ORIGINAL ARTICLE

Exploration of Antibacterial Properties of *Gnidia Glauca* (Fresen) Gilg. Leaf Saponin Fraction

Torankumar Sannabommaji[1], Vadlapudi Kumar[1*], Poornima D.V.[1], Giridhara Basappa[1,2], Gajula Hari.[1], Rajashekar J.[1]

[1]*Department of Biochemistry, Davangere University, Shivagangothri, Davangere-577007, Karnataka, India.*
[2]*Department of Biotechnology, Sahyadri Science College, Kuvempu University, Shivamogga-577203, Karnataka, India.*

Received: 12th Nov, 2019; Revised: 18th Dec, 2019, Accepted: 09th Jan, 2020; Available Online: 25th Mar, 2020

ABSTRACT

The present study deals with the evaluation of *in vitro* antibacterial profile of leaf saponin fraction of *Gnidia glauca* (Fresen) Gilg (Thymelaeaceae) against two bacterial blight Gram-negative bacterial strains *viz., Xanthomonas oryzae* pv. oryzae and *Xanthomonas campestris* pv. punicae. The antibacterial activity of saponin fraction was evaluated through zone of inhibition, minimum inhibitory concentration (MIC) by broth dilution method. Leaf saponin fraction of *G. glauca* affected the growth curve of both the bacterial strains, through affecting outer membrane permeability, causing cellular contents leakage as confirmed by the respective assays. The *G. glauca* leaf saponin fraction is rich in triterpenoid saponins, showed significant antibacterial activity with MIC at12.5 µg/ml against *X. oryzae* and *X. punicae*. Further the present study gives insights into its mode of action of *G. glauca* leaf saponins, and potential application as natural bactericidal agents to manage and control of bacterial blight causing pathogens in crop plants.

Key words: *Gnidia glauca*, Membrane permeability, Nucleotide leakage, Saponin fraction.

International Journal of Pharmacognosy and Phytochemical Research (2020); DOI: 10.25258/phyto.12.1.7

How to cite this article: Sannabommaji T, Kumar V, Poornima DV, Basappa G, Hari G, Rajashekar J. Exploration of antibacterial properties of *Gnidia Glauca* (Fresen) Gilg. leaf saponin fraction. International Journal of Pharmacognosy and Phytochemical Research. 2020;12(1):66-71.

Source of support: Nil

Conflict of interest: None

INTRODUCTION

Gnidia glauca (Fresen) Gilg (Thymelaeaceae) is an evergreen small bushy tree, commonly distributed throughout the peninsular India, tropical Africa, Sri Lanka and Madagascar. Its vernacular name is *Mukuthi, Rami* (Kan.), Fish poison bush (Eng.) and is well reputed for its medicinal value and toxic properties. It is commonly used as treatment of blisters, swellings and contusions. In Sri Lanka the ground whole plant is used as an insecticide and piscicide. In peninsular India, the bark and leaf are traditionally used for antifungal activity and treating skin diseases.[1,2] Earlier phytochemical studies on this taxon have reported to possess antifeedant acgivity,[3] anticoagulant activity,[4] anti-diabetic activity,[5] free radical scavenging activity[6,7] and *in-vitro* cytotoxic activity.[8] In Western Ghats regional farmers of Karnataka, *G. glauca* plant extracts were used traditional practice for controlling the rice bacterial blight disease caused by Gram-negative bacterial pathogen *X. oryzae* pv. *oryza*. Bacterial blight disease caused by *X. oryzae* in rice/paddy crop and by *X. campestris* pv. punicae in pomegranate crop incurs yield loss up to 70% when susceptible varieties are grown in a favorable environment to these pathogens. Exorbitant chemically synthesized pesticides, fungicides, and bactericides are the major contributors for water, soil, and ground-level contamination. Use of plant extracts as bactericides and fungicides has been in vogue for centuries, though commercially not very common.

