. chalepensis* methanol extracts inhibit *S. mutans* growth *in vitro*. There are still a number of plant compounds that remain to be evaluated at the molecular, cellular and physiological levels for their potential to treat human diseases.

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Characterization of antimicrobial resistance and related resistance genes in *Escherichia coli* strains isolated from chickens in China during 2007-2012

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In the present study, the prevalence of antimicrobial-resistant chicken *Escherichia coli* strains and the resistance genes in *E. coli* was investigated. For this purpose, 1002 chicken *E. coli* strains isolated from layer and broiler flocks in Shaanxi, Henan and Gansu provinces in China during 2007-2012 were examined. Antimicrobial susceptibility of these *E. coli* strains against 18 antimicrobials was determined by the Kirby-Bauer disk diffusion method. Eight out of the twenty antimicrobial resistance genes were detected by polymerase chain reaction (PCR). The sequences of the resistance genes in chicken *E. coli* strains were compared with the previously published sequences. Our results revealed that the antimicrobial resistance prevalence of *E. coli* strains in western China to ampicillin, doxycycline, tetracycline and nalidixic acid were consistently kept at 62-100%. The *E. coli* resistance to nalidixic acid and ciprofloxacin had an increasing trend, as high as 100% for nalidixic acid while the resistance prevalence to gentamicin had a decreasing trend. The detection rates of the genes for *tetA*, *tetB*, *blaTEM,* and *aac(3)-II* in chicken *E. coli* strains were positively correlated with their antimicrobial resistance (P <0.01) during 2007-2012. Among 1002 chicken *E. coli* strains tested, all *E. coli* strains were resistant to more than three kinds of antimicrobials. Our results revealed that 499 of the 1002 (49.8%) chicken *E. coli* strains were resistant to more than eight kinds of antimicrobials. Considering all the 1002 isolates, the detection prevalence of the genes for *tetA*, *tetB, blaTEM* in chicken *E. coli* strains were constantly over 88.9%. The detection prevalence of the genes for *floR*, *sul-I* and *cmlA* in chicken *E. coli* strains increased, while *aac(3)-II* declined from 75.0 to 28.6%.

Key words: *Escherichia coli*, antibiotic resistance, antibiotic resistance genes, polymerase chain reaction (PCR), chicken.

INTRODUCTION

Escherichia coli is one of the common pathogens in chicken production. For a long time, antibiotics have been widely used in the treatment and prevention of colibacillosis, even increasingly being used as animal growth promotion agents (Sarmah et al., 2006; Martinez, 2009). *E. coli* resistance rises with the selective pressure of the antibiotics, and then reduces the clinical efficacy of antibacterial drugs and increase the mortality of sick animals,

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Table 1. *E. coli* strains isolated from chickens in three provinces in China during 2007-2012.

Year	Number of total strains/number of strains from each source							
	Shaanxi Province			Henan Province		Gansu Province		Total
	Xi'an	Tongchuan	Yangling	Sanmenxia	Luoyang	Tianshui	Dingxi	
2007	14	20	16	18	22	24	20	134
2008	18	22	20	20	22	22	24	148
2009	12	20	16	16	18	17	16	115
2010	18	20	20	20	25	26	16	145
2011	22	42	24	25	20	26	24	183
2012	30	63	44	34	32	40	34	277
2007-2012	114	187	140	133	139	155	134	1002

thereby causing economic losses (Dho-Mouline and Fairbrother, 1998; Barnes et al., 2008; Pan et al., 2009). Avian pathogenic *E. coli* (APEC) cause aerosacculitis, polyserositis, septicemia and other mainly extraintestinal diseases in chickens, turkeys and other avain species (Dho-Moulin and Fairbrother, 1998). This disease results in significant morbidity and mortality, which gives rise to multimillion-dollar annual losses for all facets of the world's poultry industry. Plasmid-mediated antibiotic resistance genes are an important mechanism of resistance in *E. coli.* The resistance genes are not only vertical to offspring but also horizontally transmitted between different microbes, potentially affecting human health and causing economic loss in the breeding industry (Collignon and Angulo, 2006; James et al., 2007; Ben et al., 2010; Lu et al., 2010). In recent years, many reports have been published on the plasmid-mediated β-lactams, aminoglycosides, chloramphenicol, sulfonamides, quinolones and tetracyclines in *E. coli*, but few reports have been published regarding chicken sources of *E. coli* resistance and resistance genes. Therefore, study of *E. coli* resistance and resistance genes in chickens is of great significance to public health (Collignon and Angulo, 2006).

In previous studies, it was found that quinolones and the first-generation of cephalosporins resistant strains occurred in the 1990s and drug-resistant strains to the third-generation of cephalosporins were found in 2003 (Li et al., 2010). The detection prevalence of resistance gene (*blaCTX-M*) was up to 75% (Li et al., 2010). Furthermore, previous study also suggested that the majority of *E. coli* strains from swine in China were resistant to streptomycin, chloramphenicol, norfloxacin and doxycycline, showing that the detection prevalence of the genes for *cmlA* and *floR* were 65 and 57%, respectively (Wang et al., 2011). The chicken *E. coli* strains in South Australian were found to be resistant to tetracycline, ampicillin, cotrimoxazole, streptomycin and neomycin, having the detection prevalence of the genes for *tetA* (19.1%) and *blaTEM* (17.1%) (Obeng et al., 2012). Karah et al. (2010) studied the plasmid-mediated quinolone resistance gene in *E. coli* strains isolated from human in Norway and Sweden. Among isolates that were ESBL producers and were resistant to nalidixic acid and/or had reduced susceptibility to ciprofloxacin, the detection prevalences of the genes for *qnr* and *aac (6')-ib-cr* genes were 9.1 and 52.3%, respectively (Karah et al., 2010).

The aim of this study was to investigate the occurrence of antimicrobial resistance of *E. coli* strains and the correlation between plasmid-mediated resistance genes and antimicrobial resistance of *E. coli* strains isolated from chickens in Shaanxi, Henan, and Gansu provinces in China during 2007-2012.

MATERIALS AND METHODS

Source of strains

One thousand and two *E. coli* strains were isolated from liver samples of sick and dead layer and broiler flocks (Hy-Line Variety Brown) from Xi'an, Tongchuan City, Yangling Demonstration Zone in Shaanxi province; Sanmenxia and Luoyang Cities in Henan province; Tianshui, Dingxi Cities in Gansu province in China during 2007-2012. Samples were collected with visible enlargement of the liver, pericarditis, bladder inflammation and peritonitis during necropsy. The isolation information and *E. coli* source and distribution in three provinces are shown in Table 1. *E. coli* standard strain ATCC25922 was kindly provided by preventive veterinary medicine laboratory of Northwest Agriculture and Forestry University, China.

Medium, susceptibility paper and reagents

MacConkey agar and nutrient agar were purchased from Beijing Aobo Star Biotechnology Co. Bacteria trace biochemical reaction tubes were purchased from Hangzhou Tianhe Microorganism Reagent Co. Eighteen kinds of antibiotics susceptibility papers, including ampicillin (AMP), amoxicillin (AMX), ceftazidime (CAZ), cefotaxime thiophene (CEF), new neomycin (NEO), streptomycin (STR), gentamicin (GEN), kanamycin (KAN), tobramycin (TOB), amikacin (AMK), florfenicol (FFC), tetracycline (TET), doxycycline (DOX), trimethoprim sulfamethoxazole (SXT), nalidixic acid (NAL), norfloxacin (NOR), ciprofloxacin (CIP) and ofloxacin (OFZ) were purchased from Hangzhou Tianhe Microorganism Reagent Co. PCR Master Mix (containing Taq DNA polymerase, dNTP, PCR buffer) and DNA Marker DL2000 were purchased from TaKaRa Biotechnology (Dalian) Co. Plastic recycling kit was purchased from TianGen bio-technology(Beijing) Co.

Table 2. Primers used for PCR amplification of resistance genes in chicken *E. coli*.

Detected gene	Description	Fragment sizes (bp)	Primer sequences	Accession number	Position
blaSHV F[a]	β-lactams	450	CGCGAGCGGCTCATACAGG	GU732836	350-367
blaSHV R[b]			TCGTCGGGCAGCGTTTCT		778-799
blaCTX-M F	β-lactams	301	ACACGTCAACGGCACAATG	AB545872	323-341
blaCTX-M R			GAGCCACGTCACCAACTGC		605-623
blaCMY-2 F	β-lactams	470	GGGAGCTTGCCACCTACAGC	AF373218	392-411
blaCMY-2 R			CCCGCCTACCGAGTAATGC		843-861
blaTEM F	β-lactams	293	CGGTATTATCCCGTGTTG	GU550123	374-391
blaTEM R			GTCGTTTGGTATGGCTTC		649-666
aac(3)-IV F	Aminoglycosides	357	GCCGTGGTTGGCTTGTAT	EU784153	3169-3186
aac(3)-IV R			CGTTCTCGAAATCAGCTCTTG		3505-3525
aac(3)-II F	Aminoglycosides	412	GGCGACTTCACCGTTTCT	FQ482074	344-361
aac(3)-II R			GGACCGATCACCCTACGAG		737-755
ant(3')-I F	Aminoglycosides	400	GACATTGATCTGGCTATCTTGCTG	JN108887	382-405
ant(3')-I R			CTACCTTGGTGATCTCGCCTTTC		759-781
aph(3)-II F	Aminoglycosides	325	TTGCTCGGAAGAGTATGAA	JN609224	193-211
aph(3)-II R			GCCACTTACTTTGCCATCT		499-517
sul-I F	Sulfonamides	925	TCGGACAGGGCGTCTAAG	EU598449	1801-1818
sul-I R			GGGTATCGGAGCGTTTGC		2708-2725
sul-II F	Sulfonamides	792	CTTGCGGTTTCTTTCAGC	JX869967	11-28
sul-II R			CATCATTTTCGGCATCGT		785-802
cmlA F	Chloramphenicols	467	GGGTGGCGGGCTATCTTT	HM175865	2057-2074
cmlA R			GCGACACCAATACCCACTAG		2504-2523
floR F	Chloramphenicols	601	GAACACGACGCCCGCTAT	AY775258	665-682
floR R			TTCCGCTTGGCCTATGAG		1248-1265
cat-I F	Chloramphenicols	307	GTCAGTTGCTCAATCTACCTAT	AB670687	138-159
cat-I R			ACCGTAAGACGCCACATC		427-444
qnrA F	Quinolones	633	ATTGATAAAGTTTTTCAGCAAGAGG	EU195836	10-34
qnrA R			TATTACTCCCAAGGGTTCCAGC		621-642
qnrB F	Quinolones	427	CTATGATCGTGAAAGCCAGAAAGG	EU093091	171-194
qnrB R			CCGAATATCTAAGTCACCCAACTCC		573-597
qnrS F	Quinolones	300	ATCGAAGGCTGCCACTTT	EF571010	40-57
qnrS R			TGATGCACCCGCTAGGTT		322-339
aac(6')-ib-cr F	Quinolones	679	TGACCTTGCGATGCTCTAT	HM175873	76-94
aac(6')-ib-cr R			GGCTTACTTGTCTGCGTTCTT		734-754
tetA F	Tetracyclines	344	TTGGCATTCTGCATTCACTCG	FJ794040	117-137
tetA R			CCACCCGTTCCACGTTGTT		442-460
tetB F	Tetracyclines	388	TTCACCGCATAGTCCCTT	FJ917423	237-254
tetB R			TGCAATAAATCCGAGCAG		607-624
tetC F	Tetracyclines	427	TCACTATGGCGTGCTGCTA	JQ966989	15-33
tetC R			GCTGTCCCTGATGGTCGT		875-892

[a] Forward; [b]Reverse.

