Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(3): 1957-1961
Received: 24-03-2019
Accepted: 28-04-2019

Anusha S
Department of Botany, University of Kerala, Kerala, India

Riyas CT
Department of Botany, University of Kerala, Kerala, India

Reshma M Das
Department of Botany, University of Kerala, Kerala, India

Soorya S
Department of Botany, University of Kerala, Kerala, India

Namitha LH
Department of Botany, University of Kerala, Kerala, India

Vishnu B
Department of Botany, University of Kerala, Kerala, India

Arun R Pillai
Department of Botany, University of Kerala, Kerala, India

Anilkumar C
Senior Scientist and HOD, Conservation Biology Division, JNTBGRI, Kerala, India

Kamarudheen Kunju M
Associate professor, Department of Botany, University of Kerala, India

Correspondence
Anusha S
Department of Botany, University of Kerala, Kerala, India

A preliminary pharmacological evaluation of methanolic extract of *Gymnacranthera canarica* (King) warb seeds: A threatened species of myristica swamp ecosystem of southern western Ghats, Kerala

Anusha S, Riyas CT, Reshma M Das, Soorya S, Namitha LH, Vishnu B, Arun R Pillai, Anilkumar C and Kamarudheen Kunju M

Abstract
This research works deals with the screeing of methanolic extracts of *Gymnacranthera canarica* seeds for antidiabetic, antimicrobial, anti-inflammatory, antioxidant activity and total phenol, flavanoid quantification. The Myristicaceae family are well known for "Nutmegs" known to contain several polyphenol compounds like α-pinene, β-pinene…Myristicin is the key present in the Myristicaceae family. *G. canarica* is the only species of genus *Gymnacranthera* in India. It is an exclusive swamp species seen in Myristica swamp, one of the rarest and primitive ecosystem in the Southern Western Ghats, Kerala, India. On the basis of local tribal knowledge that the seeds of *G. canarica* are used as wild nutmegs this study executed to understand the pharmacological evaluation in a preliminary level. The results shows that *G. canarica* seeds methanolic extract possess a promising antimicrobial activities against both bacteria and fungi. Mostly sensitive against gram +ve bacteria. The seeds shows a good antidiabetic potential in α amylase inhibitory assay (74.68%) at a concentration of 100µg/mg of extract. But the phenol conent (8.75µg/mg) and antioxidant activities are not in a great extend, substantiating the direct correlation between phenol content and antioxidant activities. High flavanoid content in the *G. canarica* seeds of 103.3 µg/mg of extract may be responsible for the anti-inflammatory effect by *G. canarica* seeds.

Keywords: *G. canarica*, bovaine serum albumin, folin – ciocalteau, well diffusion, DPPH

1. Introduction
In India about 45,000 medicinal plant species are concentrated in the region of Eastern Himlayas, Western Ghats and Andaman and Nicobar islands. 70% population of the rural India depends on the traditional type of plants. In recent years medicinal plants have been widely deliberated for their antioxidant activity. Right uptake of natural antioxidants is associated with lower risks of degenerative diseases, especially cancer and cardiovascular diseases. Human health is mostly affected by accumulation of free radicals. Normal body function produces free radicals and these oxygen radicals can cause damages to the lipid memberane, proteins and DNA. Different chemical compounds and synthetic drug have been used as antioxidant and antimicrobial agents. Most widely used synthetic antioxidant compounds were butylated hydroxytoluene (BHA) and butylated hydroxyanisole (BHA) which have been restricted because of carcinogenic potential. But some toxic properties of synthetic antioxidants and numerous drug resistant microorganisms make an urgent need of new natural antioxidant and antimicrobial agents need to be developed (Tajkarmi *et al*., 2010). An interesting attention has been directed towards exploring plant based natural antioxidant in the recent years, especially the natural antioxidants like phenols and tocopherols. Many research projects were conducting for the phytochemical screening of leaves, roots, flowers, whole plants and stems. But the reports on seed as potent antioxidant is very few. Inspite of that the study material *G. canarica* belongs to the nutmeg family. Nutmeg family posses aromatic plants have been traditionally used in folk medicine, they are widely used as a preservatives. Nutmegs are dried kernals used as spices and in alternative medicine as it has been reported with anti-inflammatory, antioxidant and antimicrobial properties.
Gymnacranthera is a small genus which contains only 7 species distributed in the Indo - Malayan region, where the species *G. canarica* is only present in India. It is Endemic to the southern Western Ghats. Besides that they are the most dominant tree species of the Myristica swamps, a highly threatened ecosystems.

