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Full Length Research Paper

Evaluation of the antimicrobial activities of *Cymbopogon schoenanthus*

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Medicinal plants have been widely used in folk medicine. They are considered today as an interesting source of new drug due to their bioactive components. The genus, Cymbopogon (Poaceae) seems to be the most promising source of several bioactive compound. This study aimed at evaluating the antimicrobial activities of *Cymbopogon schoenanthus* on several pathogenic bacteria, fungi and virus. Antibacterial and antifungal activities were evaluated using the agar well diffusion methods. The MIC was determined by micro-broth dilution methods. The cytotoxicity of *C. schoenanthus* extracts was evaluated using MTT assay. The results showed that methanol extract of *C. schoenanthus* exhibit an antibacterial effect on several Gram-positive and negative bacteria. All extracts tested (aqueous extract, methanol extract and ethyl acetate extract) were found to have an antiviral effect on HSV1; whereas, no antifungal effect was detected on both *Candida albicans* and *Aspergillus niger.*

Key words: Antiviral, antibacterial, bioactive properties, *Cymbopogon schoenanthus*, medicinal plant.

INTRODUCTION

Since ancient times, medicinal plants have been widely used as a valuable source of medicines in several cultures. Nowadays, according to the World Health Organization (WHO), about 80% of people worldwide are currently depending on traditional medicine for their primary health care needs (WHO, 2005, 2008). Medicinal plants are considered today as an interesting source of new drug due to their bioactive components (Cragg et al., 1997; 1999; Harvey, 1999, 2001; Ali et al., 2015).

Numerous scientists have reported an increased need for novel antimicrobial drugs due to the relatively high incidence of bacterial infection as well as the capacity of pathogenic bacteria to develop a type of resistance against classical antimicrobial drugs (Ganjewala, 2009).

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In fact, the random use of multiple antibiotic drugs is considered to be the principal cause of this resistance. For this reason, numerous researches have been conducted recently to find new sources of bioactive compound (Al-Ali and El-Badry, 2010). Currently, essential oils from *Cymbopogon* species of diverse origin have been studied extensively (Sidibe et al., 2001; Ganjewala et al., 2008).

In Saudi Arabia, there are two species of the Cymbopogon genus, which are the *Cymbopogon commutatus* (also known as Sakhbar) and the *Cymbopogon schoenanthus* (also known as El-Ethkher or camel grass) (Atyat, 1995; Hilo, 1996). *Cymbopogon commutatus* has reported presence in eastern region only (Al-Doussary, 1998), while *C. schoenanthus* can be found in several regions in Saudi Arabia including Makkah Al-mukarmah region (Migahid, 1996).

According to the Arabic folk medicine, the only manner to treat with the *Cymbopogon s.* is through drinking the herb aqueous extract as a tea, in other words, by preparing herbal tea from Ethkher leafs. The traditional treatment has been used as antihelminthic, antidiarrheal, antirheumatic, carminative, diaphoretic, stomachic, diuretic, emmenagogue, for treating fever and for treating jaundice and as tonic (Rizk, 1986; Atyat, 1995). The Saudi folk medicine uses the *C. schoenanthus* for treating people with kidney stone (Hilo, 1996; Al-Ghamdi et al., 2007). In fact, beside their traditional uses in folk medicine, many scientists have reported numerous useful biological activities of the essential oils and essential oil constituents of *Cymbopogon* species. Both *in vitro* and *in vivo* studies have demonstrated how *Cymbopogon* species shows anti-inflammatory, anticancer and allelopathic activities (Li et al., 2005; Santoro et al., 2007; Alitonou et al., 2006). There are few reports available describing other bioactivities of agricultural and ecological significance such as food packaging and insect repellent of essential oils of *Cymbopogon* species (Das and Ansari, 2003; Singh et al., 2006).

The bioactive potential of *Cymbopogon* essential oils and their constituents have been rapidly recognized in the past ten years, although the conventional bioactive properties against microbes have been known for decades. Essential oils of *Cymbopogon* species are easily available, have a pleasant aroma, are non-toxic and safe; the active principles are therefore becoming increasingly popular in pharmaceuticals and medicines.

In order to evaluate the bioactive potential of essential oils and their constituents using animal models and cell systems, several species of *Cymbopogon* such as *Cymbopogon martini, Cymbopogon citrates, C. schoenanthus* and *C. winterianus* were studied recently. The obtained results demonstrated that *C. citratus* has been recognized as most promising since the essential oil of this species has exhibited a variety of fundamental and novel bioactivities. In contrast, *C. schoenanthus* essential oil demonstrated antioxidant activity, while the two other species (*C. martinii* and *C. winterianus*) showed outstanding bioactivities, particularly insect repellent and antihelmintic. The bioactive potential of other species of *Cymbopogon* have not yet been fully exploited (Das and Ansari, 2003; Li et al., 2005; Ketoh et al., 2005, 2006; Santoro et al., 2007).

In 2010, a new study was carried out by EL-Kamali and EL-Amir to exanimate the antibacterial activity of ethanol extracts obtained from eight Sudanese medicinal plants. In this study, scientists have demonstrated that *C. schoenanthus* showed relatively higher propensity to act on Gram- positive bacteria. Recently, the antibacterial activity of *C. schoenanthus* collected from the north-east area of Jeddah, Saudi Arabia has been studied by Hashim and co-workers in 2016. The *C. schoenanthus* essential oils were evaluated against Gram-positive and negative bacteria. Hashim and co-workers (Hashim et al., 2016) have demonstrated that *C. schoenanthus* essential oils represent an inhibitory effect against *S. aureus* methicillin sensitive (MSSA), *S. aureus* (MRSA), *Escherichia coli* and *Klebsiella pneumonia*.

Until now, few studies have been conducted to evaluate the anti-viral and anti-fungal activity of *C. schoenanthus* collected in Saudi Arabia.

The study was aimed at investigating the possible antifungal, antiviral and antibacterial activities of *C. schoenanthus* (L.) *Spreng* collected from Almadinah Almunawarah, in Saudi Arabia against several human pathogenic bacteria, fungi and viruses.

MATERIALS AND METHODS

Plant collection and extracts preparation

C. schoenanthus (L.) Spreng. was collected from Al Madinah Almounawarah, in the western area of Saudi Arabia. The collected plant was subjected to scientific identification using the scientific identification manuals, with the kind help of our botany specialized colleagues in the Faculty of Pharmacy at Taibah University. Voucher specimens have been deposited at the herbarium of the Faculty of Science, Taibah University, Saudi Arabia.

C. schoenanthus was rinsed twice with tab water. Leaves were separated and washed again using sterilized water and left in shade for drying. Plant material was pulverized using domestic blender to powder form. The air-dried powdered herb (1000 g) was extracted with 70% methanol (5 L × 5) till exhaustion and then concentrated under reduced pressure to yield a viscous gummy material. The residue was suspended in water H_2O (5 L) and defatted with n-hexane (4 L). The aqueous layer was then extracted with ethyle acetate (EtOAc) and butanol (1-BuOH) successively (4 L each). The ethyle acetate and butanol fractions were concentrated under reduced pressure to give EtOAc and 1-BuOH fractions, respectively.

Antibacterial and antifungal susceptibility testing

Antimicrobial susceptibility testing was done using the agar well diffusion method to evaluate the anti-bacterial and anti-fungal activities of this plant.

Bacterial and fungal cultures

Eleven bacterial pathogens and two fungal strains were used to evaluate the antimicrobial effect of *C. schoenanthus* extract. The pathogens included Gram-positive bacteria: *Enterococcus faecalis* isolate, *Staphylococcus saprophyticus* (ATCC49907), *Staphylococcus saprophyticus* isolate, *Staphylococcus aureus* (ATCC25923), *Staphylococcus aureus* isolate and *Streptococcus pyogenes* isolate) and Gram-negative bacteria: *E. coli (ATCC35218), Pseudomonas aeruginosa* isolate, *Klebsiella pneumonia* isolate, *Proteus mirabilis* and *Salmonella paratyphi B* isolates, as well as two fungal strains, *Candida albicans* and *Aspergillus niger*.

Bacterial and fungal cultures were prepared as follows. The bacterial cultures were sub-cultured in nutrient broth for 24 h at 37°C while the fungal cultures (*C. albicans* and *A. niger*) were sub-cultured in liquid Sabouraud dextrose medium for 48 h at 25°C. The turbidity of the broth cultures was equilibrated with 0.5 McFarland standard. Mature cultures of *C. albicans* and *A. niger* were inoculated unto Sabouraud dextrose broth to prepare the test inocula which were similarly equilibrated with 0.5 McFarland standard (Baker et al., 1983).

Agar plates preparation and inoculation

Antibacterial tests were carried out in triplicate. Petri dishes were prepared with 20 ml of Mueller Hinton agar. After autoclaving, the agar was allowed to cool down (45 to 50°C). Then, 0.1 ml of the diluted culture was poured on each plate using a mixer (Whirli Mixer, Fisher brand, England), and Petri dishes were left to dry for 30 min at 37°C (Bauer, 1966). Wells of 6 mm diameter were cut with sterile cork borer in the inoculated agar. Then, wells were filled with plant extract. Two positives control were used. Norfloxacin (10 µg/ml) was used as positive control-1 and Augmentin (30 µg/ml) as positive control-2. Distilled water was used as the negative control. Petri dishes were incubated at 37°C for 24 h. Antibacterial activity was evaluated by measuring the diameter of zones of inhibition excluding the hole size by using an antibiotic zone reader (Fisher-Lilly, USA).

Antifungal tests were performed by the same method in triplicate. Petri dishes with 20 ml of Sabouraud Dextrose agar were used. Amphotericin B (10 µg/ml) was used as positive control and distilled water was used as negative control. Petri dishes were incubated at 25°C for 48 h then antifungal activity was evaluated by measuring the diameter of zones of inhibition.

Minimum inhibitory concentration (MIC) test

The MIC was determined by micro-broth dilution methods (Chambers, 2001). The tested extracts were serially diluted in Mueller-Hinton broth (Oxoid) medium. Duplicate tubes of each dilution (100, 50, 25, 12.5, 6.3, 3.2, 1.6, 0.8, 0.4 and 0.2 mg/ml) were inoculated with 100 µl (5×10^5 CFU/ml) of appropriate bacterial and fungal suspension. Then, cultures were incubated at 37°C for 24 h. MICs were considered as the least concentration of each extract with no visible bacterial growth in terms of turbidity (Demarsh et al., 2001).

Antiviral activity examination

Propagation of Vero cell line by enzyme treatment

African green monkey kidney (Vero) cells incubated into culture bottle were checked using inverted microscope for its proper physical conditions. Healthy cells were propagated as follows: the media overlaying cell monolayer was poured off. Cells were released from tissue culture flask by treating with 5 ml pre-warmed trypsin-EDTA solution. EDTA chelates metal ions are involved in cell adherence, the flask was rocked so that trypsin completely cover the cell monolayer. The trypsin was aspirated with a pipette, then 2 ml of trypsin were dispensed, the bottle rocked and were incubated at 37°C. Cells were examined from time to time to avoid trypsin over action. The bottle was struck with hand to completely dislodge the cells from the bottle surface. Cells were suspended in 8 ml of growth media. 10 ml pipette was used to disperse cell aggregates by sucking up and expelling the cells about 4 times to ensure that no clumps of cells were present. After that, cells were counted using haemocytometer using trypan blue vital stain.

Ten milliliters ($2x10^5$) of Vero cells suspension were transferred to 50 cm^3 TC bottle (Falcon) tightly closed. Vero cell suspension was then incubated at 37°C and cells were sub-cultured once weekly. For seeding 96 well plate, 0.1 ml (2×10^5) of cells were transferred to each flat bottomed well and incubated at 37°C for 48 h to develop a complete monolayer sheet.

Cytotoxicity examination

Vero cells monolayer were treated with various concentrations of *C. schoenanthus* extracts for three days and the cytotoxicity of the extracts was evaluated by direct count using Neubauer hemacytometer to indicate their replication rate and checked for any physical signs of toxicity morphologically, e.g. partial or complete loss of the monolayer, rounding, shrinkage, or cell granulation by daily observation by using optical inverted microscope. Then, the maximum non-toxic concentration (MNTC) of three extracts (aqueous extract, methanol extract and ethyl acetate) was determined.

Antiviral assay

The cytotoxicity of *C. schoenanthus* extracts was evaluated by MTT assay kit. $1x10^4$ of Vero cells were plated in 96-well plates (each well contain 200 µl of media), eight wells were left empty for blank controls. Cells were incubated overnight at 37°C in a humidified incubator with 5% CO_2. Equal volume (1:1 v/v) of non-lethal dilution of Herpes simplex virus-1 suspensions (HSV1) was incubated for one hour. 100 µl of viral suspension was added and placed on a shaking platform at 150 rpm for 5 min. Then, viral suspension was incubated at 37°C in a humidified incubator with 5% CO_2 for one day to allow virus to take effect. 2 ml of MTT solution were made per 96 well plates at 5 mg/ml in PBS. 20 µl of MTT solution were added to cells, then incubated for 4 h. The media was clearly removed and MTT metabolic product was resuspended in 200 µl DMSO and thoroughly mixed on a shaking platform at 150 rpm for 5 min. Optical density (OD) was measured at 560 nm (Denizot and Lang, 1986; Hu et al., 1999).

RESULTS

Antibacterial and antifungal activity

Agar well diffusion test

The antifungal and antibacterial activities of four extract (methanol, aqueous, acetone and dichloromethane extract) of *C schoenanthus* were evaluated by using Agar well diffusion test. The antimicrobial effects were tested

Table 1. Antibacterial activity of several *C. schoenanthus* extract by measuring zones of growth inhibition (mm) using agar well-diffusion assay.

Microorganism	Methanol extract	Aqueous extract	Acetone extract	Dichloromethane extract	Augmentin[a] 30 µg/ml	Norfloxacin[b] 10 µg/ml
S. saprophyticus ATCC49907	14.7±0.22	0	0	0	32.2±0.47	20.2±0.67
S. aureus ATCC 25923	14.0±0.33	0	0	0	44.0±0.33	18.7±0.22
S. saprophyticus isolate	9.7±0.47	0	0	7.3±0.58	9.3±0.75	0
S. aureus isolate	19.3±0.58	0	0	0	38.8±0.46	16.8±0.37
S. pyogenes isolate	11.6 ±0.58	0	0	0	44.7±0.94	0
E. faecalis isolate	12.0±0.58	0	0	0	21.2±0.97	11.2±0.38
E. coli ATCC 35218	14.7±0.43	0	0	0	22.3±0.47	30.8±0.13
P. aeruginosa isolate	7.7±0.85	0	0	0	0	23.6±0.74
K. pneumoniae isolate	13.67±0.58	0	0	0	11.3±0.47	27.5±0.76
P. mirabilis isolate	20.67±0.58	0	0	0	22.6±0.48	28.8±0.37
S. paratyphi B isolate	22±1	0	0	18.6±0.58	0	23.2±0.89

[a]Positive control 1; [b]positive control 2. Values represent means ± standard deviations for triplicate experiments ($p < 0.05$). Differences between means of the zones of inhibitions were analyzed by student's t-test (SPSS version 22.0).

Table 2. Anti-fungal activity of several *C. schoenanthus* extract by measuring zones of growth inhibition (mm) using agar well-diffusion assay.

Microorganisms	Methanol extract	Aqueous extract	Acetone extract	Dichloromethane extract	Amphotericin B[a] 10 µg/ml
C. albicans	0	0	0	0	21.8±0.47
A. niger	0	0	0	0	7.8±0.47

[a]Positive control. Values represent means ± standard deviations for triplicate experiments ($p < 0.05$). Differences between means of the zones of inhibitions were analyzed by student's t-test (SPSS version 22.0).

on six Gram-positive bacteria (*E. faecalis, S. saprophyticus* ATCC49907, *S. saprophyticus* isolate, *S. aureus* ATCC 25923, *S. aureus* and *S. pyogenes* isolate) and five Gram-negative bacteria (*E. coli, P. aeruginosa, K. pneumonia, P. mirabilis and S. Paratyphi* B) as well as two fungal species (*C. albicans* and *A. niger*). Means of zones of inhibition are shown in Table 1. Results obtained showed that methanol extract of *C. schoenanthus* exhibit an antibacterial effect on all pathogenic bacteria tested .The largest inhibition zones were exhibited by Gram negative *Salmonella paratyphi B* isolate (22±1 mm) followed by *P. mirabilis* isolate (20.67±0.58mm) and Gram positive *S. aureus* isolate (19.3±0.58 mm). The lowest inhibition effect was detected on *P. aeruginosa* (7.7±0.85 mm). In contrast with the results of methanol extract, aqueous extract and acetone extract did not show any antibacterial activity on the 11 pathogenic bacteria tested. Moreover, dichloromethane extract was only active on two pathogenic bacteria. The zone of inhibitions was as follows: *Salmonella paratyphi B* isolate (18.6±0.58 mm) and *S. saprophyticus* isolate (7.3±0.58 mm). Regarding the antifungal effect of *C. schoenanthus*, the four extract showed no antifungal activity against *C. albicans* and *A. niger* (Table 2).

Determination of minimum inhibitory concentration (MICs)

Minimum inhibitory concentration was performed on eight species of pathogenic bacteria (Table 3). The obtained results demonstrated that methanol extract has an antibacterial activity against both. Gram positive and negative bacteria, MICs ranged from 0.8 to 25 mg/ml. Interestingly, methanol extract showed significant inhibitory effect on two Gram negative bacteria: *Proteus mirabilis* isolate (25 mg/ml) and *S. paratyphi* B isolate (12.5 mg/ml), while dichloromethane extract showed an antibacterial activity only against *S. paratyphi* B isolate ($P<0.05$).

Antiviral activity

Maximum non-toxic concentration

The maximum non-toxic concentration (MNTC) of three extracts (aqueous extract, methanol extract and ethyl acetate) was determined. The obtained results demonstrated that there was no significant difference

Table 3. Determination of minimum inhibitory concentration (MICs) of methanol and dichloromethane extracts.

Microorganism	Methanol extract	Dichloromethane extract
	MIC (mg/ml)	MIC (mg/ml)
E. coli ATCC 35218	1.6±2.71	0
S. saprophyticus ATCC49907	0.8± 1.36	0
S. aureus ATCC 25922	3.2± 5.43	0
S. saprophyticus isolate	1.6± 2.71	0
S. aureus isolate	6.25± 0.00	0
S. pyogenes isolate	3.75±0.02	
E. faecalis isolate	6.25± 0.00	0
K. pneumoniae isolate	3.2± 5.43	0
P. mirabilis isolate	25.00± 0.00	0
S. paratyphi B isolate	12.5.0±0.00	3.2± 5.43

Values represent means ± standard deviations for triplicate experiments ($p < 0.05$). Differences between means of the MICs were analyzed by student's t-test (SPSS version 22.0).

between cells control and the three extracts tested ($P>0.05$). The concentrations of extracts that exhibited cytotoxic effect were excluded. Results obtained are shown in Table 4.

Antiviral activity of C. schoenanthus extracts

All extracts were found to have an antiviral activity against HSV-1 at a concentration non-lethal to the cell line (Vero cells) used (Table 4). Aqueous extract showed significant antiviral effect on HSV-1, viral activity was decreased from 100 to 9.61%. Both methanol and ethyl acetate extracts have also exhibited antiviral effect on HSV-1, viral activity was decreased from 100 to 32.69 and 33.70%, respectively. Results obtained are shown in Table 5.