Antimicrobial susceptibility test

The *E. coli* strains were tested for susceptibility to 18 antimicrobial drugs by Kirby-Bauer disk diffusion method on ordinary agar plates. The standard procedure of the clinical and laboratory standards institute guidelines were strictly followed throughout the testing procedure and the determination of results (CLSI, 2008a, b). The criteria for a drug to be classified as resistant or sensitive were judged as described previously (CLSI, 2008a, b).

Primers for amplification of resistance genes in chicken *E. coli* strains

The twenty sets of primer pairs (Table 2) used for polymerase chain reaction (PCR) amplification of β-lactams, aminoglycosides,

Table 3. Antimicrobial resistance of *E. coli* isolated from chickens of Shaanxi province during 2007-2012.

Antimicrobial	Percentage of resistance % (number of resistant strains)						
	2007 (n=50)	2008 (n=60)	2009 (n=48)	2010 (n=58)	2011 (n=88)	2012 (n=137)	2007-2012 (n=441)
AMP	100.0(50)	100.0(60)	100.0(48)	100.0(58)	100.0(88)	100.0(137)	100.0(441)
AMX	26.0(13)	28.3(17)	12.5(6)	29.3(17)	0.0	6.6(9)	14.1(62)
CAZ	50.0(25)	71.7(43)	87.5(42)	89.7(52)	77.3(68)	80.3(110)	77.1(340)
CEF	26.0(13)	0.0	12.5(6)	10.3(6)	9.1(8)	13.1(18)	11.6(51)
NEO	50.0(25)	28.3(17)	25.0(12)	20.7(12)	0.0	0.0	15.0(66)
STR	88.0(44)	71.7(43)	75.0(36)	39.7(23)	38.6(34)	33.6(46)	51.2(226)
GEN	76.0(38)	56.7(34)	50.0(24)	50.0(29)	46.6(41)	53.3(73)	54.2(239)
KAN	20.0(10)	15.0(9)	12.5(6)	17.2(10)	35.2(31)	37.2(51)	26.5(117)
TOB	12.0(6)	28.3(17)	12.5(6)	10.3(6)	15.9(14)	19.7(27)	17.2(76)
AMK	12.0(6)	28.3(17)	0.0	10.3(6)	9.1(8)	0.0	8.4(37)
FFC	26.0(13)	28.3(17)	25.0(12)	29.3(17)	38.6(34)	27.0(37)	29.5(130)
TET	70.0(35)	83.3(50)	100.0(48)	100.0(58)	100.0(88)	100.0(137)	94.3(416)
DOX	100.0(50)	100.0(60)	100.0(48)	89.7(52)	92.0(81)	93.4(128)	95.0(419)
SXT	26.0(13)	28.3(17)	12.5(6)	39.7(23)	30.7(27)	27.0(37)	27.9(123)
NAL	62.0(31)	85.0(51)	87.5(42)	89.7(52)	100.0(88)	100.0(137)	90.9(401)
NOR	76.0(38)	85.0(51)	50.0(24)	60.3(35)	77.3(68)	73.0(100)	71.7(316)
CIP	50.0(25)	60.0(36)	62.5(30)	69.0(40)	92.0(81)	93.4(128)	77.1(340)
OFZ	26.0(13)	43.3(26)	50.0(24)	31.0(18)	46.6(41)	59.9(82)	46.3(204)

AMP: Ampicillin; AMX: Amoxicillin; CAZ: Ceftazidime; CEF: Cefalaxime; NEO: Neomycin; STR: Streptomycin; GEN: Gentamicin; KAN Kanamycin; TOB: Tobramycin; AMK: Amikacin; FFC: Florfenicol; TET: Tetracycline; DOX: Doxycycline; SXT: trimethoprim sulfamethoxazole; NAL: Nalidixic aid; NOR: Norfloxacin; CIP: Ciprofloxacin; OFZ: Ofloxacin.

chloramphenicol, sulfonamides, quinolones and tetracycline resistant genes, respectively were designed with Primer5.0 software based on the sequences deposited in GenBank.

All *E. coli* strains and reference strains were grown on Luria-Bertani (LB) agar plates at 37°C overnight. *E. coli* colonies were suspended in 500 µL of deionized water and boiled for 10 min, followed by chilling on ice for 5 min and centrifugation at 10,000 xg for 5 min, the supernatant was used as the DNA templates for PCR amplification. The PCR mixture contained 10 µL of 2X PCR Master mix (including 2X Taq DNA polymerase, 2X PCR Buffer and 2X dNTP mixture) (TaKaRa), 1 µL of primer pairs, 4 µL of DNA template, and deionized water to a final volume of 25 µL. PCR was completed by an initial heat activation of 5 min at 95°C and then 30 cycles of 30 s at 94°C, 30 s at annealing temperatures and 45 s at 72°C; and an extension of 10 min at 72°C. PCR products were analyzed by 1% agarose gel electrophoresis and visualized after staining with ethidium bromide on a UV transilluminator.

Sequence analysis of PCR products

Resistance gene sequences were aligned and compared with related sequences in GenBank by DNAStar program. Longitudinal data on resistance and resistance genes in *E. coli* in the same farms at Shaanxi province, China was analyzed. The six farms of Tongchuan which raised about one million of layer at Shaanxi province in China were selected for study and the changes of resistance and resistance gene in *E. coli* strains were examined during 2007-2012.

Statistical analysis

Student's *t*-test was used to measure the correlations between resistance and resistance gene and to compare resistance prevalence between years. In all tests, $p<0.05$ was considered statistically significant.

RESULTS

Susceptibility test of *E. coli* strains

The resistance information of chicken *E. coli* strains in three provinces in China to 18 common clinically used antibiotics is shown in Tables 3, 4 and 5. As shown in Table 3, the resistance prevalence of chicken *E. coli* strains to ampicillin in Shaanxi province was 100% during 2007-2012. Resistance prevalence to tetracycline and doxycycline was 70-100%. The increase in chicken *E. coli* strains resistance to nalidixic acid and ciprofloxacin was seen. The resistance rate to nalidixic acid has increased to 100% but a downward trend for neomycin and streptomycin was found. In 2007-2010, the resistance prevalence of chicken *E. coli* strains to ceftazidime increasingly was observed, but there was a slight decline in 2011-2012. A downward trend in resistance to gentamicin in

Table 4. Antimicrobial resistance of *E. coli* strains isolated from chickens of Henan province during 2007-2012.

Antmicrobial	Percentage of resistance (%) (no. of resistant isolates)						
	2007 (n=40)	2008 (n=42)	2009 (n=34)	2010 (n=45)	2011 (n=45)	2012 (n=66)	2007-2012 (n=272)
AMP	100.0(40)	100.0(42)	100.0(34)	100.0(45)	100.0(45)	100.0(66)	100.0(272)
AMX	17.5(7)	40.5(17)	41.2(14)	33.3(15)	13.3(6)	15.2(10)	25.4(69)
CAZ	32.5(13)	59.5(25)	67.6(23)	44.4(20)	51.1(23)	39.4(26)	47.8(130)
CEF	0.0	11.9(5)	0.0	6.7(3)	13.3(6)	0.0	5.1(14)
NEO	32.5(13)	23.8(10)	20.6(7)	20.0(9)	13.3(6)	0.0	16.5(45)
STR	50.0(20)	19.0(8)	41.2(14)	60.0(27)	51.1(23)	60.6(40)	48.5(132)
GEN	50.0(20)	42.9(18)	41.2(14)	40.0(18)	37.8(17)	30.3(20)	39.3(107)
KAN	7.5(3)	14.3(6)	17.6(6)	20.0(9)	15.6(7)	27.3(18)	18.0(49)
TOB	0.0	0.0	0.0	8.9(4)	13.3(6)	30.3(20)	11.0(30)
AMK	17.5(7)	0.0	0.0	0.0	11.1(5)	0.0	4.4(12)
FFC	17.5(7)	19.0(8)	20.6(7)	20.0(9)	37.8(17)	39.4(26)	27.2(74)
TET	67.5(27)	81.0(34)	100.0(34)	100.0(45)	75.6(34)	80.3(53)	83.5(227)
DOX	100.0(40)	100.0(42)	100.0(34)	100.0(45)	100.0(45)	100.0(66)	100.0(272)
SXT	0.0	19.0(8)	20.6(7)	40.0(18)	13.3(6)	19.7(13)	19.1(52)
NAL	67.5(27)	81.0(34)	100.0(34)	100.0(45)	100.0(45)	100.0(66)	92.3(251)
NOR	50.0(20)	19.0(8)	41.2(14)	80.0(36)	62.2(28)	80.3(53)	58.5(159)
CIP	32.5(13)	40.5(17)	58.8(20)	80.0(36)	86.7(39)	89.4(59)	67.6(184)
OFZ	32.5(13)	40.5(17)	20.6(7)	40.0(18)	24.4(11)	39.4(26)	33.8(92)

AMP: Ampicillin; AMX: Amoxicillin; CAZ: Ceftazidime; CEF: Cefalaxime; NEO: Neomycin; STR: Streptomycin; GEN: Gentamicin; KAN Kanamycin; TOB: Tobramycin; AMK: Amikacin; FFC: Florfenicol; TET: Tetracycline; DOX: Doxycycline; SXT: trimethoprim sulfamethoxazole; NAL: Nalidixic aid; NOR: Norfloxacin; CIP: Ciprofloxacin; OFZ: Ofloxacin.

Table 5. Antimicrobial resistance of *E. coli* strains isolated from chickens in Gansu province during 2007-2012.

