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Bioassay guided study of the flowers of *Hagenia abyssinica* against potato wilt disease

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Abstract

Bacteria wilt disease, which is caused by the bacterium Ralstonia solanacearum, mainly attacks potato, tomato and zinger. In Ethiopia, there is no effective controlling strategy yet. This disease has become the second constraint for potato production next to late blight. Therefore, to develop eco-friendly antibacterial compound, three medicinal plant extracts namely flowers of H. abyssinica, bark of C. macroschyus and fruit of P. dodecandra were screened against this disease. In vitro antibacterial tests of the ethanol extract of H. abyssinica flowers was found to be the most effective of the three plant extracts. A minimum inhibition zone of 20 mm was observed, this being more active than the commercial anti wilt substance known as kocide, which had only 15 mm inhibition zone. Kocide is the commercial anti-wilt preparation which is made up of $Cu(OH)_2$. Five batches of flowers of H. abyssinica were separately extracted with hexane, diethyl ether, ethyl acetate, ethanol and water. Only the ethanol and water extracts were active. From the ethanol extract, the most active anti-wilt substance was isolated and characterized as the known compound quercetin-3-O-glucoside.

Keywords: *Ralstonia solanacearum*, wilt disease, *H. abyssinica*, quercetin 3-O-glucoside, and in vitro.

Introduction

Potato is the most important crop in the world. The tuber is known for its high carbohydrate and low fat content and is excellent source of energy for human beings and other animals. In addition, it has minerals and vitamins. Wilt disease is the second major diseases of potato next to late blight. The causative organism for the wilt disease of potato is *Ralstonia solanacearum.* It affects many plant species worldwide particularly in warm climates and its severity of attacks varies considerably on climate, cropping practices, soil type and geographical location[1]. It is also called brown rot disease due to brown symptoms on the tubers and remained the second major constraint to potato production in most regions of the world after late blight[2].

The pathogen has extensive host range and more than 200 species are infected due to this bacteria[3,4]. The bacterium causes losses of crops in two ways: premature wilting and death of the plants which leads total yield loss is one of the factor and the other one is rotting of the tubers during transit or storage.

The pathogen is identified to be gram negative, rod shaped and strictly aerobic bacterium with its high heterogeneity and several pathogenicity[1,5-7]. *R. solanacearum* is metabolically versatile, surviving and thriving in diverse habitats such as water, soil, and infected plants[8]. It also remains viable for several years at room temperature in sterilized tap, distilled or de-ionized water. The bacterium multiplies readily in its hosts at the temperature of 27°C[7] unlike other most strains which grow at a temperature of 28 and 32°C. *R. solanacearum* has a narrow host range, and is responsible for recent outbreaks of potato wilt disease in several countries of Europe and Africa including Ethiopia[7,9,10].

Bacterial wilt disease has external and internal symptoms[1]. The most external symptoms are characterized by wilting, stunting and yellowing of the foliage of infected plants as shown in Figure-1. Sometimes, dwarfing and stunting of the plants can be the characteristics of external symptoms in the cold conditions when an infected plant does not show wilting in this condition.

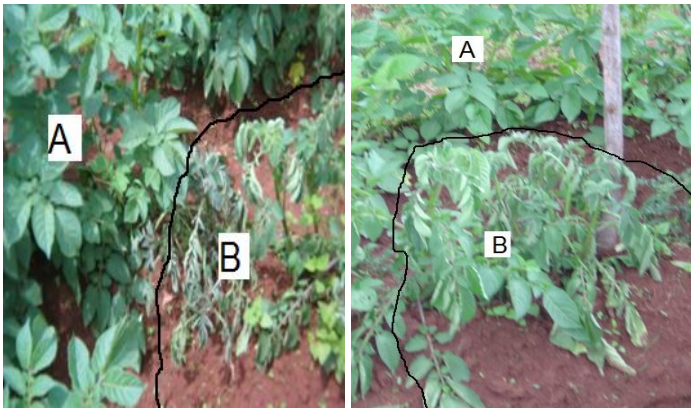


Figure-1: Pictures showing healthy (A) and infected (B) potato plants.