Myristica swamps are first reported by krishnamoorthy in 1960 as a special type of habitat of habitat from the Kulathupuzha, Anchal area of Travancore region. Local peoples use the seeds of *G. canarica* as wild nutmeg. But there is no scientific explanation about the therapeutic effect of this seed is till unknown. So the objectives of this paper deals with assessment of *in vitro* anti-diabetic assay, *in vitro* anti-inflammatory activity, *in-vitro* anti oxidant assay, evaluation of anti bacterial, anti fungal, total phenol and flavonoid contents in the methanolic seed extracts.

2. Materials and Methods

2.1 Study area

The candidate species *G. cnarica* is located in Myristica swamps forest patches of Kulathupuzha and Anchal forest ranges (8.75˚ -9.0´N and 76.75˚ -77.25˚ E) between the geo co-ordinates. The swamps are scatterd in 3 forest ranges namely Kulathuzpuzha and Anchal forest ranges and Shendurney Wild life Sanctuary. The elevation of *M. swamps* above sea level seems to be a critical as all the mapped Myristica swamps were found between 100-200m from sea level with rainfall range of 50cm -150cm in South West monsoon, 60-80cm in North East monsoon and 30cm- 50cm in rainfall other than the monsoon (published by ESS).

2.2 Preparation of methanolic plant extracts

20gram fresh seeds were carefully removed aril and thoroughly washed in distilled water. Seeds were oven dried at 40˚C for 2 days. Ground the dry plant materials, 10g were weighed and a total of 100ml of 80% methanol was added and the suspension was stirred slightly. Mixture was sonicated for 15 minutes and then transferred into the shaker for 5 days with 100rpm at room temperature. The extract was centrifuged for 10 minutes, 1500g and supernatants were collected and stored at -20˚C until the use.

2.3 *In vitro* anti-diabetic assay (α- amylase inhibitory assay)

The activity was determined using a modified assay of that described in the Worthington Enzyme Manual (Worthington, 1993; Kwon *et al*., 2006). A total of 500µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing 0.5 mg/mL of α- amylase and varing concentration(25,50, 75, & 100µg) of extract as inhibitor were pre-incubated at 25ºC for 10 min. After the pre-incubation, 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube at timed intervals. The reaction was stopped using 1.0 mL of dinitrosalicylic (DNS) acid color reagent. The test tubes were incubated in a boiling water bath for 5 min and then cooled to room temperature. Make up the volume of reaction mixture to 10mL by adding distilled water, and the absorbance was measured at 540 nm using UV-Visible light spectrophotometer. Acarbose at various concentrations (25–100 µg /ml) was used as a standard. The absorbance readings were compared with the controls that contained buffer instead of sample extract.

Calculation

$$\%\ of\ inhibition = \frac{(\mathrm{B} - \mathrm{A}) * 100}{(B - C)}$$

C- Control with starch and without alpha amylase
B- Control with starch and alpha amylase
A- Test

2.4 *In vitro* antioxidant activity- Free radical scavenging ability by the use of a stable DPPH radical

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method by Shen *et al*., 2010. Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the concentration (1mg/1000µl). The solution of DPPH in methanol 60µM was prepared fresh daily before UV measurements. This solution (3.9ml) was mixed with 100µl of test solution at various concentrations (25, 50, 75 & 100µg). The samples were kept in the dark for 15 minutes at room temperature and the decrease in absorbance was measured at 517 nm using a UV-VIS spectrophotometer. The experiment was carried out in triplicate. Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as blank.
Radical scavenging activity was calculated by the following formula.

% Inhibition = (Absorbance of Control at 0 minute - Absorbance of Test) / Absorbance of Control at 15 minutes x 100

Where C= absorption of control sample (t= 0 min), C= absorption of control (t=15 min), T=absorption of test solution.