In view of these facts, the present study is an attempt to explore the leaf saponin fraction of *G. glauca* as an antibacterial agent that offer new biocidal potential for controlling the bacterial blight disease caused by two *Xanthomonas* strains in rice/paddy and pomegranate, respectively.

MATERIALS AND METHODS

G. glauca fresh leaves material was collected during the day time in January from Western Ghats (13°33'27"N 75°10'13"E/13.55750°N 75.17028°E), Karnataka, India and got authenticated by Dr. Kumarswamy Udupa, renowned plant taxonomist, Department of Botany, Sri JCBM College, Sringeri, Chikkamagalore district, Karnataka, and a voucher specimen (No. FSB-0982) was preserved in the herbarium of the department.

**Author for Correspondence: veeke2012@gmail.com*

Isolation of Saponin Rich Extract

Leaf saponin fraction of *G. glauca* was isolated and partially purified as described in our previous publication.[9] The saponin fraction (5 mg/mL in DW) was diluted to 1:10 with distilled water, scanned at ranges 200 to 800 nm using PC based UV-Visible spectrophotometer (Systronics, Model-119) and characteristics maxima peaks were observed and recorded.[10]

Antibacterial Assays

Antibacterial potential of *G. glauca* saponin fraction was evaluated against two bacterial blight disease causing gram-negative bacterial strains *viz., X. oryzae* pv. *oryzae* and *Xanthomonas campestris* pv. *punicae* that affect rice/ paddy and pomegranate crops, respectively. *X. oryzae* pv. *oryzae* pure culture was isolated on Wokimoto's agar medium and characterized by biochemical tests.[11,12] Antibacterial efficacy of *G. glauca* leaf saponin fraction was confirmed by disc diffusion assay, bacterial growth curve kinetics, outer membrane permeability assay, nucleotide leakage assay and membrane permeability alteration by electrical conductivity measurements.[13-16] *Xanthomonas campestris* pv. *punicae* was a gifted strain from Dr. Raghavendra Sathyanarayana, University of Horticultural Sciences, Bagalakote, Karnataka.

Antibacterial activity assays were performed by agar disc diffusion method.[13] Different concentrations of *G. glauca* leaf saponin fraction was prepared in either distilled water and methanol (MeOH) ranging 2, 5, 10, 50, 100, and 150 mg/ml, were loaded onto sterile filter paper discs (5 mm). Filter paper disks were placed on WF-P agar plates seeded with *X. oryzae* and *X. punicae*. The standard antibiotic tetracycline (10 µL, 1 mg/mL in DW) was used as positive control, distilled water and methanol used as negative controls. Petri plates were incubated at 28°C for 24 hours. The diameter of inhibition zones were recorded in millimeters (mm).

MIC

Minimum inhibitory concentration (MIC) for *G. glauca* leaf saponin fraction against two bacterial strains was determined by broth micro-dilution method,[14] using 96-well microtitre plates. Two-fold serial dilutions prepared with nutrient broth medium at a volume of 200 µL per well in a 96-well plate. To each well of a microplate *G. glauca* leaf saponin fraction was added at concentrations ranging from 50 to 3.125 µg/ml. To the respective wells *X. oryzae* and *X. punicae* cells suspensions 10 µL per well at a final concentration of 5×10^6 CFU/mL bacterial suspensions were inoculated individually and incubated at 28°C for 24 hour. The MIC was evaluated by visual observations for any deposits or turbidity in the microtitre plate wells. The lowest concentration of the saponin fraction that completely inhibited the growth of individual bacterial strains was considered as the MIC value.

Bacterial Growth Curve

Time-kill kinetics for both *X. oryzae* and *X. punicae* growths were studied in sterile 96-well microtitre plate, each well containing 200 µL of NB medium supplemented with different concentrations of *G. glauca* saponin fraction at 5, 10, and 15 mg/mL of saponin fraction solution and 10 µL of freshly grown bacterial culture (10^5 CFU/mL). Tetracycline was used as a positive control, bacterial cultures without saponin fraction as growth control. Plates were incubated e at 28°C and OD_{600} was recorded every 3 hours (3, 6, 9, 12, 15, 18, 21, 24 and 48 h) intervals using microplate reader (i-Mark, Bio-Rad).