Antimicrobials	Percentage of resistance (%) (no. of resistant isolates)						
	2007 (n=44)	2008 (n=46)	2009 (n=33)	2010 (n=42)	2011 (n=50)	2012 (n=74)	2007-2012 (n=289)
AMP	100.0(44)	100.0(46)	100.0(33)	100.0(42)	100.0(50)	100.0(74)	100.0(289)
AMX	20.5(9)	26.1(12)	0.0	28.6(12)	0.0	0.0	11.4(33)
CAZ	59.1(26)	76.1(35)	81.8(27)	100.0(42)	72.0(36)	66.2(49)	74.4(215)
CEF	20.5(9)	26.1(12)	0.0	21.4(9)	0.0	21.6(16)	15.9(46)
NEO	40.9(18)	0.0	18.2(6)	28.6(12)	0.0	0.0	12.5(36)
STR	59.1(26)	50.0(23)	33.3(11)	42.9(18)	42.0(21)	55.4(41)	48.4(140)
GEN	79.5(35)	76.1(35)	81.8(27)	71.4(30)	58.0(29)	55.4(41)	68.2(197)
KAN	40.9(18)	50.0(23)	33.3(11)	45.2(19)	66.0(33)	82.4(61)	57.1(165)
TOB	20.5(9)	26.1(12)	33.3(11)	42.9(18)	42.0(21)	55.4(41)	38.8(112)
AMK	0.0	10.9(5)	0.0	0.0	10.0(5)	9.5(7)	5.9(17)
FFC	0.0	8.7(4)	33.3(11)	28.6(12)	42.0(21)	21.6(16)	22.1(64)
TET	68.2(30)	89.1(41)	97.0(32)	100.0(42)	100.0(50)	100.0(74)	93.1(269)
DOX	100.0(44)	100.0(46)	100.0(33)	100.0(42)	100.0(50)	100.0(74)	100.0(289)
SXT	0.0	26.1(12)	0.0	14.3(6)	28.0(14)	21.6(16)	16.6(48)
NAL	86.4(38)	93.5(43)	100.0(33)	92.9(39)	100.0(50)	100.0(74)	95.8(277)
NOR	59.1(26)	76.1(35)	66.7(22)	57.1(24)	72.0(36)	66.2(49)	66.4(192)
CIP	40.9(18)	76.1(35)	81.8(27)	85.7(36)	82.0(41)	82.4(61)	75.4(218)
OFZ	40.9(18)	50.0(23)	33.3(11)	42.9(18)	42.0(21)	44.6(33)	42.9(124)

AMP: Ampicillin; AMX: Amoxicillin; CAZ: Ceftazidime; CEF: Cefalaxime; NEO: Neomycin; STR: Streptomycin; GEN: Gentamicin; KAN Kanamycin; TOB: Tobramycin; AMK: Amikacin; FFC: Florfenicol; TET: Tetracycline; DOX: Doxycycline; SXT: trimethoprim sulfamethoxazole; NAL: Nalidixic aid; NOR: Norfloxacin; CIP: Ciprofloxacin; OFZ: Ofloxacin.

2007-2011 was seen, but there was a slight increase in 2012.

Table 4 shows that the resistance prevalence of chicken *E. coli* strains isolated from Henan province to ampicillin and doxycycline were 100% during 2007-2012 while tetracycline resistance prevalence was 60-100%. The resistance of chicken *E. coli* strains to tobramycin, florfenicol, nalidixic acid and cefaloxime showed an upward trend, and resistance rate of nalidixic acid has been up to 100% while resistance to neomycin and gentamicin showed a declining trend.

As seen in Table 5, the resistance prevalence of chicken *E. coli* strains isolated in Gansu province to ampicillin and doxycycline has remained at 100% from 2007-2012 while the resistance prevalence of tetracycline and nalidixic acid were 60-100%. The resistance prevalence to tobramycin showed an upward trend while gentamicin resistance prevalence showed a downward trend. Resistance rates to ceftazidime and ciprofloxacin increased in 2007-2010, but there was a slight decline in 2011-2012.

Overall, resistance prevalence of chicken *E. coli* strains to ampicillin in Shaanxi, Henan and Gansu provinces in China has been maintained at 100%. Doxycycline and tetracycline resistance prevalence were more than 80% and 60%, respectively. An upward trend to nalidixic acid and ciprofloxacin was seen while gentamicin resistance prevalence showed a downward trend. Resistance to kanamycin, tobramycin and trimethoprim sulfamethoxazole showed significant differences ($P<0.05$) and the rest of antibiotic resistance showed no significant difference ($P>0.05$).

Isolates showed multi-drug resistance (resistant to more than three kinds of antibiotics), and more than 3 were up to 100% of the drug-resistant strains, in which 8 resistant strains had the highest count for 19.2% (192/1002); isolates resistant to more than 8 antibiotics were up to 49.8% (499/1002), of which 14 chicken *E. coli* isolates resistant to 18 antibiotics, accounted for 1.4% (14/1002).

PCR detection of resistant genes in *E. coli* isolates

Among 1002 chicken *E. coli* strains, 8 of 20 resistance genes were detected by PCR. The electrophoretic patterns of these 8 resistance genes were indicated in Figure 1. The detection rates of the genes for *tetA*, *tetB*, *blaTEM*, *aac(3)-II*, *sul-I*, *cmlA*, *floR* and *qnrB* in three provinces in China during 2007-2012 were shown in Table 6. In 2007-2012, the *tetB* and *tetA* genes were detected with the highest prevalence in 86.6%-100% in chicken *E. coli* strains isolated from Shaanxi, Henan and Gansu provinces in China while the detection rates of the genes for *floR*, *sul-I*, *cmlA* and *blaTEM* increased gradually. It is interesting to note that a downward trend of detection prevalence of *aac (3)-II* gene and low detection rate of *qnrB* gene (0.4%) were seen (Table 6).

A continuous monitoring of *E. coli* resistance and resistance genes in the Tongchuan chicken farm

In 2007-2012, a continuous monitoring of *E. coli* resistance and resistance genes in the Tongchuan chicken farms was performed. The results are shown in Tables 7 and 8. Table 7 shows that *E. coli* strains had resistance to kanamycin since 2008 while that *E. coli* strains were resistant to florfenicol and ciprofloxacinb since 2009. *E. coli* strains were resistant to ceftazidime and norfloxacin since 2010 but were sensitive to gentamicin and neomycin. The number of antibiotics of *E. coli* resistance increased from 7 to 13 between 2007 and 2012. Table 8 indicates that, in 2007-2012, detection prevalence of the genes for *blaTEM*, *tetA,* and *tetB* in *E. coli* strains in this farm was more than 80%. The detection prevalence of the genes for *sul-I* and *cmlA* increased while the detection prevalence of the gene for *aac(3)-II* showed a declining trend.

The correlation between resistance and resistance genes of the *E. coli* isolates

Resistance of 1002 chicken *E. coli* strains to different antibiotics and the related resistance genes are shown in Table 9. The ampicillin, streptomycin, trimethoprim sulfamethoxazole, florfenicol, ciprofloxacin and doxycycline resistance genes in 1002 chicken *E. coli* strains are shown in Table 9. Among the 1002 *E. coli* strains detected, detection prevalence of the genes for *tetA*, *tetB*, *blaTEM*, *aac(3)-II*, *sul-I*, *cmlA*, *floR*, and *qnrB* were 97.4, 99.6, 88.9, 85.3, 41.7, 40.3, 26.5 and 0.5%, respectively.

The detection rates of the genes for *tetA*, *tetB*, *blaTEM* and *aac(3)-II* were positively correlated with the doxycycline, ampicillin and streptomycin-resistant *coli* strains, respectively ($P < 0.01$). Only 41.7% of the 223 trimethoprim sulfamethoxazole-resistant chicken *E. coli* strains carried the *sul-I* gene, showing no significant difference with the strains resistance to trimethoprim sulfamethoxazole ($P > 0.05$). In the 268 florfenicol-resistant chicken *E. coli* strains, the detection prevalence of *cmlA* and *floR* were 40.3 and 26.5%, respectively, showing no significant difference with the strains resistance to florfenicol ($P > 0.05$). Only 0.4% of the 742 ciprofloxacin-resistant strains carried the *qnrB* gene, showing no correlation with strains resistance to ciprofloxacin.

Sequence analysis of resistance genes in chicken *E. coli* strains

Among 1002 chicken *E. coli* strains tested, eight kinds of resistance genes in *E. coli* strains were detected by PCR (Figure 1). The sequences of these resistance genes have been sequenced and deposited with GenBank accession numbers JQ362472 (cmlA), JQ362473 (floR),

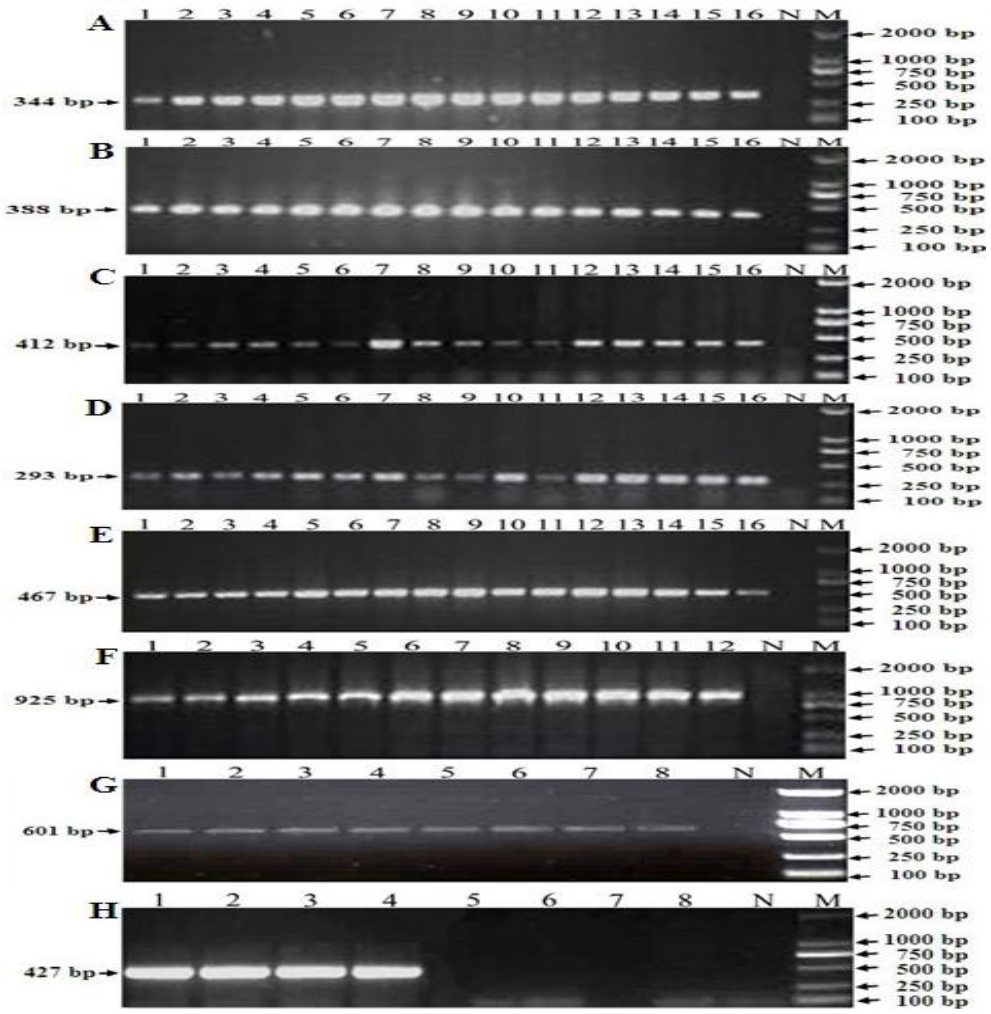

Figure 1. PCR amplification of 8 resistance genes in chicken *E. coli* strains. Panel A: lanes 1-16, *E. coli* strains carried *tetA*; Panel B: lanes 1-16, *E. coli* strains carrying *tetB*; Panel C: lanes 1-16, *E. coli* strains carrying *aac(3)-II*; Panel D: lanes 1-16, *E. coli* strains carrying *blaTEM*; Panel E: lanes 1-16, *E. coli* strains carrying *cmlA*; Panel F: lanes 1-12, *E. coli* strains carrying *sul-I*; Panel G: lanes 1-8: *E. coli* strains carrying *floR*; Panel H: lanes 1-8, *E. coli* strains carrying *qnrB*. Lanes M: DL 2000 marker and lanes N: a negative control.