2.5 *In vitro* anti-inflammatory activity- Bovine serum albumin (BSA) denaturation assay

In vitro anti-inflammatory were assayed by methods of Mizushima and Kobayashi (1968) [4] and Sakat *et al*. (2010) [5] The reaction mixture (0.5 ml) consisted of 0.45 ml bovine serum albumin (3% aqueous solution) and varing concentration of compound (250, 500, 750,1000µg/ml of final volume), pH was adjusted to 6.3 using small amount of 1N hydrochloric acid. The samples were incubated at 37ºC for 20 min and then heated at 80ºC for 2min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. The absorbance was measured using spectrophotometer at 660nm.The percentage inhibition of protein denaturation was calculated as follows:

Percentage inhibition = [(Abs Control – Abs Sample) / Abs control)] x100

Where Abscontrol is the absorbance of the DPPH radical+ solvent, Abssample is the absorbance of DPPH radical+ sample extract/standard.

2.6 Evaluation of antibacterial activity

Agar well diffusion method is used to evaluate antibacterial activity of methanolic seed extracts in strains of both gram positive and gram negative bacteria. *S. areus and Bacillus subsitils* are the gram positive strains and *E. coli*, *Klebsiella pneumonia* are the gram negative strains used. Agar well diffusion method is widely used to evaluate the antimicrobial activity of the compound. Autoclaved 15-20 mL of Mueller-Hinton agar was poured on glass petri plates and allowed to solidify. Standardized inoculum of the test organism was uniformly spread on the surface of these plates using sterile cotton swab. Four wells with a diameter of 8 mm (20 mm apart from one another) were punched aseptically with a sterile cork borer in each plate. Compound solution (40 and 80 µL) at desired concentration from 100mg/mL stock was

added to three of the wells and one well with Gentamycin (80µg/well) as positive and compound solvent as negative control. Then, the agar plates were incubated under 37°C for 24 hrs. After incubation, clear zone was observed. Inhibition of the bacterial growth was measured in mm.

2.7 Evaluation of antifungal activity

Well diffusion assay antimicrobial susceptibility testing was done using the well diffusion method to detect the presence of anti-fungal activities of the compound samples. A sterile swab was used to evenly distribute fungal culture over the PDA agar medium. The plates were allowed to dry for 15 minutes before use in the test. Compound solution (40 and 80 µL) at desired concentration from 100 mg/mL stock was added to three of the wells and one well with cotrimazole as positive and compound solvent as negative control. The plates were incubated at room temperature for 3 days after which they were examined for inhibition zones.

2.8 Total phenolic constituents study

The amount of total phenols in the methanolic seed extract was estimated by the method proposed by Mallick and Singh (1980) [6]. Different aliquots were pipette out and the volume in each tube was made up to 3.0 ml with distilled water. Folin-Ciocalteau reagents (0.5ml) was added and add 2ml of sodium carbonate solution, the tubes were placed in a boiling water bath for exactly one minute. The tubes were cooled and the absorbance was read at 650nm in a spectrophotometer against a reagent blank. Gallic acid was used as standards. Total phenol values are expressed in terms of Gallic acid equivalent (mg/g) of extract, which is a common reference compound.

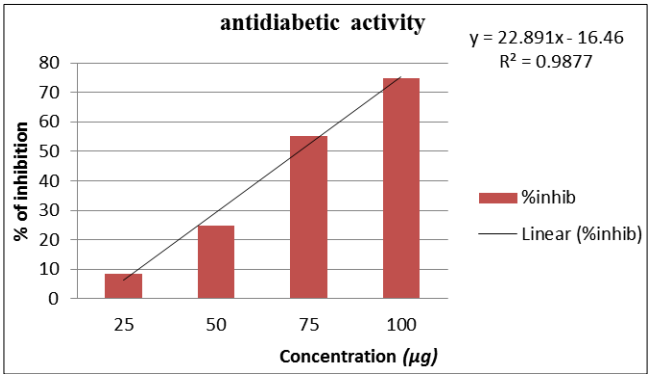
2.9 Total flavonoids determination

Total flavanoid content was measured by the aluminum chloride colorimetric assay (Chang *et al*., 2002) [9]. The reaction mixture consists of 1mg of extract and 4 ml of distilled water and was taken in a 10 ml volumetric flask. To the flask, 0.30 ml of 5 % sodium nitrite was treated and kept for 5 minutes and then 0.3 ml of 10 % aluminium chloride was added. After 5 minutes, 2 ml of 1M Sodium hydroxide was added and diluted to 10ml with distilled water. A set of reference standard solutions of morin (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with an UV/Visible spectrophotometer. The total flavonoid content was expressed as µg of ME /mg of extract.

