ANTIFUNGAL EFFECT OF CAMEL URINE AND GINGER WATER EXTRACT AGAINST *ALTERNARIA ALTERNATA* THE CAUSAL AGENT OF EARLY BLIGHT DISEASE OF TOMATO *IN VITRO.*

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ABSTRACT

Early blight caused by Alternaria alternata is one of the most important diseases of tomato in the Sudan. This study was conducted in the Plant Pathology Laboratory, Department of Crop Protection, Faculty of Agriculture, University of Khartoum. Some characteristics of the isolated fungus such as colony growth, pigmentation, sporulation, color, shape and control were studied in vitro. Different concentrations of camel urine and Zingiber officinale water extract (5%, 10% and 15%) were screened for their efficacy in inhibiting of mycelial growth, sporulation and spore germination of A. alternata. The experiments were executed in a completely randomized design. The result indicated that the isolated fungus was A. alternata. The all concentrations of camel urine and ginger tested had inhibitory effects against A. alternata. The results also indicated that inhibition of mycelial growth increased as camel urine and ginger concentration increased. Accordingly, the 15% concentration of camel urine showed the highest inhibition of mycelial growth, followed by 10% and 5% which gave78%, 67%, and 54% respectively, compared to the control. Ginger water extract gave inhibition of mycelial growth of 57%, 35% and 20%, respectively, compared to the control The sporulation of A. alternata was inhibited by camel urine and ginger at all concentrations. Sporulation inhibition percentage for camel urine concentrations were 92%, 83% and 77% and for ginger were 88%, 77% and 69% compared to the control. The spore germination of the pathogen was completely inhibited at all camel urine concentrations. The ginger extract showed full inhibition of spore germination at all concentrations except at 5%. Based on these results, camel urine demonstrated the highest antifungal activity, against A. alternata compared to ginger extract. This is the first report on the antifungal activities of camel urine and ginger water extract used against Alternaria alternata isolated from infected tomato plants in the Sudan

Key words: Biological control, Camel Urine, Ginger, *Alternaria alternate*, Tomato.

Introduction

Tomato (*Solanum lycopersicum* L. [syn. *Lycopersicon esculantum* Mill.]) belongs to the family Solanaceae along with other economically important crops such as pepper, eggplant and potato (Jones, 2008; Caicedo and Peralta, 2013).

Tomatoes are subject to a large number of pests and diseases from the time of emergence to harvest. Early Blight disease is the most important fungal disease of tomato caused by *A. alternata* which is the most common disease of many kinds of plants throughout the world.

Early blight disease is one of the most common and destructive diseases of tomato in the areas of heavy dew, rainfall and high relative humidity. The fungus can cause disease on foliage (leaf blight), stem (collar rot) and fruit, and can result in severe damage during all stages of plant development (Sabriye, 2011).

These diseases controlled mainly with agro chemicals. However, manipulation of environmental friendly fungicides to control plant disease is the new trend to reduce the contamination by synthetic compounds. Natural products are important sources of new agrochemicals for the control of plant diseases (Sallam *et al.*, 2012).

Moreover, there is an increased public demand for sustainable and chemical residue-free food production (Yeole *et al.*, 2014). Accordingly, biofungicides, of natural origin emerged as promising alternative strategies in controlling early blight disease in tomato (Prasad and Naik, 2003; Mate *et al*., 2005).

Several compounds of natural origin and their constituent have been successful in plant disease control and have been proved to be harmless and non phytotoxic unlike chemical fungicides. There are biologically active compound in camel urine and ginger these compounds attracted much attention due to their wide range of properties permitting pharmacological.

