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Efficacy of Barks Extract of Three Indigenous Trees from Zalingei Area on the Growth of *Neofusicoccum mangiferae* under Laboratory Conditions

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Abstract

This laboratory experiment was carried out to test the efficacy of plants bark extracts on the fungus *Noefusicoccum mangiferae.*Crous, whichcausesbranch wilt disease of shade, ornamentals and fruit trees. The samples were collected from Khartoum area (April- July 2019) to isolate the fungus. The laboratory experiments were conducted at the laboratory of plant quarantine- Administration of Plant Protection Ministry of Agriculture. The fungicidal potentialities of aqueous extracts of three plant barks namely: "Mahogany" (*Khaya senegalensis*), "Mokhiat" (*Boscia senegalensis)* and "Lo'at" (*Acacia nubica)* were tested against *N. mangiferae* isolated from lime (*Citruslemon*), (*F. benjamina*), (*F. nitida*) and mango (*Mangiferaindica*). The results showed that some aqueous extracts were significantly effective in inhibition of the radial growth of different pathogen isolates on Potato Dextrose Agar (PDA) media. Cold water extracts were more effective than hot water extracts, whereas the effect of *Kh. sengalensis* was more effective than the rest plant extracts. Cold water extract of *Kh. senegalensis* have shown significant effect suppresser on number of spores per 1ml spore suspension in plate treated with *Kh. senegalensis* and *A. nubica* extracts, however *B. senegalensis* was not significantly different from control. Phytochemical analysis of plant barks extracts showed that all the extracts contain secondary metabolites such as alkaloids, cumarins, flavonoids, saponins and triterpenes which act individually or in combinations as spore germination suppressers and growth inhibitors of plant pathogenic fungi.

Key Words: Efficacy,water extracts, Bark, *Noefusicoccum mangiferae,Boscia senegalensis, Acacia nubica , Khaya senegalensis*

1- Introduction

Branch wilt disease in Sudan is a serious disease in over 20 host plants in a number of plant families, including ornamentals, shade and orchard trees [1]. The disease is caused by the common air-borne fungus *Neofusicoccummangiferae* thesynanamorph of *Scytalidiumdimidiatum* previously known as *Nattrassiamangiferae*, *Fusicocumdimidiatum* and *Henndersonulatoruloidae*. This fungus has a

wide host range and causes branch wilt disease in deciduous woody plants under elevated temperatures conditions [2]. Neofusicoccum is a genus of fungi in the family Botryosphaeriaceae for which there is the single species N. mangiferae [3]. *Henndersonulatoruloidae* was originally described as a pathogen of deciduous fruit trees in Egypt [4]. This fungus has been reported to cause canker and wilt on a wide range of species including grapevine [5] and [6], *Eucalyptus*, *Psidiumguojava*, wide range of citruses and common fig (*Ficuscarica*) and wide range of *Ficus* spp. [7]; [1]; [8] and [9] and *Ficusbengalensis* [10]. It has been reported that the fungus causes serious disease in mango in India and South Africa. These include blossom blight, post-harvest diseases, and spot brown rot and stem end rot [11]. The recent trend favor the use of the alternative substances derived from natural plant extracts to control pests [12]. Plant extracts and their essential oils showed fungicidal activity against a wide range of fungi [13]. The spores of fungi cause cellular leakage and reduce the chlorophyll content in susceptible plants; the use of chemical control is either not efficient or difficult to apply. However, many practices could not effectively control the spread of the disease and it continued to spread rapidly. Therefore the use of alternative substances derived from natural plant extracts can be promising alternative to prevent disease caused by fungi and seemed to be one of the solutions [14]. Due to the paramount importance of this disease in a wide range of plant species in Sudan, efforts have been made searching for effective and environmentally friend methods of management. This work is a contribution in this aspect especially with regard to the following objective:-

1-To investigate the fungicidal activity of bark extract from three native plant of Zalingei area against *N. magiferae.*

2- Analysis of plant extracts for determination of the effect of components on the growth and sporulation of the fungal pathogen *N. magiferae.*

2- Materials and Methods

2.1 Survey and Sampling

During the dry season (April - July 2016) a general survey was carried out on shade, ornamental and fruit trees in Khartoum area to collect infected samples based on the characteristic symptoms of the branch wilt disease caused by *Neofusicoccummangiferae* on the aerial parts of the plants. Samples were collected from infected plant branches in separate plastic sacks.

