

## Research article

**Evaluation of *in vitro* antidiabetic, antioxidant, and antibacterial activities of phytochemicals from *Memecylon malabaricum* Cogn.**

Prathap H.M., Vadlapudi Kumar, Manjunatha T., Ruksana F., Vishala E., Savitharani M.

Department of Studies in Biochemistry, Davangere University, Shivagangothri, Davangere, 577007, Karnataka, India

(Received: December 2023      Revised: January 2024      Accepted: February 2024)

Corresponding author: Vadlapudi Kumar. Email: veeke2012@gmail.com

**ABSTRACT**

**Introduction and Aim:** Plant secondary metabolites have been found to have antidiabetic, antioxidant, and antimicrobial properties. *Memecylon malabaricum* Cogn., is one of endemic plant species belonging to the melastomataceae family which can be found in western ghats, sacred groves of Dakshina Kannada and Udupi districts of Karnataka, India. This study was to assess the antidiabetic, antioxidative and antimicrobial activities of alkaloids and saponins present in the plant *M. malabaricum*.

**Materials and Methods:** Leaves of *M. malabaricum* were collected from forests of Western Ghats region, Shivamogga district in Karnataka, India. Alkaloids and saponins were isolated and quantified as previously described. Antidiabetic, antioxidant, and antimicrobial activities were evaluated for alkaloids and saponins of *M. malabaricum* leaves.

**Results and Discussion:** Total alkaloid content and saponin content were found to be 147.407.00mg AE/g and 170.5347 mg QE/g and respectively. IC<sub>50</sub> of LAF and LSF for  $\alpha$ -amylase enzyme were found to be 62.117 and 151.058 mg/ml respectively, while for  $\alpha$ -glucosidase the LAF and LSF exhibited 30.008 and 43.872  $\mu$ g/ml IC<sub>50</sub> values respectively. At 100  $\mu$ g/ml concentration, the free radical scavenging activity of LSF reached around 74% for DPPH and 40% for nitric oxide. At 100  $\mu$ g/ml concentration, the scavenging activity of LAF reached around 45% for DPPH and 26% for nitric oxide. The LSF has significantly inhibited both Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus* bacteria compared to LAF.

**Conclusion:** Compared to saponin fraction, alkaloid fraction has shown better antidiabetic activity against both  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes *in vitro*. Saponin fraction has exhibited better antioxidant activity than the alkaloid fraction. The saponin fraction exhibited better antibacterial activity compared to the alkaloid fraction. From the results, it could be concluded that alkaloid fraction and saponin fraction of the *M. malabaricum* leaves could be used to manage/treat the diabetic condition, as they possess antioxidant properties.

**Keywords:** *Memecylon malabaricum*; *in vitro* antidiabetic activity; *in vitro* antioxidant activity; *in vitro* antibacterial activity.

**INTRODUCTION**

Plants produce and employ a range of secondary metabolic chemicals as a means of discouraging diseases and predators. Identifying novel bioactive molecules from these plant-based metabolites holds potential for creating powerful antimicrobial medications that can combat diseases that are resistant to pharmaceuticals (1). Several secondary metabolites demonstrated extremely strong biological activities that have been linked to antimicrobial activities; in certain instances, the phytochemical-containing plant extracts showed a higher zone of inhibition than an antibiotic, suggesting that these compounds have promising antibacterial properties (2). It has been discovered that secondary metabolites possess antibacterial, antioxidant, and antidiabetic qualities. For example, it has been demonstrated that plant-derived secondary metabolites have antidiabetic effects by targeting distinct targets in the treatment of diabetes (3,4). Furthermore, antioxidant characteristics of secondary metabolites generated from plants have been discovered to help prevent disorders linked to inflammation and oxidative stress (5).

