Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.7831

ISSN: 2582 – 2845

Ind. J. Pure App. Biosci. (2019) *7*(5), 287-296

Research Article

Antibacterial Activity of *Gnidia glauca* (Fresen) Gilg. Phytochemical Extracts against Rice Bacterial Blight Pathogen *Xanthomonas oryzae pv. oryzae*

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Received: 8.09.2019 | Revised: 13.10.2019 | Accepted: 22.10.2019

ABSTRACT

The present study was carried out to extract and screen the phytochemicals present in the aerial parts of Gnidia glauca (Fresen) Gilg. for their antibacterial activity against bacterial blight pathogen Xanthomonas oryzae pv. oryzae. Gram-negative bacterial species. Aerial parts of G. glauca were extracted for phytochemicals into water and organic solvents of different polarity such as petroleum ether, chloroform, ethyl acetate and methanol. The phytochemical screening of G. glauca extracts showed the presence of alkaloids, flavonoids, couma rins, terpenes, lignans, glycosides, saponins and steroids. Further the phytochemical extracts were evaluated for the antibacterial activity against Xanthomonas oryzae pv. oryzae by agar disk diffusion method. Bioactive extracts of leaf of G. glauca were found to be significantly effective against the X. oryzae at concentration of 10 mgmL^{-1}. Among these extracts leaf extracts were found to be most effective and showed bactericidal activities against X. oryzae with MIC ranged from 0.5 to 1 mgmL^{-1}. These solvent extracts of G. glauca which proved to be potentially effective can be used as natural alternative bioactive agents to control/manage bacterial blight disease caused by Xanthomonas bacteria not only in rice/paddy but also in other crop plants in an eco-friendly and economically feasible manner.

Keywords: *Gnidia glauca, Phytochemicals, Bacterial blight, Antibacterial activity*

INTRODUCTION

Plants produce a vast and diverse assortment of natural phytochemicals. Different plant parts such as, leaves, bark, seeds, flowers, roots, etc. are considered as reservoirs of naturally occurring phytochemicals and of structurally diverse bioactive molecules. The people residing in the rural areas are accustomed to the use of such readily available herbal medicines mainly at their raw, pure, fresh as well as crude forms. These medicines are developed through the gathered experience of people for generations (Pattanayak et al., 2012).

Cite this article: Sannabommaji, T., Kumar, V., Poornima, D.V., Basappa, G., Gajula, H., & Rajashekar, J. (2019). Antibacterial Activity of *Gnidia glauca* (Fresen) Gilg. Phytochemical Extracts against Rice Bacterial Blight Pathogen *Xanthomonas oryzae pv. oryzae*, *Ind. J. Pure App. Biosci. 7*(5), 287-296. doi: http://dx.doi.org/10.18782/2320-7051.7831

Gnidia glauca (Fresen) Gilg (Thymelaeaceae), commonly known as Mukuthi, Rame, Mukkudaka, and has been known to be toxic plant. It is a very little known plant shrub available the southern part of the Western Ghats. *G. glauca* was used in traditional medicine for cancer, sore throat, wounds, burns and snakebites. Leaves are used to treat contusions, swelling, back ache and joint aches (Kareru et al., 2005; Amarajeewa et al., 2007). *G. glauca* is also used for agrochemical applications as insecticide, pesticide and even larvicidal agent (Borris & Cordell, 1984; Franke et al., 2002; Javaregowd & Naik, 2007). It was used to be a traditional practice used in rural Karnataka of Malnad region farmers, that whole plant was decayed in cow urine then extract was sprayed to bacterial blight infected paddy crop to control bacterial blight (BB) disease caused by Gram-negative bacteria *Xanthomonas oryzae* pv. *oryzae*, that affect at different stages of crop. Bacterial blight is one of the most important and serious diseases of rice in Asia (Hasan Naqvi et al., 2014). Hence, disease damage is one of the most serious limiting factors for rice production. Rice feeds more than 50% of the world's population and constitutes more than 90% of the daily calorie intake. Rice is a worldwide staple as well as a model for cereal biology (Mew et al., 1993; Ronald & Leung, 2002; Bennetzen & Ma, 2003). The effect of synthetic chemicals can cause environmental pollution and may induce pathogen resistance. Biocontrol of bacterial blight disease plays a vital role in integrated rice pest management and eco-friendly also cost-effective.