3. Results

3.1 Anti diabetic assay – alpha amylase inhibition assay

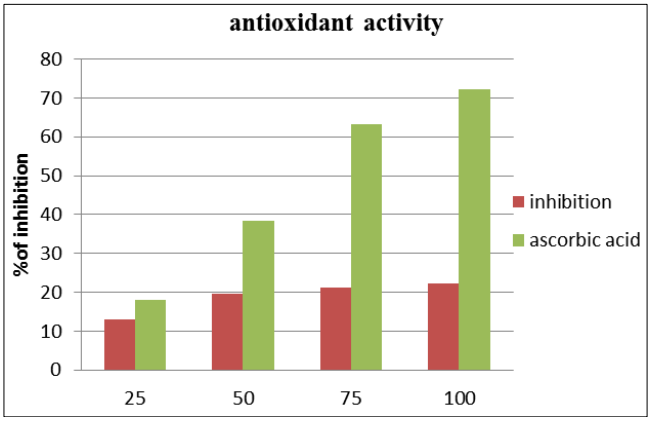
Methanolic seed extracts have were tested for alpha amylase inhibitors and they shown significant inhibition for alpha enzymes. Percentage inhibitions of alpha amylase by *G. canarica* seed extracts at various concentrations 25µg/ml shows 8.46% inhibition, 50µg/ml shows 24.84%, 75µg/ml shows 55.09% and 100µg/ml shows 74.68% inhibition respectively. Methanolic seed extract of *G. canarica* shows IC_{50} values of 72.633%. A concentration dependent increase in the inhibition of alpha amylase is graphically represented (graph 1).



Graph 1: Percentage inhibition of alpha amylase by *G. canarica* seeds methanolic extract at different concentrations.

2.2 Antioxidant activity by DPPH radical scavenging assay

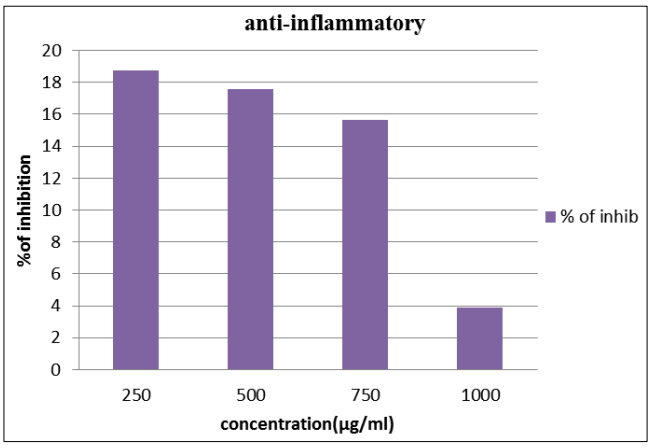
The *G. canarica* seeds extract at the dose of 100µg/ml shows 22.3% the significant free radical scavenging activity of DPPH. A noticeable concentration dependent increase in the inhibition percentage cannot be seen (graph 2).



Graph 2: Percentage inhibition of alpha DPPH by *G. canarica* seeds methanolic extract at different concentrations.

2.3 *In vitro* anti-inflammatory activity

In vitro anti-inflammatory activity shows an equal percentage of inhibition in each concentration from 250µg to 750 µg. Maximum inhibition of only 18.75% shows at 25µg/ml concentration and maximum concentration of 1000 µg/ml concentration shows very minimal inhibition percentage of 3.9% (graph 3).



