

Investigating Plant Extract in Inhibition of *Ralstonia solanacearum* Responsible for Potato Wilt

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Received: May 21, 2024

Accepted: July 30, 2024

Online Published: August 15, 2024

doi:10.5539/jas.v16n9p95

URL: <https://doi.org/10.5539/jas.v16n9p95>

Abstract

Controlling the Potato Bacterial wilt disease caused by *Ralstonia solanacearum* remains a significant challenge for Kivu's producers in Democratic Republic of Congo, especially due to limited access to healthy planting material. Therefore, this study aimed to identify effective plant species within the traditional medicine in Kivu whose extracts have the potential to inhibit the bacterium. Eighteen plant extracts were tested using the Mueller Hinton Agar diffusion method. The extract was obtained by grinding 65 grams of dry plant powder macerating in 250 ml of methanol and drying. Then 40 mg of extract was mixed with 10 µl of DiMethyl SulfOxide (DMSO) and 50 µl of sterile distilled water (SDW). The bacterium was isolated from necrotic potato vascular tissue exhibiting wilt symptoms. 500 µl of 1 cm² sterilized tissue ground in 9 ml SDW was inoculated on MacConkey agar and incubated at 28 °C. After 48 hours, biochemical tests and the disease sensitivity were tested by inoculating healthy potato plants. Results showed that *Eucalyptus globulus* emerged as the statistically most effective species, exhibiting a notable inhibition area of 19.33 mm in diameter. This was followed closely by *Capsicum frutescens*, *Manihot glasiiovii*, *Datura stramonium*, *Pteridium aquilinum*, *Galinsoga parviflora*, *Tithonia diversifolia*, and *Cupressus sempervirens*, each showing an inhibition zone of 17.33 mm. From this list, the five most effective extracts revealed an abundance of phenols after phytochemical screening. Based on these results, it would be interesting to evaluate the effectiveness of the extracts in disinfecting garden tools and to assess the role of phenol in inhibiting the bacterial growth.

Keywords: bacterial wilt, *Ralstonia solanacearum*, *Solanum tuberosum*, traditional Medicine, Kivu

1. Introduction

Potato Bacterial wilt, caused by *Ralstonia solanacearum*, is one of the major worldwide challenges, leading to significant yield losses in potatoes (Dey & Sen, 2023). The Potato bacterial wilt disease is characterized by the wilting of young green leaves, followed by partial wilting in the infection zone, and wet necrotic lesions on aerial stems, ultimately resulting in widespread plant wilting. Upon cross-section, a notable brown vascular discoloration can be observed in the vascular zone (Abdrabouh et al., 2019; Kamalakannan et al., 2020). This disease is challenging to control due to the vast diversity of its pathogens, its wide host range, its ability to survive in various environments, the latent infection of host plants, and its long lifespan in soil (Kumari et al., 2021). *R. solanacearum*, is a widespread pathogen that affects over 450 host plants from 54 angiosperms families (Genin, 2010).

R. solanacearum, pathogen of potato (*Solanum tuberosum*) (Hassan et al., 2017) is a rod-shaped, Gram-negative bacteria and tests positive for catalase and oxidase (Razia et al., 2021). On MacConkey medium, the bacteria form white colonies with a pink-violet hue at their center (Balamurugan et al., 2020). This bacteria does not produce spores, exhibiting polar flagella when present and requires strictly aerobic conditions to grow and thrives at 24-30 °C (Ravelomanantsoa, 2016; CABI, 2016). The bacteria reduce nitrate to nitrite, are glucose-positive, do not produce H₂S, no-hydrolysis starch and test positive for Simon's citrate and KOH tests (Sharma & Singh, 2019). *In vitro* Chloramphenicol and Streptocycline inhibit the bacteria (Kumari et al., 2021) and in the field, the combination of Oxyrich and Kocide is efficient for inhibition (Ghaffar et al., 2022).

Therefore, problems associated with the use of synthetic products remain an environmental concern posing notable risks (Yarou et al., 2017). Biopesticides, as alternative solutions, have emerged as sustainable options in this context. They not only decompose rapidly, minimizing ecological impacts but also exhibit a reduced effect on non-target beneficial organisms compared to synthetic pesticides (Mkenda et al., 2015; De Clerck et al., 2020).