In the present study an attempt has been made to give scientific validation for the traditional practice in order to exploit this practice and explore the potential of *G. glauca* as a bactericidal agent for control/manage the bacterial blight disease of paddy. The aim of the present study was to investigate the phytochemical screening of leaf extracts of *G. glauca*. and to evaluate the antibacterial activity of leaf extracts against bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae*. Subsequently, to know the efficacy of the extracts by determining the minimum inhibitory concentrations (MIC).

MATERIALS AND METHODS

Collection of plant material:

Gnidia glauca (Fresen) Gilg. fresh and healthy aerial parts (leaves, bark, and flowers) were collected during the day time at Kundadri hill basin of Western Ghats of Karnataka (13^o 33'27"N 75^o10'13"E/13.55750^{o}N 75.17028^{o}E), India. *G. Glauca* was authenticated by Kumarswamy Udupa E.S., Department of Botany, Sri Jagadguru Chandrashekhara Bharathi Memorial College, Sringeri, Chikkamagalore District, Karnataka, India. The fresh material washed and completely shade dried in dust free then ground to coarse powder.

Preparation of Phytoextract and removal of chlorophyll pigments:

Each dried aerial parts *viz.*, leaves, bark, and flowers of *G. glauca* fine powder (30 g) was continuously extracted with organic solvents (gradually increase the polarity to most polar) *viz.*, petroleumether, chloroform, ethyl acetate and methanol by using Soxhlet apparatus (each for 6 h). Extracts were filtered, and filtrate was concentrated under rotary evaporator (Medica, India) for recovering the solvents at 40°C. The extracts were successively separated from the chlorophyll and tissue debris that removed into the diethyl ether by solvent partition method using separatory funnel, made completely free of green pigments. Thus obtained extracts were stored at 4°C until further experimentation analysis (Harborne, 1998; Houghton & Raman, 2012).

Qualitative screening for phytochemicals:

The extracts were qualitatively tested and screened for the presence of various phytochemical constituents using standard procedures to identify the constituents as described by Harborne (1998) and Sonam Pandey (2015)**.**

Tests for Alkaloids

Dragendroff's Test: Few drops of Dragendroff's reagent (0.85 g of basic Bismuth nitrate dissolved into 40 mL of distilled water and 10 mL of glacial acetic acid

was added) was added to the 2 mL of each extract sample . The appearance of a reddish brown precipitate indicated the presence of Alkaloids.

Hager's test: Few mL of each extract was mixed with a few drops of Hager's reagent (saturated aqueous solution of picric acid). Yellow precipitate indicated the presence of alkaloids.

**Wagner`s test:** Few drops of Wagner`s reagent (2 g of potassium iodide in 5 mL of distilled water containing 1.27 g of iodine in a total volume of 1000 mL with distilled water) was added to 2 mL of each extracted sample. Appearance of dark brown precipitate indicated the presence of alkaloids.

Tests for flavonoids

Lead acetate Test: To the 2 to 3 mL of each extract was mixed with few drops of lead acetate (10% w/v in distilled water) solution. Appearance of creamy or pale yellow precipitate indicated the presence of flavonoids.

Sodium hydroxide test: To the 2 mL of each extract 2 mL of 10% sodium hydroxide solution was added and mixed. *An* intense/golden yellow precipitate indicated the presence of flavonoids.

Tests for Phenolics

Potassium dichromate test: To 2 to 3 mL of each extract few drops of potassium dichromate solution was added and mixed well. Formation of a precipitate indicated the presence of phenolics.

Test for tannins

Ferric chloride test: 2 to 3 mL of each extract was mixed with a few drops of 10% ferric chloride ($FeCl_3$) solution. The appearance of deep brownish or bluish-black color indicated the presence of tannins.

Sulphuric acid test: 2 mL of each extract was mixed with few drops of concentrated sulphuric acid and few drops of 5% hydrochloric acid and mixed well. Formation of green solution indicated the presence of tannins.

Anthracene test: To the 2 mL of each extract few drops of 5% aqueous hydrochloric acid, filtered, and then few drops of chloroform and few drops of 10% ammonia were added to the filtrate and mixed well. The appearance of aqueous solution was colorless indicated the presence of tannins.

Test for steroids and triterpenoids:

Salkowski`s test: To the 2 mL of each extract 1 mL of ethyl acetate was added, mixed into 2 mL of chloroform and few drops of concentrated sulfuric acid was carefully added alongside to form a layer. Formation of a reddish-brown coloration at the interface confirmed the presence of triterpenoids. The upper layer turned green which indicated the presence of steroids.