DISCUSSION

Medicinal plants have been widely used in folk medicine. They are considered today as an interesting source of new drug due to their bioactive components (Cragg et al., 1997, 1999; Harvey, 2001; Ali et al., 2015).

Currently, the genus, Cymbopogon (Poaceae) is renowned for their essential oils of immense commercial significance in flavours, fragrances, cosmetics, perfumery, soaps, detergents and pharmaceuticals (Ganjewala et al., 2008). The genus Cymbopogon comprises about 140 species of which most are aromatic and yield an essential oil upon the steam distillation of their aerial parts (Khanuja et al., 2005). Semi-chemical properties of the Cymbopogon essential oils have been promising in integrated pest management programme since this property may lead to development of alternatives to synthetic chemical pesticides (Kumar et al., 2008).

In addition, many scientists have evaluated the potential use of *C. schoenanthus* against gastrointestinal nematodes**.** Results obtained showed that *C. schoenanthus* essential oil had the best activity against ovine trichostrongylids **(**Katiki et al., 2011, 2012). Other studies were also performed in order to investigate the antistress properties of *C. schoenanthus*. Results obtained showed that ethanol extract has a significant antistress effect at *in vitro* and *in vivo* levels (Mahmoud Ben Othman et al., 2013). However, the bioactive potential of other species of *Cymbopogon* have not yet been fully exploited (Das and Ansari, 2003; Li et al., 2005; Ketoh et al., 2005, 2006; Santoro et al., 2007).

Conclusion

This study illustrates the importance of *C. schoenanthus* as promising sources of novel antimicrobial drugs. The results showed that methanol extract of *C. schoenanthus* exhibit an antibacterial effect on several Gram-positive and negative bacteria. Furthermore, all extracts tested (aqueous extract, methanol extract and ethyl acetate extract) were found to have an antiviral effect; whereas, no antifungal effect was detected on both *C. albicans* and *A. niger.* In conclusion, drug discovery can be significantly improved through the use of the knowledge gained from research in natural products (Gad, 2005).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Table 4. Maximum non-toxic concentration [MNTC] of aqueous, methanol and ethyl acetate extracts.

Sample	Dilution 1:2	O.D	Mean O.D	Cell viability	Selected dilution
Cell control	-	0.165 0.172 0.159 0.141	0.159	100%	
Aqueous extract	1	0.028- 0.030 - 0.022	0.026	16.35	
	2	0.037 -0.028 -0.028	0.031	19.49	
	3	0.019- 0.025-0.029	0.024	15.09	
	4	0.037- 0.026- 0.040	0.034	21.38	
	5	0.045- 0.040- 0.042	0.042	26.41	
	6	0.051- 0.049- 0.049	0.049	30.81	
	7	0.061 - 0.062- 0.070	0.064	40.25 (LD-50)	
	8	0.137- 0.148- 0.154	0.146	91.82	10^{-8} non lethal conentration
Methanol extract	1	0.017- 0.012- 0.013	0.014	8.80	
	2	0.018- 0.012- 0.015	0.015	9.43	
	3	0.028- 0.024- 0.013	0.021	13.20	
	4	0.067- 0.020- 0.057	0.048	30.18	
	5	0.059- 0.060 0.059	0.059	37.10	
	6	0.061-0.060-0.072	0.064	40.25	
	7	0.075-0.069-0.072	0.072	45.28	
	8	0.091- 0.083-0.085	0.086	54.08 (LD-50)	
	9	0.089-0.092-0.089	0.090	56.60	
	10	0.095-0.100-0.098	0.097	61.00	
	11	0.128-0.122-0.130	0.126	79.24	
	12	0.141-0.152-0.149	0.147	92.45	10^{-12} non lethal conentration
Ethyl acetate	1	0.017- 0.013- 0.013	0.014	8.80	
	2	0.024- 0.033 - 0.059	0.038	23.89	
	3	0.067- 0.078- 0.037	0.060	37.73	
	4	0.083- 0.090- 0.094	0.089	55.97 (LD-50)	
	5	0.150 - 0.160- 0.163	0.157	98.74	10^{-5} non lethal conentration
	6	0.166- 0.170- 0.145			
	7	0.167- 0.154- 0.162			
	8	0.159-0.166-0.160			

Table 5. Antiviral activity of the studied extracts against HSV 1.

Extract	Test	O.D	Mean O.D	viability	toxicity	Viral activity
	Vero cells[a]	0.154/0.155/0.156	0.155	100%	0%	0%
	HSV 1[b]	0.040/0.063/0.055/0.066/0.033	0.051	32.90	67.1	100%
Aqueous		0.141 / 0.146 / 0.148	0.145	93.54	6.45	9.61
Methanol		0.131 / 0.116 / 0.118	0.121	78.06	21.94	32.69
Ethyl acetate		0.129/ 0.111 / 0.120	0.120	77.41	22.58	33.70

[a]Negative control; [b]Positive control.

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Full Length Research Paper

Pleurotus albidus: A new source of milk-clotting proteases

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Researches have been done to discover new sources of microbial proteases with milk-clotting activity to replace the traditional rennet from calves. The aim of this study was to select a species of edible mushroom as milk-clotting enzymes producer using the submerged fermentation technology. From the six species evaluated, *Pleurotus albidus* was the one that expressed the highest value of coagulant ratio (21.60). The milk-clotting enzymes showed maximum activity at 60°C and pH 6.0. Iodoacetic acid presented the highest inhibition in proteolytic activity suggesting the presence of cysteine proteases in the crude extract from *P. albidus*. The enzymes did not present toxic action against human fibroblasts (MRC5) in the analyzed conditions and, for that reason, can be suitable to applications in food industry. This is the first report of milk-clotting enzymes production by the edible mushroom *P. albidus*

Key words: *Pleurotus albidus*, milk-clotting, submerged fermentation.

INTRODUCTION

Proteolytic enzymes represent 65% of worldwide enzymes industry market and, among them, chymosin has great application in dairy industry acting in the casein peptides linkages during the cheese making (Yin et al., 2014). The raising of world cheese production, the shortage and the high price of tradition rennet associated with the rejection of proteases genetically modified has encouraged the search of new milk-clotting sources, especially the ones from microbial source (Ahmed et al., 2010; El-Baky et al., 2011; Leite Júnior et al., 2014; Wang et al., 2015).

The microbial protease presents advantages when compared with the enzymes from plants and animal due their biochemical diversity, possibility of genetic

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manipulation and low production costs (Erjavec et al., 2012; Savitha et al., 2011).

Fungi belong to the microbial diversity and have many advantages as considered by Generally Regarded as Safe (GRAS) and their extracellular enzymes are easily recovered in bioprocess. Among fungi, the edible mushrooms are macro fungi that already produce milk-clotting enzymes usually obtained from submerged fermentation (SmF) which is the most useful method for enzymes production. The advantages of SmF are the controlling of parameters as pH, temperature, oxygen and humidity besides the possibility of large-scale production (Sandri et al., 2015; Uzuner and Cekmecelioglu, 2015; Sandhya et al., 2005).

Due to the nutritional and medicinal properties of edible mushrooms, the uses of these fungi in food and pharmaceutical industries have been raising. Among the 7000 mushrooms species that present variable edible levels only 2000 of them are considered as safe. However, a reduced number of mushrooms species are considered dangerous because they produce toxic substances which are malefic to human health and can be lethal to the individual (Jo et al., 2014).

The aim of this study was to investigate the production of milk-clotting enzymes from mushrooms and select one species to determine their enzyme profile and application in cheese production.

MATERIALS AND METHODS

Mushrooms

Pleurotus albidus DPUA1692, *Auricularia mixotricha* DPUA 1695, *Ganoderma lucidum* DPUA 1694, *Lentinus citrinus* DPUA 1535, *Lentinus crinitus* DPUA 1693, and *Pleurotus ostreatoroseus* DPUA 1720 (Culture Collection DPUA/UFAM) were evaluated as milk-clotting enzymes producers. Each culture were cultivated in Potato Dextrose Agar (PDA medium and 0.5% (w/v) yeast extract) in Petri dishes. The cultures were maintained at 25°C for 12 days.

Fermentation media and culture conditions

The production of milk-clotting enzymes was made by submerged fermentation using liquid medium composed of (g/L): glucose (20), yeast extract (5), meat peptone (5), and gelatin (5) at pH 5.6. The fermentation was performed in 125 ml Erlenmeyer flasks containing 50 ml of sterilized medium (121°C for 15 min). After cooling, each flask was inoculated using 10 micelial discs of 5 cm diameter and incubated at 30°C, on rotatory shaker (Nova Técnica, model 712, Piracicaba, São Paulo, Brazil) at 150 rpm during 72 h. The biomass was separated from the crude extract by vacuum filtration on Whatman no. 1 paper filter.

Proteolytic activity assay

The proteolytic activity was determined according to Leighton et al. (1973). Protease activity was determined in the crude extracts (150 µl) using 1.0% (w/v) azocasein (250 µl) in 0.2 M Tris-HCl buffer, pH 7.2. One unit of proteolytic enzyme was defined as the amount of enzyme that produces a 0.1 increase of absorbance in 1 h at 440 nm (U/ml). All samples were prepared in triplicate.

Milk-clotting protease assay

Milk-clotting activity was determined according to Arima et al. (1970) using 10% (w/v) bovine skimmed milk poder (Itambé®) in 0.05 M $CaCl_2$ as substrate. Briefly, 5 ml of milk solution were distributed in test tubes and pre-incubated in water bath (Gant, model 179, Cambridge, England) at 40°C for 15 min. The enzyme extract (0.5 ml) was added to the milk and counting time started. Clot formation was observed while manually rotating the test tube. The time at which the first particles were formed was measured. All samples were prepared in triplicate. One unit of milk-clotting activity (U) was defined as the amount of enzyme required to coagulate 1 ml of substrate in 40 min at 40°C. Milk-clotting activity was calculated according to Shata (2005): $U = 2400/T \times S/E$, where T (s) is the necessary time to clot formation, S is the volume of milk (ml) and E is the volume of crude extract used (ml). The coagulant ratio (R) was calculated according to the ratio of milk-clotting activity and proteolytic activity values.

To select a strain and characterize its enzymes, the samples were grouped into three classes according to the formation of compact milk clot and milk whey separation in the test tube: strong milk coagulation (distinct clot and abundant whey); weak milk coagulation (clot formation without clear separation of the whey); and milk without coagulation (clot and whey absent) (Alecrim et al., 2015).

Effect of pH and temperature on activity and stability of milk-clotting enzymes

To assay optimum pH, proteolytic activity was determined at 40°C at different pH values using the following 0.1 M buffer solutions: sodium acetate (5.0 and 6.0), Tris-HCl (7.0 and 8.0) and Glicine-NaOH (9.0 and 10.0). Optimum temperature was determined by incubating the enzyme extract at different temperatures ranging from 30 to 80°C and assaying the activity at the pH determined as optimum.

For the pH stability, the crude extract was dispersed (1:1) in the following 0.1 M buffer solutions: sodium acetate (5.0 and 6.0), Tris-HCl (7.0 and 8.0) and Glicine-NaOH (9.0 and 10.0) and maintained at 25°C for 24 h. In thermal stability, the extracts were incubated at different temperatures ranging from 30 to 80°C for 1 h. The solution of 10% (w/v) skimmed milk powder in 0.05 M $CaCl_2$ was used as substrate. All samples were prepared in triplicate. Residual enzyme activities were determined according to the optimal conditions of pH and temperature.

Effect of inhibitors and metallic ions on milk-clotting activity

The effect of inhibitors and metallic ions on milk-clotting activity was investigated by using 1 mM of pepstatin A and 10 mM of phenylmethylsulfonyl fluoride (PMSF), ethylene-diaminetetraacetic acid (EDTA), iodoacetic acid, $CaCl_2$, $CuSO_4$, KCl, $FeSO_4$, $MgSO_4$, $MnSO_4$, NaCl and $ZnSO_4$ in the reaction. Samples were incubated at 40°C for 30 min and the milk-clotting activity was determined according to the methodology discribed previously. Residual enzyme activities were determined and compared to the control, which was incubated without the inhibitors and metallic ions and corresponds to 100% of activity. All samples were prepared in triplicate.

Toxicity test

All tests were carried out at the Biological Activity Laboratory, Pharmaceutical Science College, Federal University of Amazonas.

Strain and culture conditions

In this study, human fibroblasts (MRC5) maintained in culture bottles containing Dulbeco's Modified Eagle Medium (DMEM) supplemented with 10% (w/v) fetal bovine serum (FBS) and 1% (w/v) ampicillin-streptomycin were used. The cells were maintained in bacteriological incubator with 5% CO_2 at 37°C. This study model promoted evaluation of the cytotoxic effect of *P. albidus* crude extract, at different concentrations, against strains of MRC5.

***In vitro* cytotoxicity**

The cytotoxicity of *P. albidus* crude extracts was evaluated by Alamar blue test, according to Ahmed et al. (1994). Alamar blue (resazurin), a blue dye and no fluorescent was reduced to resofurin, pink colored and fluorescent, by the viable cells. The cells were transferred to a micro plate (96 wells) in concentration of 0.5×10^4 cells/well. These plates were maintained in incubator with 5% CO_2 for 24 h. The cells were then treated in single concentration of *Pleurotus* crude extract (200 µg/ml diluted in DMSO) for 72 h. The positive control was doxorubicin (1720 µM) and the negative control was 0.1% (w/v) DMSO. Three hours before each treatment was added 10 µl of 0.2% (v/v) Alamar blue and followed by readings at 465 and 540 nm using a micro plate reader. All the assays were performed in triplicate. The data were analyzed in GraphPad prism 6.0 program and images were captured by Zen 2 program from Carl Zeiis Microscopy Gmbh Company®.

Statistical analysis

In all experiments, the data were subjected to descriptive statistical analysis of variance and the averages were compared by Tukey's test ($p<0.05$) using Minitab software, version 16.0. (Minitab, 2010).

RESULTS AND DISCUSSION

The quality of the enzymes used in cheese making has influenced the milk compounds biotransformation and in the process of clot formation, which determines the sensorial properties of the final product. Proteases with similar activities to the commercial enzymes were already identified in Basidiomycetes (Shamtsyan et al., 2014).

Another important parameter is the coagulant ratio. The relation of milk-clotting activity and proteolytic activity is associated with the enzymatic specific in the clot formation and influences in the selection of a new source of milk-clotting enzymes (Alecrim et al., 2015).

From the six edible mushrooms selected in this study, *G. lucidum* and *P. albidus* were the species that produced milk-clotting enzymes. The values of milk-clotting activity and coagulant ratio were significative to *P. albidus* (73.39 U/ml and 21.60, respectively). Silva et al. (2014) reported milk-clotting activity (60.5 U/ml) and coagulant ratio (510) in *Thermomucor indicae-seudaticae* N31. In the study of Alecrim et al. (2015), *Aspergillus flavo furcatis* DPUA 1493 presented milk-clotting activity of 68.61 U/ml and coagulant ratio of 1.81. Milk-clotting enzymes from *Termitomyces clypeatus* presented values of 333.33 U/ml and 64.70 of milk-clotting activity and coagulant ratio, respectively, in the study of Majumder et al. (2015).

Figure 1. Milk-clotting activity from *P. albidus* crude extract.

The milk-clotting enzymes from *P. albidus* formed a clot classified as strong, forming abundant whey after the addition of the enzymatic extract in milk solution (Figure 1). Similar result of clot formation was reported in the study of Alecrim et al. (2015) with enzymes from *A. flavo furcatis*, an anomorphic fungus.

The studies of optimum conditions of pH, temperature and stability can be used as indicatives of enzymes industrial application (Castro and Sato, 2013). The effect of pH in the activity of *P. albidus* milk-clotting enzymes is as shown in Figure 2. The raising of pH promoted the loss of milk-clotting activity. The enzymes exhibited 100% of activity at pH 6.0 and at pH 10.0 the activity was only 19.37%.

Pediococcus acidilactici SH enzymes also showed high milk-clotting activity at pH 6.0, however, it was observed that at pH 7.0 and 8.0 the activity decreased in 54 and 21%, respectively (Imdakim et al., 2015). Vishwanatha et al. (2010) reported high milk-clotting activity value at pH 6.3 from the enzymes produced by *Aspergillus oryzae* MTCC 5341 followed by the reduction of the activity in high values of pH.

In conditions out of the optimum pH, there was loss of enzymatic activity due to conformation changes in the protein structure caused by charges repulsion. The distribution of charge in the protein surface and the conformations are modified and, for that reason, the enzymes cannot be associated with the substrate correctly. According to Vasconcelos et al. (2004) the pH can influence in the yield of milk-clotting. In the ranges of pH from 5.7 to 6.2, the milk-clotting enzymes from microbial source present higher proteolytic activity when compared with the ones from animals and genetic modified.

The temperature had great influence in the milk-clotting

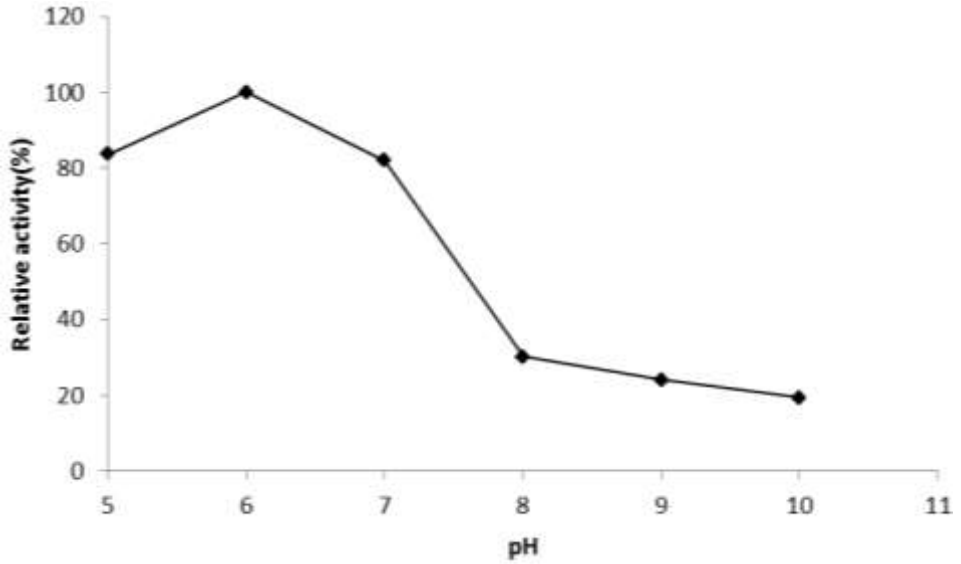

Figure 2. Effect of pH in milk-clotting activity from *P. albidus* crude extract.