External systems are not always visible on infected tubers[11]. When the disease develops, progressive discoloration of the vascular tissue, mainly the xylem, occurs as internal symptoms. This leads complete necrosis of the plant. Brown rot symptoms on tubers of potato may be exhibited at the later stages of the disease (Figure-2). A gray brown discoloration of vascular tissues indicates the complete necrosis or death of most of the cells of the plant. Early diagnosis of the disease is possible by screening tests such as planting on semi selective media and immunodiagnostic assays[6,12,13].

Figure-2: Latent infection in tubers of early and late stages.

Different controlling attempts have been made to manage bacterial wilt disease of potato worldwide[14]. These are: Breeding resistance cultivars, crop rotation, encoding proteins on the gene of the bacterium, synthetic chemicals[12,15,16]. In Ethiopia, bacterial wilt is a serious problem in particular on potato and tomato. There is no effective controlling measures available yet[11,17]. Since bacterial wilt cannot be controlled by chemicals due to damage of plant tissues, environmentally unfriend, and persists in the soil for prolonged time, it is increasingly becoming a major risk for potato production in the country[18,19]. However, the use of medicinal plant extracts may have role in suppressing this plant disease and to decrease the use of agricultural chemicals[15,20]. Controlling of this plant pathogen using biological methods is a promising strategy and attracted considerable attention with the aim due to their biodegradability and environmental friend[21-24]. Efforts to look for natural compounds from plants with anti-wilt disease properties have led to direct the presence of promising results in the area[22,25,26].

Materials and methods

TLC plates (20×20cm pre-coated aluminum sheet, silica gel 60 F_{254}), 254nm UV lamp, column, silica gel (230-400 Mesh), UV visible spectrophotometer, Bruker Advance 400 MHz NMR spectrometer, Nutrient Agar (NA), *Ralstonia solanacearum* bacterial pathogen, kocide, Autoclave Sterilizer, Vortex, Incubator, Paper disc etc were used during this work.

Extraction procedure: Berries of *Phytolacca dodecandra*, bark of *Croton macrostachyus* and flowers of *Hagenia abyssinica* were purchased from the local market (Merkato) in Addis Ababa, Ethiopia from traditional medicine vendors. Each of the materials was dried and ground into fine powder then labeled and placed in airtight bottles until used. 10g powder of berries *P. dodecandra*, and flowers of *H. abyssinica* bark of *C. macrostachyus* were extracted with 95% ethanol, each filtrate was concentrated to afford 3g (30%), 0.6g (6%), and 0.87g (8.7%) crude extracts respectively. Based on the bioassay result ground flowers of *H. abyssinica* (20g) were soaked in 200mL of 70% aqueous EtOH, placed on a shaker for 24 h and filtered. To the filtrate, water was added to make it 50% aqueous solution and then partitioned with ethyl acetate. The ethyl acetate phase was dried using anhydrous Na_2SO_4, which was filtered and concentrated to afford 3 g solid material, which was adsorbed on silica gel and applied on column chromatography packed with 12g of silica gel. Elution was done with EtOAc andEtOAc / MeOH mixtures. Fractions collected were monitored with TLC. Those fractions that had similar retention factor (Rf) were combined together. Nine fractions were collected and checked for biological activity.

Fraction 1-4, EtOAc (0.8g), fraction 5-7 (EtOAc / MeOH 4:1, (0.3g)) and (EtOAc / MeOH 1:1, (0.2g)) were obtained. 50mg from fraction seven was applied on Sephadex LH 20 for further purification to yield 4 mg of the most active compound.