Liebermann-Burchard reaction: 3 mL of each extract was mixed with 3 mL of acetic anhydride, heated in a boiling water bath and cooled at room temperature. Then few drops of concentrated sulphuric acid carefully added alongside the test tube. The appearance of a blue-green color indicated the presence of triterpenes and steroids.

Test for glycosides:

Keller Killian`s test: To the 2 mL of each extract few drops of glacial acetic acid containing few drops of 10% ferric chloride solution was mixed and then few drops of concentrated sulphuric acid was carefully added alongside the test tube. Formation of a brown, violet or greenish ring at the interface indicated the presence of glycosides.

Test for Saponins:

Foam test: In a test tube 2 mL of each extract was added 20 mL of distilled water, test tube shaken vigorously and kept for 3 min. Formation of honeycomb-like froth indicated the presence of saponins.

Thin layer chromatography (TLC)

Solvent extracts of *G. glauca* plant parts phytochemicals were separated on Silica gel G using n-butanol: glacial acetic acid:water (80:20:100, v/v/v) as mobile phase. After development, the plates were dried and sprayed with 3% RBC and vanillin sulphuric acid (5 g vanillin + 475 mL ethanol + 25 mL sulphuric acid), and heated for 3 min at 110°C, which produced colored areas in the regions indicating the presence of phytochemicals. Based on these spots

identified the tentative phytochemicals (Harborne, 1998).

Antibacterial activity

Isolation of *Xanthomonas oryzae* pv. *oryzae*

Typical bacterial blight infected rice/paddy plant leaves were collected from the field, washed under running tap water to remove dust and followed by 3-4 rinses with sterile distilled water. The leaves were cut into small pieces, surface sterilized with 1% sodium hypochlorite solution, followed by 4-5 times rinses with sterile distilled water. The infected rice leaf pieces placed onto Wakimoto's (WF-P) agar plate (containing 0.5 g of calcium nitrate, 1.82 g of disodium hydrogen phosphate, 20 g of sucrose, 5 g of peptone, 0.05 g of ferrous sulphate and 18 g of agar were added to one liter of distilled water, pH adjusted to between 6.8-7.2, autoclaved for 15 min at 121ºC and cooled) and incubated at 27 ºC for 24 h. The single yellow, round and smooth margin, non-flat, mucous colonies of *Xanthomonas oryzae* pv. *oryzae* were picked and transferred into slant WF-P medium tubes for obtaining the pure culture (Karganilla et al., 2006). Identification of the bacterial isolate was carried out following the standard biochemical tests as suggested by Shankara et al., (2017) and confirmed as *X. oryzae*. Thus obtained bacterial pure culture was preserved in tightly capped tubes as glycerol stock for further experimentation.

Disc diffusion assay

The disc diffusion method was used to determine antibacterial activity of the each crude phytochemical extract obtained from individual parts of *G. glauca* with some modifications as required (Sonam Pandey et al., 2015). The 24 h fresh culture suspension (10^5 $CFUmL^{-1}$) of *X. oryzae* strain was swab inoculated on the WF-P agar Petri plates, and sterile 5 mm diameter filter paper discs loaded with *G. glauca* each plant part extract at different concentrations (5, 10 and 15 $mgmL^{-1}$ in 5% DMSO) were placed on the WF-P agar plates. Sterile filter paper discs loaded with 5 $mgmL^{-1}$ of tetracycline were used as positive control. The plates were kept in incubation chamber at 28º C for 24 h. The zone of growth inhibition around each of phyto-extract impregnated discs measured and positive control were recorded in millimeter (mm) after incubation. A clear circular zone around the discs considered as indication for antibacterial activity.

Minimum inhibitory concentration (MIC)

The most effective *G. glauca* plant parts crude extracts which exhibiting a strong antibacterial activity at 10 $mgmL^{-1}$ was considered and further investigated to determine their MIC using disc diffusion method against *X. oryzae* (Mostafa et al., 2017). Different concentrations of the effective crude extract (10, 5, 2, 1, 0.5, 0.2 and 0.1 $mgmL^{-1}$) were prepared separately by dissolving 10 mg of each extract in 1 mL of 5% DMSO, sterilized and loaded their requisite amount over sterilized paper discs. WF-P agar Petri dishes seeded with bacterial suspension of the *X. oryzae*, were placed over with sterile filter paper discs containing different concentrations of crude extracts placed on WF-P agar, and incubated at 28º C for 24 h. The clear zones of inhibition formed around filter paper discs and individual zone diameters measured and recorded. The MIC of each extract was considered as the lowest concentration of the extract that completely inhibited the bacterial growth. The lower the MIC, the higher the activity of the extract.