Camel urine has been proven to be effective as an antimicrobial agent, and commonly used against cancer and respiratory tract infections in alternative medicine（Al-Awadi *et al*, 2014). Studies have tested the antimicrobial activity of camel urine against pathogenic microorganisms including the fungi *Aspergillus niger* , *A. flavus* ,*Fusarium oxysporum* , *Rhizoctonia solani* , *Aschocayta* sp ., *Pythium aphanidermatum* , *Sclerotinia sclerotiorum*, *Candida albicans* ; and the bacteria *Staphylococcus aureus*, *Streptococci* , *E. coli Pseudomonas aeroginosa* and *Klebsiella pneomoniae* . The results of these studies showed high antimicrobial activity against the tested microorganisms, even when accompanied by changes in anions and cations (Al-Bashan, 2011). Antimicrobial activity of camel urine is due to factors such as high salt concentrations, alkalinity, and natural bioactive compounds (Kamlu *et al*, 2004).

Ginger (*Zingiber officinale* Roscoe) rhizomes have strong aromatic and medicinal properties (Chen, 2008). Previous studies have demonstrated that plant extracts and isolated compounds from *ginger* possess strong antioxidant (Stoilova *et al*., 2007) antibacterial, antifungal, anticancer and anti-inflammatory effects (Habib *et al*., 2008). The rhizome is rich in the secondary metabolites such as phenolic compounds (gingerol, paradol and shogaoal), volatile sesquiterpenes (zingiberene and bisabolene) and monoterpenoids (curcumene and citral) (Ali *et al*., 2008).

Therefore the objectives of this study were to isolate and identify the causal agent of early blight on tomato to assessment the antifungal activities of camel urine and ginger on mycelial growth, sporulation and spores germination *In vitro.* In this study camel urine and ginger were selected for evaluation of their antifungal activities on various development stages of *A.alternata*. The findings of this study will contribute towards the knowledge on antifungal of camel urine and ginger which will be an aid towards development of bio fungicide for control plant diseases.

Material And Methods

Isolation of pathogen

Infected leaves of tomato plants were collected during winter season of 2013/2014 from an infected tomato field in Khartoum North, shambat. The infected leaves were collected in paper bags and carried to the Plant Pathology laboratory, Faculty of Agriculture, University of Khartoum, for investigation. Leaves were cut into small pieces approx.10 mm^2, surface sterilized with sodium hypochlorite (NaOCl) 1% for 1mintes; rinsed in sterilized distilled water, and then blotted with sterile filter paper under laminar air flow.

Four pieces were inoculated in a 9 cm Petri-dish containing potato dextrose agar (PDA) supplemented with chloramphenicol (0.05 g/l) as an antibacterial agent; and incubated at 27±2ºC for 7days. Plates were examined daily and pure culture was established.

Identification of the fungus

Identification was made depending on the visual characteristics of the fungus which was the culture growth pattern and pigmentation. Further investigation was made by using binocular compound microscope to view the slide prepared from the fungal growth.

The microscope was used to study the conidiophores formation, arrangement of the conidia on conidiophores, the beaks of the conidia, the transverse and longitudinal septation as well as the length and width of the conidia and the colour. The mycelial growth of the fungus was measured for 10 days.

Camel urine sample

The camel urine sample was obtained from the Camel Research Center University of Khartoum. The urine was collected directly from the camel when they urinate in sterile bottles and kept at 4ºC. Urine was collected aseptically from virgin female.

Antifungal activity of camel urine

The effect of camel urine and ginger on pathogen was determined using poisoned food technique according to (Al-Hetar *et al*, 2010 and Ravi *et al*, 2014). The stock of camel urine without dilution was tested firstly to determine the antifungal activity

before it used for different concentrations. The potato dextrose agar amended with camel urine 100% was inoculated with freshly subculture of 7 days old of *A. alternata* and incubated at 27±2ºC for 10 day.

The PDA medium was prepared as usual and autoclaved. Cooled melted media was amended with camel urine to obtain the following concentrations 5%, 10%, 15%, flasks received no camel urine served as control. Then the medium was poured into sterilized Petri dishes. Then plates were inoculated with fungal discs of 5mm in diameter from the margin of 7days old cultures. Plates were incubated at 27±2°C .The radial colony growth was measured daily and Inhibition percentage was calculated as follows:

IPRG% = [(R1-R2)/R1] × 100%

Where, R1 = radial growth of fungus in control Plates; and R2 =radial growth of fungus in treatment plates (Abd-Elkareem *et al.*, 2006 and Guo *et al.*2006).