Outer Membrane Permeability

The experiment for outer membrane permeability assay was carried out with some modifications.[15] Overnight grown bacterial cultures of *X. oryzae* and *X. punicae* in nutrient broth were (20 µL each culture of 10^8 CFU/mL) inoculated individually to each respective well in a microplate, and 80 µL of *G. glauca* saponin fraction at concentrations ranging from 0.65 to 10 µg/mL were added. Microplate was incubated at room temperature for 24 hours, and the bacterial growth was measured at 450 nm using a microplate reader (i-Mark, Bio-Rad).

Nucleotide Leakage Assay

The assay for nucleotide leakage was essentially performed.[15] The overnight grown culture of *X. oryzae* and *X. punicae* were washed and resuspended in 10 mM PBS (pH 7.4). Final density of cells adjusted at 10^6 CFU/mL, and treated with 100 mg/mL of *G. glauca* saponin fraction for 24 hours at 28°C along with respective control sets. After incubation, bacterial cell suspensions were centrifuged at 10,000 rpm for 10 min and supernatants were diluted appropriately. The presence of nucleic acids in the supernatants was recorded using UV-visible spectrophotometer (Systronics, India) at 260 nm.

Conductivity Measurement

The membrane permeability assays were carried out by electrical conductivity measurements using conductivity meter.[16] Freshly grown *X. oryzae* and *X. punicae* bacterial cells were harvested by centrifugation at 8,500 rpm for 10 minutes, washed three times with 10 mM PBS (pH 7.4), and diluted appropriately to obtain 10^6 CFU/mL bacterial cells. Leaf saponin fraction of *G. glauca* ranging from 50 to 250 mg/mL was added to 25 mL of each bacterial cell suspension. Samples were mixed and incubated at 28°C in a shaker-incubator at 120 rpm for 24 hours, and the change in electrical conductivity was recorded using conductivity meter (Systronics, India).

Statistical Analysis

All the experiments were performed in triplicates under the same conditions individually. Data were analyzed and reported as mean values ± standard deviation. All statistical comparisons were done by using Microsoft Excel. Differences at $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Isolation and partial purification of *G. glauca* leaf saponin fraction was described in our previous publication[9] by Sannabommaji *et al*. (2018), It has been shown that, *G. glauca* leaf saponin fraction is rich in triterpenoids saponins with

8.6% of saponin fraction and 392 µg/mL total saponin extract equivalent to *Quillaja* saponin (a triterpenoid saponin). In the present study results of the UV-visible spectrophotometry suggested that, *G. glauca* leaf saponin fraction displayed the absorption maxima peaks at 224.0, 221.6, 209.6, and 202.4 nm with the absorbance 1.107, 1.169, 1.136, and 1.067, respectively (Figure 1). Saponins lack chromophores in their structure, if the UV spectrophotometry detection is limited at 205-230 nm range indicating the presence of triterpenoid saponins.

Saponins are glycosides of triterpens and steroids, many of these phytochemicals have antimicrobial and/or antiherbivore activity and seem to be efficient role in plant defense.[17-21] They are promising source of triterpenoids that are generally act by permeabilising cell membrane and in various stress conditions render protection against pathogens and pests.[22-23]

Antimicrobial Activity

The biocidal nature of triterpenoid saponins seems to involve membranolytic properties, rather than simply altering the surface tension of the extracellular medium, disrupting the membranes and causing leaking of cellular contents.[14,24] Results of the antibacterial activity assays suggest that, *G. glauca* leaf saponin fraction inhibited the growth of both bacterial blight causing Gram-negative pathogens *viz., X. oryzae* pv. *oryzae* and *X. campestris* pv. *punicae*. The saponin fraction showed inhibition zone around the discs against *X. oryzae* (15.3 ± 0.5 mm) and *X. punicae* (16.0 ± 0.5 mm) at 150 mg/mL concentration (Table 1).