Various studies in the Democratic Republic of Congo have highlighted the potential of several plants including *Azadirachta indica* A. Juss., *Allium capsicum* L., *Zingiber officinalis* Roscoe, *Allium sativum* L., *Tephrosia vogelii* Hook. f., *Tetradenia riparia* (Hochst.) Codd, *Carica papaya* L., *Tithonia diversifolia* (Hemsl.) A. Gray, *Eucalyptus citriodora* L'Hér., *Cupressus lusitanica* Mill. for their effectiveness against phytopathogenic microorganisms and human pathogens (Shalukoma et al., 2015; Bashige et al., 2020; Kaboyi et al., 2021; Korangi et al., 2021; Syamasamba et al., 2022; Masengo et al., 2023).

The *in vitro* growth inhibition test of *R. solanacearum* has revealed the effectiveness of several plants in combating this bacterium such as *Nicotiana tabacum*, *Tagetes minuta*, and *Allium sativum* that demonstrated superior efficacy, producing inhibition zones greater than 20 mm of diameter (Mutimawurugo, 2020). This is a promising way for managing bacterial wilt.

However, very little research has been done in the Kivu region on alternative control of bacterial wilt, which continues to constraint potato cultivation, particularly concerning access to healthy planting material and to control disease spread in field by garden tools. This study aims to bridge the existing research gap by identifying plant species capable to inhibit *R. solanacearum*'s growth *in vitro*, specifically targeting the bacterial wilt of potatoes. The study seeks to contribute to the development of sustainable, locally-sourced solutions for managing this pervasive agricultural challenge by focusing on species recommended by traditional practitioners in the Kivu region.

2. Material and Methods

2.1 Area Descriptions

The study was carried out in the laboratory of microbiology at the Université Libre des Pays des Grands Lacs (1°39'33"S and 29°11'00"E). The medicinal plants were collected in the Mugunga area of Goma city, North Kivu coordinates 1°40'57.2"S and 29°13'54.5"E. The soils from the plant harvest site in North Kivu are classified as andosols. Properties of these soils in 0 and 15 cm layer are as follows: pH of 6.8 to 7.15; organic matter content: 4.5 to 5.2%; Nitrogen rate content of 0.2 to 0.32%; and a sandy-loamy texture (Mwanjalolo Jackson-Gilbert et al., 2015). The potatoes plants affected by wilting were harvested from a farmer's field in the North of Goma city (North Kivu, DRC) at coordinates 1°36'52"S and 29°15'51"E.

2.2 Potato and Medicinal Plant Species Sources

The healthy potato material (*Solanum tuberosum*) was sourced from the SOBETRA variety, and purchased from the Luhutu center for adaptation of improved seeds. The diseased potato material was identified by the wilting leaves and a white exudate drains from vascular tissue 3 minutes after sectioning the plant. This exudate seeped out of the potato stem in the form of threads, when placed in a beaker of water (Dey et al., 2023).

Plant extracts were sourced from 18 medicinal plant species reported from Ethnobotanical studies (Balagizi, 2021; Korangi et al., 2021). These were the following species: *Ageratum conyzoides* AC (Asteraceae), *Capsicum annum* CA (Solanaceae), *C. frutescens* CF (Solanaceae), *Cupressus sempervirens* CS (Cupressaceae), *Datura stramonium* DS (Solanaceae), *Eucalyptus globulus* EG (Myrtaceae), *E. cinerea* EC (Myrtaceae), *Euphorbia hirta* EHi (Euphorbiaceae), *Heliantus annum* HA (Asteraceae), *Galinsoga parviflora* GP (Asteraceae), *Jatropha curcas* JC (Euphorbiaceae), *Lycopersicon esculentum* LE (Solanaceae), *Manihot glaziovii* MG (Euphorbiaceae), *Psidium goyava* PG (Myrtaceae), *Pteridium aquilinum* PA (Dennstaedtiaceae/Fern), *Ricinus communis* RC (Euphorbiaceae), *Tithonia diversifolia* TD (Asteraceae), and *Zealandia pustulata* ZP (Polypodiaceae/Fern). These species were identified in the Lwiro Research Center.