Table 6. Detection of 8 resistance genes in 1002 chicken *E. coli* strains during 2007-2012.

Resistance gene	Detection rates of resistance genes (%) (number of isolates carrying resistance genes)						
	2007 (n=134)	2008 (n=148)	2009 (n=115)	2010 (n=145)	2011 (n=183)	2012 (n=277)	2007-2012 (n=1002)
qnrB	0.0	0.7(1)	0.9(1)	0.7(1)	0.0	0.4(1)	0.4(4)
floR	0.0	6.1(9)	7.0(8)	6.9(10)	8.2(15)	10.5(29)	7.1(71)
sul-I	0.0	2.0(3)	5.2(6)	7.6(11)	13.1(24)	17.7(49)	9.3(93)
cmlA	3.7(5)	5.4(8)	9.6(11)	11.7(17)	13.7(25)	15.5(43)	10.9(109)
aac(3)-II	53.0(71)	50.0(74)	51.3(59)	47.6(69)	36.1(66)	31.8(88)	42.6(427)
blaTEM	64.2(86)	78.4(116)	85.2(98)	94.5(137)	97.8(179)	99.3(275)	88.9(891)
tetA	86.6(116)	89.2(132)	93.0(107)	96.6(140)	100.0(183)	100.0(277)	95.3(955)
tetB	91.0(122)	93.2(138)	96.5(111)	100.0(145)	100.0(183)	100.0(277)	97.4(976)

AMP: Ampicillin; AMX: Amoxicillin; CAZ: Ceftazidime; CEF: Cefalaxime; NEO: Neomycin; STR: Streptomycin; GEN: Gentamicin; KAN Kanamycin; TOB: Tobramycin; AMK: Amikacin; FFC: Florfenicol; TET: Tetracycline; DOX: Doxycycline; SXT: trimethoprim sulfamethoxazole; NAL: Nalidixic aid; NOR: Norfloxacin; CIP: Ciprofloxacin; OFZ: Ofloxacin.

Table 7. Antimicrobial resistance of chicken *E. coli* strains isolated from the Tongchuan farms at Shaanxi province during 2007-2012.

Year	Number of isolates	Sensitive drugs	Resistant drugs
2007	20	AMX, CAZ, CEF, KAN, TOB, FFC,AMK, SXT, CIP, OFZ, NOR	AMP, NEO, STR, GEN,TET, DOX, NAL
2008	22	AMX ,CAZ, CEF, AMK, TOB, FFC, SXT, NOR, OFZ, CIP	AMP, NEO, STR, GEN, KAN, TET, DOX, NAL
2009	20	AMX, CAZ, CEF, TOB, AMK, SXT , NOR, OFZ	AMP, NEO, STR, GEN, KAN, FFC, TET, DOX, NAL, CIP
2010	20	CEF, AMX, NEO, GEN, TOB, AMK, SXT , OFZ	AMP, CAZ, STR, KAN, FFC, TET, DOX, NAL, CIP, NOR
2011	42	AMX, CEF, NEO, GEN, TOB, AMK, OFZ	AMP, CAZ, STR, KAN, FFC, TET, DOX, SXT , NAL, NOR, CIP
2012	63	AMX, NEO, STR, GEN, AMK	AMP, CAZ, CEF, KAN, TOB, FFC, TET,DOX, SXT , NAL, NOR, CIP, OFZ

AMP: Ampicillin; AMX: Amoxicillin; CAZ: Ceftazidime; CEF: Cefalaxime; NEO: Neomycin; STR: Streptomycin; GEN: Gentamicin; KAN Kanamycin; TOB: Tobramycin; AMK: Amikacin; FFC: Florfenicol; TET: Tetracycline; DOX: Doxycycline; SXT: trimethoprim sulfamethoxazole; NAL: Nalidixic aid; NOR: Norfloxacin; CIP: Ciprofloxacin; OFZ: Ofloxacin.

Table 8. Resistance genes in chicken *E. coli* strains isolated from the Tongchuan farms at Shaanxi province during 2007-2012.

Year	Number of isolates	Detection rates of resistance genes (%) (number of isolates carrying resistance genes /no. of total isolates)							
		qnrB	***floR***	***sul-I***	***cmlA***	***aac(3)-II***	***blaTEM***	***tetA***	***tetB***
2007	20	0	0	0	0	75.0(15)	85.0(17)	85.0(17)	90.0 (18)
2008	22	0	4.5(1)	0	0	72.7(16)	81.8(18)	86.4(19)	95.5 (21)
2009	20	0	0	5.0(1)	10.0(2)	65.0(13)	90.0(18)	90.0(18)	100.0(20)
2010	20	5.0(1)	5.0(1)	15.0(3)	15.0(3)	55.0(11)	95.0(19)	95.0(19)	100.0(20)
2011	42	0	14.3(6)	19.0(8)	21.4(9)	31.0(13)	100.0(42)	100.0(42)	100.0(42)
2012	63	0	12.7(8)	25.4(16)	25.4(16)	28.6(18)	100.0(63)	100.0(63)	100.0(63)

Table 9. Resistance and related resistance genes in 1002 chicken *E. coli* strains to different antibiotics.

Types of antimicrobials	Number of resistant isolates	Resistance genes	Number of isolates carrying resistance genes (detection prevalence)
Ampicillin	1002	blaTEM	891 (88.9%)
Streptomycin	498	aac(3)-II	425 (85.3%)
Trimethoprim sulfamethoxazole	223	sul-I	93 (41.7%)
Florfenicol	268	cmlA	108 (40.3%)
		floR	71 (26.5%)
Ciprofloxacin	742	qnrB	4 (0.5%)
Doxycycline	980	tetA	955 (97.4%)
		tetB	976 (99.6%)

JQ362474 (qnrB), JQ362475 (sul-I), Q362476 (blaTEM), JQ362477 (tetA), JQ362478 (tetB) and JQ362479 (aac(3)-II). The sequences homology of *cmlA*, *floR*, *qnrB*, *sul-I*, *blaTEM*, *tetA*, *tetB* and *aac (3)-II* sequences with the previously published sequences of *cmlA* (HM175865), *floR* (AY775258), *qnrB* (EU093091), *sul-I* (EU598449), *blaTEM* (GU550123), *tetA* (FJ794040), *tetB* (FJ917423) and *aac (3)-II* (FQ482074) are 99.30, 98.92, 97.14, 99.89,

100, 99.66, 98.84 and 97.29%, respectively.

DISCUSSION

Antibiotics are widely used in the treatment and prevention of disease and can also promote the growth of animals. Under the pressure of antibiotic selectivity, drug-resistant bacteria appear. To date, there are many reports regarding *E. coli* resistance in many countries and regions (Harada et al., 2012; Holzel et al., 2012; Johns et al., 2012; Ryu et al., 2012a,b). The *E. coli* resistance has become a global problem (Alan et al., 2007; Szmolka and Nagy, 2013). To date, the comprehensive studies on chicken *E. coli* resistance genes are relatively few. Soufi et al. (2011) studied resistance of 166 chicken *E. coli* strains in Tunisia and found that resistant rates of different strains to ampicillin, streptomycin, nalidixic acid, sulfonamide and tetracycline are 66-95%. To date, *E. coli* resistance problem is very serious in China. Dai et al. (2008) found that the resistant rates of chicken *E. coli* strains in China between 2001 and 2006 to ampicillin and doxycycline is more than 70%. The resistance of chicken *E. coli* strains to ampicillin and doxycycline are very serious. Our results revealed that resistant rates of chicken *E. coli* strains isolated from three provinces in China between 2007-2012 to ampicillin and doxycycline was 100 and 80%, respectively while the resistant prevalence of amikacin was below 30%. In the present study, we also found that quinolone resistance among *E. coli* from chicken in China is rising, which is consistent with a previous study (Zhang et al., 2010).

The occurrence of antibiotic resistance of chicken *E. coli* strains isolated from three provinces in China during 2007-2012 is different, and this may be related to the use of different antibiotics in the farms in different provinces. In-feed or therapeutic antibiotics were used in these farms for all major classes of antibiotics except vancomycins. Ampicillin, tetracycline, doxycycline and nalidixic acid and ciprofloxacin were usually added into animal feed or drinking water in each sampling farm of these provinces, and the resistance of chicken *E. coli* strains isolated to the above antibiotics increased gradually. For example, a chicken farm in Gansu province in China used kanamycin to prevent and treat layer yolk peritonitis caused by *E. coli* for four years, the resistance rate of *E. coli* strains to kanamycin during 2009-2012 increased from 11.0 to 65.0%.