*Memecylon malabaricum* Cogn., is an endemic species of plant in the melastomataceae family that grows in the western ghats of Karnataka, India, as well as the sacred woods of Dakshina Kannada and Udupi districts (6). The antibacterial, antioxidant, and antidiabetic properties of the methanolic crude extracts were assessed (7,8). Information about the phytochemicals in the plant that are involved in its antioxidant, antibacterial, and antidiabetic properties is not currently accessible. Till-date, few pharmacological or phytochemical studies have been carried out. The present study involved the extraction and estimation of total alkaloids and saponins, as well as the measurement of their antidiabetic and antioxidant activities using the DPPH and nitric oxide radical scavenging test methods. Agar well diffusion was employed to assess the antimicrobial effects.

**Materials and Methods****Collection of plant material and preparation of plant extracts**

Leaves of *M. malabaricum* were gathered in Western Ghats region of Shivamogga district, Karnataka, India,

specifically in the Chakranagar of Hosanagar taluk. After washing the fresh leaves under running tap water to get rid of any surface pollutants, leaves were rinsed with distilled water and allowed to dry in the shade for 15 days, or until their dry weight remained consistent. Using an electric blender the dried leaves were grounded to reducing the particle size. After that, the ground leaves powder was kept in an airtight container. The powder after defatting by using n-hexane, was subjected for phytochemicals extraction using a Soxhlet device. Then using the extracted alkaloid fraction, saponin fractions were isolated by standard procedures. Every cycle of the solvent extraction process was carried out for roughly 24 hours. After concentrating through a rotary evaporator, the resultant extracts (alkaloid fraction and saponin fractions) were refrigerated until that were processed and used further.

### Isolation of alkaloid fraction

The defatted *M. malabaricum* leaf powder was then Soxhlet extracted for 24 hours using 30% acetic acid in ethanol (9). The extract was then filtered and concentrated using a rotary evaporator. The chlorophyll pigments were removed by subjecting the extract to diethyl ether using a separatory funnel. The extract was then separated into numerous diethyl ether aliquots to guarantee that the chlorophyll entered the non-polar phase and collected the filtered material from the polar phase. Drop-wise additions of concentrated ammonium hydroxide were made to the filtrate until precipitation was achieved.

### Estimation of total alkaloids

The amount of alkaloid content in *M. malabaricum* LAF was determined using Bromocresol green (BCG) solution and standard atropine of various concentrations was used (10). The overall alkaloid content was reported as mg of AE per gram of extract.

### Quantitative assessment of saponins and alkaloids

#### Isolation of saponin fraction

The methanolic extract of *M. malabaricum* leaf was diluted to 20% using distilled water. Then, to exclude green pigments like chlorophyll, the extract was subjected to liquid-liquid partition using ethyl acetate. The resultant partitioned chlorophyll free methanolic extract was reduced to 40 ml using distilled water and 60 ml of n-butanol. Using 10 ml of 5% sodium chloride the combined n-butanol extract was washed twice and aqueous dark brown saponin fraction was separated in a separating funnel. The polar phase was then collected, concentrating and drying the resulting saponin fraction at 37 °C. The percent recovery of saponins was determined and maintained at 4°C (9).

### Estimation of total saponins

The saponin concentration in *M. malabaricum* LSF was determined by Vanillin reagent method (11). The test sample's total saponin content was measured using

*Quillaja* bark saponin equivalents, and expressed as Qs equivalents of SE in µg/mg extract.

### Evaluation of antidiabetic activity

The leaf alkaloid fraction (LAF) and leaf saponin fraction (LSF) of *M. malabaricum* were tested for  $\alpha$ -amylase inhibition using the 3,5-dinitrosalicylic acid (12). Ability of LAF and LSF's to block  $\alpha$ -glucosidase was assessed by using *p*-nitrophenyl- $\alpha$ -D glucopyranoside as substrate (13). Acarbose was employed as the positive control. The inhibitory action was described as the half maximum inhibitory concentration (IC<sub>50</sub>), which is a measure of the extract's/LAF and LSFs' efficacy in suppressing enzyme activity.