The anti-*Xanthomonas* potential of *G. glauca* leaf saponin fraction was assessed quantitatively determining the minimum inhibitory concentration (MIC), the lowest concentration that completely inhibited visible growth of *X. oryzae* (12.5 µg/mL) and *X. punicae* (12.5 µg/mL). Results are depicted in Table 2.

Previous research studies reported potent antibacterial activity of *G. glauca* root extract against gram-negative bacterial pathogens.[25] The leaf and bark extracts of *Lasiosiphon eriocephalus* (syn. *Gnidia glauca*) showed bactericidal effect at 5 mg/mL against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella sps*., and *X. oryzae*.[2] Triterpenoid saponins of *Caesalpinia pulcherrima* Linn, displayed significant antibacterial activity against gram-positive and gram-negative bacterial strains.[26]

Results of the present study suggest that, leaf saponin fraction of *G. glauca* rich in triterpenoids at 15 µg/mL concentration inhibited the growth of both the bacterial strains *X. oryzae* and *X. punicae*, the efficacy could be compared to positive control tetracycline. Growth curves of *G. glauca* leaf saponin fraction treated bacterial strains *X. oryzae* and *X. punicae* are shown in Figures 2(A) and (B).

Alteration of cytoplasmic membrane permeability is an important bactericidal mechanism.[27] Results of the present study revealed that, *G. glauca* leaf saponin fraction able to increase the outer membrane permeability in both the *Xanthomonas* strains (Figure 3A). Cell membrane permeability and cellular leakage assays are associated with disruption/damage to the bacterial cell membranes. Certain extent, DNA and RNA are released after membrane disruption, in the present study these nucleotides were quantified by monitoring the absorbance at 260 nm (Figure 3B). The leaf saponin fraction of *G. glauca* probably induced the disruption of cytoplasmic membrane and increased absorbance at 260 nm as indicated by the leakage of nucleotides and consequently reflects a loss the membrane integrity. Similar findings were also reported for the other phytochemical extracts against certain bacterial strains.[28,29] Furthermore, it has been observed during the present study that, the electrical conductivity was observed to be increased significantly from 9.76 to 10.36 µS/cm for *X. oryzae* and 9.66 to 10.16 µS/cm for *X. punicae* (Figures 3C and D). Increased

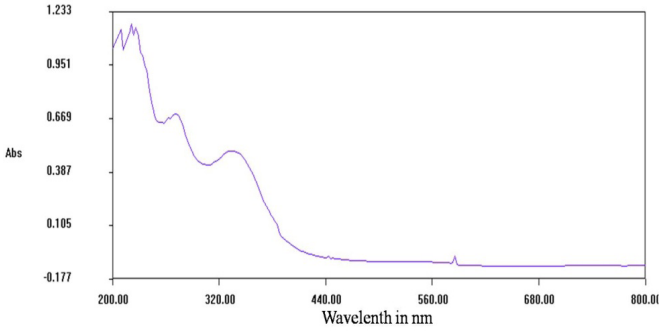

Figure 1: UV-Vis spectral scan of leaf saponin fraction

Table 1: Antibacterial activity of the leaf saponin fraction of *G. glauca* on *X. oryzae* and *X. punicae* strains at various concentrations by disc diffusion method

	Concentration of triterpenoid saponin fraction (mg/mL)							
	2	*5*	*10*	*50*	*100*	*150*	*Tetracycline (PC)*	*MeOH and DW (NC)*
Test Organism	*Zone of inhibition (mm)*							
X. oryzae	-	7.3 ± 0.5	10.6 ± 0.5	11.3 ± 0.57	13.3 ± 0.5	15.3 ± 0.5	17.6 ± 1.52	-
X. punicae	-	7.0 ± 0	10.8 ± 0.76	13.5 ± 0.5	14.5 ± 0.5	16.0 ± 0.5	17.3 ± 0.57	-

Note: MeOH = Methanol; DW = Milli-Q water; PC = Positive control; NC = Negative control; '-' sign indicates no zone of inhibition.