2.2 Preparation of the Media

2.3 Isolation and Conservation of the Pathogen

Segments prom infected branches were washed thoroughly with tap water and surface sterilized with 5% sodium hypochlorite solution and plated

onto Potato Dextrose Agar (PDA) medium supplemented withstreptomycin (g/l) as bacteriostatic agent. Plates were incubated for 7 days at 28 ± 2ºc and the culture were sub cultured for purification. Samples from the positive plates were transferred to previously prepared PDA plates and incubated in the same conditions for seven days and investigated under binocular until pure cultures from each isolate was obtained. Single spore isolation was carried out by dividing the newly prepared PDA plate to 5 chambers from the bottom using a marker. One big square in the center of the plate and four small ones in one row. Small amount of specimens were drawn from a 24hrs age culture of each isolate of *N. mangiferae* by sterilized needle to the centered square. Under binocular microscope a single spore was transferred from the specimen in the centered square to each small square. The plates were incubated at 27± 2°c for 3 days. The square which show mycelial growth were considered positive and cut by sterilized scalpel blade and transferred to newly prepared PDA plates and incubate for 7 days at 27± 2°c. The pure culture from single spores were transferred to PDA Slants on McCartney bottles and preserved at 4° c for further experiments.

2.4. Laboratory Experiments

2.4.1 In vitro Testing of Three Plant Bark Extracts as Control Agents of *N.mangiferae*

2.4.2 Collection and Preparation of Plant Extract

Bark of three indigenous plants of Zalingei area namely Mahogany (*Khayasenegalensis*), Mokhiat (*Boscia senegalensis*) and Lu'ot (*Acacianubica*) were peeled from the main stem and collected in separate plastic bags. Then, they were spread on laboratory bunch for 10 days at room temperature to dry and crashed manually then grounded using mortar and pestle and blended by domestic blender to get a fine powder. The obtained powder from each plant bark was passed through a sieve (25 meshes) and stored in darkness at room temperature in tightly-closed polythene bags. Bark powders were sterilized by mixing 20g from each bark powder with 5 ml 95% ethanol for 5 minutes then evapurated under Hood for 10 minutes.

Cold water extracts were prepared by mixing 20 g for each powder with 100 ml of sterilized distilled water the mixures were shacked overnight. The extracts then filtered through 3 layers of cheese cloth and kept in a refrigerator at 4ºC. For hot water extracts 20 g from each bark powder was surface sterilized and mixed with 100 ml sterilized water and boild in water path for 90 minutes at 100ºC, and then filtered through 3 layers of sterilized cheese cloth. The products were kept at 4 ºC for further experiments.

2.4.3 Testing of the Fungicidal Potentiality of Plants Extracts

The fungicidal effect of barck extracts aganst *N. mangiferae* was assessed *Invitro*. Five ml of each extract were mixed with pre-autoclaved PDA medium in liquid conditions to give final concentration 5%. A 5 mm diameter disc from the edge of 7 day-old culture of the pathogen was placed on the surface of PDA-PE plates. PDA without plant extract was included as control. Each treatment was replicated 4 times and arranged in a completely randomized design (CRD) and incubated at 28 ±2 ℃ for one week. The leaner growth of mycelium and the averages were calculated.

2.4.4 Determination the Effect of Plant Extracts on the Numbers of Spores per ml Spore Suspension

The Petri dishes were treated with the different plant extract and inoculated with one of the *N. mangiferae* isolates. After 10 days the spores form each treatment was harvested in 100 ml sterilized distilled water by scratching the mycelial growth and the spores on the media using sterile scalpel blade. Then the suspensions were blended and sieved through double layered sterile cheese cloth. Spores count was carried out by means of three haemocytometer readings recorded from each treatment and compared with the reading in the control to determine the effect of plants bark extracts on numbers of spores produced by the pathogen isolate.

2.4.5 Phytochemical Analysis of Plant Extracts

2.4.5.1 Preparation of the Extracts for Analysis

A total of 10g of the powdered plant barks sample were refluxed for4 hours with 100ml of 80% ethanol. The excess ethanolwas evaporated. This prepared extract (PE) was used for the various tests.

2.4.5.2 Test for Unsaturated Sterols and Triterpenes

10 ml of the PE was evaporated to dryness in a water path and the cooled residue was stirred several times with petroleum ether to remove most of the coloring materials. The residue was then extracted with 20ml chloroform. The chloroform solution was dehydrated over sodium sulphateanhydrous. 5ml portion of the chloroform solution was mixed with 0.5ml of acetic anhydride followed by 2 drops of concentrated. Sulphuric acid. The gradual appearance of green, blue pink to purple color was taken as an evidence of the presence of sterols (green to blue) and or triterpenses (pink to purple) in the sample [15].