Antifungal activity of ginger water extract

Ginger was collected from local market in Khartoum north and washed thoroughly under running tap water, shade dried and then powdered. Stock solution was prepared by socking 10g of powder in 100 ml sterilized distilled water for 48 hr at room temperature, then filtered through tow layer of cheese-cloth and autoclaved. Different concentrations of 5%, 10% and 15 % were prepared by incorporation of ginger extract into sterilized moltened PDA , and poured into sterilized Petri dishes. Plates were seeded with 5 mm fungal plugs from the margin of 7-days old culture, and incubated at 27±2ºC. Radial growth (mm) of the colony was measured and mycelial inhibition percentage was calculated using the formula mentioned above.

Effects of camel urine and ginger water extract on sporulation

Sporulation was assessed using amended media as described above. Mycelial growth in control plates were permitted to extended to the edges. All plates were flooded with sterile-distilled water and checked for 20 min; then filtered through two layers of cheese-cloth. Spore concentration per mL was determined using haemacytometer according to Al-Hetar *et al*, (2010). Sporulation inhibition-percentage was calculated using the formula described above for mycelial growth.

Effects of camel urine and ginger water extract on spore germination

Spore germination was assessed by spreading 300 µl of a diluted spore suspension (300spores/mL) onto a PDA plate amended with different concentrations of camel urine and ginger water extract of (5%, 10%, and 15%). Plates were incubated at 27±2 for 48 h and the numbers of colony formed were counted. The inhibition percentage of spore germination (IPSG %) was determined using the formula described above for mycelial growth measurements.

Statistical analysis

Experiment was laid out in completely randomized design; and collected data were subjected to the analysis of variance (ANOVA) and means were separated using Duncan's Multiple Range Test at the 5% level of significance Analysis of variance and mean separation were performed by the statistical software SPSS version16.0.

Result

Isolation of the causal agent:

The primary cultures which isolated from the infected tomato leaves showing the early blight disease symptoms produced numbers of fungal species. The dominant species was *Alternaria* sp. The fungus growth was obvious from the infected leaves after 7-10 days. The pure culture growth of the causal agent of the early blight disease was conspicuous by grey, dark blackish brown or black pigmentation.

Identification of the causal agent:

The causal agent of early blight disease isolated from the infected leaves was identified as *A. alternata* using binocular compound microscope. The identification was based on the colony growth, pigmentation and sporulation (conidia and conidiophores) color and shape of the vertical and horizontal division and the beak of the conidia as well as the presence of the spores in the chains. The fungus colonies where characterized by a slow to medium growing pattern. The diameter of the colony reached 9cm in 10 days incubated at 27ºC. The face side of the colonies growth in PDA showed a dark green color at the start, later darkening to olive-black while the reverse side was typically black in the center surrounded with a grey brown growth. The microscopic examination revealed that conidiophores arise directly from substrate pale brown consisting of a catenulated conidia chain. The conidia where club-shaped and dark brown, formed chain with a short beak and highly septate. Accordingly, the causal agent was identified as *A. alternata*.

The effect of camel urine on mycelial growth of *A. alternata:*

In the present study, the efficacy of camel urine was evaluated against *A. alternata in vitro.* The stock of camel urine was tested without dilution showed full inhibition within 10 day. At all concentrations tested, the colony diameter of *A. alternata* was significantly reduced by camel urine ($P<0.05$) compared to the control (Fig.1). It was observed that the inhibitory effect increased as camel urine concentration increased. Analysis of variance showed that there were highly significant differences among camel urine concentrations. At concentration of 5%, 10%, 15%, the percentage of mycelia growth inhibition was: 54%,

67%, and 78%, respectively compared to the control. The maximum inhibition of camel urine was recorded at concentration 15%, (Table, 1and Fig. 1).

Table 1: The efficacy of camel urine against mycelial growth of *A. alternata in vitro*

Treatments	Mycelial diameter (mm)	Percentage of inhibition %of mycelia growth
5%	28.38[b]	54%
10%	20.11[c]	67%
15%	13.73[d]	78%
control	60.83[a]	0

Means followed with same letter(s) in the same column are not significanly different at (p=0.05) according to Duncan Multtiple Range Test (DMRT) .