2.3 Inoculums

To isolate *R. solanacearum*, a 1 cm² section of vascular tissue from an infected plant was used following the principle that diseased vascular tissues contain pure strains of the bacterium (Denny, 2006). 500 µl of sterilized and crushed tissue solution in sterile distilled water (SDW) was inoculated on MacConkey Agar and incubated at 28 °C for 48 hours (Nash & Krenz, 1991; Bridson, 1998; Safini et al., 2014).

The pure colonies of *R. solanacearum* obtained, characterized by white appearance with a pink-violet in center (Balamurugan et al., 2020; Sahu et al., 2020) were further purified on MacConkey Agar. To confirm the bacterial identity nine biochemical tests were conducted, including oxidase, catalase, Gram staining, use of carbohydrates (Glucose and Lactose), urea production, motility, and citrate test (Simmons, 1926; Kovacs, 1956; He et al., 1983; Hayward, 1995; Lelliot & Stead, 1987; Safini et al., 2014). The isolates of *R. solanacearum* were characterized using biochemical tests in accordance with previous studies (Rohiniet al., 2017; Balamurugan et al., 2020).

The final stage in identifying the isolated strain involved inoculating 1 ml of a solution of pure colonies of the strain, which consisted diluted in sterile distilled water to 10⁵ colony-forming units (CFU). This inoculation was carried out in the vascular tissues of healthy 21-day-old potato plants (Vragas, 2023). To determine the concentration of 10⁵ CFU of pure *R. solanacearum* strains, the method of serial dilutions was performed. The colonies were diluted in distilled water at concentrations ranging from 10⁻¹ to 10⁻¹⁰. Then, 100 µl of the solution (distilled water-colonies) at concentrations ranging from 10⁻³ to 10⁻⁷ were inoculated onto 90 mm Petri dishes containing 25 ml of MacConkey Agar medium. The number of colonies observed on the plates allowed us to calculate the colony-forming unit contained in one milliliter of the solution (Jett et al., 1997).

Disease severity was assessed by counting the number of withered leaves, and constantly monitoring the results recorded from the 7th day after inoculation, and every 3 days for 45 days. The severity of wilting was recorded, for potato, at intervals of each 3 days (He et al., 1983; Horita & Tsuchiya, 2001) after inoculation on the following scale: 1 = no symptoms, 2 = the inoculated leaflet wilted, 3 = 5 wilted leaflets, 4 = less than 10 wilted leaflets and 5 = dead plant (Abdrabouh et al., 2019).

2.4 Production of Plant Extracts

The various plant parts were collected and dried at room temperature in the shade. The dried plants were pulverized. From dry powder, 65 grams of each plant material were weighed into glass containers and diluted in 250 milliliters of the extracting solvents (methanol) and labeled. The samples were shaken constantly and filtered using Whatmann No 1 filter paper after 24h. The container of filtrate was put in a Water bath until a thick pomace remained. The thickened extract was stored in sterile plastic bottles of 50 ml volume in the refrigerator at 4 °C.

2.5 Evaluation of Plant Extracts for Inhibition of *R. solanacearum*

The experiment was conducted on Mueller Hinton Agar (MHA) using Whatman filter paper discs. These discs were impregnated with a solution composed of the plant extract, Dimethyl Sulfoxide (DMSO), and sterile distilled water in the proportions of 20 mg, 5 µl, and 25 µl, respectively, and left to soak for one hour. The MHA medium was then inoculated with 1 ml of *R. solanacearum* at a concentration of 10⁵ CFU/ml, before the complete cooling of the sterile Petri dishes in a laminar flow environment. The extract-soaked discs were then placed onto the MHA. Following this, the diameter of bacterial inhibition was measured using a ruler on the MHA plates.

2.6 Screening for the Presence of Phytochemical Groups

Phytochemical screening was conducted on the extracts obtained from the top 5 plant species identified as most effective in bacterial inhibition. This screening aimed to detect and quantify of various molecular groups including alkaloids, flavonoids, terpenoids, saponins, and tannins, essential for understanding their antibacterial properties.