Bioassay procedure: Antibacterial activities of the crude EtOH extracts of Berries of *P. dodecandra*, bark of *C. macrostachyus* and flowers of *H. abyssinica* were studied using agar well diffusion and spot on lawn methods *in vitro*. These are two different methods, which are used as a preliminary screening of efficient plant extracts to the target bacteria and fungi. The strain of race 3 *Ralstonia solanacearum* bacteria was isolated from potato in Holeta agricultural research center. This pure isolate of the pathogen was used for *in vitro* bioassay study.

Nutrient agar (NA), which is prepared from mixtures of peptone (5g), beef extract (3g), agar (15g) and distilled water, was used as a growing medium for the bacteria. 0.1g berries of *P. dodecandra* was dissolved in 500µL DMSO. Flowers of *H. abyssinica* and bark of *C. macrostachyus* extracts each 0.1g) were dissolved in 500µL of ethanol separately.

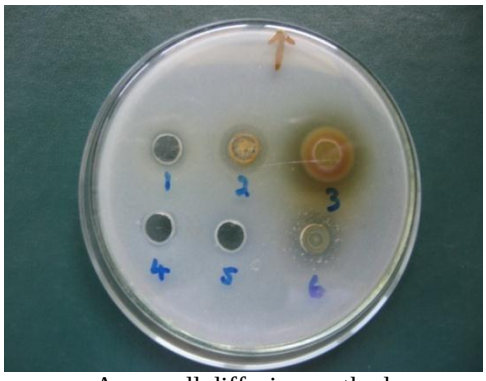
Agar well diffusion method: 2g nutrient agar was added to Erlenmeyer flask containing 100mL of distilled water and mixed well by vortexing. The solution was divided into 5 test tubes (20mL each). All five test tubes were autoclaved at 120^{o}C for 15 minutes and then cooled in water bath to 50^{o}C for 45 minutes. 50µL culture broths of the bacteria were inoculated into the above-prepared sterile nutrient agar medium. It was mixed by vortexing and poured into Petri dish plates. After solidification, Sterilecork borer (8mm diameter) was used to make wells on it and each well was filled with 50µL of each diluted extracts. The solvents (ethanol and DMSO) were used as a vehicle (-ve control). Kocide was used as a standard. After air-dried for certain munities, all Petri dish plates were incubated at 30^{o}C for 24 h. The experiment was performed in duplicate and the average inhibition diameter zone was measured in mm.

Test 2. Spot on lawn method: 2g nutrient agar was added to 100mL of water containing flask and mixed well by vortexing. The solution was divided into 5 test tubes (20mL each). All the five solution containing test tubes were autoclaved at 120^{o}C for 15 minutes and then cooled in water bath to 50^{o}C for 45 minutes. After mixing by vortexing, it was poured to sterile Petri dish plates and allowed to dry. Soft agar was prepared by mixing 0.33g nutrient broth (peptone and beef extract mixture), 0.17g agar and 25mL of distilled water. This was mixed well and added into five test tubes each containing 5mL. 20µL culture broths of the bacteria were inoculated into each of the test tubes containing 5mL soft agar. It was mixed by vortexing and poured into Petri dish plates. 10µL of diluted extracts was applied as a drop. All the test Petri dish plates were incubated at 30^{o}C for 24h. The experiment was performed in duplicate and the average diameter of zone of inhibition was measured in mm.

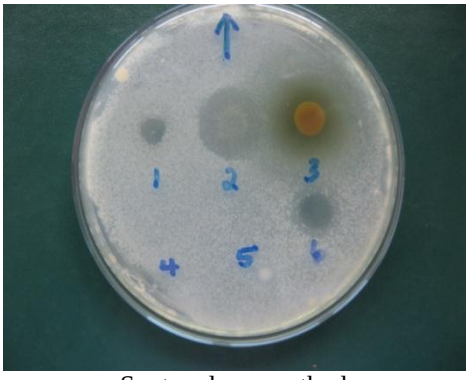
With the same experiment, hexane, diethyl ether, ethyl acetate, ethanol and water extracts of flowers of *Hagenia abyssinica* were tested against the *R. solanacearum* bacteria using agar well diffusion and spot on lawn methods. Ethyl acetate soluble and insoluble parts of crude ethanol extracts were also studied to their biological activities.