Table 2: MIC values of *G. glauca* leaf saponin fraction treated to *Xanthomonas* strains at various doses.

	Serial dilution (µg/mL)					
Test organism	*50*	*25*	*12.5*	*6.25*	*3.12*	*MIC*
X. oryzae	-	-	-	+	+	12.50
X. punicae	-	-	-	+	+	12.50

"-" No growth; "+" growth

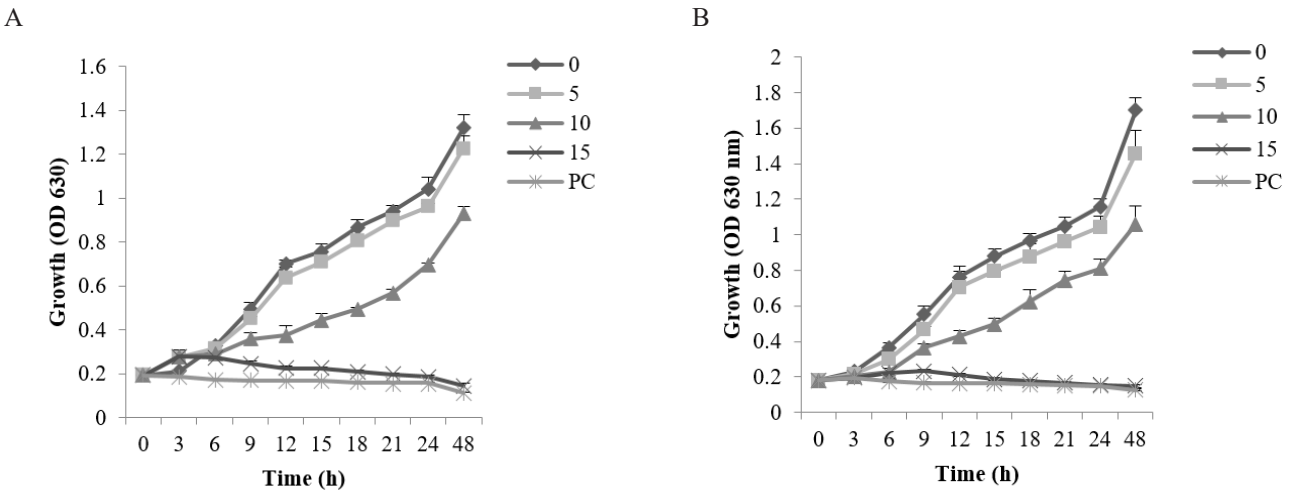

Figure 2: Effect of leaf saponin fraction on growth of (A) *X. oryzae* and (B) *X. punicae*