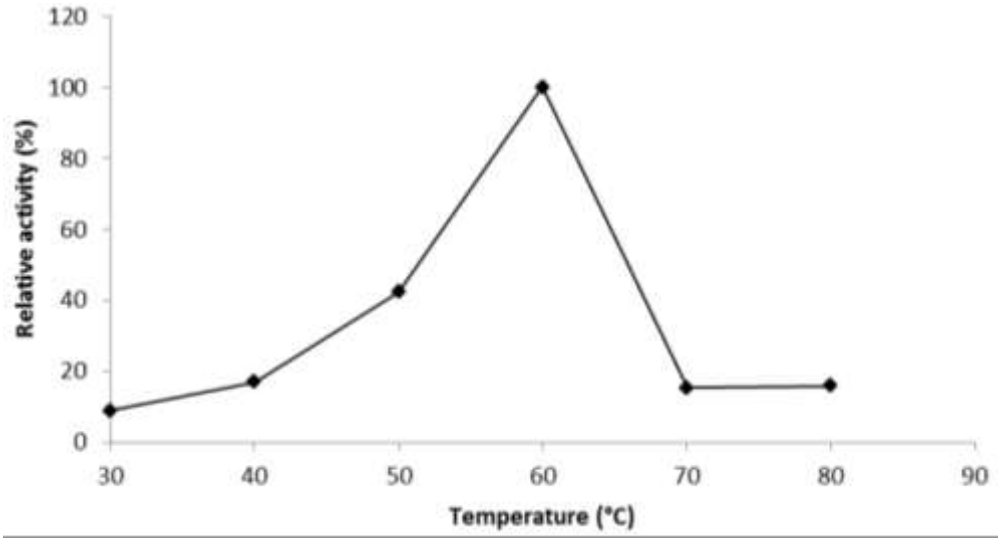

Figure 3. Effect of temperature in the milk-clotting activiy from *P. albidus* crude extract.

enzymes activity (Figure 3). The optimum temperature was determined at 60°C. Milk-clotting enzymes from other fungi as *Rhizopus oryzae* (Kumar et al., 2005), *Penicillium oxalicum* (Hashem, 1999) and *Rhizopus microsporus* var*. rhizopodiformis* (Sun et al., 2014) also presented optimum activity at 60°C.

According to Dybowska and Fujio (1996) at high values of temperature, the process of coagulation is decreased. The increase in milk temperature affects the protein aggregation, increase the velocity of gel formation (McMahon et al., 1984) and cause the shortening of the protein matrix due the raise of hydrophobic interactions (Ahmed and Helmy, 2012). The main factor responsible for the increase of the heated milk coagulation time is the complex formed between κ-casein and β-lactoglobulin or α-lactalbumin although the equilibrium alterations of the salt are also involved (Balcones et al., 1996).

Figure 4 shows the stability of milk-clotting enzymes from *P. albidus* at different pH values. The enzymes presented activity in all study conditions, however, a reduced stability was observed with the increase of pH values. The highest milk-clotting activities were observed at pH 4.0. At pH 5.0 and 6.0, the enzymes maintained 51.34 and 30.82% of activity, respectively. The highest

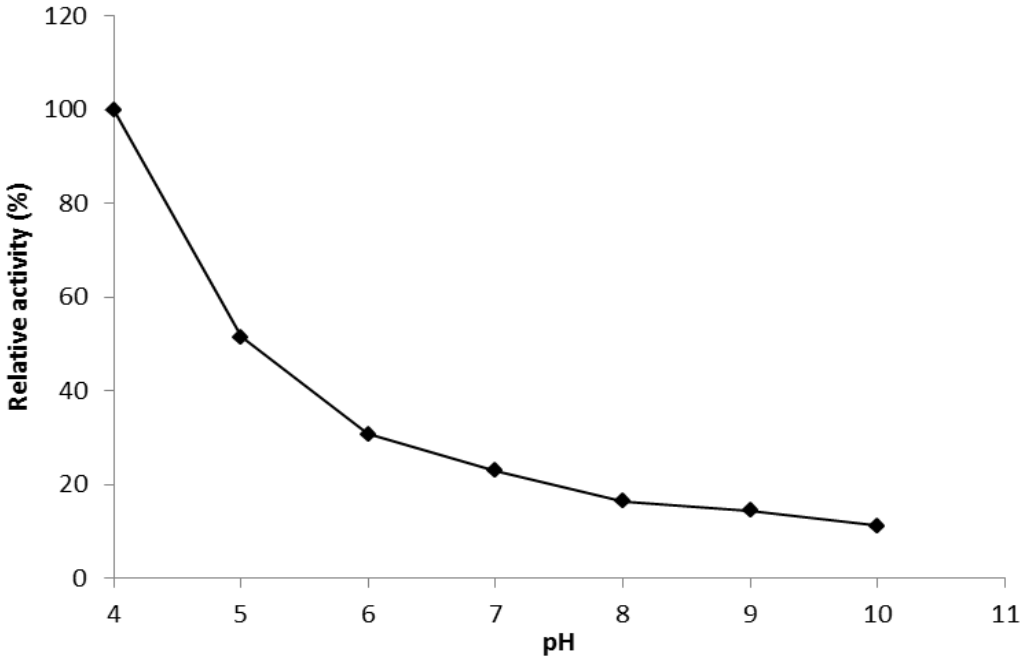

Figure 4. Effect of pH on the stability of milk-clotting enzymes from *P. albidus* crude extract, after 1 h of incubation.

loss of activity was observed at pH 10.0 present only 11.21% of activity. Merheb-Dini et al. (2010) reported similar results using milk-clotting enzymes from *T. indicae-seudaticae* N31. In this study, the highest milk-clotting activity were determined at pH 3.5. Yegin et al. (2012) reported enzymatic stability of *Mucor mucedo* DSM 809 in the pH range from 5.0 to 5.5 with loss of milk-clotting activity at pH higher than 6.0. The different levels of resistance in thermal treatment by milk-clotting enzymes vary according to the protein origin (Hayaloglu et al., 2014). Figure 5 shows the crude extract enzyme stability to different values of temperature. After 1 h of incubation at 40°C, the enzyme presented the highest milk-clotting activity, but at 50°C there was decrease to 34%. The enzymes lost 72.71, 75.85 and 78.80% of activity at 60, 70 and 80°C, respectively. *T. clypeatus* MTCC 5091 was stable between 35 and 50°C, retaining more than 80% of the activity, with a fast decrease according to the increase of temperature (Majunder et al., 2015). In the study of Alecrim et al. (2015) the enzymes from *A. flavo furcatis* presented thermal stability higher than 70% between 40 and 60°C, however in the highest temperature was observed a decrease to 28% of activity.

The effect of metallic ions and inhibitors in the milk-clotting enzymes activity are shown in Table 1. The ion Zn^{2+} raised the milk-clotting activity in 78%. The ions Mn^{2+}, Fe^{2+} and Cu^{2+} caused a reduction of 97.60, 78.75 and 35%, respectively. Mg^{2+}, Ca^{2+}, Na^{+} and K^{+} promoted a complete inhibition of the milk-clotting enzymes (Figure 5). Sun et al. (2014) showed that Zn^{2+} ions raised the milk-clotting enzyme activity from *R. microsporus* var. *rhizopodiformis*. Ahmed and Helmy (2012) also observed the raise of activity in the presence of Zn^{2+} ions (67.7%) in the study with *Bacillus licheniformis* 5A5. Cu^{2+} and Mg^{2+} ions stimulated milk-clotting enzymes from *P. oxalicum* in the report of Hashem (2000). According to Merheb-Dini et al. (2009) the ions can link in amino acid residues and modify the protein structure which can have positive or negative influence in the proteolytic activity.

In the tests using different inhibitors, the results showed the presence of different milk-clotting protease groups from *P. albidus* with the highest prevalence of cysteine-proteases. Milk-clotting proteases from *P. albidus* maintained stability with EDTA. They retained 93.7% of milk-clotting activity. Pepstatin A and PMSF caused inhibition of 35 and 49.5%, respectively. Iodoacetic acid caused the highest inhibition of milk-clotting proteases (37.7%). These results demonstrated that SH groups probably can be involved in the catalytic mechanism and it is suggested that milk-clotting enzymes from *P. albidus* belong to cysteine protease family.

After 72 h of treatment, MRC5 cell viability was equal or superior to 100% and the cells did not present any morphological changes when compared with negative control (Dimethyl Sulfoxide [DMSO]). The positive control (doxorubicin) presented 23% of cell viability when tested with 20 µM or 34 µg/ml (Figure 6). These results show that the extract from *P. albidus* does not presenttoxicity and can be used with safety in food industry. Al-Temimay et al. (2015) and Lai et al. (2013)

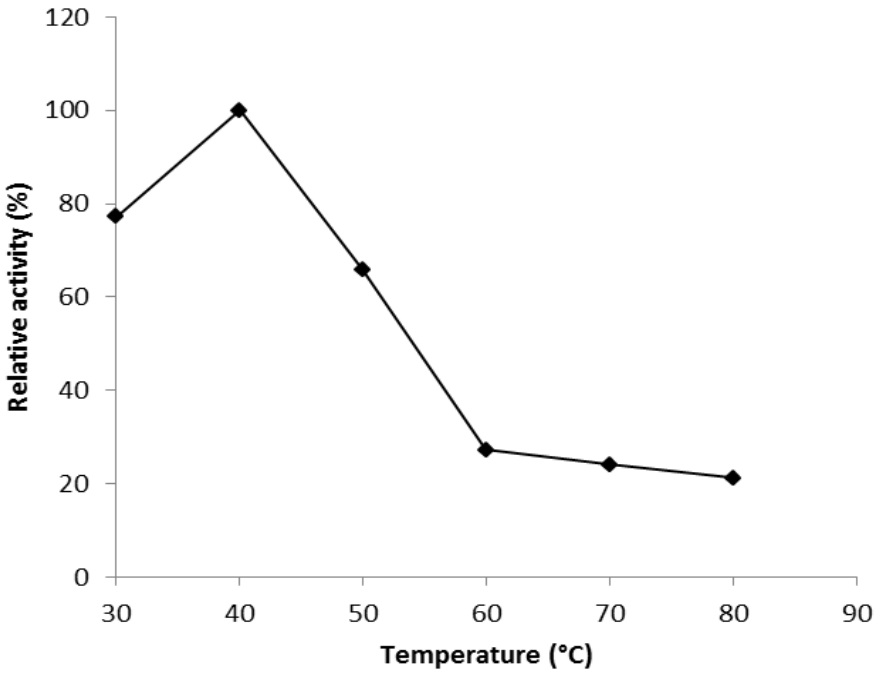

Figure 5. Effect of temperature on the stability of milk-clotting enzymes from *P. albidus* crude extract, after 1 h of incubation.

Table 1. Effect of ions and inhibitors in milk-clotting activity of *P. albidus* milk-clotting enzymes.

Chemicals	Concentration (mM)	Relative activity (%)
Control	—	100 ± 0.00
Zn^{2+}	10	178 ± 0.20
Mn^{2+}	10	98 ± 0.17
Fe^{2+}	10	79 ± 0.04
Cu^{2+}	10	35 ± 0.02
Mg^{2+}	10	0 ± 0.00
Na^{+}	10	0 ± 0.00
Ca^{2+}	10	0 ± 0.00
K^{+}	10	0 ± 0.00
EDTA	10	94 ± 0.13
Pepstatin	1	65 ± 0.22
PMSF	10	51 ± 0.29
Iodoacetic acid	10	38 ± 0.20

reported that the extracts from *Pleurotus ostreatus* and *Hericium erinaceus* did not present toxicity effect against MRC5 fibroblasts strains. The aqueous extract from *Lignosus rhinocerotis* was also not toxic to MRC5 cells (Lau et al., 2013).

Conclusions

From the five edible mushrooms evaluated, *P. albidus*, a species nontoxic, from Amazonic ecosystem, produces milk-clotting enzymes. This potential coagulant expresses optimum activity at 60°C, pH 6 with higher stability at 40°C, pH 4, with the presence of cysteine proteases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

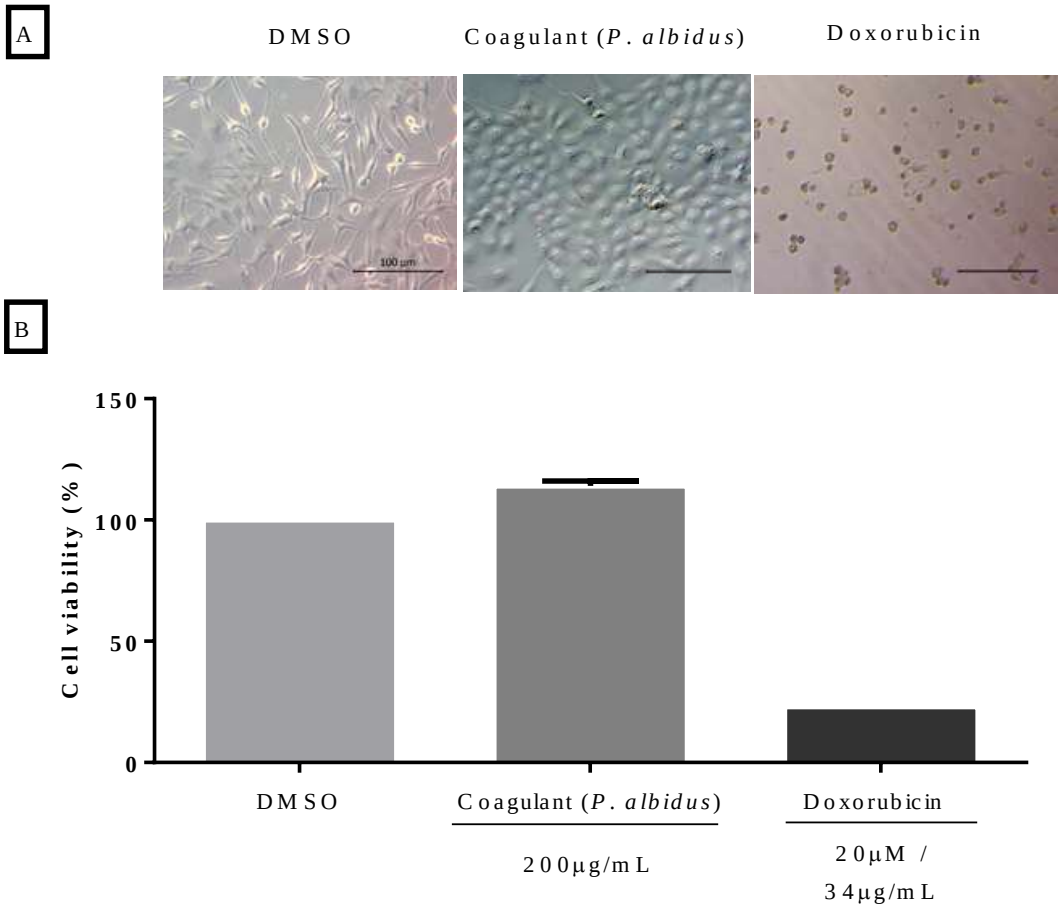

Figure 6. Result of cytotoxic assay of *P. albidus* coagulant extract against human fibroblasts MRC5 after 72 h of treatment. A: MRC5 cells after treatment with DMSO, *P. albidus* coagulant extract, Doxorubicin. B: DMSO (negative control), cell viability of *P. albidus* extract, 0.01% (w/v) and Doxorubicin (positive control -20 µM or 34 µg/mL).

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Full Length Research Paper

Phomopsis sp. as an endophyte of *Turnera subulata* L.: Isolation, identification and antimicrobial and antioxidant activity of their extracts

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***Turnera subulata* L. is a plant that belongs to the Turneraceae family and is popularly known in Brazil as "Chanana"; it is used as an alternative medicine. Among all microorganisms, fungi are mostly associated with plants. The aim of this study is to isolate, identify and evaluate the antifungal and antioxidant activity of extracts of *Phomopsis* sp. isolated from *T. subulata*. From the leaf fragment obtained from *T. subulata,* the filamentous endophytic fungus *Phomopsis* sp was isolated. The fungal isolate had a higher growth in potato dextrose agar (PDA) and potato sucrose agar (PSA) culture medium, as well as in the presence of light. In the antagonism test of the endophytic *Phomopsis* sp. against human pathogens, there was inhibition zone against *Escherichia coli, Staphylococcus aureus, Candida albicans, C. tropicalis* and *C. glabrata*. Concerning the antioxidant activity, it was observed that the chloroform extract was more effective than hexane. On the other hand, all the extracts from the mycelium of *Phomopsis* sp. and its ethyl acetate extract from the cultured filtrate had low antimicrobial activity against strains of *E. coli, S. aureus, C. albicans, C. tropicalis* and *C. glabrata*. Therefore it was concluded that *Phomopsis* sp. may act as an endophyte of *T. subulata.* Extracts from *Phomopsis* sp. promoted inhibition zone of growth when tested against human pathogen. Its hexanic and chloroform extracts showed lower antioxidant activity.**

Key words: Biological control, endophytic fungus, secondary metabolites, filamentous fungus, fungal extract.

INTRODUCTION

Endophytic microorganisms are potentially useful to industries, particularly in food and pharmaceutical industries. Several selected species of endophytes are used in crop protection industries, besides being used as

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genetic vectors (Souza et al., 2004). These microorganisms are important genetic source for biotechnology; they have caught the attention of the scientific community because they produce secondary metabolites which are used in food industry and pharmaceuticals (Strobel, 2003).

Valsaceae mitosporico belongs to the genus, *Phomopsis;* phylumm Ascomycota; subphylum, Pezizomycotin; class, Sordariomycetes; subclass, Sordariomycetidae; order, Diaporthales; family, Valsaceae. *Diaporthe* is the telemorphic form of the genus, Valsaceae (Hanlin and Menezes, 1996). This genus has been isolated as an endophyte in several vegetable crops such as *Theobroma cacao* L. (Rubini et al., 2005), *Spondias mombin* L. (Rodrigues et al., 2000), *Aspidosperma tomentosum* (Corrado and Rodrigues, 2004), *Heterosmilax japonica* Kunth (Gao et al., 2005), among others.

Fungi are considered as promising sources of new drugs for therapeutic use and in fact, several medicines used in health centers are derived from fungal metabolites. In the past two decades, a group of microorganisms that stood out for the production of bioactive metabolites were endophytes, especially fungi. Endophytic microorganisms are found in many species of medicinal plants. Stierle et al. (1995) reported that the fungus *Taxomyces andreanae*, taxol-producing endophytic and other taxanos, that live in the interior of the medicinal plant, *T. brevifolia*, is used for cancer treatment.

According to Arbo (2004), the family Turneraceae has 10 genus and around 190 species with a wide distribution in tropical and subtropical regions of the world, with tropical America being the diversity Center of these species. The genus *Turnera* presents about 120 species scattered in the Americas and Africa, being the most representative of the family Turneraceae (Barbosa et al., 2007).

For every moment, more is known about the interaction between plants and endophytic microorganisms, as well as the new species found in symbiosis and the wealth of metabolites produced by them. Chen et al. (2014), in his review, discusses the evolution and discoveries of endophytic fungi. Malhadas et al. (2017) describe the antimicrobial activity of *Penicillium* species isolated from olive leaves, as well as the identification of their compounds. In view of the above, the goal of this study is to isolate and evaluate the antimicrobial potential of *Phomopsis* spp. isolated from *Turnera subulata* leaves.