The resistance mechanism of *E. coli* is complicated. The resistance genes mediated by plasmid can make the resistance spread among different bacteria, which make bacteria obtain resistance genes more easily and thus produce multiple resistances (Li, 2005; Roberts, 2005; Zhang et al., 2009; Liu et al., 2012; Mosquito et al., 2012). This mechanism is that resistance genes can directly code enzymes which result in damage antibiotic effect (Skold, 2000; Yoo et al., 2003; Li et al., 2007; Ramirez and Tolmasky, 2010). Yu et al. (2009) found that the aminoglycoside resistance gene in human *E. coli* strain is main *aac (3)-II*. Previous studies on β-lactamase genes in the French *E. coli* strains indicated that the detection prevalence of the genes for *blaTEM and blaCTX-M* among 8 ceftiofur-resistant strains were 62.5 and 100%, respectively (Meunier et al., 2006). The results are similar to a previous study suggesting that *blaTEM* is the main β-lactamase resistance gene in the human *E. coli* (Yang et al., 2011). Tang et al. (2011) detected the *E. coli* drug-resistant gene in pigs in China during 2004-2007 and found that the β-lactamase resistance gene is mainly *blaTEM* and the detection rate is 87%. They also found that the resistance genes of aminoglycoside, tetracycline, and Sulfa are mainly *aphA*, *tet*B and *sul-II*, respectively. The detection prevalence for these resistance genes were 82.6, 49.8 and 55.4%, respectively (Tang et al., 2011). The detection rates for *E. coli* drug-resistant genes show differences which may be due to the strains from various countries and regions and the difference of serum type or antibiotic usage mode. During 2007-2012, we conducted the detection of resistance and resistance genes at chicken farms in Tongchuan City of Shaanxi province in China for 6 years, and found that the numbers of antibiotic resistance increased from 7 to 13. The detection prevalence of resistance genes for *sul-I* and *cmlA* increased gradually. The resistance genes of *tetB*, *tetA*, *blaTEM* and *aac(3)-II* in *E. coli* strains are positively correlated with the resistance of bacterial strain ($P < 0.01$).

In addition, only few quinolone resistance genes were detected from quinolone resistant strains in this study. Whether its resistance is associated with other types of resistance genes or other mechanisms of resistance remains to be further elucidated. Resistance genes were detected from several aminoglycosides and florfenicol-sensitive strains, indicating the resistance genes in a silent state under the pressure of antibiotic. These strains are likely to develop into drug-resistant strains. Therefore, the detection of resistance and associated resistance genes in animal source of pathogenic isolates will be of great significance to the rational use of antibiotics in clinical and public health.

In summary, our results revealed that 1002 chicken *E. coli* strains isolated in three provinces in China during 2007-2012 showed multiple drug resistance. Of all isolated strains, 499 of 1002 *E. coli* strains (69%) were resistant to more than eight kinds of antibiotics, of which resistance gene *tetB*, *tetA*, *blaTEM* and *aac(3)-II* showed a positive correlation ($P < 0.01$) with the *E. coli* strains resistance to antibiotics. The current results provide useful information on the drug prevention of chicken colibacillosis in China and resistance mechanisms of *E. coli.*

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Full Length Research Paper

Time kill-kinetics antibacterial study of *Acacia nilotica*

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***In vitro* time-kill kinetics antibacterial study of *Acacia nilotica* was assessed against *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* determined by plate count technique and analyzed by percentage and log reduction. All test organisms were susceptible to the aqueous methanolic extract. The minimum inhibitory concentration ranged between 0.5 and 1 mg, while minimum bactericidal concentration ranged between ≥1 and ≥2 mg/ml. Average log reductions in viable cell counts for the extract ranged between 0.18log_{10} and 0.35log_{10} cfu/ml for *P. aeruginosa*, 0.27log_{10} and 1.95log_{10} cfu/ml for *S. aureus* and 0.27log_{10} and 0.45log_{10} cfu/ml for *E. coli* after 10 h interaction at 0.5x MIC and 1x MIC. Most of the extracts were rapidly bactericidal at 2x MIC achieving a complete elimination of most of the test organisms within 12 h exposure. A good correlation was found between the killing curves and the MIC of *A. nilotica* against the test organisms.**

Key words: Time-kill kinetics, antibacteria, *Acacia nilotica*, percentage reduction.

INTRODUCTION

Medicinal plants are sources of enormous quantities of chemical substances which are able to initiate different biological activities including those useful in the treatment of human diseases (Kew Gardens, 2013). Most of the synthetic antibiotics now available in market have major setbacks due to the accompanying side effects on patients and the multiple resistances developed by pathogenic microorganisms to them (Nkomo, 2010). Hence, there is a justifiable need to explore for new and more potent antimicrobial compounds of natural origin to combat these pathogens. *Acacia nilotica* (L.) Willd. ex Delile is a plant used ethnomedically in the traditional treatment of tuberculosis in Northern Nigeria (Oladosu et al., 2010). It is reported to be bactericidal against wide range of microorganisms such as *Mycobacterium tuberculosis* and other HIV/AIDS opportunistic infections such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *C. albicans* (Oladosu, 2012). It is commonly called Gum Arabic tree, Egyptian mimosa, Egyptian thorn, red thorn, Babool, babul in India (Kew Gardens, 2013).

Geographically, *A. nilotica* is widely spread in sub-tropical and tropical Africa from Egypt to Mauritania southwards to South Africa, and in Asia eastwards to Pakistan and India. It has been introduced in China, the Northern Territory and Queensland in Australia (where it is considered to be a pest plant of national importance), in the Caribbean, Indian Ocean islands, Mauritius, United States, Central America, South America and the Galápagos Islands. It has naturalized in several countries where it has been introduced as a medicinal, forage and fuel wood plant (Bennison and Paterson, 1994, Kew Gardens, 2013). Its principal constituents are gallic acid, (+)-catechin and methyl gallate (Oladosu, 2012). It has been reported to contain l-arabinose, catechol, galactan, galactoaraban, galactose, N-acetyldjenkolic acid, N-acetyldjenkolic acid and sulphoxidespentosan. Seeds contain crude protein (18.6%), ether extract (4.4%), fiber (10.1%), nitrogen-free extract (61.2%), ash (5.7%) and silica (0.44%); phosphorus (0.29%) and calcium (0.90%) of DM (Pande et al., 1981). From phytochemical analysis of stem bark of *A. nilotica*, it showed the presence of

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carbohydrates, saponins,annins and cardiac glycosides (Ogbadoyi et al., 2011). Toxicologically, *A. nilotica*, at 2 and 8% levels, has a low toxicity potential (Al-Mustafa et al., 2000; Oladosu et al., 2012). In a survey of potentially allergenic plants in Pondicherry, it was reported likely to cause pollen allergy (Anonymous, 1998). *A. nilotica* has a wealth of medicinal uses. It is used for stomach upset and pain, the bark is chewed to protect against scurvy, an infusion is taken for dysentery and diarrhea (Kew Gardens, 2013). In Nigeria, it is one of the standard drugs for treating diarrhea and it has been authenticated to have an antidiarrhoeal property (Misar et al., 2008). Saba et al. (2011) reported the antibacterial activity of *A. nilotica* lysates against neuropathogenic *E. coli* K1, MRSA and *K. pneumonia.* Time kill kinetics study is a way of determining the spectrum and kill rate of an antibacterium.

The aim of the study is to determine the speed of cidal activity of *A. nilotica* within a given contact time.

MATERIALS AND METHODS

Plant extraction

The fruit pulp of *A. nilotica* was prepared according to NIPRD/NIH Standard Operating Procedure No. 1 (2006). The plant was air dried to constant weight at room temperature (35±2°C) and pulverized using a crushing machine (Trapp metallurgical, Trapp Ltd, Brazil). Seventy percent (70%) aqueous methanolic extraction was done so as to extract both polar and non polar components of the plants.

Organism preparation

The test organisms viz: *S. aureus*, *P. aeruginos* and *E. coli* were prepared as described by Dominiguez et al. (2001). Two to three colonies of 20 h growth on Mueller- Hinton Agar of the organisms to be studied were suspended on 50 ml prewarmed (37°C) Mueller-Hinton broth. The suspension was incubated overnight at 37°C, diluted 1/2500 in the same prewarmed medium and incubated in waterbath with agitation (50 rpm). The absorbance of the culture was monitored with a spectrophotometer (6405 Jenway, Barloworld Scientific Ltd. Dunmow, Essex CMB 3LB), using a wavelength 450 nm and 19 mm diameter spectrophotometer tubes until absorbance of 0.1 was reached and a plotted standard curve (equivalent 2.5 - 3.0 × 10^7 cfu/ml for *E. coli* and *P. aeruginosa* and 1.8 - 2.0 × 10^7 cfu/ml for *S. aureus*).

Procedure

The time- kill kinetics antibacterial study of the fruit extract of *A. nil*otica was carried out to assess the killing rate of the extract within a given contact time. This study was done according to standard guide for assessment of antimicrobial activity using time-kill kinetics procedure of (Antimicrobial Susceptibility Testing Method, 2008). Microbial population at the initiation and completion was determined by spectrometric and plate count methods at interval of 2 h. To 200 ml of Mueller- Hinton broth in three conical flasks was added 400, 200 and 100 mg of extract to obtain 2, 1 and 0.5 mg/ml extract, respectively. The solutions were centrifuged at 13,226 x g for 15 min to remove impurities. The extract solutions in flasks were inoculated with 100 µl of inoculum suspensions of test organisms (*S. aureus*, *E. coli* and *P. aeruginosa*) of 10^6 cfu/ml and incubated at 37°C for 24 h. The optical density of each dilution was recorded on uv/spectrophotometer at 540 nm (Jenway, 6405) at initiation time (0 h) and every 2 h for 10 h. For surviving organism count, an aliquot of each dilution (1 ml) was transferred and plated on 20 ml Tryptic Soy agar at interval of 2 h.

Plates were incubated at 37°C for 24 h. Number of viable organisms was counted as cfu/plates. Average duplicate (2 plates from each replicate dilution) counts were multiplied by the dilution factor to arrive at cfu/ml. The results were analyzed using ANOVA with software SSPS version 16. Differences in the mean values of $p < 0.05$ were considered to be statistically significant and were separated using Duncan new multiple range test.