RESULTS AND DISCUSSION

Medicinal plants are the essential source of bioactive compounds for the development and discovery of new antimicrobial compounds with diverse chemical structures. Bioactive compounds of *G. glauca* plant parts were successfully extracted through organic solvents *viz.*, petroleum ether, chloroform, ethyl acetate and methanol and the physical characteristics of each extract are recorded in Table 1.

Table 1: Physical characteristics and yield of *G. glauca* plant parts phytochemical extracts.

Sl. No.	Solvent for extraction	Plant part extracted	Color	Odor	Consistency	Yield %
1	**Petroleum ether**	Leaf	Dark brown	Characteristics	Greasy	1.5
		Bark	Yellowish brown	Characteristics	Greasy	8.11
		Flower	Brownish yellow	Characteristics	Powder	1.0
2	**Ethyl acetate**	Leaf	Dark green	Characteristics	Greasy	1.5
		Bark	Greenish brown	Characteristics	Sticky	1.0
		Flower	Brownish	Characteristics	Powder	0.55
3	**Chloroform**	Leaf	Yellowish	Characteristics	Powder	0.70
		Bark	Dark yellow	Characteristics	Powder	0.30
		Flower	Yellowish brown	Characteristics	Powder	0.04
4	**Methanol**	Leaf	Green	Characteristics	Sticky	1.5
		Bark	Reddish brown	Characteristics	Sticky	5.2
		Flower	Reddish brown	Characteristics	Greasy	0.04

Results of the phytochemical extracts qualitative screening tests revealed the presence of saponins, flavonoids, alkaloids, tannins, glycosides, steroids in petroleum ether, ethyl acetate, methanolic and extracts of *G. glauca* plant parts. Results are depicted in Table 2.

Table 2: Phytochemical screening of various solvent extracts of *G. glauca* plant parts

Tests	Solvent extracts														
	Pet. Ether			Chloroform			Ethyl Acetate			Methanol			Water		
	L	B	F	L	B	F	L	B	F	L	B	F	L	B	F
Dragendroff's test (Alkaloids)	-	+	-	-	+	+	-	+	+	-	+	-	+	+	+
Wagner's test (Alkaloids)	-	+	-	-	-	+	-	+	-	-	+	+	+	-	+
Hager`s test (Alkaloids)	-	-	-	-	-	+	-	-	+	-	+	+	+	+	+
Lead acetate test (Phenolics)	-	-	+	-	+	-	+	-	+	-	+	+	+	+	+
$K_2Cr_2O_7$ test (Phenolics)	-	-	+	-	-	-	-	-	+	-	-	+	+	+	+
NaOH test (Flavonoids)	-	-	+	-	-	-	-	+	-	-	+	-	+	+	-
Ferric chloride Test (Phenolics/Tannin)	+	+	-	-	+	-	-	+	+	-	+	+	+	+	+
Salkowski test (Steroids)	+	-	-	-	-	+	+	+	+	-	-	+	-	-	-
Liebermann's reaction (Steroids)	-	+	+	-	-	+	+	+	+	+	+	+	+	-	+
Keller-Killian test (Glycosides-)	+	-	+	-	-	+	+	-	+	-	+	+	+	+	+
Foam test (Saponins)	+	-	-	+	+	-	+	+	-	+	+	+	+	+	+
Anthrancene test (Tannins)	-	-	-	+	+	+	-	+	-	+	+	-	-	-	-
Sulphuric acid test (Tannins)	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-

Note: (-) Absence and (+) Presence of the compound. L-Leaf; B-Bark; F-Flower

Results suggest that, the methanolic and aqueous extracts of leaf, bark, and flower of *G. glauca* contain bioactive compounds such as saponins, steroids, flavonoids, alkaloids, glycosides, and tannins. The ethyl acetate extracts showed the presence of alkaloids, steroids, glycosides, phenolics and tannins in all parts. While petroleum ether extracts were confirmed to possess alkaloids, steroids, and glycosides. Among the different solvent extracts methanolic and water extracts of aerial parts of *G. glauca* were found be rich in phytochemicals. These results were in coincident with the results that previously reported by Netravathi et al., (2010), Kharat et al., (2013), Godghate et al., (2015), and Sannabomaji et al., (2018).