Fig. (1): Effect of different concentrations of camel urine on mycelial growth of *A. alternata*

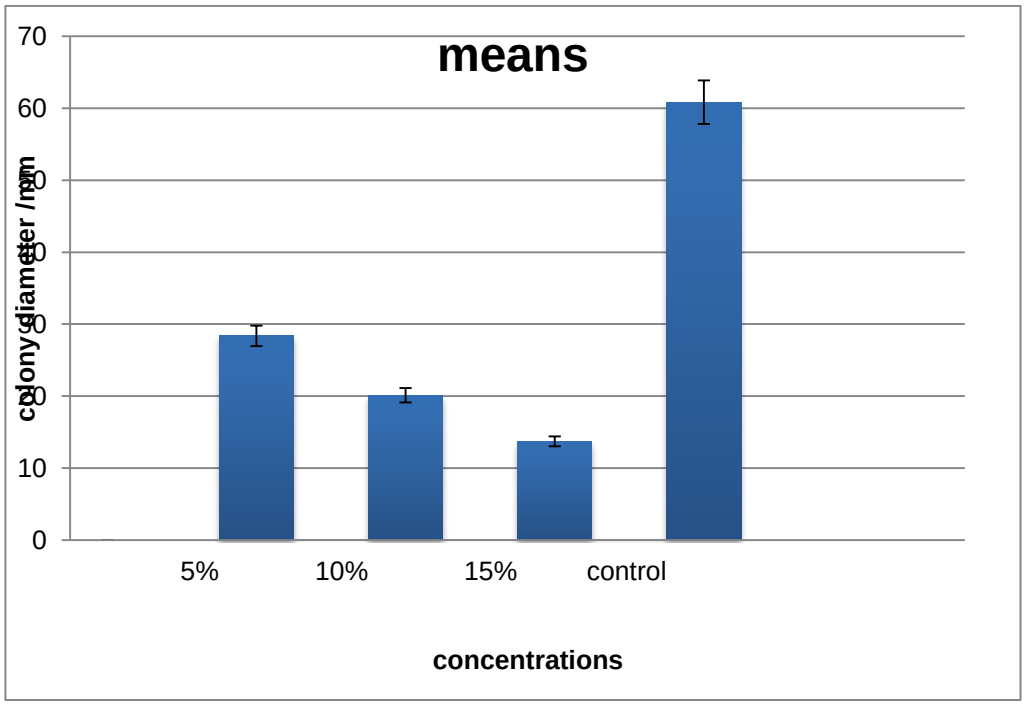
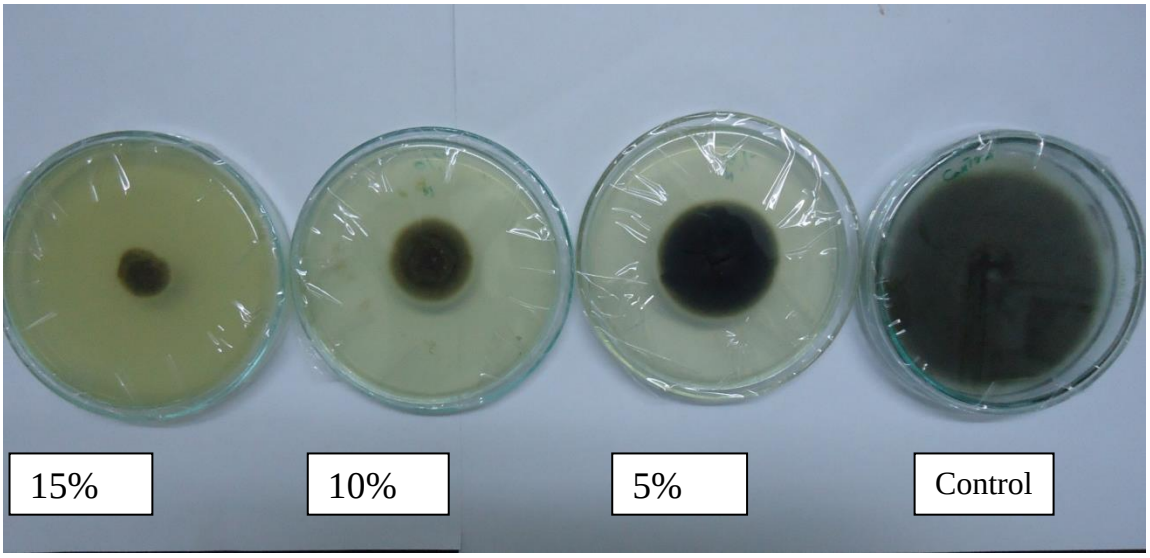