2.4.5.3 Test for Alkaloids

7.5ml of the PE was evaporated to dryness on a water bath.5 %ml of 2N Hcl was added and stirred while heating on the water bath for 10minutes, cooled filtered and divided into two

test tubes. To one test tube few drops of Mayer's reagent was added while to the other tube few drops of Valser's reagent was added. A slight turbidity or heavy precipitate in either of the tow test tubes was taken as presumptive evidence for the presence of alkaloids [16].

2.4.5.4 Tests for Flavonoids

17.5ml of the PEwas evaporated to dry ness by boiling in a water bath, then cooled and the residue was defatted by several extractions with petroleum ether and the defatted residue was dissolved in 30ml of ethanol 80% and filtered [16].

2.4.5.5 The filtrate was used for following tests

Add to 3ml of the filtrate in a test tube 1ml of 1% aluminum chloride solution was in methanol was added. Formation of a yellow color indicated the presence of Flavonoids. Flavones or and chalcone.

B/ to 3ml of the filtrate in a test tube 1ml of 1% potassium hydroxide solution was added. A dark yellow color indicated the presence of Flavonoids compounds (flavones or flavonenes) chalcone and or flavonols.

C/ to 2ml of the filtrate 0.5ml of magnesium turnings were added. Producing of defiant color to pink or red was taken as presumptive evidence that flavonenes were present in the plant samples.

2.4.5.6 Tests for Tannins

For this test 7ml quantity of the PE was evaporated to dryness on water bath. The residue was extracted several times with n-hexane and filtered. The insoluble residue was stirred with 10ml of hot saline solution. The mixture was cooled, filtered and the volume of the filtrate was adjusted to 10ml with more saline solution. 5ml of this solution was treated with few drops of gelatinsalt reagent. Formation of immediately precipitate was taken as evidence for the presence of tannin in the plant sample. To another portion of this solution, few drops of ferric chloride test reagent were added. The formation of blue, black or green was taken as an evidence for the presence of tannins [17].

2.4.5.7 Test for Saponins

One 1 g of the original dried powder plant material was placed in a clean test tube.10 ml of distilled water was added and the tube was stoppered and vigorously shaken for about 30 seconds. The tube was then allowed to stand and observed for the formation of (honeycomb) the appearance of honeycomb, which persisted for least an hour, was taken as evidence for presence of Saponins [15].

2.4.5.8 Test for Cyanogenic Glycoside

Three 3g of the powdered plant sample were placed in erlenmeyer flask and sufficient water was added to moisten the sample, followed by 1ml of chloroform (to enhance every activity). A piece of freshly prepared sodium picrate paper

was carefully inserted between a split crocks which was used to stopper the flask. Change in the color of the sodium picrate paper from yellow to various shades of red was taken as an indication of the presence of cyanogenic glycoside.

2.4.5.9 Test for Anthraquinone Glycoside

Ten 10g of the powdered plant sample were boiled with 10ml of 0.5N KoH containing 1ml of 3% hydrogen peroxide solution. The mixture was extracted by shaking with 10ml of benzene. 5ml of the benzene solution was shaken with 3ml of 10% ammonium hydroxide solution and the two layers were allowed to separate. The presence of anthraquinones was indicated if the alkaline layer was found to have assumed pink or red color.

2.4.5.10 Test for Cumarins

Three 3g of the original powdered plant sample boiled with 20ml distilled water in test tube and filter paper attached to the test tube to be saturated with the vapor after a spot of 0.5N KOH put on it. Then the filter paper was inspected under UV light, the presence of cumarins was indicated if the spot have found to be adsorbed the UV light.

3- Results

3.1 Effect of Plant Barks Extracts on the Growth of *N. mangiferae*

3.2 Effect on the Radial Growth of *N. mangiferae* Isolates

The results of growth inhibition percent due to the effect of plant bark extracts in Table 1 showed that hot water extracts did not affect significantly on the radial growth of the pathogen compared to the control, the cold water extracts of *Kh. senegalensis* and *B. senegalensis* have the similar effect on the growth of *N. mangiferae* isolated from C. lemon and *F. benjamina*. However, cold water extract of *A. nubica* has significantly reduced the growth of the pathogen isolated from *F. nitida.* Cold water extract of *B. singalensis* was not significantly effective as growth inhibitor of *N. mangiferae* isolated from Mango (*Mangiferaindica*), whereas *Kh. singalensis* and *A. nubica* extract were significantly reduced the radial growth of *N. mangeferae*.