To identify Alkaloids, 20 mg of the sample was dissolved in 2% HCl and filtered with a glass funnel. One milliliter of Wagner's reagent (1.27 g of iodine + 2 grams of potassium iodide in 100 ml of distilled water) was added to 2 ml of the filtrate. The redish or brown solution was an indication for the presence of alkaloids (Harbone, 1983; Okeniyi et al., 2013; Anitha & Sudarsanam, 2013; Yemata et al., 2019).

For flavonoids, 0.5 g of extract in 10 ml of distilled water was added to the test tube and mixed by shaking and filtered. Then 3 ml of the aqueous filtrate was mixed with 5 ml of ammonia (10%) added with 1 ml of concentrated sulphuric acid, yellow coloration denoted a positive result (Harbone, 1983; Patle et al., 2020).

Phenolic compounds were detected by mixing 2 ml of the plant extract with 3-4 drops of 5% Ferric Chloride (FeCl_3) solution with black color indicating the presence (Harbone, 1983; Yemata et al., 2019; Patle et al., 2020).

Terpenoids were tested by mixing 0.5 gram of plant powder with 10 ml of methanol and centrifuged. 5 ml of the supernatant was mixed with 2 ml of chloroform added with 3 ml of sulphuric acid. A brown layer between the two solutions indicated their presence (Harbone, 1983; Sharma et al., 2020).

Saponins were identified by boiling 1 gram of plant extract in 5 ml of distilled water, and then shaken vigorously for five minutes. The persistence of the frothing confirmed the presence of saponin (Harbone, 1983; Muharrami et al., 2020).

For Tannins screening, 0.2 grams was dissolved in 5 ml of distilled water, heated in a water bath and filtered. Then 2 ml of 5% ferric chloride solution was added to 1ml of the filtrate. The blue color meant positive test (Harbone, 1983; Muharrami et al., 2020).

2.7 Experimental Design and Data Analysis

The experiment was designed to assess the antibacterial activity of various plant extracts against *R. solanacearum*. The experiment for Inhibition Zone for Plant Extracts was carried out in a completely randomized experimental design, comprising 22 treatments in total. These included 18 different plant extracts, three positive controls (tetracycline, penicillin, and doxycycline), and one negative control (a mixture of distilled water and DMSO). Each treatment was replicated thrice (Opande et al., 2022). The antibacterial activity was recorded in mm of clear zones of inhibition after 48 h. The resulting data were analyzed for variance (ANOVA) using Sisvar 5.6 software, and mean differences were determined using the Tukey's test at a significance level of 0.05 (Viana et al., 2019).

3. Results and Discussions

3.1 Yield of Plant Extracts

Figure 1 shows that the extract yield obtained from *Ageratum conyzoides* leaves was higher than that of other species, followed by *Eucalyptus globulus* and *Eucalyptus cinerea*. However, *Cupressus sempervirens* and *Zealandia pustulata* expressed the lowest values in extracts, which could be attributed to their belonging to less evolved taxa, resulting in the possession of different phytochemical groups that require different extraction methodologies (Cao et al., 2017).