MATERIALS AND METHODS

Isolation and purification of endophytic fungi from *T. subulata*

Leaves of *T. subulata* without symptoms of diseases or mechanical injuries were collected in the urban region of Maceio city and taken to Laboratory of Biotechnology of Plants and Endophytic Microorganisms where they were promptly washed with running water. All the materials were disinfected superficially through the following protocol: 1) the vegetal sample was washed in alcohol (70% v/v) for 1 min; 2) the material was washed in sodium hypochlorite (0.20% w/v) plus two drops of commercial detergent for 20 min and 3) triple washing in sterile distilled water.

After cleaning and disinfecting the *T. subulata* samples with 1 cm of diameter, they were inoculated on petri plates containing potato dextrose agar (PDA) medium, and incubated for five days at room temperature on mycelial growth. After incubation, each microorganism colony was taken as a sample of the vegetative mycelium and inoculated in PDA medium for six days under continuous light at temperature of 25°C (±3°C). After growth, the samples were checked on optical microscopy to verify the purity of the isolated fungal colonies.

Prior to the identification of the fungi isolated, micro culture methodology was done. The colony was scraped before it was grown in petri dishes containing PDA medium. Thereafter it was inoculated on slides with a drop of the same culture medium on an equilateral triangle and damp cotton with sterile water to grant moisture required for fungal growth. It was incubated for three days under continuous light.

After this procedure, slides were stained with bromophenol blue and observed by optical microscopy to determine the presence of the fungus *Phomopsis* spp. based on its morphologic characteristics.

DNA extraction from the endophytic fungus

The endophytic fungus isolated from *T. subulata* L. was grown in 100 ml of liquid medium potato dextrose (PD), at 23 to 25°C without agitation for four days. Such samples were forwarded to Forensic DNA Laboratory at the Natural History Museum of the Federal University of Alagoas for DNA extraction and subsequent sequencing of the prevalence of ITS2 regions (internal transcribed spaces 1 and 2, respectively) of rDNA.

The mycelium of *Phomopsis* sp. was isolated from the culture medium by centrifugation for 10 min at 7000 rpm (after the fourth day of growth) and subjected to DNA extraction using the CTAB protocol (Ferreira and Grattapaglia, 1995). After the extraction, DNA was stored at 4°C. The amplification of the rDNA was performed by PCR. Specific primers of Write et al. (1990) were used for the amplification of the prevalence of ITS1 and ITS2 regions.

The fragments produced were separated by capillary electrophoresis in automatic sequencer ABI Prism 310 Genetic Analyzer, using polymer POP6 (Applied Biosystems, Foster City, CA, USA). An analysis of the Electropherograms was made using the program SeqScape v2.5. The mixed sequence obtained was 515 nucleotides and was analyzed using the BLAST program of NCBI, which allowed for the comparison percentage of similarity found with the data available in NCBI (National Center for Biotechnology Information) (www.ncbi.nlm.nih.gov).

Chemical prospecting from the mycelium of *Phomopsis* sp.

Erlenmeyer, containing PD medium and inoculated with a fungal disk in a diameter of approximately 5 mm, was incubated in the dark without aeration at 25 (±3°C) for the production of biomass. After 30 days of cultivation, the biomass was filtered and taken to kiln drying at 45°C until dry mass was obtained. The dry biomass was removed through maceration with n-hexane (5 x 250 mL), chloroform (5 × 250 mL), ethyl acetate (5 x 250 mL) and methanol (5 x 250 mL).

Preparation of samples and determination of IC_{50}

The stock solutions of the samples (dry extracts; 40 mg/mL) were

prepared from the dissolution of each sample in methanol (HPLC grade). From these solutions were prepared dilutions in concentrations of 30, 20 and 10 mg/ml. Five experiments of dried extracts were performed with each of the solutions (10 to 40 mg/ml). Reaction mixtures consisting of 0.1 ml of the test solution and 0.9 ml of stock solution of DPPH 40 mg/ml (~100 Î¼mol/L) were prepared and the measures of absorbance of each were made at 515 nm, in triplicate every 15 min, for 1 h.

Antimicrobial activity of extracts of *Phomopsis* sp against human pathogenic microorganisms

Hexane, chloroform, ethyl acetate and partition of the mycelium and extract in ethyl acetate of the culture filtrate of *Phomopsis* sp were tested against *E. coli* (ATCC 8739), *S. aureus* (ATCC 6538), *Candida albicans* (URM 4126), *C. tropicalis* (URM 4977) and *C. glabrata* (CCIBM), through the agar diffusion method, described by Bauer et al. (1996). All strains are deposited in the culture bank of Microbiology Laboratory of the Institute of Biological Sciences and Health (ICBS).

The extracts were diluted in six different concentrations. Then 20 µL of each concentration was added to Whatman filter discs paper No. 5 (0.5 cm) that was properly sterilized and put in holes of approximately 5 mm with the aid of a puncher of agar. For inoculation, we used 100 µL of each bacterial suspension/yeast, depositing the same through agar nutrient in petri dishes. Then paper discs containing extracts were added. Each test was done in three repetitions in a completely randomized design.

RESULTS AND DISCUSSION

From the leaf fragments of *T. subulata, it* was possible to isolate a filamentous fungus that has been identified by classical methods of microscopy and molecular as the fungus *Phomopsis* sp.. Until now, this is the first report on the isolation of the fungus *Phomopsis* sp. as endophyte of *T. subulata* leaves.

The fragment from the region of *Phomopsis* sp. isolate obtained from *T. subulata* presented 439 pb (pairs of bases) arranged in the following way: 5'CCCTTTGTGAACTTATACCTATTGTTGCCTCGGCG AGGCCGGCCTCTTCACTGAGGCCCCCTGGAAACAG GGAGCAGCCCGCCGGCGGCCAACTAAACTCTTGTTT CTATAGTGAGTCTCTGAGTAAAAAACATAAATGAATC AAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCG ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAA TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA CATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGT TCGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTG ATGGGGCACTGCCTTCTAGCGAGGGCAGGCCCTGA AATCTAGTGGCGAGCTCGCTAGGACCCCGAGCGTAG TAGTTATATCTCGTTCTGGAAGGCCCTGGCGGTGCC CTGCCG 3'

Using bioinformatics tool BLAST (Basic Local Alignment Search Tool) on the site http://blast.ncbi.nlm.nih.gov/ blast, this sequence was compared to the other existing ones in this region strings; it was recorded in the database of the program where the isolates of *Phomopsis* and *Diaporthe* were obtained. It was also possible, through this tool, to obtain a sequence dichotomous tree compared to sequences from the NCBI database (Figure 1).

After cultivating the isolate in a liquid medium for 30 days, with a total of approximately 16 g of dry biomass isolated from *Phomopsis* sp. (about 0.4 g per 100 ml of culture medium), the following quantities of extracts were obtained: n-hexane, 1.68 g; chloroform, 1.28 g; ethyl acetate, 0.72 g; and methanol, 1.92 g, showing an approximate sum of 35% extracts obtained from the total dry biomass (Table 1).

In the antimicrobial activity tested, the extracts, hexane, chloroform, ethyl acetate, mycelium of *Phomopsis* sp., and the extract in acetate filtered showed low antimicrobial activity against the tested micoorganisms: *E. coli* (ATCC 8739), *S. aureus* (ATCC 6538), *C. albicans* (URM 4126), *C. tropicalis* (URM 4977-) and *C. glabrata* (CCIBM). This contradicts the results obtained by Chareprasert et al. (2006), where *Phomopsis* sp., was inhibitory against *Bacillus subtilis, S. aureus*, *E. coli* and *C. albicans*.

However, the processes of cultivation and extraction can exert a high influence on the target microorganisms, as well as the metabolites produced by the fungus. In addition, the amount of metabolites varies, according to the host plant, environment in which the fungus is found, species and other factors, both environmental and genetic. Based on this, it is necessary to test other pathogens and fungal isolates, as well as to diversify the use of culture media, temperature, luminosity and carbon source. Genetic variations in the genus or even at species level are also capable of contributing to antimicrobial activity.

Corrado and Rodrigues (2004) observed the bactericidal activity of strains of the endophytic fungus *Phomopsis* sp. isolated from the leaves of *Aspidosperma tomentosum* and petioles of medicinal plant *Spondias mombin*. All the evaluated extracts inhibited the growth of bacteria, fungi and yeasts tested, showing the great potential of this fungus as a source of bioactive products.

The yeasts of the genus *Candida* have great importance because of the high frequency with which they colonize and infect the human host (Colombo and Guimarães, 2003). The appearance and increase in the incidence of several pathogens caused by species of a white-robed throng and other pathogens, and the resistance of the same therapies already existing led to the search for other active principles.

Therefore, the cellular extracts n-hexane and chloroform de *Phomopsis* sp., endophytic of *T. subulata* had low antioxidant activity; the extract in chloroform was slightly higher and got a maximum uptime of 20%.

Hexanics and chloroformic extracts, in ethyl acetate and methanolic extract of mycelium of *Phomopsis* sp. and filtered acetate extract showed low antimicrobial activity against the strains of *E. coli*, *S. aureus*, *C. albicans*, *C. tropicalis* and *Candida glabrata*. Other tests should be conducted with the purpose of increasing the

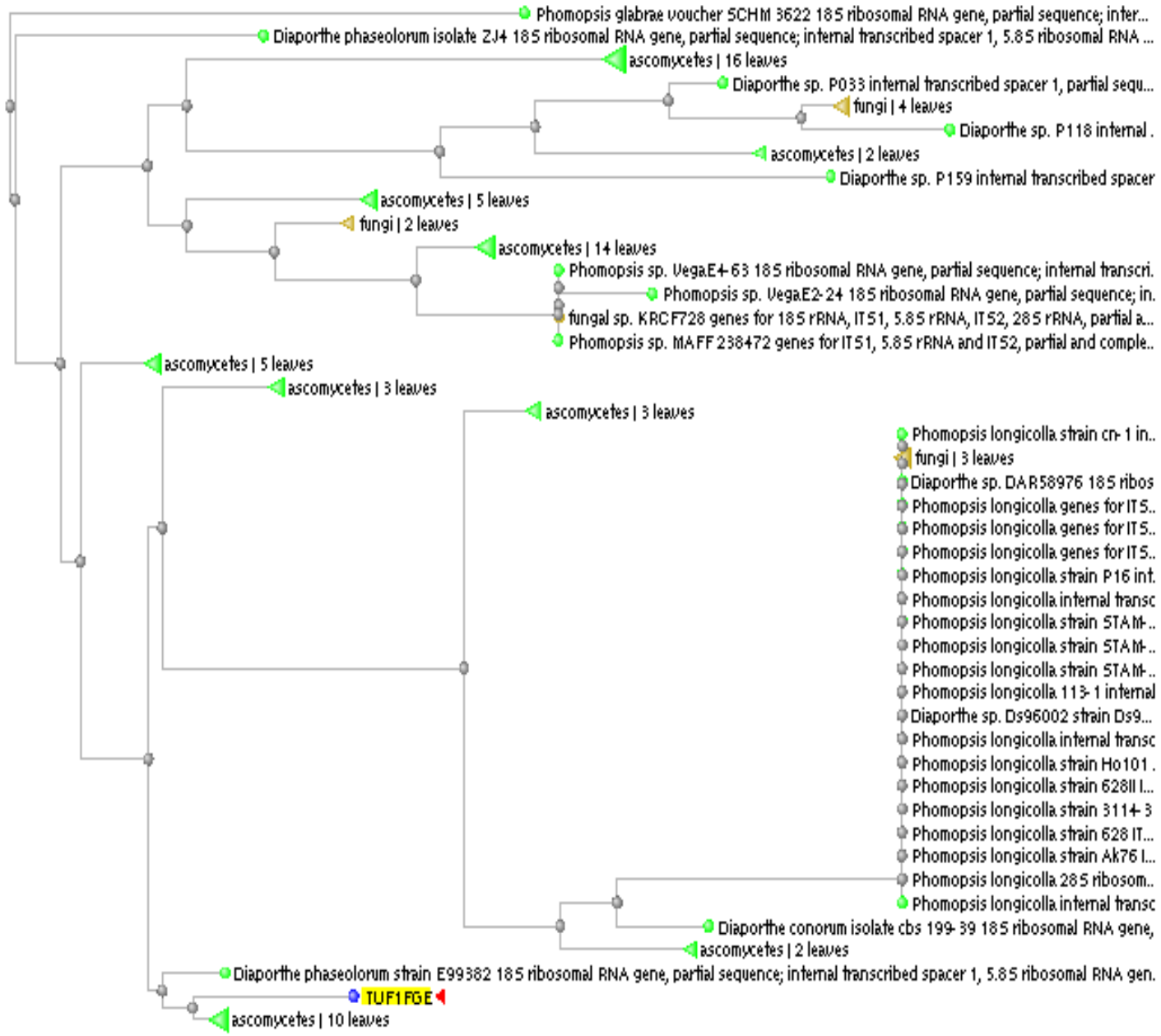

Figure 1. Neighbor joining phylogenetic tree obtained by the insertion of the sequence of bases of ITS region of rDNA from the fungal isolated from leaves of *T. subulata* in comparison with the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi#58396904). Identified as *Phomopsis* SP. (teleomorph phase: *Diaporthe* sp.).

Table 1. Yield of extracts of biomass of *Phomopsis* sp. (30 Days) obtained from *T. subulata* in dry biomass.

Isolate	Solvent	Dry weight (g)	Yield (%)
	n-hexane	1.68	10.5
	Chloroform	1.28	8.00
Phomopsis sp (16 g)	Ethyl acetate	0.72	4.5
	Methanol	1.92	12.0
	Total		**35.0**

action spectrum of *Phomopsis* sp. against other target microorganisms.

Conclusion

Phomopsis sp. fungus may be able to act as an endophyte of the plant *T. subulata*, as shown in this first report of its isolation in leaves of this plant. Although it has low antimicrobial activity, the same fungus must be tested with other target microorganisms, besides using other methodologies to identify its compounds.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

PDA, Potato dextrose agar; **ITS**, internal transcribed spaces; **PSA,** potato sucrose agar.

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Full Length Research Paper

Antagonistic effect of lactobacilli isolated from camel (*Camelus dromedarius*) milk on food borne pathogens

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Camel milk is a suitable substrate for the growth of protective bacterial flora. Detection of lactic acid bacteria producing antimicrobial substances from camel (*Camelus dromedarius*) milk in south Algeria against some food-borne pathogens is the subject of this work. Morphological, physiological and biochemical tests have identified four *Lactobacillus* isolates belonging to the following species: *Lactobacillus fermentum, Lactobacillus helviticus, Lactobacillus plantarum* and *Lactobacillus acidophilus*. In order to demonstrate the inhibitory effect of these bacteria *in vitro*, their antagonistic property was tested against six pathogenic strains often involved in food-borne illness: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella enteritidis* and *Shigella flexneri* using the disc diffusion method. The antagonistic effect was manifested by the appearance of inhibition zones around the discs. The potential inhibitor was estimated by calculating diameter of inhibition zones which extend from 02 to 16 mm. All *Lactobacillus* isolates secreted into the culture medium inhibitory substances were able to inactivate the growth of pathogenic strains tested. *L. plantarum* has shown the largest inhibition zone against *S. aureus* (16 mm). These two strains were chosen to determine the nature of *L. plantarum* secreted substances responsible for the antagonistic effect. The obtained results have shown that *L. plantarum* inhibitory property against *S. aureus* resulted from the combined effect of several biological agents originating from their metabolic activities, especially organic acids and bacteriocins.

Key words: Antagonism, camel milk, Lactobacilli, food borne pathogens, mixed culture, pure culture.

INTRODUCTION

Food borne pathogenic bacteria such as *Escherichia coli, Staphylococcus aureus and Salmonella* are the cause of various pathologies and food borne illness. Pasteurization, fermentation and refrigeration are mostly used to preserve and prolong the shelf-life of food products. However, these methods do not constitute a sufficient guarantee to fight against microbial contamination. Excessive and uncontrolled use of chemical additives may create health risks for the consumer (Mami et al., 2010). Lactic acid bacteria have

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been used successfully, with few adverse effects to prevent antibiotic associated diarrhea, to treat severe infantile diarrhea and to treat various diarrheal illnesses (Tadesse et al., 2005). Several studies have exploited microbial interactions to protect food quality and to fight against undesirable microorganisms (Allouche et al., 2010). Hence search for new strains of lactic acid bacteria producing antimicrobial substances is a universal objective for creation of lactic leaven intended for a better food bio-preservation (Mami et al., 2010; Franz et al., 2010).

It has been shown that camel milk possesses a specific protective system, powerful against contamination flora. This system is composed of antagonistic substances such as protective proteins ((lysosomes, lactoperoxidases and lactoferrin), organic acids, hydrogen peroxide (H_2O_2), bacteriocins produced by lactic acid bacteria which limit the growth of certain pathogenic microbes (Barbour et al., 1984; Klaenhammer et al., 1994; Siboukeur, 2007; Jrad et al., 2013). However, only few studies reported the effect of lactic acid bacteria isolated from camel milk on pathogenic bacteria. Therefore, the aim of this study was to highlight lactic strains isolated from camel (*Camelus dromedaries*) milk belonging to nomads in the Wilaya of Ouargla (South Algeria) and to evaluate *in vitro,* their antimicrobial activities against various pathogenic strains encountered in dairy products and involved often in food-borne diseases.

MATERIALS AND METHODS

Biological material

Milk sample

The collection of milk was carried out on a camel (*C. dromedaries*), belonging to nomads in the wilaya of Ouargla (South Algeria), 6 years old, after her first pregnancy and one calving.

Pathogenic strains

The antimicrobial activity of lactic acid bacteria isolated from camel milk was tested on the growth of the following pathogenic strains: *Salmonella enteritidis* (ATCC 25928), *Staphylococcus aureus* (ATCC 6538*)*, *Escherichia coli* (ATCC 4157), *Pseudomonas aeruginosa* (ATCC 25853), *Bacillus subtilis* (ATCC 6633) and *Shigella flexneri* (ATCC29903) obtained from Microbiology Laboratory of Saidal Group and Microbiology Laboratory of Pasteur Institute, Algiers.

Isolation and identification of lactic acid bacteria from camel milk

The isolation was performed on Man-Rogosa and Sharp (MRS) agar, medium suitable for specific research of Lactobacilli. The cultures were incubated for 48 to 72h at 30°C under anaerobic conditions. The purification was done by carrying successive streaking of individual colonies on MRS agar and incubation at 30°C for 48 h until colonies with same size, shape, color, were obtained, indicating therefore the purity of the strain. The identification was based on the determination of macroscopic characters (colonies aspect on MRS agar), microscopic characters (shape, Gram reaction, mobility and sporulation), physiological characters (catalase test, the growth at different temperatures, salt tolerance and production of CO_2) and biochemical characters (fermentation of carbohydrates using the API 50 CH gallery). The fermentation results of the 49 sugars in the API50CH gallery were treated by ApiWeb software (BioMérieux) to identify lactic acid bacteria with a similarity rate of 100% (Alaoui et al., 2016). The conservation of the pure strains was performed on inclined solid medium (MRS agar). Then, the cultures were maintained at 4°C and every four weeks, the strains were inoculated in new medium (MRS agar).

The antagonism test

Preparation of bacterial pre-cultures

A young lactic culture of 18 h of each isolate was inoculated into a test tube containing 10 mL of MRS broth. The tube was then incubated at 37°C for 18 h under anaerobic conditions. The pathogenic strains were each inoculated in a test tube containing 10 mL of nutrient broth and incubated at 37°C for 18 h.