In general these data indicated that cold water extracts were more effective than hot water extracts. The results in Table 2 showed the ranges of inhibition percentages attributed to the effect of cold and hot water plant barks extracts of *K. senegalensis, B. senegalensis* and *A. nubica* which were 10.21% to 42.23% and 2.36% to 7.09 %, respectively. The growth inhibition due to cold and hot water extracts of plant barks of *Kh. senegalensis*, *B. singalensis* and *A. nubica* ranged between 13.02% to 40.23%, 11.60% to 22.91% and 10.21% to 42.23% respectively, whereas the ranges in hot water extracts were

2.36 %to 7.09%, 3.02% to 6.25% and 3. 48% to 6.03%, respectively.

Fig. 1. Effect of hot aqueous extracts of K. *senegalensis*, B. *senegalensis* and A.n barks on the sporulation of *N. mangiferae*

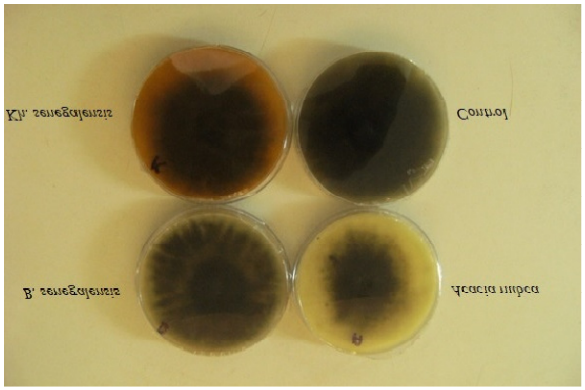

Table1. Effect of cold and hot water extracts of *K. senegalensis, B. senegalensis* and *A. nubica* on radial growth of four isolates of *Neofiscoccummangiferae*

Treatments(P.E)	Isolates *of N. mangiferae*							
	Host plants							
	Citruslemom		*F. benjamina*		*F. nitida*		*M. indica*	
	CWE	HWE	CWE	HWE	CWE	HWE	CWE	HWE
	Radial growth in (cm)							
Kh.s	7.75b	8.39a	6.98b	8.48a	6.69b	8.69a	5.29c	8.25a
Bo.s	7.14b	8.39a	7.85b	8.71a	6.83b	8.38a	7.69ab	8.46a
Ac.n	8.00ab	8.41a	7.08 b	8.58a	5.03c	8.59 a	7.34b	8.40a
Control	8.91a	8.95a	8.88a	8.93a	8.86a	8.90a	8.85a	8.88a
LSD	1.10	0.76	0.93	0.50	1.22	0.86	1.39	0.76

CWE = Cold Water Extract

HWE = Hot Water Extract

P.E = Plant Extract

Kh.s = Khaya *senegalensis*

Bo.s = *Bosciasenegalensis*

Ac.n = *Acacianubica*

Mean with the same letter(s) in the column are not significantly different at P< 0.05.

3.2 Effect of Cold and Hot Water Extracts on the Numbers of Spores per (spores/ml) ml Spore Suspension

The effect of plant extracts on the density of spores mass is presented in Figure 1. The results indicated that the color was darker in the treated plates as evidence of extensive spore masses compared with control plates. Data in Table 3 and Figure 1 showed that the plant bark extracts have different effects on the sporulation of the fungi. Cold water extracts effect was significant as sporulation inhibitor in all *N. mangiferae* isolates compared with control. On the other hand, hot water extract of *Kh. singalensi* and *A. nubica* have the same effect, but in contrast *B. singalensis* effect was not significantly different from the control at P< 0.05.

Table 2. The means number of spores per ml spore suspension of four *N.mangiferae isolates* treated with cold water and hot water bark extracts of *K. senegalensis, B. senegalensis* and *A. nubica* bark

Treatments (P.E)	Isolates *of N. mangiferae*							
	Host plants							
	Citruslemom		*F. benjamina*		*F. nitida*		*M. indica*	
	CWE	HWE	CWE	HWE	CWE	HWE	CWE	HWE
	Numbers of spores per 1ml spore suspension							
Kh.s	6.13 c	10.33c	11.27 b	18.74b	5.85b	8.90b	3.86b	5.68c
Bo.s	12.42b	18.05b	17.08 b	27.09ab	4.75b	8.29b	5.20b	8.75ab
Ac.n	8.38bc	12.81c	10.98 b	20.58ab	3.88b	8.92ab	3.89b	7.16bc
Control	22.63a	22.63a	28.75 a	28.75a	12.70a	12.70a	10.77a	10.77a
LSD	5.66	4.41	6.72	8.72	2.86	3.79	2.98	2.62

CWE = Cold Water Extract

HWE = Hot Water Extract

P.E = Plant Extract

Kh.s = Khaya *senegalensis*

Bo.s = *Bosciasenegalensis*

Ac.n = *Acacianubica*

Mean with the same letter(s) in the column are not significantly different at P< 0.05.