Thin layer chromatography (TLC) analysis plays an important role, and it has been introduced in all the modern pharmacopoeias for the identification of bioactive compounds. The tissue extracts of *G. glauca* phytochemicals were separated by TLC when sprayed with 3% RBC, the chromatograms showed hemolysis indicating presence of saponins. Ethyl acetate extracts of leaf, bark and flower, methanolic extracts of leaf and petroleum ether extracts of leaf and bark showed green colored spots confirm the presence of saponins and triterpenoids (Figure 1a). In addition, vanillin sulphuric acid reagent sprayed onto the plates to detect brown, dark green and purple color spots indicated the presence of phenolics, steroids and terpenoids (Figure 1b). Thus in the present study, presence of bioactive compounds such as alkaloids, flavonoids, steroids, terpenoids, and saponins in the tissue extracts confirmed by TLC analysis.

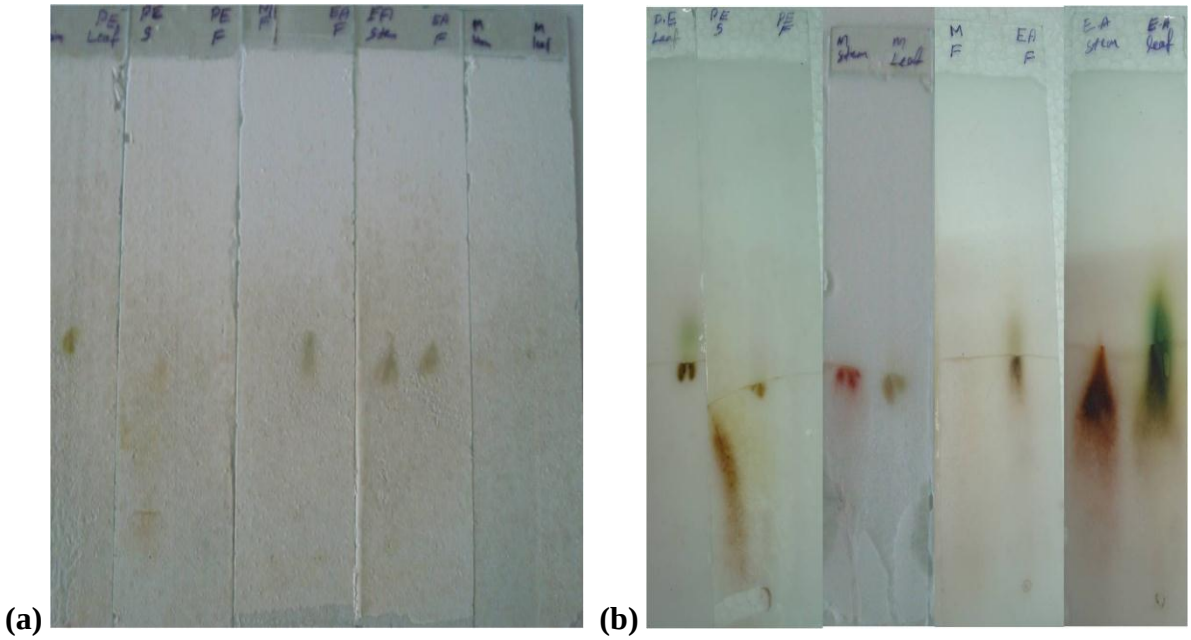

Fig. 1: TLC profile of various extracts of *G. glauca*: sprayed with (a) 3% RBC and (b) Vanillin-sulphuric acid reagents

Note: PE – Petroleum ether, M – methanol, EA – Ethyl acetate extracts; L – leaf, F – flower and S – Bark-stem

Antibacterial activity:

The bacterial isolate formed yellow mucous colonies on WF-P medium, the features confirm the *X. oryzae* (Samanta et al., 2014). The antibacterial efficacy of *G. glauca* each tissue extract was evaluated by disc diffusion method through measuring clear zones in the WF-P medium that were formed due the growth inhibition of *X. oryzae*. Results of the antimicrobial activity studies suggest that leaf extracts prepared in petroleum ether, ethyl acetate have shown better bacterial growth inhibition activity (Figure 2).