Standardization of bacterial pre-cultures

The inoculum size of each pathogen and lactic acid bacteria was standardized against the McFarland turbidity standard No. 0.5 using a spectrophotometer at a wave length of either 600 or 625 nm. The McFarland 0.5 standard was used in the preparation of standardized bacterial inoculums for the susceptibility test to antimicrobial agents. It corresponds approximately to a homogenous bacterial suspension of 1.5 x 10^8 cells/mL (McFarland, 1907).

Detection of antagonism by the disc diffusion method

This method consists of pouring 20 mL of Trypic Soja Agar (TSA) medium into sterile Petri dishes. After solidification, the dishes were flooded with 1 mL of pathogenic strains pre-cultures. After drying, sterile filter discs (9 mm) were dipped in the lactic acid bacteria pre-cultures incubated for 18 h and placed on solidified TSA agar seeded with test microorganisms pre-cultures. The diffusion of substances responsible for bacterial interactions was improved by incubation at 37°C for 24 h (Tadesse et al., 2005).

Estimation of inhibitory potential of lactic acid bacteria

The antimicrobial activity is revealed by the appearance of inhibition zones around the discs. Inhibition zones (Z_i) were measured according to the Equation 1.

$$Z_i(\text{mm}) = \text{Inhibitory zone diamter (mm)} - \text{Disc diameter (09 mm)} \quad (1)$$

According to Guessass (2007), a positive result is when the inhibition zone diameter (Z_i) is greater than 2 mm. Antibacterial activity tests were done in triplicates and the mean values were recorded.

Confirmation of the inhibition and determination of the inhibitor nature

The antibacterial activity was confirmed *in vitro* by a test on

Table 1. Morphological characteristics of camel milk isolates.

Groups	Morphological characteristics			
	Size	Shape	Colour	Contour
1	Medium (2 à 3 mm)	Circular and flat	Whitish	Regular
2	Small (1 mm)	Lenticular and cambered	Yellowish	Regular

reconstituted bovine milk in interaction conditions between the two types of microorganisms (lactic acid bacteria of camel milk and pathogenic bacteria). The experiment was carried out with lactic strains that showed the greatest inhibitor potential (high value of Z_i). For that, a sterile sample of 150 mL reconstituted bovine milk was sterilized at 120°C for 20 s then contaminated with 1 mL of *S. aureus* pre-culture. After, the contaminated sample was divided into three series of tubes. In tube 1, the sample was directly incubated at 37°C for 8 h (Pure culture). In tube 2, the contaminated bovine milk, was inoculated with 1 mL of *L. plantarum* pre-culture (Lb3) and incubated at 37°C for 8 h (mixed culture); whereas, the contaminated sample in tube 3 was inoculated with 1 mL of neutralized supernatant of *L. plantarum* pre-culture resulting from centrifugation at 8000 rpm for 10 min at 4°C (Siboukeur and Siboukeur, 2013). The resulting supernatant was adjusted to pH 6.5 with NaOH (1 N) to remove the antibacterial activity which could be exerted by organic acids and incubated in the same conditions (Jrad et al., 2013). Growth and variation in cell number of *S. aureus* in pure and mixed cultures were estimated by counting the colonies of *S. aureus* on Chapman agar every 2 h of the incubation period for the 3 series. For that, decimal dilutions (10^{-1} to 10^{-5}) of each sample were made. Only the dishes containing between 30 and 300 colonies were taken into consideration (Guessas et al., 2006). A volume of 0.1 mL from the dilution 10^{-1} for each sample was plated on the surface of the Chapman agar in order to count *S. aureus* colonies (Kaban et al., 2006). The colonies were counted to determine the number of CFU/mL using the following equation:

$$The\ number\ of\ colonies\left(\frac{CFU}{mL}\right) = Colonies\ number * \frac{1}{V} * \frac{1}{D}$$

V: The inoculated volume, D: concerned dilution.

The final result of colonies count was converted into logarithm (Log_{10} cfu / mL) according to the established recommendations of Alomar et al. (2008). In this experiment, the authors aimed to confirm the inhibition of *S. aureus* by *L. plantarum* by comparing the variation in *S. aureus* cells number during 8 h of incubation in pure culture (series 1) and mixed culture (series 2 and 3).

Then, in order to show the nature of the agents responsible for the inhibition of *S. aureus* by *L. plantarum,* the authors tried to show the effect of organic acids produced by *L. plantaum* in first stage, and to eliminate the effect of these organic acids by neutralizing the supernatant of *L. plantarum* pre-culture with NaOH (1N) to prove the existence of the inhibiting agents other than the organic acids. The enumeration of viable *S. aureus* cells and pH measurement were done in triplicate every 2 h from the dilution 10^{-1} for the three series and the mean values were recorded.

Statistical analysis

Microsoft Excel software (Microsoft Excel 2010) was used to plot the curve of inactivation. Experiments were done in triplicates and the means of the three data sets are presented. Analysis of variance was performed using statistical software SPSS. In all cases, significant difference was based on the 5% level ($P \leq 0.05$).

RESULTS

Isolation and identification of lactic acid bacteria from camel milk

Macroscopic appearance

In the present study, a total of 32 isolates were recovered on MRS agar medium from camel milk sample. They were divided into two groups with different morphological characteristics as shown in the Table 1.

Microscopic appearance

Gram reaction revealed that among the 32 isolates, 14 were retained as Gram (+), rod shaped, individual, in pairs and in chains. Therefore, Lactobacilli was potentially isolated as shown in Figure 1.

Biochemical characterization

Among the 14 Gram (+) isolates, four of them were catalase (-) and were retained. The results of morphological, physiological and biochemical tests of isolated Lactobacilli from camel milk are shown in Table 2.

The macroscopic, microscopic and physiological characteristics showed that all camel milk isolates belong to the genus Lactobacillus. Fermentation of carbohydrates using API 50 CH Gallery system showed that Lactobacillus isolates belong to the following species*: L. fermentum* (Lb1), *L. helveticus* (Lb2)*, L. plantarum* (Lb3) and *L. acidophilus* (Lb4). This biochemical identification was based on the comparison with fermentation profiles of Biomerieux database for these species with 100% similarity.

Results of antagonism

The antagonistic effect was manifested by the appearance of clear and translucent inhibition zone around the discs. Diameter of inhibition zones produced by *Lactobacillus* isolates on the test pathogenic strains are shown in Table 3.

All *Lactobacillus* isolated from camel milk inhibited the

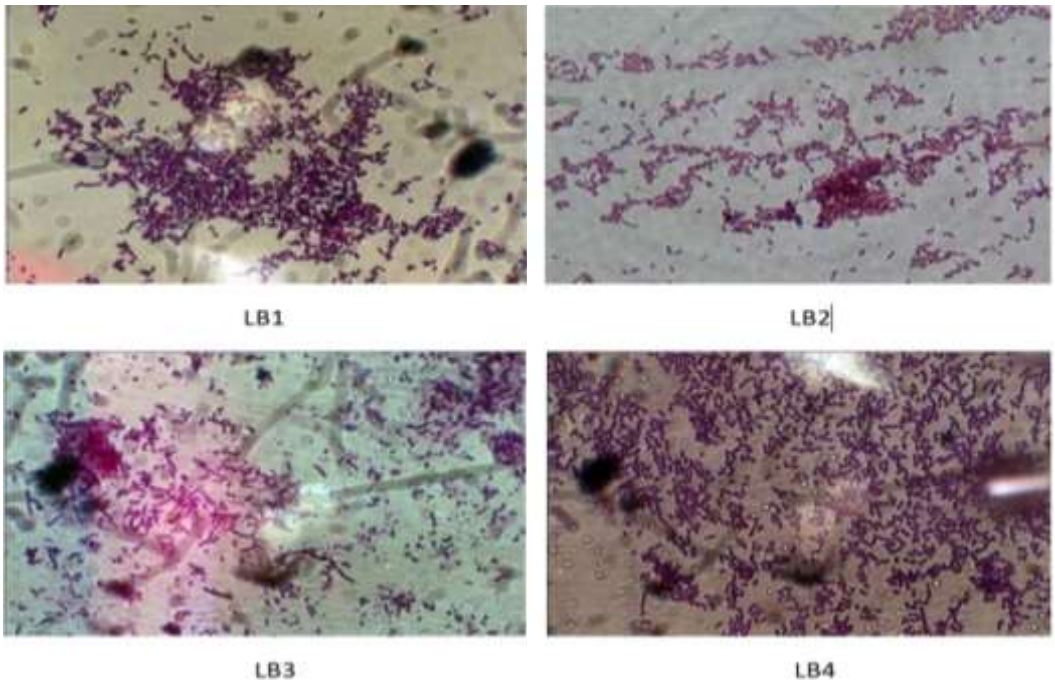

Figure 1. Microscopic appearance of lactobacilli isolates from camel milk after Gram staining (G x 100). Lb1: *L. fermentum*, b) Lb2*: L. helviticus,* c) Lb3*: L. plantarum,* d) Lb4: *L. acidophilus.*

Table 2. Morphological, physiological and biochemical characteristics of the Lactobacilli isolated from camel milk.

Morphological, physiological and biochemical characters		Isolated lactic strains			
		Lb1	Lb2	Lb3	Lb4
Gram		+	+	+	+
Catalase		-	-	-	-
Morphology		Bacilli	Bacilli	Bacilli	Bacilli
Gas production (CO_2)		+	-	-	-
Growth at 10°C		-	-	+	-
Growth at 45°C		+	+	-	+
Growth at 2% NaCl		+	+	+	+
Growth at 4% NaCl		+	+	+	+
Growth at 6.5% NaCl		+	+	+	+
Thermoresistance at 60°C/30 min		+	+	+	+
Arginine hydrolysis (ADH)		+	-	+	-
Esculin hydrolysis		-	-	+	+
	Glucose	+	-	+	+
	Raffinose	+	-	+	+
	Lactose	+	+	+	+
Sugar fermentation	Cellubiose	+	-	+	+
	Mannitol	+	-	+	+
	Arabinose	+	-	+	+
	Xylose	+	-	+	-

(+): Positive reaction; (-): negative reaction.

growth of all pathogenic strains tested to varying degrees, except Lb1 which was inactive against *B. subtilis.* This concluded that *Lb. fermentum, L. helveticus, L. plantarum* and *L. acidophilus* are characterized as

Table 3. Diameter of inhibition zones (mm) produced by *Lactobacillus* isolates on the tested pathogenic strains assessed by the disc diffusion method (essays are in triplicates)**.**

Pathogenic strains	Lactobacilli isolates	Diameters of the inhibition zones (mm) Mean ± standard deviation
Staphylococcus aureus	Lb1	12.66±1.52
	Lb2	11±2
	Lb3	16±2
	Lb4	10.33±0.57
Bacillus substilis	Lb1	0
	Lb2	8.3±3.51
	Lb3	2±2.64
	Lb4	7.33±0.57
Salmonella enteritidis	Lb1	10±1.73
	Lb2	11±2
	Lb3	13±1.73
	Lb4	14.5±1.41
Escherichia coli	Lb1	5.6±1.52
	Lb2	7.6±1.52
	Lb3	10±1
	Lb4	5.3±0.57
Pseudomonas aeruginosa	Lb1	10±1
	Lb2	12.33±0.57
	Lb3	11±1
	Lb4	12±1.73
Shigella flexneri	Lb1	12.33±0.57
	Lb2	10.33±1.52
	Lb3	14.66±0.57
	Lb4	11±1.73

producers of inhibitory substances against pathogenic strains. The inhibition zones are clear with distinct borders; diameters of inhibition zones varied from 02 (lower activity observed for *L. plantarum* against *Bacillus subtilis*) to 16 mm (higher activity observed for *L. plantarum* against *S. aureus*). Therefore*, L. plantarum* was retained as the strongest inhibitor against the tested pathogenic strains.

Confirmation of the inhibition and determination of the inhibitor nature

Confirmation of the inhibition

The growth kinetics of *S. aureus* in the presence and absence of *L. plantarum* is shown in Figure 2. The bacterial count was carried out every 2 h during 8 h of treatment at 37°C. In the sample of tube 1 (reconstituted milk + *S. aureus)*, the survival fraction of *S. aureus* did not significantly increase or decrease since the contaminated milk was not treated by Lb3.

However, when comparing the bacterial count of tube 2 (reconstituted milk + *S. aureus* + *L. plantarum*) and 3 (reconstituted bovine milk + *S. aureus* + *L. plantarum* + NaOH 1N) to tube 1, a drastic *S. aureus* reduction was noticed just after 2 h of treatment. The number of viable cells per milliliter declined from 4.42 to 2.9 $\log_{10}$ cfu/mL for the tube 2 and 2.69 $\log_{10}$ cfu/mL for tube 3 after 4 h of treatment. And *S. aureus* cells number became less than 2 $\log_{10}$ cfu/mL within 6 and 8 h for the tubes 2 and 3, respectively (Figure 2). These differences were not statistically significant on the 5% level ($p \geq 0.05$), (0.77 >0.05) and (0.87>0.05).

Nature of inhibitor

The milk pH measurement was done every 2 h of treatment. A pH decrease was shown in Figure 3 for the

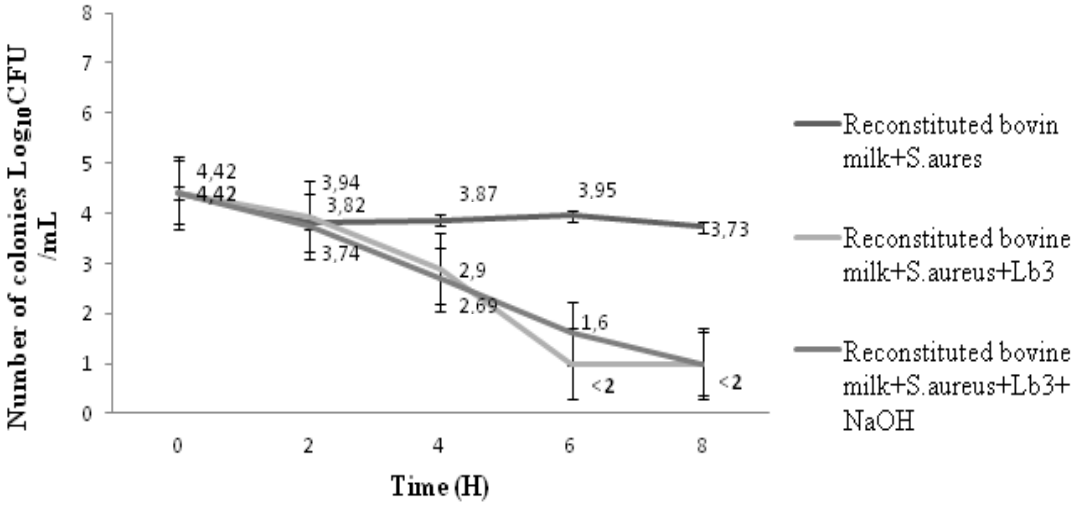

Figure 2. Kinetics of growth of *S. aureus* in pure and mixed cultures with Lb*. Plantarum* in reconstituted bovine milk.

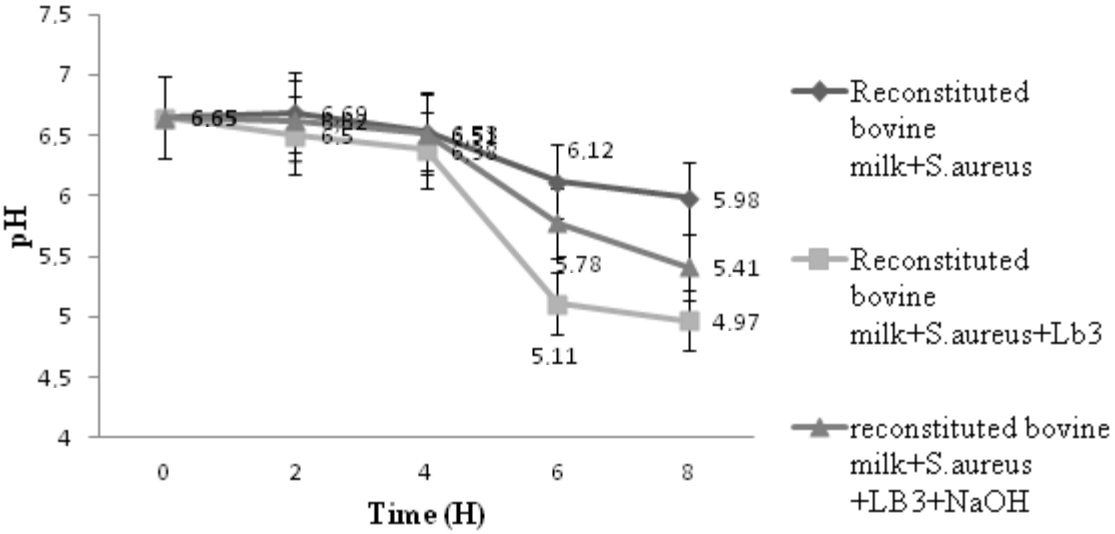

Figure 3. Evolution of pH versus time in pure and mixed culture of *S. aureus* with *Lb. plantarum.*

three milk samples (tubes 1, 2 and 3). In tube 1 (untreated sample), pH was changed insignificantly during 8 h of incubation. In contrast, for tubes 2 and 3 (treated samples), pH decreases considerably from the 4th h to reach a value of 4, 97 and 5, 41 respectively. These differences were not statistically significant on the 5% level ($p \geq 0.05$), (0.81>0.05 and 0.56>0.05).