RESULTS

The time-kill kinetics profile of the test bacteria by *A. nilotica* is shown in Tables 1 to 3. This study unlike an MBC/MIC assay, allows the determination of the speed of cidal activity of the extract (Aiyegoro et al., 2009). The extract exhibited bactericidal effect at 2 mg/ml concentration against all the test bacteria. The number of surviving microorganisms in the extract was determined by plate count method at sampling time and enumerated. The percentage reduction and log reduction from initial microbial population for each time point was calculated to express the change (reduction or increase) of the microbial population relative to a starting inoculum. The change was determined as follows:

$$\% \text{ Reduction} = \frac{\text{Initial count} - \text{count at x interval}}{\text{initial count}} X\ 100$$ (ASTM E, 2008)

The Log reduction was calculated as follows:

Log_{10} (initial count) – Log_{10} (× time interval) = Log_{10} reduction

A significant decrease ($p<0.05$) in population of test organisms was observed at each interval. In the time-kill kinetics antibacterial study against *P. aeruginosa,* Table 1 shows average log reduction in viable cell count ranged between 0.32 Log_{10} to 6.63 Log_{10} cfu/ml after 6 h of interaction, and between 6.61Log_{10} and 6.63 Log_{10} cfu/ml after 24 h of interaction in 1 and 2 x MIC. Percentage reduction in viable cell count was observed to be from 52.2 to 99% between 2 to 24 h of interaction. In the time-kill kinetics antibacteria study of *A. nilotica* extract against *S. aureus* in Table 2, average log reduction in viable cell count ranged between 0.32 Log_{10} to 1.95 Log_{10} cfu/ml, 2.14 log_{10} to 6.61 cfu/ml and 6.63 Log_{10} cfu/ml after 24 h of interaction in 0.5, 1 and 2 x MIC. Percentage reduction in viable cell count (99.99%) at 2 and 1 mg/ml respecttively throughout the period of exposure indicated a very high significant decrease ($p<0.05$) as compared to 0.5 mg/ml (52.2 to 70.7%). In the time kill kinetics antibacterial study of *A. nilotica* against *E. coli*, there was a very high significant ($p<0.05$) percentage reduction of viable

Table 1. Time-kill kinetics antibacterial study of *A. nilotica* against *P. aeruginosa.*

Initiation time (h)	Population of organism (cfu/ml)			Percentage (%) reduction			log reduction		
	0.5 mg/ml	1 mg/ml	2 mg/ml	0.5 mg/ml	1 mg/ml	2 mg/ml	0.5 mg/ml	1 mg/ml	2 mg/ml
0	$3.6 \times 10^6 \pm 0.3$	$4.1 \times 10^6 \pm 0.3$	4.3×10^6	N/A (%)	N/A (%)	N/A (%)	N/A	N/A	N/A
2	$2.4 \times 10^6 \pm 0.2$	$3 \times 10^6 \pm 0.3$	0	≥52.2	≥99.2	≥99.9	0.32	2.14	6.63
4	$1.6 \times 10^6 \pm 0.1$	$2.4 \times 10^6 \pm 0.2$	0	≥60.8	≥99.4	≥99.9	0.41	2.23	6.63
6	$1.7 \times 10^6 \pm 0.11$	$2.0 \times 10^6 \pm 0.2$	0	≥70.1	≥99.5	≥99.9	1.95	2.31	6.63
8	$1.7 \times 10^6 \pm 0.11$	0	0	≥80.0	≥99.9	≥99.9	2.01	6.61	6.63
10	$1.8 \times 10^6 \pm 0.1$	0	0	≥71.8	≥99.9	≥99.9	1.95	6.61	6.63
12	$2.0 \times 10^6 \pm 0.1$	0	0	≥70.7	≥99.9	≥99.9	1.95	6.61	6.63
24	$2.0 \times 10^6 \pm 0.1$	0	0	≥70.7	≥99.9	≥99.9	1.95	6.61	6.63

Values are means ± S.E.D.

Table 2. Time-kill kinetics antibacterial study of *A. nilotica* against *S. aureus.*

Initiation time (h)	Population of organism (cfu/ml)			% reduction			log reduction		
	0.5 mg/ml	1 mg/ml	2 mg/ml	0.5 mg/ml	1 mg/ml	2 mg/ml	0.5 mg/ml	1 mg/ml	2 mg/ml
0	$2.0 \times 10^6 \pm 0.1$	$4.1 \times 10^6 \pm 0.3$	4.3×10^6	N/A (%)	N/A (%)	N/A (%)	N/A	N/A	N/A
2	$1.0 \times 10^6 \pm 0.07$	$3.0 \times 10^6 \pm 0.2$	0	≥52.2	≥99.2	≥99.9	0.32	2.14	6.66
4	$9.4 \times 10^6 \pm 0.14$	$2.4 \times 10^4 \pm 0.2$	0	≥60.8	≥99.4	≥99.9	0.41	2.23	6.66
6	$6.4 \times 10^6 \pm 0.3$	$2.1 \times 10^4 \pm 0.3$	0	≥70.1	≥99.5	≥99.9	1.95	2.31	6.66
8	$5.2 \times 10^6 \pm 0.14$	0	0	≥80.0	≥99.9	≥99.9	2.01	6.61	6.66
10	$4.5 \times 10^6 \pm 0.2$	0	0	≥71.8	≥99.9	≥99.9	1.95	6.61	6.66
12	$3.4 \times 10^6 \pm 0.14$	0	0	≥70.7	≥99.9	≥99.9	1.95	6.61	6.66
24	$4.4 \times 10^6 \pm 0.3$	0	0	≥70.7	≥99.9	≥99.9	1.95	6.61	6.66

Values are means ± S.E.D.

cell count of *E. coli* (92.0%) in 2 mg/ml than 1 mg/ml (45.74 to 60.99%) within 2 h of exposure as seen in the result in Table 3. Total bacteria elimination (≥99.99%) observed at the highest concentration (2 mg/ml) at the 6 h of exposure to the extract of *A. nilotica* was a minimal bacteriocidal effect.

DISCUSSION

Time-kill kinetics antibacterial study has been used to investigate numerous antimicrobial agents and they are also often used as the basis for *in vitro* investigations for pharmacodynamic drug interaction (Ogunwonyi et al., 2010). The time kill antibacterial assay of the extract of *A. nilotica* gave variable kinetics against susceptible bacteria tested as seen in Tables 1 to 3. The extract demonstrated both bacteriostatic and bactericidal effects as it shows a concentration-dependent killing. The bactericidal concentration of the extract was 2 mg/ml against *P. aeruginosa* and *E. coli*

Table 3. Time-kill kinetics antibacterial study of extract of *A. nilotica* against *E. coli.*

Expo time	Plate count (cfu/ml)		Percentage (%) reduction		Log reduction	
	1 mg/ml	2 mg/ml	1 mg/ml	2 mg/ml	1 mg/ml	2 mg/ml
0	$2.1 \times 10^6 \pm 0.3$	$3.1 \times 10^6 \pm 0.2$	N/A (%)	N/A (%)	N/A	N/A
2	$1.1 \times 10^6 \pm 0.1$	$2.4 \times 10^6 \pm 0.2$	≥45.74	≥92.0	0.27	1.10
4	$9.9 \times 10^6 \pm 0.63$	$1.0 \times 10^6 \pm 0.02$	≥51.73	≥96.67	0.32	1.48
6	$7.4 \times 10^6 \pm 0.14$	0	≥64.16	≥99.99	0.45	6.48
8	$8.6 \times 10^6 \pm 0.1$	0	≥58.07	≥99.99	0.38	6.48
10	$8.7 \times 10^6 \pm 0.3$	0	≥ 57.07	≥ 99.99	0.37	6.48
12	$8.8 \times 10^6 \pm 0.3$	0	≥57.09	≥99.99	0.37	6.48
24	$8.0 \times 10^7 \pm 0.01$	0	≥60.99	≥99.99	0.41	6.48

Values are means ± S.E.D.

and this is not surprising as Pseudomonas species have been reported to be resistant to many antimicrobial agents (Orishadipe et al., 2005). However, a bacteriostatic effect was observed at a lower concentration (1 mg/ml); only *S. aureus*was susceptible to the extract at 0.5 mg/ml. A complete elimination of *P. aeruginosa* was achieved after 10 h of exposure, while complete elimination after 2 h of exposure was observed in *S. aureus* and *E. coli.* In the overall study, the trend of cidal activities is also time and dose dependent. At higher concentration and longer duration of time (12 h), more bacteria were killed. Inhibitory levels of the crude extract could be bacteriostatic and bactericidal independent of Gram position of test organisms. This study revealed that the extract was rapidly bactericidal at 2 x MIC achieving complete elimination of test organisms after 12 h exposure. The result obtained in this study corroborates the previous study of Aiyegoro et al. (2008) in the time-kill study of *Helichrysum pedunculatum* that the time-kill is time and concentration dependent. A concentration of 1 mg/ml activity agrees with the findings of Saib et al. (2011) that 1 mg/ml concentration of the pod of *A. nilotica* exhibited >90% bactericidal activity. Furthermore, Suma et al. (2012) in their study of antimicrobial activity of *A. nilotica* against *Xanthomonas malvacearum* bacteria indicated the effect of the extract at 500 µg/ml, which is similar to our result against *S. aureus*.

Overall, this time-kill study corroborates the reported efficacies of preliminary antibacterial study of selected plant extracts as reported by Oladosu et al. (2010) and this support the folkloric uses of these plants in treatment of different ailments among the traditional people. In this study, the 70% aqueous methanolic extracts of the test plant exhibited a broadspectrum antibacterial activity at the test concentration of 2, 1 and 0.5 mg/ml and the rate of killing of the extract appear to be time and concentration dependent. The antibacterial activities exhibited by this plant suggest it as a potential candidate in bioprospecting for antimicrobial drugs and the isolation and the identification of the active principles of the plant will be a step forward in drug discovery.

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Full Length Research Paper

Herbal control of prevalent microorganisms in buck (male goat) semen in Bauchi State, Nigeria

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A study was carried out to evaluate the antimicrobial properties of some common herbs in controlling the prevalent microorganisms affecting Buck semen in Bauchi. Ten semen collections were done using the artificial vaginal method from three Bucks (BI, BII, and BIII) of the Red Sokoto breed (average age 20 months). Semen samples were cultured aerobically on Nutrient agar and MacConkey agar (Oxoid) to test the presence of bacteria and on Potato Dextrose Agar (Oxoid) for fungi. Two bacteria species: *Staphylococcus aureus* and *Bacillus cereus*, together with moulds: *Aspergillus niger* and *Aspergillus fumigatus*, as well as yeast *Torulopsis Spp.*, were isolated. The corresponding pH values and nitrite content of semen samples were indicative of abnormality and infection, respectively. A cold ethyl acetate extract of the leaf and stem bark of three different herbs (*Careca papaya, Vernonia amygdalina* and *Jatropha curcas*) was tested against the isolates from the semen samples. Herb extracts showed appreciable inhibition activity on all the isolates except the *J. curcas* leaf extract that showed no effect. These could therefore be used in the control of microorganisms in buck semen in Bauchi, and beyond.

Key words: Artificial insemination, buck semen, microorganisms, nitrite, herbal, antimicrobials.

INTRODUCTION

Microorganisms are important contaminants of many body fluids, including semen of animals and humans. Consequently, the extent of microbial contamination is an important parameter to consider in the quality control of semen that is used for artificial insemination or direct mating. Nigeria is the leading producer of goats in Africa with a reported population of 24.5 million (Bamikole and Ikhatua, 2009). Artificial insemination is a very important practice by modern animal breeding systems. It is a vital tool for the rapid improvement of livestock, allowing for maximum use of the best sires on numerous dams (Butswat and Choji, 1995). It is one of the animal production technologies that augment production and returns from livestock at a faster rate and enhance cross breeding programmes. Artificial insemination (AI) could therefore hold great prospects to enhance goat breeding, although only 30 animals were inseminated in Bauchi from 1979-1989 and they were mostly cattle (Voh, 1990).