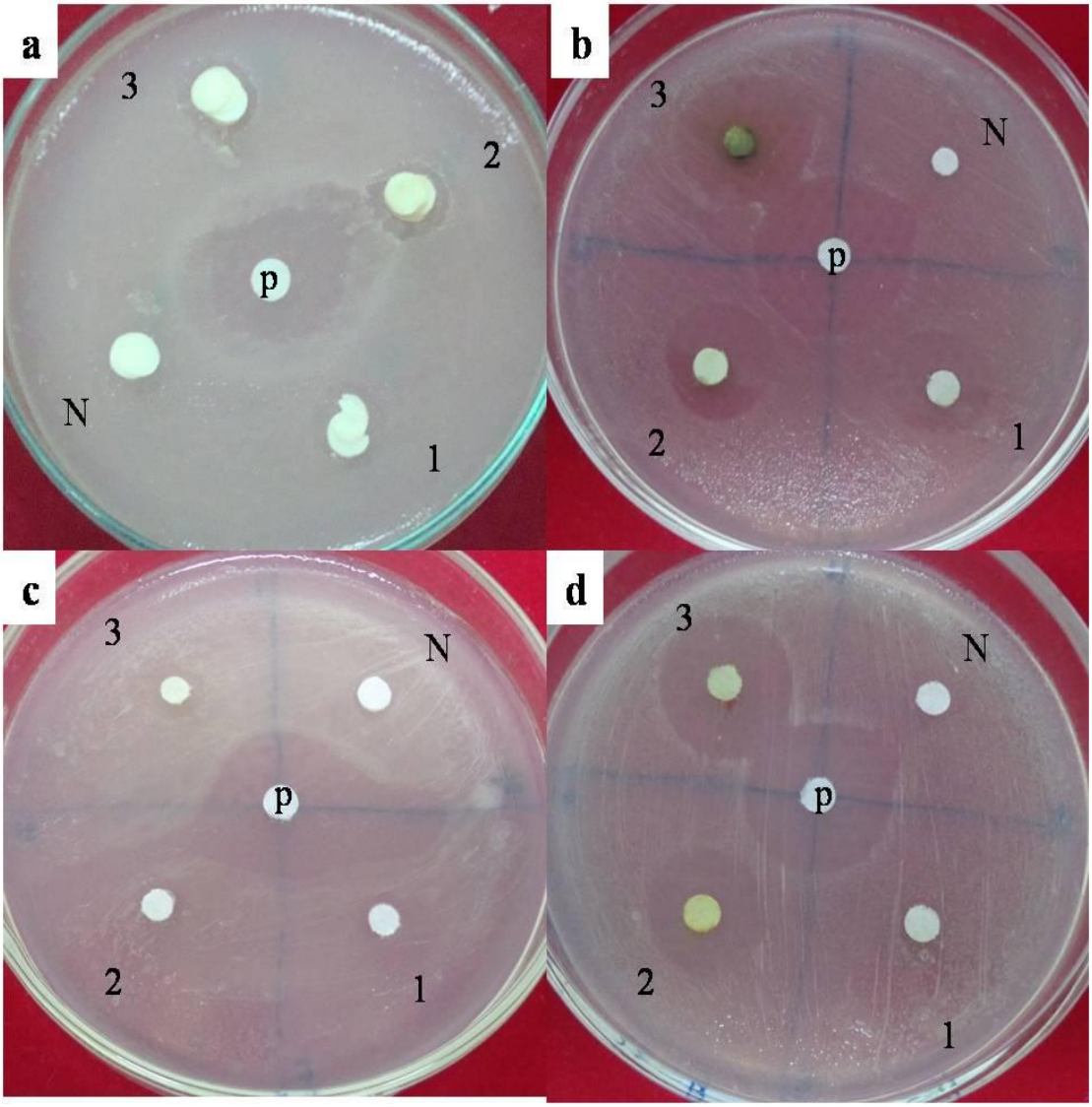

Fig. 2: Anti-bacterial activity of *G. glauca* Leaf extract showed clear zones of inhibition. a) Pet. ether; b) Ethyl acetate; c) Chloroform and d) methanol leaf extract.

Note: P (Positive) and N (Negative) control; extract concentration (1) 5 $mgmL^{-1}$, (2) 10 $mgmL^{-1}$ and (3) 15 $mgmL^{-1}$.