Results and discussion

In this work, flowers of *Hagenia abyssinica*, fruits of *Phytolacca dodecandra* and bark of *Croton macrostachyus* were selected based on the fact that these plants are known for their varied biological activities. The plant materials were extracted with ethanol. Each crude extract was tested for their activities targeting *R. solanacearum* bacteria using agar well diffusion and spot on lawn methods**.** The inhibition zones measured for fruit of *P. dodecandra* extract on agar well diffusion and spot on lawn methods were 4 mm and 2 mm in diameter respectively. Bark of *C. macrostachyus* extract showed relatively a better inhibition zone than fruit of *P. dodecandra* extract with 8 mm and 15 mm inhibition zone in agar well and spot on lawn methods respectively. However, flowers of *H. abyssinica* extract showed the highest inhibition zone in both methods (20 mm in diameter). Kocide, which is 70% $Cu(OH)_2$ solution is used as a positive control with 10 mm inhibition zone. Hence, the crude EtOH extract of flowers of *H. abyssinica* was the most active and therefore worthy of further investigation to identify the responsible compound for the activity (Figure-3). This extract was therefore, subjected to subsequent bioassay guided isolation.



Agar well diffusion method



Spot on lawn method

Figure-3: Picture showing growth inhibition zones of extracts of *P. dodecandra* (1), *C. macrostachyus*(2), *H. abyssinica*(3), Ethanol (4) and DMSO (5) for negative control and kocide (6) as a positive control.

The summary of antibacterial activities of extracts are shown in Table-1 using Agar well diffusion method (method 1) and Spot on lawn method 2 (method 2).

Table-1: Inhibition zone results of crude extracts.

95% ethanol extracts of	Average growth inhibition zones	
	Method 1	Method 2
P. dodecandra	4 mm (x)	2 mm (x)
H. abyssinica	20 mm (xxx)	20 mm (xxx)
C. macrostachyus	8 mm (x)	15 mm (xx)
Kocide	10 mm (xx)	10 mm(xx)

X = Below 10 mm inhibition zone, xx = 10 -15 mm inhibition zone and xxx = above 20 mm inhibition zone.

Bioassay- guided extracts of *H. abyssinica:* The hexane, diethyl ether, ethyl acetate, ethanol and water extracts of flowers of *Hagenia abyssinica* were screened for their antibacterial activities using methods 1 and 2. The very polar ethanol and water extracts were found to be active with 24mm and 17mm diameter inhibition zones on method 1and 2 respectively. However, non- polar and less polar extracts exhibited less activity against the growth of the bacteria.

The active ethanol extract was fractionated with ethyl acetate (EtOAc) and both fractions (the EtOAc soluble and insoluble) were screened against the pathogen. The EtOAc soluble part growth inhibition of the bacteria with 25mm while the insoluble part showed 29mm GI in agar well diffusion method. The positive control kocide showed 15mm inhibition zone (Table-2).

Table-2: *In vitro* antibacterial activities of flowers of *H. abyssinica* crude extracts against *R. solanacearum* bacteria using methods 1 and 2.

Extracts of *H. abyssinica*	Average growth inhibition zones	
	Method 1	Method 2
EtOAc insoluble part of ethanol extract	29 mm (xxx)	15 mm (xx)
EtOAc soluble part of ethanol extract	25 mm (xxx)	12 mm (xx)
70% aqueous ethanol extract	18 mm (xxx)	17 mm (xxx)
Water extract	17 mm (xxx)	11 mm (xx)
Kocide	15 mm (xx)	15 mm (xx)

x= minimum, xx= average, xxx= highest inhibition.