Fig.2: Effect of camel urine concentrations on mycelial growth of *A.alternata*.



The effect of different concentrations of ginger water extract on mycelial growth of *A. alternata*:

The colony diameter of *A. alternata* was significantly reduced by the three ginger concentrations ($P<0.05$) compared to the control (Fig.2). It was observed that the inhibitory effect increased as concentration increased. Results showed that ginger extract concentrations of 5%, 10% and 15%, were inhibited the mycelia growth of *A. alternata* by 20%, 35% and 57%, respectively (Table 2, Fig. 2). It was observed that the concentration of 15%, showed the strongest inhibitory effects over other concentrations.

Table 2: The efficacy of ginger extract against mycelial growth of *A. alternata in vitro*

Treatments	Mycelial growth diameter (mm)	Percentage of inhibition %of mycelia growth
5%	49.10^{b}	20%
10%	40.27^{c}	35%
15%	26.88^{d}	57%
Control	61.33^{a}	0

Means followed with same letter(s) in the same column are not significanly differentat (p=0.05) according to Duncan Multiple Range Test (DMRT)

Fig. (3): Comparison between different concentrations of ginger on mycelial growth of *A.alternata*

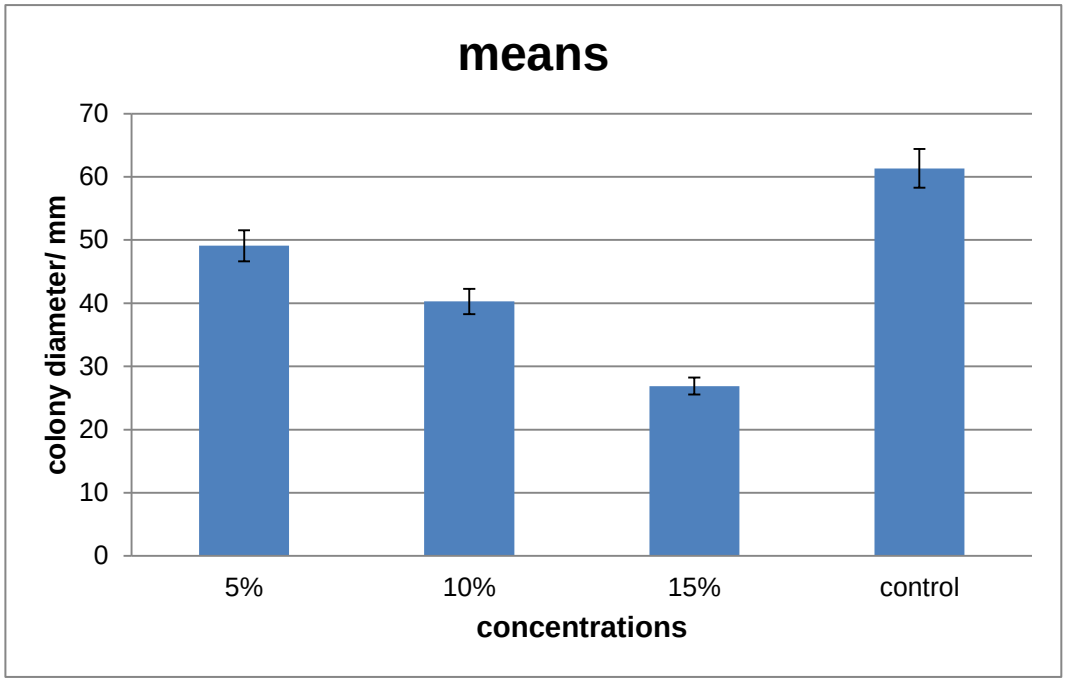
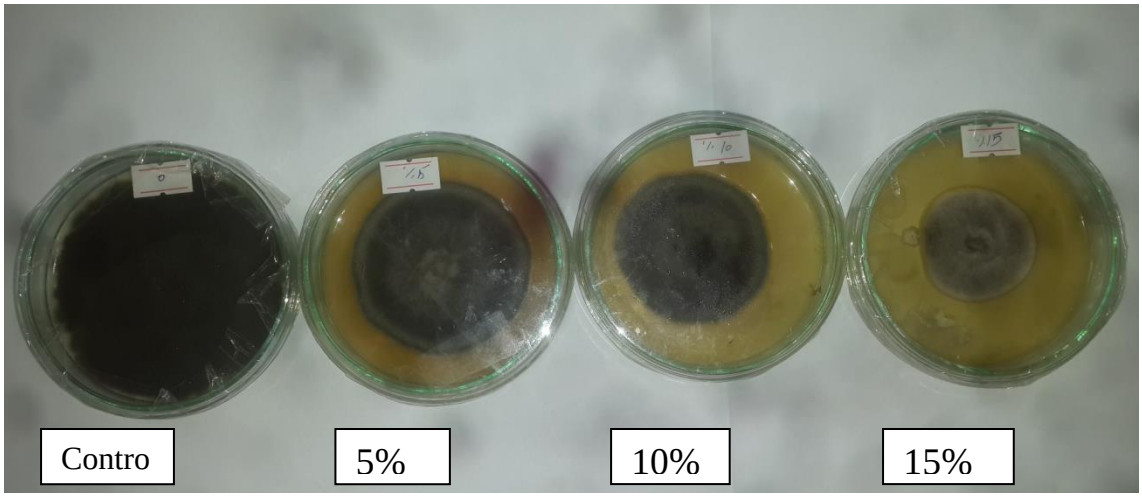


Fig.4: Effect of ginger concentration on mycelial growth of *A.alternata*



Effects of camel urine and ginger on sporulation of *A. alternata*