The benefits of this technology are, however, derived only when it is readily available to the livestock owner and is effectively and sustainably utilized by him. Though the technology has been accepted and practiced in the developing countries for most of the farm animal species, in the tropics, AI has come into serious conflict with environmental, cultural and geographical conditions that adversely affect production (Futmayr, 1979). However,

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certain constraints have militated against the adoption of AI in this zone (Butswat and Choji, 1995) and the only method of livestock reproduction has been natural mating, which exposes the females to contamination from males carrying pathogenic organisms within their urogenital tract. The scarcity of veterinary services, the high cost of drugs particularly antibiotics and antimycotics for inclusion in diluents as well as for treating animals, and insufficient knowledge on microbial spectrum of semen, are some of the factors that have affected the pace of the adoption of the AI option.

During the practice of AI, semen collected is normally preserved in specially compounded diluents pending insemination (Niba et al., 2002). Antibiotics and sometimes antimycotics are often added to the diluents before conservation. This is done to protect the semen content from destruction before insemination. Quite often, antibacterial and antimycotic application is done without prior knowledge of the type of microorganisms prevalent in semen. Above all, some inseminators do not include antimycotics even when their pathogenic role in semen with regard to the release of toxins (aflatoxins and mycotoxins) is crucial for the reduction of semen fertility (Csuka, 1998; Luis et al., 2010). Microorganisms have a deleterious effect on sperm function, both directly by altering the structure of the sperm, by affecting its motility (Depuydt et al., 1998) or by provoking a premature acrosome reaction (Kohn et al., 1998), the putrefaction of diluents components of biological origin, and the utilization of metabolic substrates (Lamming, 1984), and indirectly stimulating the production of antibodies that can be directed against the sperm glycocalyx complex (Kurpisz and Alexander, 1995). Most ejaculates collected from healthy animals are contaminated with bacteria to some extent, for example, ejaculates from boars (Wallgren, 1996; Luis et al., 2010), stallions (Malmgren et al., 1998) or bulls (Smole et al., 2010). Some reports indicate that metabolic products such as endotoxins from some bacteria appear to have detrimental effects on the survival of sperm (Almond and Poolperm, 1990). Hence, semen quality and the quantity of viable sperm cells may be reduced with bacterial contamination.

Some bacteria have however, developed resistance to many conventional antibiotics. Even a small amount of antibiotic usage can result in considerable levels of antibiotic resistance within an animal species, for example tetracycline-resistant strains of *Clostridium perfringens* in Swedish broilers (Johansson et al., 2004). There is a considerable interest worldwide in reducing the use of antibiotics to stem the development of antibiotic-resistant strains of bacteria. Separation of spermatozoa from bacterial contaminants by Single Layer Centrifu-gation (SLC) is possible (Morrell and Wallgren, 2011). Ethno-veterinary practices also hold a way out in solving this dilemma especially with the high cost of efficient antibiotics that are out of the reach to most peasant farmers. Many plants and plant parts have been shown to exhibit antibiotic and antimicrobial properties. Anosa and Okoro (2011) reported anticoccidial activity of the methanolic extract of *Musa paradisica* root in chickens while, Ademola and Eloff, (2011) reported anthelminthic activity of acetone extract and fractions of *Vernonia amygdalina* (bitter leaf) against *Haemonchus contortus* eggs and larvae in small ruminants. Reports have shown that about 80% of the world population relies solely on medicinal plants for the treatment of diseases (Dwivdedi, 1999). Some ethno veterinary practices have been reported in the northern part of Nigeria by Abdu et al. (2000).

The objective of this study therefore, was to investigate the prevalence of microorganisms in buck semen of the Red Sokoto breed and to exploit indigenous means of controlling them with medicinal herbs through *in vitro* evaluation of some plant extracts.

MATERIALS AND METHODS

Preliminary screening of herbs with antimicrobial property on isolates

Experimental plants

Herbs were collected from villages around Bauchi; Yelwa Tudu, Kagadama, Doka and Dumba in Gwallameji area in the month of June 2002. Plants were identified by the criteria described by Audu (1989) and authenticated in the herbarium of the Botany Programme, of the same University, where a voucher specimen was deposited.

Extraction of plant materials

Parts of *C. papaya, V. amygdalina* and *J. curcas* were dried under shade for two weeks and then carefully packed in large khaki (brown) envelopes and dried in an oven at 37°C for 12 h before milling. The plant materials were reduced to a fine powder by grinding using a Macsalab Model 200 LAB grinder (Eriez-Bramley, South Africa). The crude extracts were prepared by cold solvent extraction method (Akinyanju et al., 1986). 10 g of each of the fine powder was weighed and soaked in 50 ml of ethyl acetate with a 5:1 solvent to dry weight ratio and allowed to stand for 48 h at 25°C.

Gravity filtration was done using filter paper (Whatman No. 13) and evaporated to dryness in the sun and later stored at 4°C until use.

Animals and husbandry

Ejaculates from three mature bucks, 18-20 months old, of the red sokoto breed were used for this study. Each buck was housed individually in an open sided netted pen with straw bedding and fed a standard breeders diet and also given access to water *ad libitum*. The animals were kept in accordance with the recommendations in the Guide for the Care and use of Laboratory Animals (DHHS, NIH publication no.85-23; National Research Council (NRC) 1985).

Semen was manually collected using the Artificial Vagina (AV) method (Hafez, 1995). The AV was normally washed with soap and hot water after each collection and disinfected with 70% alcohol before drying. Collection was done every three days and lasted for one month (July 2002). Freshly collected semen was placed in plastic bags that were sealed and transported to the Microbiology

Table 1. Bacteria isolated from the Seminal fluid of three Buck in triplicate.

Replicate	I	II	III
Buck I	*S. aureus*	*S. aureus*	*S. aureus*
	B. cereus	*B. cereus*	*B. cereus*
Buck II	*S. aureus*	*S. aureus*	*S. aureus*
Buck III	*S. aureus*	*S. aureus*	*S. aureus*
	B. cereus	*B. cereus*	*B. cereus*

Table 2. Fungi isolates from the seminal fluid of three Buck, in triplicate

Replicate	I	II	III
Buck I	*Torulopsis spp.*	*Torulopsis spp.*	*Torulopsis spp.*
Buck II	*Aspergillus fumigatus*	*Aspergillus fumigatus*	*Aspergillus fumigatus*
Buck III	*Aspergillus niger*	*Aspergillus fumigatus*	*Aspergillus niger, Aspergillus fumigatus*

Laboratory of the University at ambient temperature (approximately 16°C) in an insulated box to protect it from draughts. The samples were processed approximately 30 min after collection due to the time spent in transit.

Isolation and identification of microorganism

Bacteria

A loopful of the test semen was aseptically streaked on nutrient agar and blood agar plates in triplicates and incubated aerobically at 37°C, for 24 h. This was repeated for the ten semen collections from the three bucks. Bacterial colonies were carefully picked and purified by repeated sub-cultures on nutrient agar and blood agar plates and their morphologies were studied.

Pure cultures were then preserved on nutrient agar slants and used for gram staining using the method described by Peters et al. (2010), and the isolates were identified.

The pH and nitrite values of the semen were determined using diagnostic urinalysis test strip (Combi-9 test strip) according to standard medical diagnostic methods (Chesbrough, 1984).

Fungi

A loopful of the test semen was aseptically inoculated onto already prepared potato dextrose agar plates (Oxoid Ltd) in triplicates and incubated at 25°C for 5 days. This was repeated for the ten collections from the three bucks.

Following incubation, the growth of fungi was examined using a hand lens (x10). Specimens were picked out in lactophenol cotton blue, examined on a microscope slide, and later identified (Brass et al., 1979).

Antimicrobial screening test on isolates

The bacterial isolated were cultured in peptone water for 18 h. 0.3 ml of each of the bacterial suspension was mixed with 15 ml of nutrient agar in sterile Petri plates and allowed to solidify. A sterile steel borer was used to punch wells into the agar and each well was filled in with either 0.1 ml extract (that is 2% concentration) or with 0.1 ml distilled water as control. Petri dishes were incubated at 37°C for 24 h and the diameter in centimeters of the zone of growth inhibition was measured using a vernier caliper.

Spores of the isolated fungi were harvested from stock and seeded onto potato dextrose agar plates at 0, 2, 4 and 16% concentrations of the herbal extracts. Spore germination and growth were monitored for a week.

RESULTS AND DISCUSSION

The semen samples of the three bucks yielded isolates of *S. aureus* and *B. cereus,* as bacteria, *A. niger* (*vartiegham*), *A. fumigatus* as moulds and *Torulopsis spp.* as yeast (Tables 1 and 2). All the bucks, showed a remarkably acidic semen, with buck I revealing significant acidity (pH = 5.1).

The nitrite values for the three bucks were significant; with Buck I revealing the highest value (Table 3).

The susceptibilities of the bacterial isolates to the ethyl acetate extracts of tested herbs (*C. papaya, V. amygdalina* and *J. curcas*) are shown in Table 4, and the antimicrobial evaluation of the combined ethyl acetate extract of the individual herbs on both the bacteria and fungi isolates is shown on Table 5.

There are many factors that adversely affect the quality of semen used for artificial insemination, of which bacterial contamination is a very important one. Contamination can be the result of urinary tract infection (Serrano et al., 1994) but it may also occur during semen collection (Arauz et al., 2000). *S. aureus* and *B. cereus* were the only bacteria isolated from the semen samples. This finding agrees with the results of Althouse and Lu (2005) who identified about 25 different genera of bacteria in boar semen among which were *E. coli*, *Pseudomonas*, *Staphylococcus* and *Proteus spp*. This is because semen is an ideal medium for the establishment and growth of many microorganisms including bacteria and fungi. Lombardo and Thorpe (2000) also isolated *E. coli*, *Salmonella spp*,, *Shigella spp*., and *Yersinia spp.*, in swallow semen while Mangiagalli et al. (2012) and Martino et al. (2006) isolated only *E. coli* and *S. faecalis*

Table 3. The pH and Nitrite Values of the Semen.

Parameter	Buck I	Buck II	Buck III
Number of samples collected	10	10	10
pH	5.1	6.1	6.1
Nitrite	+++	++	++

+++ = highly positive; ++ = positive.

Table 4. The diameter of Zone of Inhibition of Ethylacetate extract of Herbs on Bacterial Isolate from Buck Seminal Fluid.