DISCUSSION

In the present study, a total of 32 lactic acid bacteria were isolated from raw camel milk sample. Morphological, physiological and biochemical tests identified four Lactobacillus isolates belonging to the following species: *L. fermentum, L. helviticus, L. plantarum* and *L. acidophilus*. Carbohydrate degradation tests by the API 50 CH galleries allowed us to differentiate these *Lactobacillus* species with fermentation profiles of 100% similar to those given by ApiWeb software (BioMérieux) for these same species. The positive reading is given by a change of the color under the effect of pH variation in the API 50 CH galleries wells. These classic galleries API 50 CH were used to differentiate 15 lactobacillus isolates

from the 112 lactic isolates of camel milk that belonged to the species: *L. brevis*, *L. delbrueckii* and *L. fermentum* in the work of Alaoui et al. (2016). Similar to our findings, *L. fermentum* (Lb1) isolated in this study were among the lactobacilli isolated from camel milk in the work of Khandelwal et al. (2015) and Alaoui et al. (2016). For *L. helveticus* (Lb2) and *L. acidophilus* (Lb4), they were isolated from goat milk in the work of Badis et al. (2005), and isolation of *L. plantarum* (Lb3) from raw goat milk and raw camel milk was described in several studies (Guessass and Kihal, 2004; Badis et al., 2004 a, b; Mami et al., 2008). All *Lactobacillus* isolated from camel milk inhibited the growth of majority of the tested pathogenic strains in varying degrees. The inhibition zones of diameter of *L. fermentum* (Lb1) against the tested pathogenic strains varied from 5.6 to 12.66 mm. The greatest inhibition recorded was against *S. aureus* without any inhibition against *B. subtilis.* Foster and Hall (1991) reported that some microorganisms produce an acid-tolerance response system that protects them against severe acid stress for longer periods. According to Cogan et al. (1997), the good acidifying lactic acid bacteria are those which are able to reduce the pH of the milk from its initial value of approximately 6.6 to 5.3 over a period of 6 h. However, the pH variation technique (ΔpH) allows classifying the lactobacilli into rapidly acidifying strains, with medium acidification rate and low acidification rate. On this basis and from the data shown in Figure 3, *L. fermentum* was able to reduce the pH of bovine milk from 6.65 to 5.11 after 6 h of incubation (severe acid stress). Therefore, *L. fermentum* is a rapidly acidifying strain. This may be the reason for resistance of *B. subtilis* to *L. fermentum* as compared to the other tested strains which probably belong to the other less acidifying categories. For *L. helveticus* (Lb2), inhibition zones diameters varied from 7.6 to 12.33 mm. In comparison with the work of other investigators that reported the weak antibacterial activity against *P. aeroginosa* by *L. casei* and *L. bulgaricus* isolated from various foods (Nigatu et al., 2015), the current results showed good inhibitory effect against *P. aeruginosa* by *L. helveticus.* Whereas, *L. helveticus* (Lb2) was more active against *E. coli* as compared to those isolated from cow's milk in the work of Allouche et al. (2010). For *L. acidophilus* (Lb 4), inhibition zones diameters varied from 5.3 to 14.5 mm. The greatest inhibition was against *Salmonella enteritidis.* Itoh et al. (1995) and Tahara and Kanatani (1996), showed that it is also possible that *L. acidophilus* exerts inhibitory activity only against bacteria taxonomically closest to the producing strain. *L. plantarum* (Lb3) showed the larger zone of inhibition against *S. aureus* (16 mm). The inhibition of *S. aureus* and *E. coli* by *L. plantarum* isolated from goat milk have already been described by Mami et al. (2008), Todorov et al. (2004) and Karthikeyan and Santosh (2009). According to Trias et al. (2008), the diameter of the inhibition zones varies with the type of culture medium used and the species used as a target strain or indicator strain.

In series 1 (untreated sample), the survival fraction of *S. aureus* did not significantly increase or decrease during the incubation period. The low growth of *S. aureus* in untreated sample of reconstituted bovine milk medium can be explained by the existence of an intrinsic antimicrobial activity of the lactic acid bacteria found in this milk against *S. aureus.* In contrast, the important reduction in *S. aureus* cells number (Log $_{10}$cfu/mL<2) in mixed culture with *L. plantarum* (series 2 and 3) after 8 ho of treatment confirmed the bactericidal effect of *L. plantarum* on *S. aureus*. This same event was observed after 72 h in the work of Mami et al. (2008). According to Lairini et al. (2014), *Lactobacillus* are rapidly acidifying strains. The observed decrease in pH for the series 2 and 3 resulted from the production of organic acids (lactic and acetic acid) by *L. plantarum* isolated from camel milk. However, for series 1 (untreated sample), pH reduction was due to acid production by *S. aureus.* The inhibition of *S. aureus* in parallel with the pH decrease in the medium means that the acidity caused by organic acids produced by *L. plantarum* is an inhibitory agent for the growth of *S. aureus.* This decrease in pH has as consequence, a significant inhibition of *S. aureus* (less than 2 Log $_{10}$cfu /mL) from the 6th h of treatment for series 2. McLean and McGroarty (1996) showed that about 60% of the antimicrobial activity of lactic acid bacteria was removed when the filtrates were neutralized to pH 6.5 with NaOH. However, addition of the neutralized *L. plantarum* pre-culture supernatant to the *S. aureus* culture has only delayed this event (charge less than 2 Log $_{10}$cfu /mL) in series 3. It can be concluded that organic acids are probably not the unique agent responsible for this phenomenon, other metabolites could be implicated. Inhibition of *S. aureus* by the production of hydrogen peroxide is excluded because this one has a catalase (Alomar et al., 2008). The H_2O_2 released by lactic acid bacteria strains inhibit bacteria which do not possess defenses against oxidative stress (Ouwehand and Vesterlund, 2004). The production of bacteriocins by *L. plantarum* is widely accepted (Ouwehand and Vesterlund, 2004; Todorov et al., 2004; Karthikeyan and Santosh, 2009). Therefore, inhibitory properties of *L. plantarum* against *S. aureus* are also owed to the production of bacteriocins. Lactobacillus strains isolated from camel (*Camelus dromedaries*) milk in this study showed different inhibitory activities against the tested pathogenic strains, this can also be explained by the quantity and the structural variability of bacteriocins produced (Richard, 1996).

The results obtained showed that the antibacterial property of *L. plantarum* isolated from camel milk against *S. aureus* result from the synergistic (combined) effect of several biological factors originating from their metabolic activities, especially the organic acids and unknown bacteriocins.

Conclusion

Lactic acid bacteria isolated from camel milk in this study are able to produce antibacterial substances to eliminate the presence of tested pathogenic bacteria. Camel milk differs from milk of other species by the presence of a powerful protector system; it constitutes a source of new antimicrobial strains. It appeared that the synergistic effect of several biological factors originating from their metabolic activities (organic acid, bacteriocins, H_2O_2) produced by lactic acid bacteria derived from camel milk was responsible for the self-purification effect of stored camel milk which is kept for many hours in relatively high temperatures (about 28°C). This study showed also the inhibitory activity of lactic acid bacteria derived from camel milk observed *in vitro* against pathogenic species that may accidentally contaminate milk, indicating the possibility of exploiting this activity for use as a means of food bio-preservation and to preserve the health of the consumer against pathogenic bacteria involved in food poisoning deemed in the summer season.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Helicobacter pylori cytotoxin-associated gene A protein among adult dyspeptic patients in South-western Nigeria

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***cagA* gene, a marker for the *cag* pathogenicity island (CagPAI) and a virulent factor in *Helicobacter pylori* infection codes for 120 to 145 kDa protein that is associated with cytotoxin production and more severe clinical outcomes. The aim of this study was to determine whether any correlation exists between *H. pylori* CagA protein and the endoscopic findings among dyspeptic patients from South-western, Nigeria and also to investigate the evolutionary relationships among *H. pylori* cagA gene with the GenBank strains. A total of one hundred and twenty four *H. pylori* positive isolates were amplified for the detection of *cagA* by polymerase chain reaction (PCR) and *H. pylori* isolates positive for *cagA* were further evaluated for protein expression using western blotting analysis. Also, DNA sequencing, blasting and phylogenetic analysis were performed on twelve selected isolates positive for *cagA. cagA* gene was detected in all the 124 samples (100%) and protein expressions of *cagA* by western blotting analysis was 88.7% (110/124). There was a high prevalence (91.7%) of expressed *cagA* strains in patients with positive endoscopic lesions than those with normal endoscopic findings (85.7%); however, association was not statistically significant (P>0.05). Also, the expressed *cagA* had no statistically significant association with all the positive endoscopic findings (P>0.05). Phylogenetic analysis of the selected *H. pylori* cagA gene showed high similarity with some GenBank strains of western *cagA H. pylori.* This study shows that CagA is high among dyspeptic patients in Nigeria but with no statistically significant association with the endoscopic findings.**

Key words: Protein expression, CagA, protein, GenBank, dyspeptic.

INTRODUCTION

Helicobacter pylori is a bacterial pathogen that colonizes exclusively the mucous layer of the human stomach and classified as carcinogenic to humans by the International Agency for Research on Cancers (IARC, 1994). However, *H. pylori* organisms vary in their carcinogenic potential and the common type of gastric cancers is

Table 1. PCR primers used in this study.

Region amplified	Primer sequence (5′ to 3′)	Size of PCR products (bp)	References
16S rRNA- forward	GCGCAATCAGCGTCAGGTAATG	502	Mona et al. (2015)
16S rRNA – Reverse	GCTAAGAGAGCAGCCTATGTCC		
CagA- Forward	TTGACCAACAACCACAAACCGAAG	183	Smith et al. (2002)
CagA- Reverse	CTTCCCTTAATTGCGAGATTCC		

adenocarcinoma which occurs most commonly in East Asian countries than western countries (Higashi et al., 2002; Suzuki and Mori, 2016). Since the discovery of *H. pylori* by Marshall and Warren (1984), it has been confirmed that *H. pylori* infection results in chronic active gastritis and can lead to gastric ulcer, duodenal ulcer and gastric cancer (Malfertheiner et al., 2007; Yamaoka, 2010). Infection with *H. pylori* is more common and acquired at an earlier age (30 to 50%) in children and attain over 90% during adulthood in developing countries (Salih, 2009). In South-west, Nigeria, studies reported high prevalence rates (60.5 to 73%) of *H. pylori* among dyspeptic patients (Ndububa et al., 2001; Adesanya et al., 2002; Ola et al., 2006). Cytotoxin associated gene A (*cagA*) is one of the important virulence factors of *H. pylori.* The CagPAI contains 31 genes, including *CagA* gene, which is one of the markers for the cag pathogenecity island (CagPAI). *CagA* codes for 120 to 145 kDa protein that is associated with cytotoxin production and more severe clinical outcomes such as peptic ulcer and gastric cancer (Arents et al., 2001; Hatakeyama, 2006).

Previous studies have reported the prevalence of *H. pylori* and the virulence genes in relation to disease outcome among dyspeptic patients in Nigeria (Smith et al., 2002; Olokoba et al., 2013). However, data relating to the information on the association of the *cagA* gene with the disease outcome is still controversial and there is dearth of information on protein expression of putative *cagA* genes in relationship to clinical outcomes. This information is important as CagA protein is one of the major virulence factors of *H. pylori.* Hence, this study was aimed at confirming whether any correlation exists between the CagA protein of these strains and the endoscopic findings among dyspeptic patients from South-western, Nigeria and also to investigate the evolutionary relationships among *H. pylori* cagA gene with the GenBank strains.

MATERIALS AND METHODS

A total number of one hundred and twenty-four isolates of *H. pylori* from dyspetic patients were obtained from the laboratory of Dr. S. I. Smith of the Nigeria Institute of Medical Research, Yaba. Medical history and the endoscopic findings of the patients were obtained from four different hospitals based on the records provided by the gastroenterologist between April 2012 - December 2015, by which approval to conduct the study was obtained from Ethics Committee of the Nigerian Institute of Medical Research Yaba, Lagos.

Culturing of *H. pylori*

The isolates were sub-cultured on Columbia blood agar base (Oxoid), containing laked horse serum (7%) and Dent's antibiotic supplement (Oxoid) in a microaerophilic atmosphere conditions at 37°C as described previously (Toledo and Lopez-Solis, 2010). Colonies were identified by Gram staining and biochemical tests such as oxidase, catalase and urease reactions.

DNA extraction and PCR amplification

Genomic DNA of the isolates were extracted with the QIAamp® DNA kit (Qiagen, Germany) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until use. The PCR amplification of *H. pylori* 16s rRNA was carried out in 25 µl reaction mixture and PCR conditions were: 35 cycles at 94°C for 1 min, annealing 60°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 5 min. The DNAs of J99 and P12 were used as positive controls. *H. pylori* 16S rRNA gene-positive samples were subjected to PCR to detect *cagA* gene using the primers and PCR conditions as described previously by Smith et al. (2002) and the PCR primers are listed in Table 1.

Protein expression of *cagA* gene

Antigens were prepared from lysates of strains of *H. pylori* positive for *cagA* genes. Single gel system for protein separation was prepared according to a protocol of Ahn et al. (2001) and the procedures for Western blotting were performed following the methods of Figueroa et al. (2002) with some modifications. Briefly, the proteins from the lysates were electrophoretically separated in polyacrylamide gel electrophoresis (SDS-PAGE) according to molecular weight (MW) of the proteins. Proteins were then blotted to nitrocellulose membrane (PVDF). For the development of immunoblots, polyvinylidene difluoride (PVDF) filters were blocked in TBS containing 3% (w/v) bovine serum albumin. *CagA* protein expression was analyzed using a 1:2,000 dilution of *CagA* specific polyclonal antibodies. Anti-rabbit antibody was used as a secondary

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Table 2. Association between expressed *cagA* and the endoscopic findings.

Disease	CagA+	CagA-	X^2	P-value
NUD	24	4	0.878	0.349
GERD	16	0	1.690	0.194
Erosive gastritis	66	10	0.684	0.408
Ulcer	4	0	0.103	0.748

Table 3. Association between expressed *cagA* and positive endoscopic lesions.

cagA status	Positive endoscopic lesions		Normal endoscopic findings	
	n	%	n	%
cagA+	88	91.7	24	85.7
cagA-	8	8.3	4	14.3
Total	96	100	28	100
P-value		0.3488		

antibody and immunoreactive bands were visualized using staining solution.

Sequencing and bioinformatic analysis

The amplified PCR products of *cagA* gene from twelve selected strains based on each centre used were purified and subjected to sequencing reactions by inqaba™ biotech, South Africa (BigDye® Terminator v3.1 Cycle Sequencing). Blasting and Genebee (http://www.genebee.msu.su/services/phtree_reduced.html) were used for phylogenetic prediction.

Statistical analysis

The data obtained were subjected to descriptive statistical analysis using EPI info 7. Chi squared test was used to determine associations between the protein status of the isolates and the endoscopic findings and the differences were considered to be statistically significant when the p value obtained was less than 0.05.

RESULTS

One hundred and twenty four *H. pylori* isolates were amplified for the detection of *H. pylori* 16S rRNA and *cagA* gene by polymerase chain reaction (PCR) and all were 100% positive for both *H. pylori* and cagA gene. CagA protein was expressed in 88.7% (110/124) and the positive rate of *CagA* protein was 85.71% (24/28) in the isolates from patients with non ulcer dyspepsia (NUD), 100% (16/16) from those with GERD, 86.84% (66/76) from those with erosive gastritis, and 100% (4/4) from ulcer patients (Table 2). However, no statistical relation was found between CagA positivity and the endoscopic findings (p >0.05 by Chi-square test) (Tables 2 and 3). Immunoreactive bands for *CagA* protein expression is shown in Figure 1.

The phylogenetic relationships among the 12 twelve selected strains and four *cagA* genes of *H. pylori* strains (PHL 121, LSU 2003-1, CAM23 and 01-1086) obtained from GenBank based on high sequence similarity with blasting, revealed that *cag A* genes were divided into two major groups: Strains LU 14 to 01-1086 and strains LA2 to LA4 (Figure 2), having four sub groups: LU 14 – IF9; LA1-IB6; CAM23-10-1086 and LA2-LA4 (Figure 2)

DISCUSSION

From these findings, *CagA* gene was found in all the isolates (100%) studied and the result is in agreement with previous studies in Africa and East Asia countries where *cagA* genes were present in more than 90% of cases (Tanih et al., 2010; Yamaoka et al., 1999). However, this result is higher than those observed from western populations where *cagA* were present in about 60 to 70% of *H. pylori* causing infections (Podzorski et al., 2003; Ribeiro et al., 2003). This indicates that *cagA* prevalence varies in every part of the world (Ozbey and Aygun, 2012). Several studies have demonstrated that the genotype varies among *H. pylori* strains isolated from different geographic regions (Kersulyte et al., 2000; Faundez et al., 2002), indicating that there are important geographic differences. In this study, *CagA* proteins was expressed in 88.7% (110/124) isolates and expression was observed with *CagA* specific antibodies by SDS-PAGE and Western blot (WB) analysis. This study showed that most *H pylori* strains isolated were positive for Cytotoxin associated gene (*CagA*) proteins. Also, Hatakeyama (2004) reported that many strains of *H.*

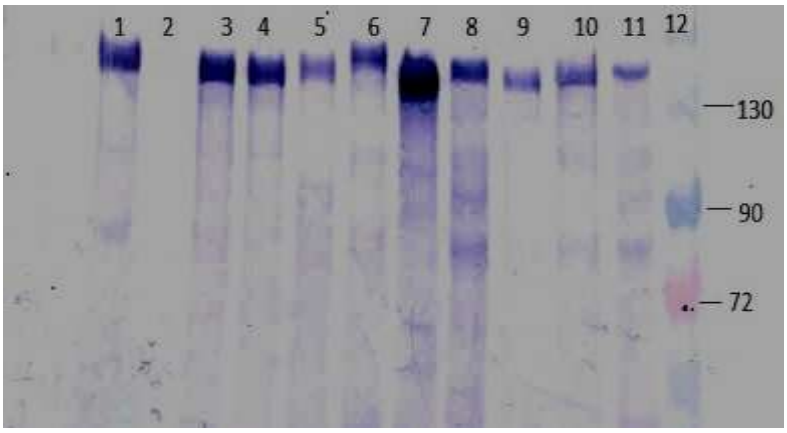

Figure 1. *cagA* protein expression of 130-140kD. Lane 1, positive control; lane 2, negative control; lanes 3 to 11, samples; lane 12, pre stained ruler.

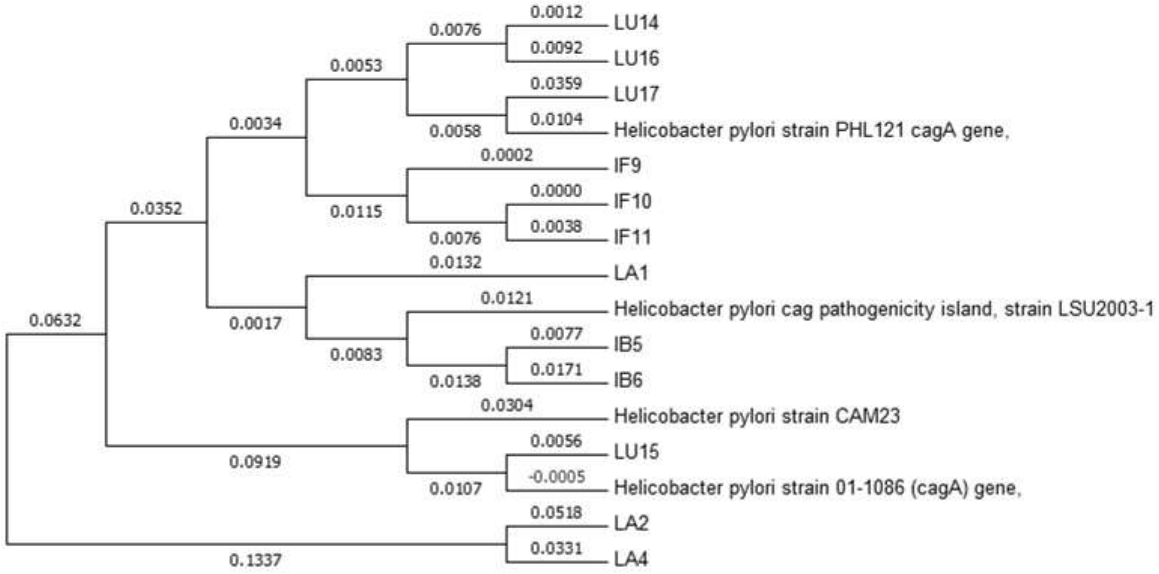

Figure 2. Phylogenetic tree of *cagA* genes of twelve strains from southwest Nigeria and four strains from the GenBank.

pylori produce *cagA* protein, which is a 120 to 145 kDa hydrophilic protein that induces several alterations in the signalling pathways. From these findings, there was no significant association between expressed *cagA* and the clinical outcomes and this is in agreement with earlier reports on *cagA* genotypes (Smith et al., 2002; Ozbey et al., 2013). Whereas in Western countries, the occurrence of *vacA*s1 and *cagA* genotypes have been reported to be significantly associated with peptic ulcers (Ribeiro et al., 2003). However, the reported studies were based on the association of cagA genes with the endoscopic findings, but this present study focuses on the association of CagA protein with the endoscopic findings.