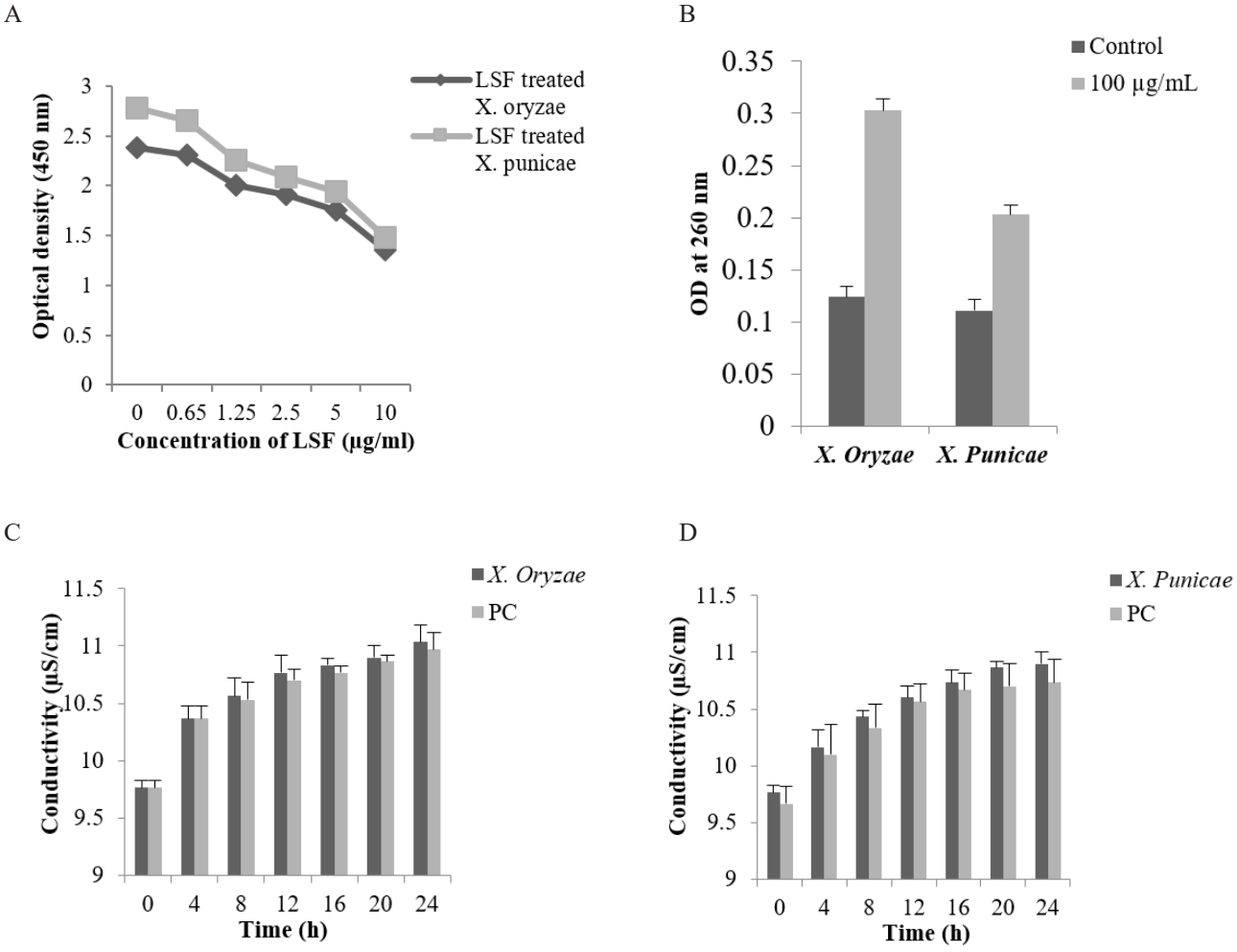

Figure 3: Effect of *G. glauca* leaf saponin fraction on *Xanthomonas* strains. Outer membrane permeability activity on *Xanthomonas* strains (A): Total nucleotide leakage assay (B): Outer membrane permeability; (C & D): Conductivity of ionizable solutes leakages in *X. oryzae* and *X. punicae* respectively.

Note: Culture without leaf saponin fraction was used as untreated control (control) and tetracycline was used as positive control (PC).

absorption at 260 nm and electrical conductivity clearly indicate the disruption of membrane permeability and loss of viability in both the bacterial strains.

It has been reported that, saponins have the ability to induce the formation of pores within the cell membrane which becomes more permeable for ions as the ion pumps are unable to restore the physiological conditions and finally cause the lysis of cells.[16] Results of all the assays carried out during the present study suggest that, *G. glauca* leaf saponin fraction was effective against both the *Xanthomonas* bacterial strains. The growth has been affected in both the bacterial strains through affecting the cell membrane integrity and disrupting membrane permeability. The triterpenoids saponins present in the *G. glauca* leaf saponin fraction probably interact with the lipids present in the cell membrane, increases the permeability and to kill the *Xanthomonas* bacterial growth and reproduction.