	Average diameter of zone of inhibition (cm) for 3 replicates at 2% concentration	
Herb	***Staphylococcus aureus***	***Bacillus cereus***
Carica papaya (pawpaw leaf)	0.1	0.1
Carica papaya (pawpaw stem bark)	0.3	0.3
Vernonia amydalina (Bitter leaf, stem bark)	0.3	0.3
*Vernonia amydalina (*Bitter leaf, leaf)	0.3	0.45
*Jactropha curcas (*Physic nut stem bark)	0.4	0.3
Jactropha curcas (Physic nut leaf)	Resistant	Resistant
Control (water treatment)	Resistant	Resistant
Mixture of all the Herbs (1:1)	0.35	0.65

Table 5. The effect of all the extract mixed together (1:1) on fungi isolates at varying concentrations.

Fungi	2%	4%	16%
Aspergillus niger	No growth from 1 – 7 days	No growth from 1 – 7 days	No growth from 1 – 7 days
Aspergillus fumigatus	No growth from 1 – 7 days	No growth from 1 – 7 days	No growth from 1 – 7 days
Control	Profuse growth with sporulation	Profuse growth with sporulation	Profuse growth with sporulation

in rabbit and rooster semen respectively. In a study on bacterial contamination of boar semen (Dede, 1981), *Staphylococci* were isolated along with ten other genera of bacteria in the tropical environment. However, Zgorniak-Nowosielska et al. (1984) studying horse semen samples from Poland isolated only saprophytic flora.

The presence of nitrite in the semen samples as indicated by combi – 9 test strip, and above all the significantly higher nitrite concentration of buck I (+++) are not only indicative of the presence of bacteria but also the differences in bacterial contents of the semen samples.

Arthur et al. (1982) observed that fresh semen samples are slightly acidic, although the pH of normal semen is in the range 7.2 to 7.6, and that excessively high values occur with inflammation of the accessory glands. They also showed that a pH value of Friesian bull semen above 7.0 is needed for excellent fertility (Arthur et al., 1982). The observed acidic nature of the studied buck semen (pH = 5.1 and 6.1) not only confirms an abnormality, but the degree of abnormality in the semen.

Before restraining the bucks for the research period, the animals had been feeding wildly and perhaps might have acquired *Bacillus cereus* through this means or even through the feed used during the period of this study, as there were no quality controls on the feed. The same might have happen with the mould, *Aspergillus spp*, or even it may have been already in the animals.

Chesbrough (1984) reported that *B. cereus* is widely distributed in nature and lives as saprophytes in the soil, dust, water, on vegetation and cereals and also observed that it is a major pathogen in cattle, sheep and goat and the bacilli are excreted in the faeces, urine and saliva of infected animals.

A. niger (Vartieghem, 1867) and *Aspergillus fumigatus* (Fresenius, 1863) are ubiquitous saprophytes in soil, on plants, man and animals, while the yeast, *Torulopsis, spp* appear on foodstuffs and genitals of both man and animals (Brass et al., 1979). Fungi have been noted to exert a major effect on the hormonal activity within the reproductive system of an animal. For instance, *Aspergillus* genera are known to be associated with the secretion of a toxic fungal metabolite, aflatoxin. More so, Clarke et al. (1987) observed that ingestion of aflatoxin-contaminated feed can lead to widespread reproductive abnormalities in male chicken, including a reduction in circulating levels of testosterone.

The discovery of new therapeutic substances of natural origin with possible low or no toxicity to animals, humans and the environment, based on ethnomedical and ethnoveterinary practices is the focus of researches today.

From antimicrobial screening tests, pawpaw leaf and stem bark (*C. papaya*), bitter leaf and stem bark (*V. amygdalina*) and *J. curcas* stem bark (but not leaves) show appreciable inhibitory properties against the bacterial isolates *in vitro* as compared to the water control treatment. Various authors have reported on the medicinal value of *V. amygdalina* (Ademola and Eloff, 2011; Yeap et al, 2010). Nalubega (2010) also confirms the superior antibacterial property of *V. amygdalina* over *C. papaya* and *Jatropha curcas*. The ether extracts of *V. amygdalina* showed considerable antibacterial activity on all four bacteria isolated (*S. faecalis*, *S. aureus*, *E. coli* and *S. typhymurium*). *Carica papaya* was only effective against three of the four identified bacteria (*S. faecalis*, *S. aureus* and *S. typhymurium*) while *J. curcas* was active against *S. faecalis* and *S. aureus* only. The acetone extract of *V. amygdalina* was also shown to possess antibacterial activity towards *B. cereus, B. pumilus, B. subtilis, Micrococcus kristenae, S. aureus, E. cloacae* and *E. coli* growth with minimum inhibition concentration (MIC) of 5 mg/ml (Kambizi and Afolayan, 2001). The essential oil of Lavandula multifida exhibited significant antimicrobial activity against tested representative disease bacteria, that is *S. aureus*, *E. coli*, *P. aeruginosa*, *B. cereus*, and antifungal activity against phytopathogenic strains such as *Alternaria spp:*, *Pencillium expansum*, *Rhizopus stolonifer* and *Botrytis cinerea (*Sellam et al., 2013*).*

Similarly, the extracts of herbs combined in equal ratio showed a much more appreciable diameter of zone of inhibition than the individual extract on the bacteria isolates growth, and completely inhibited the growth of the mould fungi spores at all test concentrations (except at 0%) for 7 days. This is explained by Gunics et al. (2000) as synergy between antibiotics. He reported a positive influence of interaction between antibiotics and non-conventional antibiotics on bacteria. Ademola and Eloff (2011) also observed synergy of action from the different fractions of acetone extracts of *V. amygdalina* on the control of helminthes in small ruminants in Nigeria; he suggested that differences in the chemical nature of the various components could have multiple mechanisms of action which may be useful for the antihelmintic efficacy.

This conclusion was also confirmed by Sellam et al. (2013) who concluded that the significant efficacy of the essential oil of Lavandula multifida against bacteria and fungi was due to the numerous compounds it contains.

The addition of antibiotics to semen extenders is stipulated both nationally and within the European Union (Morrell and Wallgren, 2011). However many antibiotics and antimicrobials have a detrimental effect on spermatozoa, and the choice of agents for use in semen extenders is limited. Furthermore, even small amounts of antibiotic usage can result in considerable levels of antibiotic resistance within an animal species. Results from this study shows that ethno veterinary control through the use of herbs and herbal extracts could hold an alternative solution for the control of micro-organisms in buck semen.

Conclusion

This study shows that the knowledge of microorganisms before insemination and their sensitivities to antibiotics and antimycotics in compounding diluents is very important. This above all proves the possibility of indigenous knowledge of herbs in controlling these pathogens so as to ease and enhance artificial insemination practice in this environment. Although the three herbs were effective, the superior effect of *V. amygdalina* (bitter leaf-leaf) was outstanding, followed by *C. papaya* and *J. curcas* respectively.

However, more studies should be carried out to map out the specific bioactive ingredients and ways of including the herbs directly in the animal feed.

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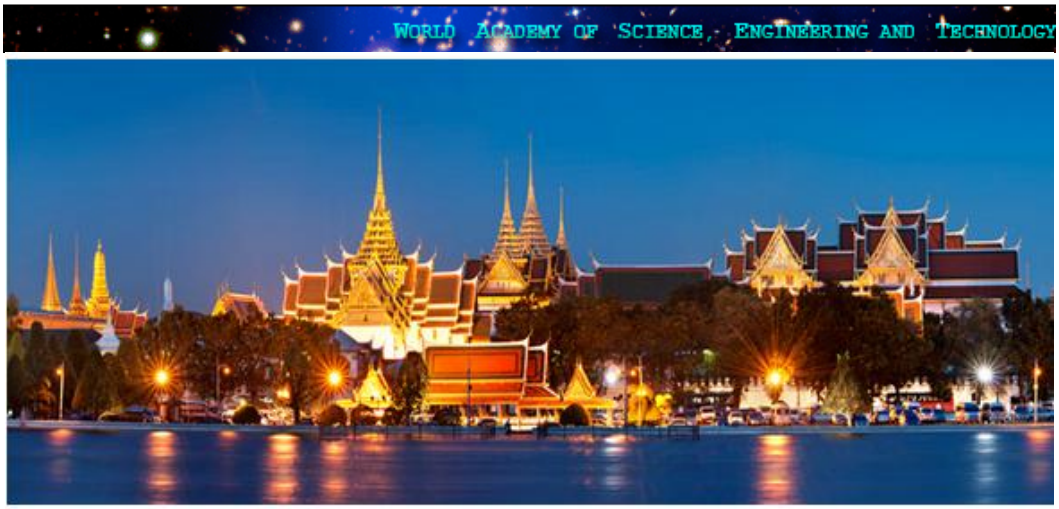

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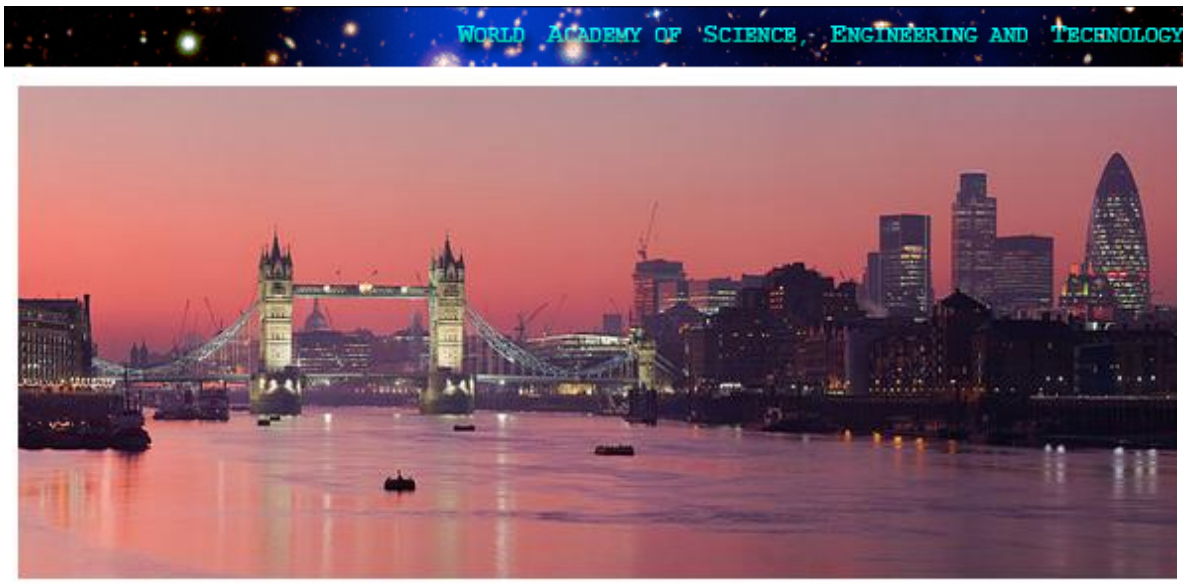

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