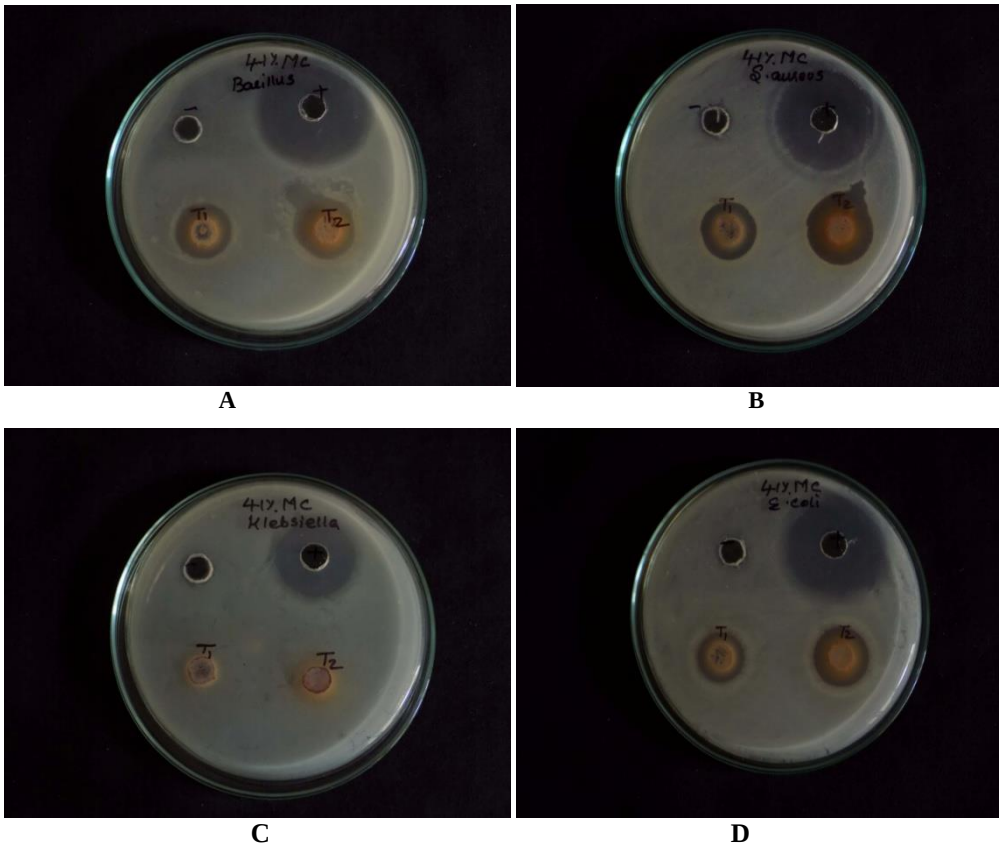
Graph 3: Effect of methanolic extract of *G. canrica* seeds in DPPH radical scavenging activity.

2.4 Antibacterial activity

The methanolic seed extract is subjected to screening against gram positive and gram negative bacteria. Compound solution of 40µg/ml and 80 µg/ml at desired concentration 100mg/ml was added to the wells. Positive control Gentamycin shows 34 mm clear zone of inhibition at 80 µg/ml against *S. aureus*. Maximum inhibitory action shown against gram positive bacteria *S. aureus* (fig1) and no inhibitory action is seen in *Klebsiella pneumonia* shown in table 1.

Table 1: Antibacterial activity of *G. canarica* seeds methanolic extract

Sample	Organism	Diameter of zone		
		+ (80µg)	T1(4mg)	T2(8mg)
Methanolic seed extract	*S. aureus*	34	18	22
	E. coli	36	16	19
	Klebsiella pneumonia	25	-	-
	Bacillus substilis	37	18	20



A B C D

Fig 1: Antibacterial activity of *G. canarica* seeds methanolic extract against A: *Bacillus subtilis*, B: *Staphyloccocus aureus*, C. *Klebsiella pneumoniae* D: *Eschericia coli*.