The results in (Table 3) showed that camel urine and ginger was inhibited the sporulation at all concentrations. The number of spores treated with camel urine and ginger at concentrations 15%, 10% and 5% steadily decreased with an increase in concentration. Sporulation inhibition percentage for camel urine concentrations were 92%, 83% and 77% and for ginger were 88%, 77% and 69%, respectively compared to the control Sporulation inhibition at all concentrations was significantly different ($P<0.05$). The camel urine at concentration 15% significantly inhibited sporulation percentage compared with other treatments ($P<0.05$).

Table 3: Effect of camel urine and ginger extract at various concentrations on sporulation of *A. alternat*a after 9 days of incubation

Treatments	Concentration	No. of spores/mL	PIS%

Camel urine	5 10 15	6.3×10^5 4.7×10^5 2.2×10^5	77% 83% 92%
Ginger extract	5 10 15	7.8×10^5 6.1×10^5 3.2×10^5	69% 77% 88%
Control		26.5×10^5	

Where PIS% = Percentage inhibition of sporulation. Means followed by the same letter(s) within column are not significant at P < 0.05, according to (DMRT)

Effects of camel urine and ginger water extract on pathogen spore germination

After 48 hour of incubation, spore germination in control plates were 81 colonies in both experiment. Camel urine at all concentrations tested completely inhibited spore germination of *A. alternata* (100%), but ginger extract showed complete inhibition at 10%and 15%, compared to control (Table 4).

It was clear that from the results, different concentration of camel urine and ginger caused highly significant inhibition in the spore's germination.