Among the different solvent extracts prepared, methanol and ethyl acetate found to be more effective than chloroform and petroleum ether. The individual solvent extracts of leaves were found to be potentially effective in suppressing bacterial growth of rice bacterial blight pathogen at 10 $mgmL^{-1}$ of phytochemical extract as compared with positive control. Results of present study suggest that different solvent leaf extracts of *G. glauca* effectively inhibited the *X. oryzae* bacterial growth at 10 $mgmL^{-1}$ of crude extract. Probably the volatile constituents extracted into petroleum ether, similarly saponins, alkaloids, and flavonoids in chloroform, ethyl acetate and methanol extracts might be responsible for growth inhibition of *X. oryzae*. While ethyl acetate and methanolic extracts showed strong antibacterial activity against bacterial blight pathogen. Hence, further investigations were carried out to evaluate the minimum inhibitory concentration (MIC) for extracts against the *X. oryzae*.

The MIC of the most effective solvent extracts (ethylacetate andof *G. glauca* was employed by disc diffusion method to evaluate their bactericidal properties. The inhibitory concentration effects of the effective solvent extracts were depicted in Table 3.

Table 3: MIC (Minimum inhibitory concentration) of the solvent extracts of leaf of *G. glauca* against *X. oryzae*

Solvent Extract	Concentration in $mgmL^{-1}$						
	0.1	0.2	0.5	1	2	5	10
	Inhibition zone (mm)						
Pet. Ether	0	0	0	6	6	7	8
Ethyl acetate	0	0	0	7	10	13	16
Chloroform	0	0	0	6	7	8	10
Methanol	0	0	7	9	11	14	18

The inhibitory effect of *G. glauca* leaf methanol crude extracts started at 0.5 $mgmL^{-1}$ with inhibition zone of 7 mm against *X. oryzae* while petroleum ether, chloroform and ethyl acetate extracts suppressed bacterial growth at 1 $mgmL^{-1}$ with inhibition zones of 6, 6 and 7 mm respectively. The results of MIC of effective solvent extracts suggested that *G. glauca* plant parts particularly leaves possess the potential phytochemicals that can be exploited and used to control and manage the bacterial blight disease in rice/paddy crop. *X. oryzae* causing bacterial blight disease is one of the most destructive afflictions of cultivated rice. *G. glauca* leaf extracts suppressing bacterial growth, which appear to be significantly effective against *X. oryzae*. A variation in MIC of different solvent extracts of *G. glauca* may arise from their variation in their phytochemicals and their volatile nature of their constituents. These results are in accordance with those of Arvind et al. (2012), Shivalingaiah et al. (2013) and Gowrish et al. (2016).

Bioactive pesticide products are developed from medicinal plants to control numerous animal and plant diseases, and to provide useful tools for growers to decrease the incidence and/or severity of diseases (Chunxue Cao et al., 2010). Results of the present study suggested that, *G. glauca* leaf phytochemicals prepared as solvent extracts that proved to be significantly effective against *X. oryzae*, can be used as antibacterial agents to control/manage the bacterial blight disease. Further efforts are needed to explore the possible potential of *G. glauca* as a bio-pesticide that can be applied at the field level on rice/paddy crop, along with currently being used controlling agents for the bacterial blight.

CONCLUSION

From the results of the present study it could be concluded that, *G. glauca* plant parts possess phytochemicals that belong to alkaloids, phenolics and terpenoids classes. These phytochemicals can be extracted into aqueous and organic solvents of different polarity. Of the phytochemical extracts prepared from different parts, leaf extracts possess most potent antibacterial phytochemicals against bacterial blight pathogen *X. oryzae*. All solvent leaf extracts which proved potentially effective against *X. oryzae* can be used as natural biocidal agents to control bacterial blight disease and avoiding healthy hazards of chemically synthesized antibacterial products. Further studies are needed in future to exploit the *G. glauca* leaf extracts as bactericidal agent not only against *X. oryzae* but also against other related *Xanthomonas* bacterial species that cause bacterial blight diseases on other agricultural and horticultural crops. Further studies are warranted to understand the exact mechanism of action.

Acknowledgement

Sannabommaji T., expresses sincere thanks to Department of Backward Classes Welfare, Government of Karnataka for financial assistance through Devaraja Urs fellowship.

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