From these findings, none of the patients studied were diagnosed with gastric cancer, more so, with the presence of cagA positive isolates. Abdulkareem et al. (2015) reported that, there is usually low incidence of gastric cancer in Africa (Africa enigma) despite a high prevalence of *H. pylori* with most strains positive for the virulent genes such as *cagA* gene and *vacA* gene. A lot of factors may contribute to this, and one of which could be mixed infection of *cagA* negative and *cagA* positive strains which could lower disease burden, and diets rich in anti-oxidants which reduces risk of gastric cancer (Abdulkareem et al., 2015). A study from Soweto, South Africa indicated that host response to *H. pylori* among Africans may be protective against virulent form of *H. pylori* (Segal et al., 2001). Also, the ability of CagA

secreted by different strains of *H. pylori* to perturb host-cell functions and to trigger gastric cancer is determined by the binding potential of Src homology 2-containing protein-tyrosine phosphatase-2 (SHP-2) which is high in *H. pylori* strains from East Asian countries than those from Western countries (Higashi et al., 2002).

The phylogenetic tree of *cagA* genes revealed that the strains were clearly delineated into 2 major phylogenetic clades with strains LA2 and LA4 distantly separated from others. However, blasting of the edited sequences of strains LA2 and LA4 in NCBI platform, revealed that they were 89 and 87% respectively, most similar to the western type of cytotoxin associated protein A (*cagA*) gene, partial cds of *H. pylori* strain 01-1086 from Colombia (Accession no: JQ318034.1). The other strains clustered in the second clade but with significant genetic variability. This clade further subdivided into 3 minor clades with strain LU15 most distant and stands as a bridge linking the clade with strains LA 2 and LA4. Strains LA1, IB5 and IB6 clustered to form a minor clade with IB 5 and IB6 more related. Also, the results from blasting indicated that IB 5 and IB6 were 95 and 96%, respectively, most similar to *H. pylori* cag Pathogenicity Island, strain LSU2003-1 originated from African with Accession number of FR666843.1 (Olbermann et al., 2010). Strains from Ife (IF 9, IF 10 and IF 11) clustered forming a minor clade and were all related (96, 95 and 95%). While cluster from LU 14, LU 16 and LU 17 were related to Western *cagA* gene of *H. pylori* strain PHL121 (accession no: GU173869) obtained primarily from gastritis patients in philippines (Cortes et al., 2010).

Conclusion

This study demonstrated a high prevalence of *H. pylori* infection of the *cagA* genotype and the phylogenetic analysis revealed that *cagA* gene was related to the western *cagA.* However, there was no significant association between CagA protein and the clinical outcomes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Phosphate solubilizing fungi isolated and characterized from Teff rhizosphere soil collected from North Showa zone, Ethiopia

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Phosphorus is one of the major bio elements limiting agricultural production. About 95 to 99% phosphorus in agricultural soil is unavailable form for plant growth. Phosphate solubilizing microorganisms can increase soil phosphate availability. This study was aimed to identify and evaluate phosphate solubilizing fungi from Teff rhizosphere soil. Fungi were identified using lactophenol cotton blue staining confirmation and Biolog Microstation identification system. Fungi isolates were screened and transferred to biolog universal yeast agar media. Pure yeast cells and filamentous fungi were suspended in sterile water and filamentous fungi (FF) inoculum fluid at 49±2 and 75±2 turbidity measured by biolog turbidimeter, respectively. 100 µL transferred from each suspension into 96 wells of the biolog yeast microplate and filamentous fungi microplate tagged with different carbon source and incubated at 26°C for 24 to 72 h and read by micro station at a single wavelength of 590 nm, results were recorded and processed for identification by micro log3 software ver. 4.20.05. Biolog microstation read 24 fungi species. Filamentous fungi ≤0.5 similarity index (62.5%), yeast ≥0.5 similarity index (25%), yeast ≤0.5 similarity index (12.5%). The identified fungi were tested for phosphate solubilization by the Pikovskaya's agar (PVK) selective media. Seven species were positive in phosphate solubilizing ability: *Trichosporon beigelii B, Rhodotrula aurantiaca A, Cryptococcus luteolus, Zygoascus hellenicus, Penicillium purpurogenum var. rubrisclerotium, Neosartorya fisheri var. fischeri,* and *Candida montana.* At 15 days incubation, *T. beigelii B* and *R. aurantiaca A* was able to solubilize phosphate with solubilizing index of 5.3 and 2.6, respectively. *T. beigelii B,* were superior in phosphate solubilization. Therefore, these species can be candidated and exploited after further evaluation as biofertilizers for agriculture productivity.

Key words: Biolog Microorganisms, micro station, phosphorus, rhizospher, soil, solubilization, Teff.

INTRODUCTION

Phosphorus (P) is the second essential macronutrient for plant growth and development. It accounts 0.2% of plant dry weight, limits the growth of plants and crop yield (Sharma et al., 2013). Phosphorus contributes remarkably to photosynthesis, energy and sugar production, nucleic acid synthesis, and promotes N_2 fixation in legumes (Saber et al., 2005).

The mineral nutrition of plants mainly depends on soil P content that can be assimilated as a soluble phosphate (Ehteshami, 2011). Phosphorous increases the strength

of cereal straw, promotes flower formation and fruit production, stimulates root development and also essential for seed formation (Sharma et al., 2011). It also plays a role in stalk and stem strength, maturity and production crop quality and resistance to plant diseases (Richardson, 2007). Mobility of phosphate ions in the soil is very low due to their high retention in soil. Stevenson (1986) and Holford (1997) reported that the recovery rate of P fertilizer by plants is only about 10 to 30%. The remaining 70 to 90% is accumulated in soil or in the form of immobile that is bound by Al or Fe in acid soils, or Ca and Mg in alkaline soils (Prochnow et al., 2006; Yang et al., 2010). Phosphorus is highly insoluble and unavailable to plants. It must be converted into soluble form. Phosphate solubilizing microorganisms can play an important role in dissolving both fertilizer phosphorus and bound phosphorus in the soil that is environmentally friendly and sustainable (Khan et al., 2007). Several groups of microorganism including fungi, bacteria and actinomycetes are known as efficient fixed P solubilizers (Sundara et al., 2002). Fungi are the important components of soil microbes typically constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions. Fungi have been reported to have greater ability to solubilize insoluble phosphate than bacteria (Nahas, 1996). A wide range of soil fungi are reported to solubilize insoluble phosphorous such as *Aspergillus niger* and *Penicillium* species, which are the most common fungi capable of phosphate solubilization (Whitelaw et al., 1999). Exploration of phosphate solubilizing microorganisms has been conducted by many researchers from soils (Chen et al., 2006; Widawati et al., 2008; Gupta et al., 2012), mangrove (Vazquez et al., 2000; Holguin et al., 2001), and rhizosphere (Chung et al., 2005; Poonguzhali et al., 2008; Oliveira et al., 2009). From such explorations various types of phosphate solubilizing microorganisms have been successfully identified. In last few decades, a large array of rhizosphere bacteria and fungi including species of *Penicillium, Azotobacter chroococcum, Bacillus subtilis, Bacillus cereus, Bacillus megaterium, Arthrobacter ilicis, Escherichia coli, Pantoea agglomerans, Pseudomonas putida, Pseudomonas aeruginosa, Enterobacter aerogenes, Microbacterium laevaniformans,* and *Micrococcus luteus* have been identified as P-fertilizers (Kumar et al., 2014). The principal mechanism for many soil fungi and bacteria can solubilize inorganic phosphate into soluble form through the process of acidification, chelation, exchange reactions and production of organic acids (Han, 2006). Acid phosphatases play a major role in the mineralization of organic phosphorus in soil phosphate solubilization effect is mainly through the reaction between organic acids excreted from organic matters with phosphate binders such as Al, Fe, and Ca, or Mg to form stable organic chelates to free the bound phosphate ion (Arcand and Schneider, 2006; Gupta et al., 2012). Phosphorus deficiency is the most important problem of Ethiopian soil and more than 70 to 75% of highland soils are characterized by phosphorus deficiency (Beyene, 1982). The deficiency is very severe in the acidic soils of the southern, southwestern and western regions. Areas Al^{3+} and Fe^{3+} high are totally incriminated with phosphorus fixation (Sertsu and Ali, 1983). The fixed forms of P in acidic soils are aluminum and iron phosphates, while in alkaline soils they are calcium phosphates (Rfaki et al., 2014). Around 70% of Ethiopian vertisol have available phosphorus below 5 ppm, which is very low for supporting good plant growth and fixation in vertisols is related more to calcium, which is the predominant cation in all profiles than Al^{3+} and Fe (Mamo et al., 1988). Vertisols are dark, montmorillonite-rich clay soils with characteristic shrinking and swelling properties. They have high clay content (>30% to at least 50 cm depth from the surface) and when dry they show cracks of at least 1 cm wide and 50 cm deep. They have high calcium and magnesium contents (FAO, 2000). Teff [*Eragrostistef* (Zucc.) Trotter] is the major indigenous cereal crop of Ethiopia, where it was originated and diversified. It is a highly demanded and a staple food grain for majority of the Ethiopian people. In a country of over 80 million people, teff accounts for about 15% of all calories consumed in Ethiopia (Bekabil et al., 2011). The teff grain is ground to flour which is mainly used for making popular pancake-like local bread called injera and sometimes for making porridge. The grain is also used to make local alcoholic drinks, called tela and katikala. Tef straw, besides being the most appreciated feed for cattle (Ketema, 1997). Teff is the only cultivated of all 300 *Eragrostis* species. Its agro ecological adaptability has resulted in its cultivation as an important crop in 10 of 18 agro ecological zones of the country. It can be grown in altitudes ranging from near sea level to 3000 ms, but the best performance occurs between 1100 and 2950 masl (Hailu and Seyfu, 2000). Annual rainfall of 750 to 850 mm, growing season rainfall of 450 to 550 mm and a temperature range of 10 to 27°C. A very good result can also be obtained at an altitude range of 1700 to 2200 m and growing-season rainfall of 300 mm (Seyfu, 1993). The crop performs well in both water logged vertisol in the highlands as well as water-stressed areas in the semi-arid regions throughout the country and consequently it is preferred over other grain crops such as maize or barley (Zeleke, 2010). Teff production and productivity have been far below the potential (Demeke, 2013). Currently, the average national productivity is estimated to be less than 0.5 ton per ha. This is very low

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compared to other cereals such as wheat and sorghum grown in the region. Lower grain yield is mainly attributed to low soil fertility, especially nitrogen (N) and phosphorus (P) deficiencies and weed control practices (Seifu, 1993). Declining soil fertility as a result of continuous cropping without replenishing soil nutrients, continues application of phosphate fertilizer and soil erosions is the major factors that reducing production and productivity of the crop in Ethiopia. Higher grain yield of teff was recorded by applying inorganic fertilizers (Abate, 1993). However, chemical fertilizers are neither easily available nor affordable for the majority of poor Ethiopian farmers and not environmentally friendly (FAO, 1987). Such economic considerations necessitate for an alternative less expensive and environmentally friendly agricultural technologies to improve yield and quality of grain. Screening and characterization of phosphate solubilizing microorganisms are important for proper utilization of their beneficial effects to increase crop production and sustain agricultural productivity of the country without contaminating environments. In Ethiopia, only few studies on teff root-associated microorganisms have been undertaken. The effect of phosphate solubilizing some fungus on growth and yield of teff was studied by Asfaw (1993). Inoculation of teff by vascular arbuscular mycorrhazal (VAM) and plant growth promoting rhizobacteria (PGPR) give good result on teff productivity. So previous research works tell us using biofertilizer is better indicative to improve teff productivity to a significant level. However, there are some trials on rhizobacteria and vascular arbuscular mycorrhazal using as biofertilizer, phosphate solubilizing fungi were not studied well. This study was aimed to isolate, identify and evaluating of phosphate solubilizing fungi from teff rhizosphere soil collected from North Showa farm land and selecting superior solubilizing fungi that will be candidated for bio fertilizer after further evaluation for agricultural productivity.

MATERIALS AND METHODS

Study area

The study was conducted in North Showa zone in five selected districts, particularly in Kewot, Tarmaber, Efratana gidim, and Siadeberna wayu. North showa zone is one of the 10 zones of Amhara regional state. The elevation ranges from 1100 to 3009 m above sea level. Geographic coordinate latitude: 9°46'8.4" and longitude: 39°40'4.8". The zone is located in approximately average 200 km far from Addis Ababa (Figure 1).

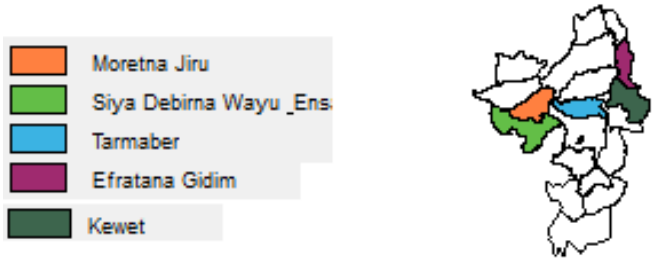

Figure 1. Map of study area.

Sample collection

Twenty five (25) teff farmland site were selected based on three teff varieties, two soil types and 200 m difference within 1200 to 2200 m.a.s.l altitude in the study area. Seventy five rhizosphere soil samples were collected through drillings at 5, 10, and 15 cm depth (Figure 2). Approximately, 15 g of soil were taken from each depth of sampling point and a total of 45 g composite soil per sampling farmland were stored in sterile sample tube and icebox during April 08 to 28/2016 and transported to microbial directorate laboratory in Ethiopian Biodiversity Institute to Addis Ababa and kept in +4°C until processed.

Screening and isolation of fungi from teff rhizosphere soil

One gram of soil from each sample was serially diluted up to 10^{-6} mL in distilled water. About 0.1 mL inoculum sample was transferred to yeast extract peptone dextrose agar media (YPDA), rose bengal agar, potato dextrose agar by cotton swab and streaked using nichrom loop. Primary cultures were incubated for 26°C in digital incubator for 48 h. Isolates were subculture twice until pure colony was obtained for morphological identification. A single yeast colony and pure filamentous fungi was streaked to Biolog universal yeast agar (BUY agar plate (60 g/1 L) and incubated for 48h at 26°C for yeast and filamentous fungi micro plate (YT/FFMicroplate) inoculum preparation). The yeast and filamentous fungi were identified according to the Biolog micro station reading and procedure.

Colony morphology identification

The colony morphology of the isolated fungi were examined after grown on yeast extract peptone dextrose agar media and biolog universal yeast agar media at 26°C for 48 h and its colony morphology, form, size, elevation, margin/edge, and colony color were observed using hand lens as well as its percentage frequency were recorded.

Identification of yeast from teff rhizosphere soil

Pure yeast isolates after being grown on yeast extract, potato dextrose agar were transferred to biolog universal growth agar and incubated at 26°C for 48 h. Pure colony of yeast suspensions were prepared in 9 mL sterile distilled water and adjusted to 47±2T using biolog turbidiameter. 100 µL of inoculum was dispensed using digital pipettor to each of 96 wells of yeast micropate (YT) and incubated at 26°C 24 to 72 h.

The YT micro plate is tagged with 96 carbon source. An isolate ability to metabolize each carbon source is measured in the presence or absence of purple hue in the wells. Tetrazolium violet a redox dye forms a purple color when oxidized by cellular respiration of microorganisms. The YT micro plate measures both metabolic reactions as well as turbidity growth to produce identifications. YT micro plate was read by the micro station reader at 24, 48, and 72 h at a single wavelength of 590 nm. The biolog software micro log3 ver. 4.20.05 compared the results obtained with the test strain to the database and provided identification based on distance value of match and separation score produces similarity index value and

Figure 2. Activites during teff rhizosher soil collection.

probability for species identification (Biolog1993).

Identification of filamentous fungi from teff rhizosphere soil

Filamentous fungi screened and isolated on rose bengal agar and potato dextrose agar were stained by lactophenol cotton blue in order to confirm to which genera the fungi is belonged to then pure filamentus fungi were transferred into biolog universal growth agar media and incubated at 26°C for 48 h. Pure sporulate filamentous fungi suspension were prepared using 15 mL filamentous fungi inoculum fluid and adjusted to 75±2T using biolog turbidiameter. 100 µL of inoculum was dispensed using digital pipettor to each of the 96 wells of filamentous fungi micropate (FF) tagged with different carbon source and incubated at 26°C and 24 to 240 h. After incubation, the FF micro plate measures both metabolic reactions as well as turbidity growth to produce identifications. Filamentous fungi micro plate (FF) was read by the micro station reader at 24, 48, and 72 h at a single wavelength of 590 nm. The biolog software micro log3 ver. 4.20.05 compared the results obtained with the test strain to the database and provided identification based on distance value of match and separation score produces similarity index value and probability for species identification (Biolog1993).

Identification of phosphate solubilizing microorganisms

Fungal isolate identified by biolog microstation were tested for their phosphate solubilizing ability. Pure fungi colonies were collected using a needle nose and spotted at 4 quadrants on sterile solid Pikovskaya media (2.5 g $Ca_3(PO_4)$, 0.5 g $(NH_4)_2SO_4$, 0.2 NaCl, 0.1 g $MgSO_4.7H_2O$, 0.2 g KCl, 10 g glucose, 0.5 g of yeast extract, 20 g agar, 0.0001 g $MnSO_4$, 0.0001 g $FeSO_4$, and 1000 mL distilled water) (Rao, 1982). $Ca_3(PO_4)_2$ was used as a source of phosphate. Observations were made until the formation of a clear zone around the colonies of fungi that indicated the occurrence of phosphate dissolution. At 5 days intervals, solubilization index (SI) was measured using the following formula (Premono et al., 1996). Fungi that formed the fastest clear areas with the greatest diameter indicate the most superior phosphate solubilizing fungi.

SI = Colony diameter + Halozone diameter/Colony diameter

Statistical analysis

The data analysis involved various descriptive statistics such as means and percentages frequency. STATA ver.13 was used for phosphate solubilization index data analysis.

RESULTS

Percentage frequency of fungal species isolated from teff rhizosphere soil

A total of 450 fungal colonies were grown and counted on different growth media and identified pure colonies having similar morphology were clustered in order to detect the incidence frequencies of the microorganisms encountered. Sixty five percent were filamentous fungi and 35% were non filamentous fungi. From filamentous fungi, *Aspergillus* species were dominant (33%), *Penicillium* species (29%), *Fusarium* species (16%), *Trichoderma* (13%), and *Colletotrichum* (9%). The phosphate solubilizer fungi isolates were also identified based on their colony morphology that is pigmentation, shape, size, texture, elevation and margin) (Table 1).

Identification of filamentous fungi species using lacto phenol cotton blue staining (LPCB) and biolog micro station

Representative filamentous fungal isolates from clustered group were stained using lacto phenol cotton blue to confirm to which genera filamentous fungi belonged to

Table 1. Colony morphology for phosphate solubilizer fungi.