Isolation of active compounds from *H. abyssinica* flowers: The 70% aqueous ethanol extracts off lowers of *H. abyssinica* (3g) was allowed to pass with column chromatography. Nine fractions were collected and screened for the antibacterial test. Fraction 7 was found to have the highest inhibition zone (26 mm). Fraction 8 (18mm) and fraction 9 (17mm) were also significant to control the growth of the test bacteria on agar well diffusion method. From the most active fraction, fraction 7(50 mg) was applied on silica gel and Sephadex LH-20 to obtain the most active compound. Elution was done with MeOH:$CHCl_3$ (1:1) solvent system and seven sub-fractions were collected. The TLC profile of these sub-fractions were analyzed with $CHCl_3$/MeOH (4:1) solvent system and a single spot with Rf valve 0.3 was observed on sub-fraction six (Figure-4). It was concentrated and 4 mg of a colorless compound was obtained which was labeled as *H. abyssinica* flower extract fraction 6 (HAF6).

It has recently been reported that the polar extracts of flowers of *H. abyssinica* contain flavonoids[27]. When a drop of $FeCl_3$ was added to the extract a dark green color was developed. The UV spectrum of the extract was generated and when a drop of 1 M NaOH added a red shift occurred. This group tests indicated the presence of flavonoids.

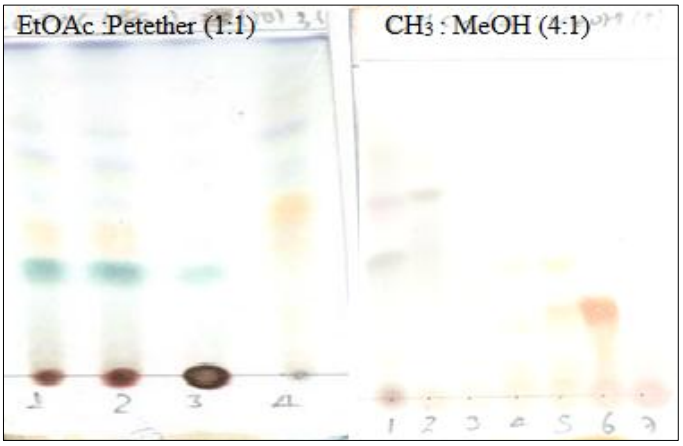


Figure-4: TLC profile of fractions collected from Sephadex LH-20 and the pure compound.

Characterization of HAF6: The compound is an amorphous solid, melts at 230-238^oC. On TLC, it gives red color when sprayed with 1% vanillin in H_2SO_4 reagent. Its UV spectrum gave two absorption maxima at 359 nm and 258 nm, typical UV-Vis spectra of flavonoids[28]. Therefore, HAF6 may probably be a flavonoid or flavonoid derivatives.

The ^1H NMR spectrum of HAF6 suggests the presence of five protons in the aromatic region of the spectrum. The signal appeared as doublet at δ4.7 integrates for one hydrogen is most likely because of a proton attached on anomeric carbon. The signals appearing in the region δ 3.1 - 3.5 are due to protons attached on carbon bearing oxygen. The signals that appear around δ 3.3 which integrate for four protons due to hydrogen attached on oxygen. ^1H NMR δ/ppm (400 MHz, MeOD/ $CDCl_3$): 7.7 (1H, d, H-2', *J*= 2.4 Hz), 7.3 (1H, dd, H-6', *J*= 2.4, 2 Hz), 6.7 (1H, d, H-5', *J*= 8.4 Hz), 6.3 (1H, d, H-8, *J*=2 Hz), 6.2 (1H, d, H-6, *J*=2 Hz), 4.7 (1H, d, *J*= 7.6, H-1'), 3.5 (1 H, t, *J*= 2.8, 4.4 Hz, H-2''), 3.4 (1H, m, H-3''), 3.4 (1H, m, H-4''), 3.3 (1H, m, H-5''), 3.2 (1H, m, H-6'').