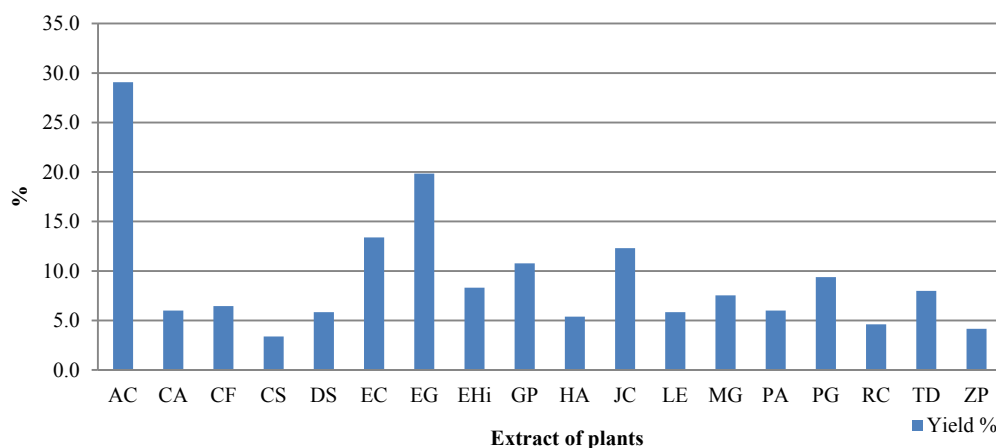


Figure 1. Yield of plant extract

Note. *A. conyzoides*: AC, *C. annum*: CA, *C. frutescens*: CF, *C. sempervirens*: CS, *D. stramonium*: DS, *E. globulus*: EG, *E. cinerea*: EC, *E. hirta*: EHi, *H. annum*: EA, *G. parviflora*: GP, *J. curcas*: JC, *L. esculentum*: LE, *M. glaziovii*: MG, *P. goyava*: PG, *P. aquilinum*: PA, *R. communis*: RC, *T. diversifolia*: TD, and *Z. pustulata*: ZP.

Previous studies have shown that extraction process and age of the leaf significantly influence the plant extract yield, with young leaves producing more extract than mature ones thus, the extraction process and the age of the leaf significantly influenced the plant extract yield (Chen et al., 2018). The *Ageratum conyzoides* leaves used in this study were collected from a cultivated field after weeding, which gave an advantage in terms of the age of

the plant. Methanolic extractions are known to have considerable yields and optimal composition among phytochemical groups.

Solvent recycling systems (Soxhlet) produce higher extract yields (45-55%) than those carried out without using this system ($\leq 21\%$) (Valle et al., 2019). However, in this trial, the extract yields were obtained under standardized harvest conditions without solvent recycling. These results could have different outcomes if the age of the plant was standardized at harvest and solvent recycling was used.

3.2 Morphological and Biochemical Characteristics of *R. solanacearum*

The characteristics of the identified bacterium include morphological and biochemical characteristics of *R. solanacearum* are presented in Table 1 and Figure 2, and indicate that, the bacterium is Rod (Bacilli), Gram-negative, motile, with the presence of oxidase and catalase enzymes. It degrades glucose and lactose, is non-H₂S producing, produces urea, and utilizes Simon's citrate as a carbon source. These results align with the findings of previous studies (CABI, 2016; Ravelomanantsoa 2016; Sharma & Singh 2019; Balamurugan et al., 2020; Razia et al., 2021), who reached the same conclusion after conducting biochemical tests.



Figure 2. Gram stain results

Table 1. Results of Biochemical Tests on *R. solanacearum*

Tests	Gram	Oxidase	Catalase	Glucose	Lactose	H ₂ S	Mobility	Citrate	Urea	Form
Results	-	+	+	+	+	-	+	+	+	Rod (Bacilli)

3.3 Disease Severity of Potato Bacterial Wilt After Inoculation in Healthy Plant of Potato

From results illustrated in Figure 3, symptoms first appeared on the 8th day on the SOBETRA variety which completely succumbed 42 days after the appearance of the initial symptoms. The 5 scales of expression of disease severity were observed differently on healthy inoculated plants (Abdrabouh et al., 2019). As described in Figure 3, the appearance of symptoms corresponding to scale 1 occurred on day 8 after inoculation, the second scale was only observed from day 8 to day 10, while scale 3 spanned from day 10 to approximately day 13-14. Scale 4, the longest phase, extended over more than 25 days. The final scale started on the 38th day after inoculation. Thus, it is obvious that the bacteria used for this study is *R. solanacearum*, pathogen of potatoes, causing bacterial wilt, also known as brown rot.