P-solubilizing fungi	Shape	Elevation	Size	Marigin	Surface texture	Color
Trichosporon beigelii B	Irregular	Flat	Large	Lobate	Concentric	White yellow
Rhodotrula aurantiaca A	Rhound	Flat	Large	Undulate	Radiate	White
Penicillium purpurogenum Var.rubrisclerotium	Circular	Umbonate	Large	Filamentous	Radiate	Gray
Neosartoryafisheri var. fischeri	Circular	Umbonate	Large	Filamentous	Rugose	Olive green
Cryptococcus luteolus	Irregular	Flat	Lobate	Obate	Radiate	Yellow
Zygoascus hellenicus	Irregular	Flat	Lobate	Obate	Radiate	Yellow
Candid montana	Round	Flat	Large	Smooth	Radiate	White pink

Table 2. Biolog micro station filamentous fungi identification result read.

Index value	Fungus species	LPCB Staining result	Probability	Similarity	Distance	Teff farm land districts
Filamentous fungi ≤0.5 Similarity index (62.5%)	*Colletotrichum lindemuthianum(Saccardo & Mangus) Briosi*	+	-	0.001	32.96	Ejersa Qubete
	Emericella quadrilineata (Thom & Raper) C.R. Benjamin	+	-	0.001	29.89	Kewot (Worentele)
	Fusarium melanochlorum (Caspary) Sacc.	+	-	0.001	32.37	Efratana Gidm (Karalgoma)
	Aspergillus brevipes G. Sm.	+	-	0.002	28.24	Tarma Ber (Asfachew)
	Fusarium juruanum	+	-	0.000	48.72	Tarma Ber (Armania)
	Trichoderma piluiliferum Webster & Rifai	+	-	0.000	48.48	Efratana Gidm (Karalgoma)
	Fusarium avenaceum s.sp.nurragi Summerell & L.W. Burgess	+	-	0.002	27.20	Kewot (Korebta)
	Penicillium vulpinum (Cooke & Massee) Seifert & Samson	+	-	0.003	25.98	Tarma Ber (Chira Meda)
	Neosartorya fischeri var. Fischeri (Wehmer) Malloch & Cain	+	-	0.001	32.18	Ejersa Qubete
	Fusarium udum E.Butler	+	-	0.000	39.36	Tarma Ber (Chira Meda)
	Hypocrea pseudokoningii	+	-	0.000	33.5	Tarma Ber (Chira Meda)
	Trichoderma cittrinoviride Bissett BGA	+	-	0.000	46.16	Efratana Gidm (Karalgoma)
	Trichoderma aureoviride Rifai	+	-	0.000	39.81	Efratana Gidm (Karalgoma)
	Penicillium purpurogenum var. Rubrisclerotium Thom	+	-	0.004	24.46	Ejersa Qubete
	Penicillium pinophilum Hedge. BGB	+	-	0.002	26.74	Mendida (Moyesilasie)

and read by biolog micro station equivalent to molecular method. The result revealed that 15 filamentous fungi species associated teff rhizosphere soil. Both lacto phenol cotton blue staining result and biolog microstation read showed that a filamentous fungi ≤0.5 similarity index (62.5%) *Colletotrichum lindemuthianum*, *Emericella quadrilineata*,*Fusariummelanochlorum*, *Aspergillus brevipes, Fusarium juruanum, Trichoderma piluiliferum*, *Fusarium avenaceum*, *Penicillium vulpinum, Neosartorya fischeri var. Fischeri, Fusarium udum, Hypocrea pseudokoningii, Trichoderma cittrinoviride, Trichoderma aureoviride, Penicillium purpurogenum var. rubrisclerotium,* and *Penicillium pinophilum* (Table 2).

Identification of yeast species using biolog micro station

Biolog microstation read at 24, 48 and 72 YT microplate incubation result revealed that yeast ≥0.5 similarity index (25%) *Rhodotorula aurantiaca A, Candida etchellsii, Kluyveromyces delphensis, R. aurantiaca A, Cryptococcus luteolus,* and

Table 3. Biolog micro station yeast identification result read.

Index value	Fungus species	Probability	Similarity	Distance	Teff farm land districts
Yeast≥Similarity index 25 %)	*Rhodotorula aurantiaca A*	100	0.604	6.1	Kewot (Abay Atir)
	Candida etchellsii	74	0.545	4.01	Deneba (Dacho)
	Kluyveromyces delphensis	77	0.533	4.75	Kewot (Worentele)
	Rhodotorula aurantiaca A	100	0.512	7.77	Kewot (Tere)
	Cryptococcus luteolus	-	0.728	4.13	Tarmaber (ChiraMeda)
	Trichosporon beigelii B	99	0.523	7.51	Mendida (Moyeselase)
Yeast≤0.5 Similarity index (12.5%)	*Cryptococcus albidus var aerius*	-	0.226	14.34	Mendida (Moyeselase)
	Zygoascus hellenicus	-	0.216	14.1	Tarmaber (ChiraMeda)
	Candida montana	-	0.47	3	Kewot (Abay Atir)

Table 4. Phosphate solubilization index (PSI).

Fungus species isolated from teff rhizosphere soil	Phosphate solubilization index (PSI)		
	5th days	10th days	15th days
Trichosporon beigelii B	3.8	4.3	5.3
Rhodotrula aurantiacaA	1.425	2.2	2.6
Cryptococcus luteolus	1.33	1.88	2.01
Penicillium purpurogenum var.rubrisclerotium thom	1.318	1.4	1.5
Zygo ascus hellenicus	1.47	1.56	1.7
Neosartorya fisheri var. fischeri	1.53		1.88
Candida montana	1.2	1.3	1.6

Trichosporon beigelii B. and yeast ≤0.5 similarity index (12.5%), *Cryptococcus albidus var aerius, Zygoascus hellenicus,* and *Candida Montana* (Table 3).

Phosphate solubilization test

A total of 24 fungus species were evaluated for their phosphate solubilization efficiency on Pikovskaya's agar selective media. Among all 7 isolates were positive for phosphate solubilization (Table 4). From 1.0 to 3.4 cm clear zone diameter were recorded within 15 days of incubation (Figure 4). *T. beigelii B* showed superior solubilization index (PSI) of 5.3, followed by *R. aurantiaca A* which is 2.6, the smaller solubilization index recorded 1.5 by *P. purpurogenum var. rubrisclerotium* (Table 4 and Figure 4).

DISCUSSION

Phosphorus deficiencies are wide spread on soil throughout the world and one of the limiting factors for crop productivity. Phosphorus fertilizers represent major cost for agricultural production. Many bacteria, fungi and a few actinomycetes are potential solubilizers of bound phosphates in soil thus playing an important role making it available to plants in the soluble form (Halder et al., 1991; Abd-Alla, 1994; Whitelaw, 2000; Goldstein, 1986). Solubilization of insoluble phosphorus by microorganisms was reported by Pikovskaya (1948). During the last two decades knowledge on phosphate solubilizing microorganisms increased significantly (Richardson, 2001; Rodriguez and Fraga, 1999). The 3 main phosphate solubilization mechanisms employed by soil microorganisms are (1) release of organic acid anions, siderophores, protons, hydroxyl ions, CO_2, that release of complexing or mineral dissolving compounds, (2) extracellular enzymes, and (3) the release of phosphatase enzyme (McGill and Cole, 1981). Fungi have been reported to possess greater ability to solubilize insoluble phosphate than bacteria (Nahas, 1996). Many fungal species can solubilize rock phosphate, aluminium phosphate and tricalcium phosphate, such as *Aspergillus niger, Aspergillus tubingensis, Aspergillus fumigatus, Aspergillus terreus, Aspergillus awamor, Penicillium italicum, Penicillium radicum, Penicillium rugulosum, Fusarium oxysporum, Curvularia lunata, Humicola* spp., *Sclerotium rolfsii, Pythium* spp., *Aerothecium* spp., *Phoma* spp., *Cladosporium* spp., *Rhizoctonia* spp., *Rhizoctonia solani, Cunninghamella* spp., *Rhodotorula* spp., *Candida* spp., *Schwanniomyces occidentalis, Oideodendron* spp., and *Pseudonymnoascus* spp. (Isbelia et al., 1999; Sparks, 1999; Whitelaw et al., 1999; Didiek and Sugiarto, 2000; Helen et al., 2002). Mäder et al. (2002) also reported *Penicillium albidum*, *Penicillium*

thomii, Penicillium restrictum, Penicillium frequentans, Gliocladium roseum, *Myrothecium roridum*, *Penicillium jensenii* and *Eupenicillum javanicum* have been phospho fungi, Varsha et al. (2010) reported that yeast belonging to genus *Saccharomyces, Hansenula, Klockera, Rhodotorula* and Debaryomyces spp. were phosphate solubilizing yeast. Fungi commonly reported to effectively solubilizing phosphorus include species of *Aspergillus candidus, A. niger, Aspergillus parasiticus, Aspergillus rugulosus, Aspergillus terreus*, *Penicillium, Pseudeurotium, Trichoderma* spp. and some mycorrhizal fungi (Aseri et al., 2009; Muraleedharan et al., 2010). Firew et al. (2016) reported that from haricot bean, faba bean, cabbage, tomato, and sugarcane phosphate solubilizing fungi *Aspergillus* spp. (55.69%), *Penicillium* spp. (23.35%), and *Fusarium* spp. (9.58%) were isolated from Jimma Ethiopia. Increased growth and P uptake of several crop plants due to PSB inoculation have been reported in a number of studies conducted under both growth chamber and greenhouse conditions (Dey et al., 2004). In this study, a total of 24 fungus were isolated from teff rhizosphere soil collected from North Showa, Ethiopia (Tables 3 and 4) and tentatively evaluated for their phosphate solubilization efficiency on Pikovskaya (PVK) selective media. Among all, 7 isolates were positive for phosphate solubilization: *T. beigelii B, R. aurantiaca A, C. luteolus, P. purpurogenum var.rubrisclerotium, Z. hellenicus, Neosartorya fisheri var. fischeri,* and *C. montana* (Table 4). Woyessa and Assefa (2011) reported bacteria isolated from teff rhizosphere soil from agricultural fields of Alemgena and Bushoftu Ethiopia, isolates tef rhizosphere contains a diverse flora of microorganisms. The genera were *Pseudomonas, Chryseomonas, Burkholderia, Bacillus, Brevibacillus, Stenotrophomonas* and *Aeromonas*. These 4 species *B. subtilis, Burkholderia cepacia, Pseudomonas fluorescens,* and *Bacillus coagulans* were superior phosphate solubilizer bacteria. However, many rhizospheric bacteria and fungi isolated from different crop rhizosphere soil, there is little information regarding teff rhizophere fungi and potential phosphate solubilizer. This study will confirm that there are a diverse teff rhizosphere fungi and superior phosphate solubilizer fungi isolated from North Showa teff farm land (Table 3). The soil yeasts *Candida tropicalis, Geotrichum candidum, Geotrichum capitatum, Rhodotorula minuta* and *Rhodotorula rubra* solubilized insoluble phosphate reported by Al-Falih (2005). The fungi species *P. purpurogenum var. rubrisclerotium* and *R. aurantiaca A* are phosphate solubilizer fungi species discovered in this study are also similar with the work of Yasser et al. (2014) and Isbelia et al. (1999). In this study, phosphate solubilization index were measured within 5 days intervals for 15 days and they showed 1.5 to 5.3 PSI clear zone diameter over colony diameter ratio (Table 4). Narsian et al. (2008) reported yeast belonging to genus Saccharomyces *Hansenula, Klockera, Rhodotorula* and *Debaryomyces* exhibited the highest SI (1.33 to 1.50). The study by Yasser et al. (2014), phosphate solubilization index recorded 1.05 to 1.45. *A. japonicas* (SI=1.45), *A. niger* (SI=1.12), *Penicillium expansum* (SI=1.20), *Penicillium funiculosum* (SI=1.40), *Penicillium variable* (SI=1.13), and *P. purpuragenum* (SI=1.30). In this study, the largest solubilization index recorded by *T. beigelii B* (PSI, 5.3), *R. aurantiaca A* (PSI, 2.6), the smallest solubilization index recorded by *P. purpurogenum var. rubrisclerotium* (PSI, 1.5) (Figure 3 and Table 4). According to De Freitas et al. (1997), good phosphate solubilizers produce halos around their colonies with diameters higher than 1.5 cm. Most efficient phosphate solubilizer on Pikovskaya's agar plates with PSI = 3.29. Whereas among fungi *P. canescens* showed the highest solubilizing index (Chabot et al., 1993; Nahas, 1996). Phosphate solubilization index (PSI) values up to 2.4 have been recorded for *A. niger*, with values of 3.1 for *Penicillium italicum* and 3.0 for *Paecilomyces lilacinus* (El-Azouni, 2008; Hernandez-Leal et al., 2011). Fungal strains isolated from sugarcane and sugar beet rhizosphere showed SI in range of 1.13 to 1.59 (Mahamuni et al., 2012). Alam et al. (2002) reported PSI of the fungal strains isolated from maize rhizosphere that ranged from 1.53 to 1.80. In this study, *P. purpurogenum* recorded the smallest SI valve 1.5, but Anju et al. (2015) reported that *P. purpurogenum* SI is 2.25±0.65. In this study, new phosphate solubilizer yeast *T. beigelii* isolated from teff rizhosphere soil with superior solubilization index (PSI) is 5.3 in 15 days incubation. Therefore, demonstration of high phosphatase activity and releasing high amount of phosphorus may be due to the specificity of the phosphatase (Deepa et al., 2010). Therefore, these strains can be candidated and exploited as bio fertilizers through further evaluation and optimization test to increase agricultural productivity of teff crop.

Conclusion

Twenty four (24) fungi isolated from teff rhizosphere soil using lactophenol cotton blue staining and biolog microstation identification system where equivalent to molecular techniques and the dominant species were filamentous fungi. Seven fungi species *T. beigelii B, R. aurantiaca A, C. luteolus, P. purpurogenum var. rubrisclerotium, Z. hellenicus, N. fisheri var. fischeri,* and *C. montana* were positive for phosphate solubilization efficiency. *T. beigelii B* was the superior among the isolated fungi in solubilizing index of 5.3 followed by *R. aurantiaca A* with 2.6 and good candidate after further evaluation on *in vitro* test, green house and field trials as bio fertilizer. The rise in the cost of chemical fertilizer, the lack of fertilizer industries in developing countries and the growing environmental issue and biodiversity loss using chemical fertilizer are timely important concern using alternative ecofriendly bio fertilizer to increase yield and productivity of teff crop.

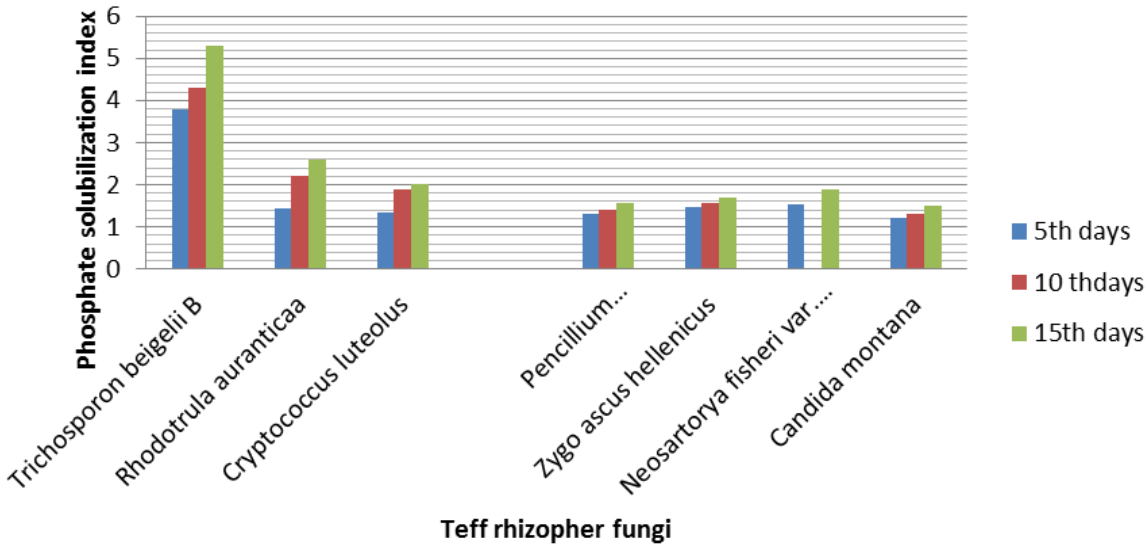

Figure 3. Graph of phosphate solubilization index at 5, 10 and 15 days incubation.

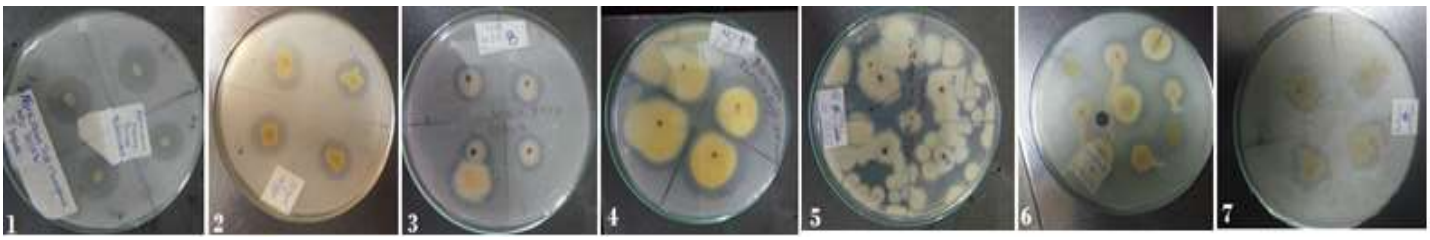

Figure 4. Phosphate solubilizing fungi photo from back side of culture plate: (1) *Trichosporon beigelii B,* (2) *Cryptococcus luteolus,* (3) *Rhodotrula aurantiaca A*, (4) *Pencillium purpurogenum var.rubrisclerotium,* (5) *Neosartorya fisheri var. fischeri*, (6) *Candida montana*, (7) *Zygo ascus hellenicus.*

RECOMMENDATION

The beneficial effects of plant growth promoting microorganisms (PGPM) have not been exploited well. In the past, some microbial inoculants prepared from *Rhizobium* for leguminous crops, *Azotobacter* and *Azospirillium* for cereal crops and *Frankia* for tree crops have been used as nitrogen providers in many developed and developing countries. However, enormous interest increase in research in recent years in PGPM such as nitrogen fixer, phosphate solubilizer, and pathogen suppressor. There is no well-organized microbial inoculant industry for bio fertilizer production especially for phosphate solubilizer and there is no link with researcher working on microbial bio fertilizer in Ethiopia, therefore, Agricultural Research Institute, microbiologist, soil scientist agronomist, and stockholders in general must work together in depth on structural and functional diversity of PGPM and selecting superior biofertilizer, biopesticide, biostimulant to increase crop yield and prductivity. Further research should be continued with selecting efficient phosphate solubilizer microorganism (PSM) isolates. These may be used for inoculum production and their inoculation effect on the plant growth must be studied *in vitro*, green house and field trials.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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