CONCLUSION

G. glauca leaf saponin fraction has been explored as potential source for triterpenoid saponins with constitutive antibacterial activity. This bioactive saponin fraction showed significant antibacterial activity and mode of action in permeabilising membrane causing leakage of cellular components and finally inhibiting the reproductive growth of *Xanthomonas* strains. Results suggest that, *G. glauca* leaf triterpenoid saponins are effective bioactive compounds that could be useful for the development of new biocidal agents to control the bacterial blight diseases in both rice/paddy and pomegranate crops.

REFERENCES

1. Vinayaka KS, Hegde SV, Banakar S, Kekuda TR. Antifungal potency of *Gnidia glauca* (Fresen) Gilg and *Pothos scandens* L. Nat Prod. 2009; 5(3):146-148.
2. Gupta A, Goldie O, Annika D, Sharon M. Bactericidal effect of crude extracts of an endangered plant: *Lasiosiphon eriocephalus* Decne. J. Microbiol. Biotech. Res. 2012;2(6):866-870.
3. Sundararajan G, Kumuthakalavalli R. Antifeedant activity of aqueous extract of *Gnidia glauca* Gilg and *Toddalia asiatica* Lam. on the gram pod borer, *Helicoverpa armigera* (Hbn). Journal of Environmental Biology. 2001;22(1):11-14.
4. Joshi PC, Mandal S, Das PC, Adhikari P. Preliminary studies (*In-vitro*) on anticoagulant activity of naturally occurring bis-coumarins. Aryavaidyan. 2004; 18(1): 33-36.
5. Ghosh S, Ahire M, Patil S, Jabgunde A, Bhat Dusane M, Joshi BN, Pardesi K, Jachak S, Dhavale DD, Chopade BA. Antidiabetic activity of *Gnidia glauca* and *Dioscorea bulbifera*: potent amylase and glucosidase inhibitors. Evidence-Based Complementary and Alternative Medicine. 2012;2012.
6. Ghosh S, Derle A, Ahire M, More P, Jagtap S, Phadatare SD, Patil AB, Jabgunde AM, Sharma GK, Shinde VS, Pardesi K. Phytochemical analysis and free radical scavenging activity of medicinal plants *Gnidia glauca* and *Dioscorea bulbifera*. PLoS One. 2013; 8(12):e82529.
7. Rao SB, Jayanthi M, Yogeetha R, Ramakrishnaiah H, Nataraj J. Free radical scavenging activity and reducing power of *Gnidia glauca* (Fresen.) Gilg. Journal of Applied Pharmaceutical Science. 2013;3(6):23.
8. Gowrish A, Vagdevi HM, Rajashekar H, Kumar MV, Shobha KS. In-Vitro Cytotoxic and Antioxidant Activity of *Gnidia glauca* (Fresen) Gilg Root Extract. Journal of Applicable Chemistry. 2013;2(5):1362-1369.
9. Sannabommaji T, Kumar V, Poornima DV, Gajula H, Rajashekar J, Manjunatha T, Basappa G. Phytochemical Analysis with Special Reference to Leaf Saponins in *Gnidia glauca* (Fresen.) Gilg. InBiotechnological Approaches for Medicinal and Aromatic Plants. Springer, Singapore, 2018, 271-287.
10. Kalaichelvi K, Dhivya SM. Screening of phytoconstituents, UV-VIS Spectrum and FTIR analysis of *Micrococca mercurialis* (L.). Benth. International Journal of Herbal Medicine. 2017; 5(6):40-44.
11. Karaganilla A, Natural MP, Ou SH. A comparative study of culture media of *X. oryzae* pv. *oryzae*. Philippines Agriculture. 1973;57:141-152.
12. Shankara K, Patil MB, Pramesh D, Sunkad G, Yenjerappa ST, Ibrahim M, Rajesh NL, Chikkannaswamy. Isolation and Characterization of Bacterial Leaf Blight of Rice (*X. oryzae* pv. *oryzae*) isolates from Southern India. International Journal of Pure Applied Bioscience. 2017;5(4):452-461.