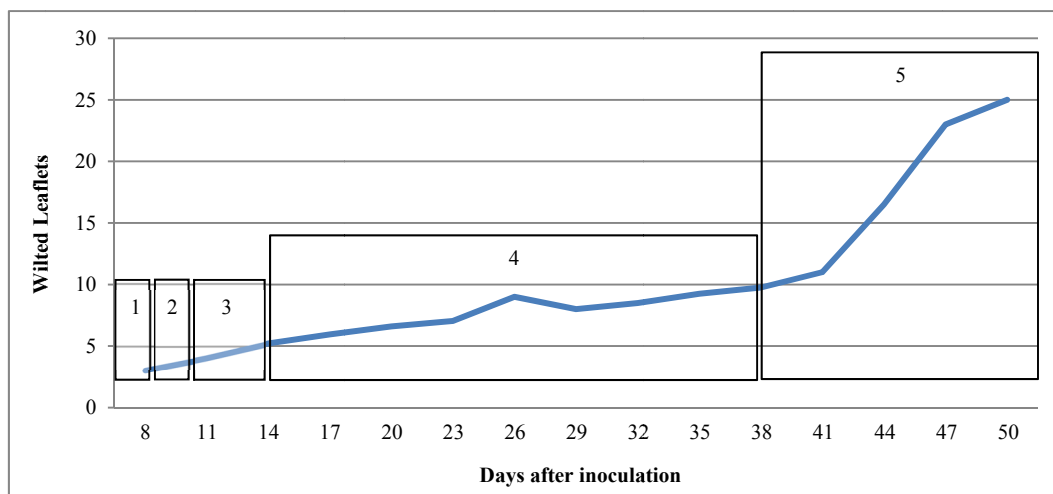


Figure 3. Daily Evolution of Potato bacterial wilt severity on inoculated plant

Note. The numbers 1 to 5 are indicating the scales of disease severity wilt on potatoes plants.

Under hydroponic conditions, wilting symptoms in the infected plants started to appear 4 days post inoculation (dpi). By 5 dpi, almost all plants exhibited severe wilting symptoms. This suggest that increased evapotranspiration by opening the growth container's lids could accelerate plant wilting. This finding further suggests that *in vitro* infection could be used to test the virulence of *R. solanacearum* in potato (Wang et al., 2019).

When various African Great Lake Region varieties were tested significant differences were observed in both days to symptom expression (ranging from 7 to 20 dpi) and the days to complete wilting (From 28 to 40 dpi). The Kirundo variety exhibited the quickest onset of disease symptoms followed by Gikungu, whereas Cruza and Sangema were the slowest to display disease symptoms (Uwamahoro et al., 2020).

3.4 Inhibition Test by Plant Extract

The results of the inhibition test are presented in Table 2.

Table 2. Inhibition zone for plant extracts

Treatments	Inhibition zone (mm)	
Negative control	7.00	a1
EHi (<i>E. hirta</i>)	7.00	a1
CA (<i>C. annum</i>)	9.00	a1 a2
PG (<i>P. goyava</i>)	9.33	a1 a2 a3
JC (<i>J. curcas</i>)	10.00	a1 a2 a3 a4
AC (<i>A. conyzoides</i>)	11.33	a2 a3 a4 a5
EC (<i>E. cinerea</i>)	12.33	a2 a3 a4 a5 a6
HA (<i>H. annum</i>)	13.00	a3 a4 a5 a6
LE (<i>L. esculentum</i>)	13.00	a3 a4 a5 a6
D Doxycycline	13.00	a3 a4 a5 a6
P Penicillin	13.67	a4 a5 a6 a7
T Tetracycline	13.67	a4 a5 a6 a7
RC (<i>R. communis</i>)	14.00	a5 a6 a7
ZP (<i>Z. pustulata</i>)	15.33	a6 a7
TD (<i>T. diversifolia</i>)	15.67	a6 a7 a8
CS (<i>C. sempervirens</i>)	15.67	a6 a7 a8
GP (<i>G. parviflora</i>)	17.33	a7 a8
PA (<i>P. aquilinum</i>)	17.33	a7 a8
DS (<i>D. stramonium</i>)	17.33	a7 a8
MG (<i>M. glaziovii</i>)	17.33	a7 a8
CF (<i>C. frutescens</i>)	17.33	a7 a8
EG (<i>E. globulus</i>)	19.33	a8

Note. The means followed by the letter 'a' with the same index are not different according to the Tukey's test at 0.05.