2.5 Antifungal activity

Antifungal activity of *G. canarica* was evaluated on two different strains at 40µl and 80µl concentration. Only 80 µl concentration shows 11mm clear inhibition on both *Candida albicans* and *A. niger* and 40 µl do not shows any inhibition in both tested fungi. The positive control cotrimazole, 80 µl concentration shows 20mm clear inhibition zone for candida albicans and 30 mm inhibition zone for Aspergillus niger shown in Table 1.

Table 2: antifungal activity of *G. canarica* seeds methanolic extract

Organism	Diameter of zone		
	+	T1	T2
Candida albicans	20	-	11
A. niger	30	-	11

2.6 Quantification of total phenolics and flavanoid content in methanolic extract of *Gymnacranthera canarica* seeds.

Total phenolic content in the mature seeds of *G. canarica* methanolic extract was found to be 8.75µg GAE/mg of methanolic extract and the total flavanoid content was found to be 103.3µg ME/mg of methanolic extract (table 3).

Table 3: Total phenolic and flavanoid contents in mature G. canarica seeds methanolic extracts.

Sample	Total phenol µg/ mg	Total flavanoid µg/ mg
G. canarica seeds	8.75	103.3

3. Discussion

Many herbal extracts have been reported for their antidiabetic activities and being used in the traditional medicine system. Various extracts from plants have been investigated with respect to suppression of glucose production from carbohydrayes in the glucose absorption from the intestine. (Mangesh *et al*., 2018). The enzyme alpha amylase catalyses the hydrolysis of 1, 4- glucosidic linkage of starch, glycogen and various oligosaccharides into simple sugars that can be easily absorbed by the body. So inhibition of alpha amylase ezyme is an effective way to control diabetes. Methanolic extract of *G. canarica* seeds shows about 74.68% inhibition at a concentration of 100µg.

Methanolic extract of seeds of *G. canarica* posses strong antimicrobial activity against gram positive bacteria, active against gram negative as well as against fungal strains. Antibacterial activities in nutmegs are more seen in

chloroform and ethanolic extracts. They only posses fungal activities in methanolic extract (Cho *et al*. (2007). But the methanolic extract of *G. canarica* seeds shows a significant inhibition in very small concentration against gram positive bacteria *S. aureus* followed by *Bacillus subtilus*. To a certain limit it shows activity against *E. coli* but do not have any effect on *Klebsiella pneumonia.* In comparison with conventional antibiotic Gentamycin, it showed more sensitivity against all organisms. Plant extracts generally shows activity against gram positive bacteria due to the presence of single peptidoglycan layer. Nutmeg M. Fragrans and other seeds do not posses any antimicrobial activity in comparison with leaves. In nutmegs antimicrobial properties are due to the many antimicrobial compounds (Sabulal *et al*. (2006) [12] like α-pinene and β-pinene (pinene type monoterpene hydrocarbons) have been reported to have antimicrobial activity (Dormans and Deans, 2000) [13]. Myristicaceae members widely posses these compounds and may *G. canarica* seeds also posses antimicrobial and antifungal properties due to this phenolic compounds. But the amount of total phenolics in fully mature seeds was found to be 8.75 µg GAE/ mg dry weight. Many studies focused on the correlation of antioxidant activity to phenolic compounds contents. In 2008, Kumar *et al* reported that there is a very strong correlation between DPPH and total phenolic content in the extracts of *Kappaphycus alvarezii.* From the results obtained the statement is substantiating because very poor antioxidant activity only seen in the methanolic seed extracts. Total phenolic content of the seeds are also very low. So there is an evident correlation between the antioxidant activity and total phenolics.

Inflammation is a protective response induced during the tissue injury or any infection to defend the invaders in the body. The anti-inflammatory result obtained from *G. canarica* seeds is only about maximum inhibition percentage of 18.75% at a concentration of 250 µg and there is a concentration dependent decrease in concentration can be seen in the assay. BSA denaturation assay is used for the *in vitro* anti-inflammatory study. BSA assay seeks to eliminate the use of live specimens as far as possible. Chloroform extracts are better for Myristicacea anti-inflammatory assay. Even in methanolic extracts *G. canarica* seeds shows a minimal level of anti-inflammatory activity due to the presence of high flavanoid content. A clear conclusion of flavanoid on human health is till unknown, but existing evidence indicating that flavanoids potentially display a multitargeting anti-inflammatory function. Further studies need to be taken to understand the compounds responsible for the therapeutic effect of *Gymnacranthera canarica.*

4. Conclusion

In summary, for the first time we have demonstrated the *Gymnacranthera canarica* seeds preliminary pharmaceutical effect on the basis of a local traditional knowledge that it have been used as wild nutmeg. And the study shows it shows high antidiabetic activity followed by high phenol content. Since it is the methanolic extract, shows a good antimicrobial effect. Further pharmacodynamic investigation are required to understand the precise mechanism exhibited by *G. canarica*.