The ^{13}C NMR spectrum with the help of Dept-135 showed ten quaternary carbons. Among these, the carbon signal at δ 177 is attributed to carbonyl carbon. The remaining nine quaternary carbons are because of carbon resonating in the aromatic region or olefinic regions. The spectrum also displayed the presence of five carbons resonating in the region of carbon bearing oxygen. In particular, the signal at δ 99.1 is most likely of anomeric carbon. ^{13}C NMR (MeOD: $CDCl_3$ δ in ppm): 177.9 (C-4), 164.4 (C-7), 161.1 (C-5), 158.1 (C-9), 156.9 (C-2), 148.3 (C-4'), 144.1 (C-3'), 134.7 (C-3), 121.5 (C-6'), 121.3 (C-1'), 116.7 (C-5'), 114.6 (C-2'), 105 (C-10), 98.6 (C-6), 99.1 (C-1''), 94 (C-8), 76.4 (C-2''), 76 (C-3''), 73.8 (C-4''), 68.9 (C-5'') and 61.0 (C-6'').

When the ^{1}H and ^{13}C-NMR data of compound HAF6 were correlated with literature values of quercetin-3-*O*-glucoside, there is close similarity between the two compounds. The ^{1}H and ^{13}C NMR correlation was supported by the UV-Vis spectrum. HAF6 absorbed at 359 nm and 258 nm with closer valve quercetin-3-*O*-glucoside, which absorbs (366 and 259 nm). Moreover the melting point of compound HAF6 was 238 ^{0}C which is comparable withquercetin-3-*O*-glucoside which melts at 241 ^{0}C[29]. The appearance of each carbon and hydrogen of compound HAF6 with their corresponding spectrum and the correlation of these spectrum with the literature value of quercetin-3-*O*-glucoside (Figure-5) are indicated in Table-3.

Table-3: ^{1}H NMR and ^{13}C NMR spectral data of compound HAF6 correlation.

No of Carbons	^{13}CNMR	Literature values[31]	^{1}HNMR	Literature values[31]
2	156.9	158.92		
3	134.7	135.59		
4	177.9	179.36		
5	161.1	162.92		
6	98.6	99.80	6.2	6.2
7	164.4	165.91		
8	93.9	94.63	6.3	6.39
9	158.1	158.36		
10	104.9	105.58		
1’	121.3	122.94		
2’	114.6	117.44	7.7	7.70
3’	144.1	145.79		
4’	148.3	149.77		
5’	116.7	115.90	6.8	6.88
6’	121.5	123.08	7.3	7.58
1’’	99.1	104.18	4.7	5.25
2’’	76.4	75.69	3.5	3.47
3’’	76.0	78.03	3.4	3.42
4’’	73.8	71.20	3.4	3.42
5’’	68.9	78.37	3.3	3.34
6’’	61.0	62.53	3.2	3.21

Therefore, based on the UV-Vis, melting point, ^{1}H NMR, ^{13}C NMR and DEPT-135 data and its correlation with the literature value the compound isolated from the biologically active extract of *H. abyssinica* was most likely quercetin-3-O-glucoside.

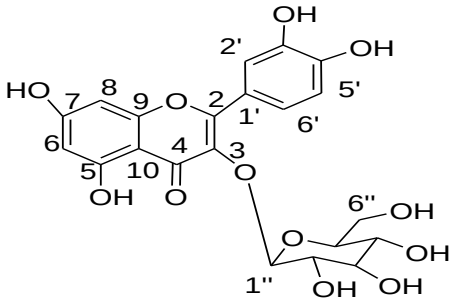


Figure-5: Proposed structure of quercetin-3-*O*-glucoside.

Thomson in 1911 was isolated this and other polar components of flowers of *H. abyssinica* as a remedy for intestinal warms, especially tape warms in humans[27, 30, 31].

Conclusion

This preliminary work indicates that the polar extracts of flowers of *H. abyssinica* exhibits significant activity against the pathogen that causes potato wilt disease. Quercetin-3-*O*-glucoside is isolated as a bioactive ingredient from the active fraction to suppress the growth of *R. solanacearum* bacteria.

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