Based on these results, the solvent (DMSO) was suitable and had no effect on the normal growth of microbial strains in negative control. According to the Tukey's test at 5% precision (Table 2), *Eucalyptus globulus* was found to be the most effective species in inhibiting *R. solanacearum*, followed by *C. frutescens*, *M. glaziovii*, *D. stramonium*, *P. aquilinum*, *G. parviflora*, *T. diversifolia*, and *C. sempervirens* in the second statistical classification. It is worth noting that *E. globulus* also proved to be effective to inhibit the same bacterium in a similar research conducted in Iran (Nezhad et al., 2012). Interestingly results observed an inhibition zone of *R. solanacearum* of 2.01 cm², corresponding to a diameter of 16 mm, in their *in vitro* tests on Mueller Hinton Agar (Hassan et al., 2009).

The wide spectrum of biological activities of *E. globulus* are mainly attributed to the diversity of phytochemical constituents in the plant parts. Eucalyptus leaf decoctions are traditionally used for various medicinal purposes, including treating the mouth infections, rinsing wounds, applying compresses for trauma, and suitable for inhalations in upper respiratory tract diseases (bronchitis). The most important phenolic compounds that largely determine the antiradical and reductive activity of eucalyptus leaves are phenolic acids and, quercetin glycosides (Balčiūnaitienė et al., 2022). Various mechanisms have been suggested to explain the mode of action of plant extract of *E. globulus*. Generally, these include damaging the bacterial cell membrane, inhibiting efflux pumps, and inhibiting DNA and protein biosynthesis.

3.4 Phytochemical Screening

The phytochemical screening results are presented in Table 3.

Table 3. The results of Phytochemical Screening of 5 efficiencies plant in inhibition of *R. solanacearum*

Extract	Alkaloids	Terpenes	Phenols	Flavonoids	Saponin	Tannins
PA	+	-	+++	+	+	+
DS	+++	+	+	+	+	+
MG	+	+	+++	+++	+	+++
CF	+++	+	+++	-	+	-
EG	-	+	+++	+	+	+

Note. +++ = abundant; + = moderate; - = absent.

C. frutescens: CF, *D. stramonium*: DS, *E. globulus*: EG, *M. glaziovii*: MG, *P. aquilinum*: PA.

The results in Table 3 reveal that the top 5 species that are most effective against the bacterium responsible of potato bacterial wilt have a confirmed abundance of phenol. Phenol is a polar compound that acts as an antibacterial agent. Its mechanism of action involves denaturing proteins in bacterial cells, leading to the cessation of all metabolic activities catalyzed by proteins (Juariah et al., 2023). Other similar results were finding for phytochemical screening of *P. aquilinum* (Adou et al., 2016), for *M. glaziovii* (Nduche et al., 2018; Aguirre et al., 2021), for *C. frutescens* (Gurnani et al., 2016), and for *D. stramonium* (Sreenivasa et al., 2012; Amabye et al., 2016).

4. Conclusion

The primary objective of this study was to identify plant species capable of effectively inhibiting *R. solanacearum*, a significant pathogen impacting potato production. Among the eighteen plants species evaluated, *E. globulus* demonstrated superior efficacy, as evidenced by its statistically significant bacteria inhibition. While these findings are promising, it is necessary to extend this research beyond the laboratory. Conducting greenhouse trials is the next crucial step to verify the effectiveness of *E. globulus* in a more controlled, yet realistic agriculture setting. This could pave the way for proposing a method of use for the farmers of Kivu who are struggling with the management of potato wilt.

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Acknowledgments

We greatly appreciate the valuable contributions of our community advisory committee members. We would also like to thank every team member who took the time to participate in this study.

Authors Contributions

Prof Gakuru Semacumu Jean Baptiste was responsible for study design and data collection. Prof. Angele P. Ibanda drafted the manuscript and Prof. Monde Godefroid revised it. Prof Dedh'a Benoit facilitated the collection of medicinal plants. All authors read and approved the final manuscript.

Funding

There is no financier for this work.

Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Informed Consent

Obtained.

Ethics Approval

The Publication Ethics Committee of the Canadian Center of Science and Education. The journal's policies adhere to the Core Practices established by the Committee on Publication Ethics (COPE).

Provenance and Peer Review

Not commissioned; externally double-blind peer-reviewed.

Data Availability Statement

The data supporting this study's findings are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Data Sharing Statement

No additional data are available.

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