5. References

1. Chandran MDS, Mesta DK. On the conservation of the Myristica swamps of the Western Ghats. Forest genetic resources: status, threats, and conservation strategies, 2001, 1-19.
2. Gul P, Bakht J. Antimicrobial activity of turmeric extract and its potential use in food industry. Journal of food science and technology. 2015; 52(4):2272-2279.
3. Phoboo S, Bhowmik PC, Jha PK, Shetty K. Anti-diabetic potential of crude extracts of medicinal plants used as substitutes for Swertia chirayita using *in vitro* assays. Botanica Orientalis: Journal of Plant Science. 2010; 7:48-55.
4. Mizushima Y, Kobayashi M. Interaction of anti-inflammatory drugs with serum proteins, especially with some biologically active proteins. Journal of Pharmacy and Pharmacology. 1968; 20(3):169-173.
5. Sakat SS, Juvekar AR. Comparative study of *Erythrina indica* Lam. (Febaceae) leaves extracts for antioxidant activity. Journal of Young Pharmacists. 2010; 2(1):63-67.
6. Malick CP, Singh MB. Analysis of total phenols. Plant enzymology and histo enzymology, Kalyani Publishers, New Delhi, 1980, 286.
7. Saxena SN, Karwa S, Saxena R, Sharma T, Sharma YK, Kakani RK *et al*. Analysis of antioxidant activity, phenolic and flavanoids content of fenugreek (*Trigonellafoenum-graecum* L.) seed extracts. Int J Seed Spices. 2011; 1(1):38-43.
8. Chen HJ, Liu X, Yu ZC, Gan ZB, Qiu J, Lu JZ. Active flavanoids protect plasmid DNA damage induced by cigarette smoking. Centr South Pharm. 2013; 11:506-9.
9. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. Journal of food and drug analysis. 2002; 10(3).
10. Pourmorad F, Hosseinimehr SJ, Shahabimajd N. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. African journal of biotechnology. 2006; 5(11):16.
11. Sivasothy Y, Chong WK, Hamid A, Eldeen IM, Sulaiman SF, Awang K. Essential oils of Zingiber officinale var. rubrum Theilade and their antibacterial activities. Food Chemistry. 2011; 124(2):514-517.
12. Sabulal B, Dan M, Kurup R, Pradeep NS, Valsamma RK, George V. Caryophyllene-rich rhizome oil of Zingiber nimmonii from South India: Chemical characterization and antimicrobial activity. Phytochemistry. 2006; 67(22):2469-2473.
13. Dormans HJD, Deans SG. Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. J Appl. Microbiol. 2000; 88(2):308-316.
14. Meng F, Zuo G, Hao X, Wang G, Xiao H, Zhang J, Xu G. Antifungal activity of the benzo [c] phenanthridine alkaloids from Chelidonium majus Linn against resistant clinical yeast isolates. Journal of ethno pharmacology. 2009; 125(3):494-496.
15. Rahman MA, Sultana R, Akter R, Islam MS. Antidiarrheal and antidiabetic effect of ethanol extract of whole Ageratum conyzoides L. in albino rat model. African Journal of Pharmacy and Pharmacology. 2013; 7(23):1537-1545.
16. Yadav P, Singh D, Mallik A, Nayak S. Madhuca longifolia (Sapotaceae), a review of its traditional uses, phytochemistry and pharmacology. International Journal of Biomedical Research. 2012; 3(7):291-305.
17. Lachman J, Šulc M. Phenolics and antioxidant activity of wines during the winemaking process. Bornimer Agrartechnische Berichte. 2006; 55:161-168.