13. Andriani Y, Ramli NM, Syamsumir DF, Kassim MNI, Jaafar J, Aziz NA, Marlina L, Musa NS, Mohamad H. Phytochemical analysis, antioxidant, antibacterial and cytotoxicity properties of keys and cores part of *Pandanus tectorius* fruits. Arabian Journal of Chemistry. 2015.
14. Lunga PK, Qin XJ, Yang XW, Kuiate JR, Du ZZ, Gatsing D. Antimicrobial steroidal saponin and oleanane-type triterpenoid saponins from *Paullinia pinnata*. BMC comple. Alte. Med. 2014;14(1): 369.
15. Pinto N, Campos LM, Evangelista ACS, Lemos AS, Silva TP, Melo RC, Fabri RL. Antimicrobial *Annona muricata* L. (soursop) extract targets the cell membranes of Gram-positive and Gram-negative bacteria. Industrial crops and products. 2017; 107:332-340.
16. Zhao Y, Chen M, Zhao Z, Yu S. The antibiotic activity and mechanisms of sugarcane (*Saccharum officinarum* L.) bagasse extract against food-borne pathogens. Food Chemistry. 2015; 185:112–118.
17. Morrissey JP, Osbourn AE. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. Microbio. Mol. Bio. Rev. 1999;63(3):708-724.
18. Francis G, Kerem Z, Makkar HP, Becker K. The biological action of saponins in animal systems: a review. British journal of Nutraceutical. 2002;88(6):587-605.
19. Sparg S, Light ME, Van Staden J. Biological activities and distribution of plant saponins. Journal of Ethno-pharmacology. 2004;94(2-3):219-243.
20. Hassan SM, Haq AU, Byrd JA, Berhow MA, Cartwright AL, Bailey CA. Haemolytic and antimicrobial activities of saponin-rich extracts from guar meal. Food Chemistry. 2010;119(2):600-605.
21. Nabinejad A. Antibacterial effects of *Saponaria officinalis* extracts against avian pathogenic *Escherichia coli* (APEC). African Journal of Agricultural Research. 2013;8:2068-2071.
22. De Geyter E, Lambert E, Geelen D, Smagghe G. Novel advances with plant saponins as natural insecticides to control pest insects. Pest Technology. 2007;1(2):96-105.
23. Ramesha T, katrin G, Thomas L, Paul O, Anne O. Triterpene Biosynthesis in Plants. Annual Review of Plant Biology. 2014;65:225-257.
24. Killeen GF, Madigan CA, Connolly CR, Walsh GA, Clark C, Hynes MJ, Power RF. Antimicrobial saponins of *Yucca*

schidigera and the implications of their in vitro properties for their*in-vivo* impact. Journal of Agriculture and Food Chemistry. 1998;46(8):3178-3186.

25. Gowrish A, Vagdevi HM,Rajashekar H. Antibacterial and antitubercular activity of *Gnidia glauca* (Fresen) Gilg root extracts. International Journal of Pharmaceutical Chemistry. 2016;6(7):186-191.
26. Asati N, Yadava RN. Antibacterial activity of a triterpenoid saponin from the stems of *Caesalpinia pulcherrima* Linn. Natural Product Research. 2018;32(5):499-507.
27. Silverman JA, Perlmutter NG, Shapiro HM. Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*. Antimicrobial Agents Chemotherapy. 2003;47:2538-2544.
28. Sampathkumar B, Khachatourians GG, Korber DR. High pH during trisodium phosphate treatment causes membrane damage and destruction of *Salmonella enterica* serovar Enteritidis. Appl. Environ. Microbiol. 2003;69:122–129.
29. Alvarez-Ordonez A, Carvajal A, Arguello H, Martinez-Lobo FJ, Naharro G, Rubio P. Antibacterial activity and mode of action of a commercial citrus fruit extract. Journal of Applied Microbiology. 2013;115:50-60.