3.3 Phytochemical Analysis of Three Plants Bark Extracts

Three plants bark extracts have been analyzed chemically to identify their active bio-agents content which has a fungicidal effect. The results in Table 3 showed that the three plant extracts contains alkaloids. This result was confirmed by appearance of turbidity and precipitation in the test tube. However, the test for anthraquenone shown a negative result in all plant bark extracts whereas the laboratory test for cumarins absorbed under UV lamp 254 has given positive result .The laboratory examination for esterols for the three plant extracts revealed that *A. nubica* was positive, indicated by appearance of blue-green color in the test tube. Esterols were not detected in *B. senegalensis* and *K. senegalensis.* Laboratory test for flavonoids resulted in appearance of yellow color in the test tube with all plant extracts. Testing for saponins showed that foams were observed as positive results with all plant extracts. In examination of tanins blue-green color was observed in the test tubes which contain *A. nubica* and *B. senegalensis* as a positive result, but in case of *K. senegalensis* the resultwas negative. In the test for triterpenes content, all the extracts examined have shown positive results and pink color was developed as indication. The phytochemical analysis of plant bark extracts shown negative result for cyanogenic glycoside and anthroquenone glycosids (Table 3).

Table 3. Preliminary phytochemical analysis of *K. senegalensis, B. senegalensis* and *A. nubica* bark extracts of having biological activity against *N. mangiferae*

Test	Acacia	Boscia	Khyaya	Observation
Alkaloids	Positive	positive	Positive	Turbidity and precipitate
Anthraquenone glycosids	Negative	Negative	Negative	No observation
Cumarins	Positive	Positive	Positive	UV absorption
Esterols	Positive	Negative	Negative	Blue-green color
Flavonoids	Positive	Positive	Positive	Yellow color
Saponins	Positive	Positive	Positive	Foam formation
Tanins	Positive	Positive	Negative	Blue – green color
Triterpenes	Positive	Positive	Positive	Pink color
Cyanogenic glycoside	Negative	Negative	Negative	No observation

4. DISCUSSION

Based on the survey and the laboratory investigations the causal agent of branch wilt *N. mangiferae* was dominant in Khartoum area.

The extracts of higher plants have been reported to exhibit antimicrobial and antifungal properties under laboratory trails. Plant metabolites and plant based fungicides appears to be one of the better alternatives as they known to have minimal environmental impact and less danger to consumers in contrast to synthetic compounds [18]. Based on this view, the laboratory experiments revealed that the aqueous extract of plant barks namely: *Kh.senegalensis*, *B. senegalensis* and *A. nubica* have no significant inhibition potentiality on the radial growth of the pathogen under laboratory conditions. However the cold water extracts were more effective than

hot water extracts of plant barks tested in the laboratory. This result refers to the evidence that hot water treatmentscause degradation of the components which have antifungal activity. The above mentioned result related to the finding of Namsi et al., [19] with the methanol leaf extract of the three different tested plants have been studied against many fungal species [20]; [21]; [22] and have showed a broad antifungal activity. [23] reported similar conclusion that the fungicide components of plant extracts, denatured by heat. The results also confirmed that different plants bark extract have different inhibition percent on the pathogens isolated from different tree hosts. Differences between means of spores per ml spore suspension in different extracts is proved that cold water extract is superior to hot water extracts in reduction of number of spores per 1ml spore suspension; this may be due to denaturation of plant biocide by heat treatment. [24] Studied the effect of the extracts of *M. oliefera, S. aromaticum* and *C. zeylanicum* as antifungal activitiestheir results showed statistically significant antifungal activity against mycelial growth of tested pathogens and SER development. Phytochemical analysis was carried out for the three plant extracts to determine the biocides contained having fungi static action. The results showed that the different effect of extracts on the pathogen reefer's to containing the main growth inhibitory, namely alkaloids, cumarins, flavonoids, saponins, and triterpenes. These compounds have different fungicidal action even at very low concentrations. These results agreed with the findings of [25], [26] and [27] stated that saponins are a major family of secondary metabolites in a wide range of plant species. This might be a promising result. These compounds, called phytoanticipins seemed to be involved in plant disease resistance because of their well-known antimicrobial activity. In spite of these positive results of plant biocides, their concentrations and formulations adjustment still inconclusive.

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