Table 4: Effects of camel urine and ginger water extract at various concentrations on germination of *A. alternata* spores after 2 days of incubation

Treatments	Concentration	No. of colonies	PIS%
Camel urine	15 10 5	0 0 0	100% 100% 100%
Ginger extract	15 10 5	0 0 1	100% 100% 99%
Control		81	

Where PIS% = Percentage inhibition of germination.

Discussion

In this study the causal agent of early blight disease was isolated and identified as *A. alternata* (Fr.) Keissler, this result agrees with Abbo, (2011) who characterized the causal agent of early blight disease on Solanaceous crops in Sudan as *A. alternata*.

In the present study the efficacy of camel urine and ginger extract was evaluated against *A. alternata in vitro.* Camel urine significantly inhibited mycelial growth of *A. alternata* at all concentrations used. The high antifungal activity of camel urine

reflected on inhibition of mycelial growth of tested fungi, this result agreed with Al-Judaibi, (2010) who found that camel urine inhibit the growth of *Aspergillus niger* and *Candid albicans* compared with the antifungal agents mycostatin. Mycelial growth, sporulation, and spore germination were affected by camel urine on various stages in the growth and development of *A. alternata*.

The study indicated that high inhibition of sporulation could be associated to a stress response caused by camel urine. Conidia of *A. alternata* were very sensitive to camel urine and no germination was observed on media amended with different concentrations. These findings agreed with (Al-Awadi and Al-Jedabi, 2000; Al-Talhi and Al-Bashan, 2006) who demonstrated the antimicrobial activity of camel urine which contain antibiotics, salts and urea, that affect fungus, bacteria, viruses. (AL-Awadi and AL-Jedabi, 2000).

This result also agreed with Khalifa *et al*. (2005) who noted that no pathological signs appeared on liver or kidney tissues after using camel urine. The high salt concentration of the urine causes plasmosis and analysis of mycelium (AL-awadi and AL-Jedabi, 2000). Similar results were obtained from Al-Zahrany (2002) who recorded growth inhibition of *A. niger* after its treatment with camel urine for 14 - 18 months. Our result also agrees with AL-awadi and AL-Jedabi (2000) who recorded the effect on the dry weight of the yeast and fungi. Shoeib and Ba-hatheq (2008) proved through electron microscopic studies, the effect of urine on the morphological properties of some human pathogenic bacteria.

The results of this study also provided new information regarding the inhibitory effects of various concentrations of ginger extract on the growth of *A. alternata*. Plates assay showed that the fungus was sensitive to ginger extract because radial growth was significantly reduced at all concentrations. However, highly inhibition was achieved at concentration 15%.

Radial mycelial growth and sporulation were reduced as ginger water extract concentration increases. Amadi (2014) in his screening on the phytochemical effectof ginger extracts showed the presence of some secondary metabolites namely: tannins, alkaloids, flavonoids, anthraquinones, saponins and steroids. Presence of these secondary metabolites is suggestive of the presence of antifungal property in these extracts. In addition, the results of antifungal activity assays showed that the extract of ginger had inhibitory effects on the growth of *A. alternata* fungi. Our results are agreed with him. In the light of the above recorded results, the present study may suggest that camel urine and ginger are potential compounds for application in disease management. The effect of camel urine ginger may be due to exhibit chelating activity of spore elements and essential nutrient which lead to membrane permeability.

The present study showes effectiveness of camel urine and ginger water extarct against *A. alternata*

Conclusion and recommendation

These results show promise and potential use of camel urine for effective control of *A. alternata* in vitro. This is the first report on the antifungal activities of camel urine and ginger water extract used against *A. alternata* isolated from infected tomato plants in the Sudan. The present study suggested that camel urine and ginger could encourage for control early blight depending on findings.

Our findings confirm the antifungal effects of camel urine and ginger on mycelial growth, sporulation and spore germination. To enhance the utility an intense study on camel urine and ginger may help to use them as active biofungicides in commercial scale therefore further studies should be done to determine whether camel urine is able to induce defense mechanism reaction, enhancing plant growth and control the causal disease under green houses and field conditions. There seems to be no report in literature on the effect study of camel urine on plant pathogen except *Fusarium oxysporium* therefore present study focused on the antifungal effect *in vitro*.

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