Inhibitory activity (%) =  $(1 - A_s/A_c) \times 100$

where, A<sub>s</sub>= absorbance of sample, A<sub>c</sub> = absorbance of control

### Evaluation of antioxidant activity

The 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) technique is widely used to assess the free radical scavenging capacity of natural substances (14). The free radical scavenging activity of *M. malabaricum*'s LAF and LSF was assessed against DPPH. Nitric oxide radical scavenging was also employed to test the antioxidant capacity of the LAF and LSF (15).

% Inhibition =  $\frac{\text{Absorbance of control} - \text{Absorbance of sample X}}{\text{Absorbance of control}} \times 100$

### Evaluation of antibacterial activity

With minor modifications, LAF and LSF were assessed for antibacterial activity using the agar-well diffusion method (16). Tests were conducted on the samples using the Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* bacterial strains. Using sterile saline solution, the inoculum was adjusted to roughly  $5 \times 10^5$  CFU/ ml. As a stock solution, samples were dissolved at a rate of 10 mg/ ml in DMSO. Various concentration ranges, ranging from 200 µg/ml to 800 µg/ml were placed into distinct wells. Mueller-Hinton (1941) agar medium (17) was used for antibacterial activity evaluation. Bacterial cultures were incubated at 37°C, and the inhibitory zones' diameters (in millimeters) were determined.

### Determination of minimum inhibitory concentration (MIC)

The broth dilution method (18) was used to determine the Minimum Inhibitory Concentrations (MIC). The LAF and LSF were dissolved in DMSO by serially diluting from the stock solutions, ranging from 0 µg/ ml to 800 µg/ ml (w/v). Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* were the two pathogens against which the samples were tested. Using a micropipette the growth medium was mixed with an equivalent volume (10 µl) of inoculum adjusted to  $5 \times 10^5$  CFU/ ml in the designated wells.

Plates were incubated for 48 hours at 37°C. The lowest concentration that prevented the tested microorganisms from growing was indicated by the MIC values.

**RESULTS**

Alkaloids and saponins were extracted from the leaves of *M. malabaricum*. Alkaloids and saponins found in LAF and LSF respectively were quantified using a slightly modified version of the previously recommended protocol. Total alkaloid content was  $147.407 \pm 15.00$  mg AE/g, while the total saponin content was  $170.5347 \pm 22.57$  mg QE/g.

**Evaluation of antidiabetic activity**

$\alpha$ -amylase and  $\alpha$ -glucosidase enzymes were used to perform antidiabetic activities on LAF and LSF. The

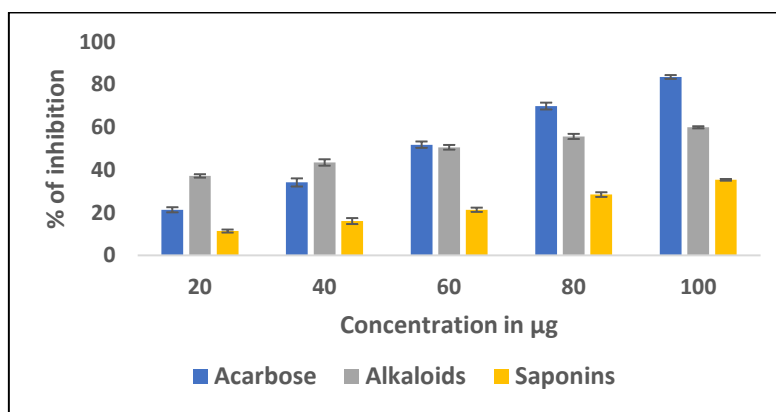
results are shown in Table 1. Results pertaining to inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzyme activities by LAF and LSF are depicted (Fig. 1 and 2). The outcomes were compared with those of the standard acarbose.

**Evaluation of antioxidant activity**

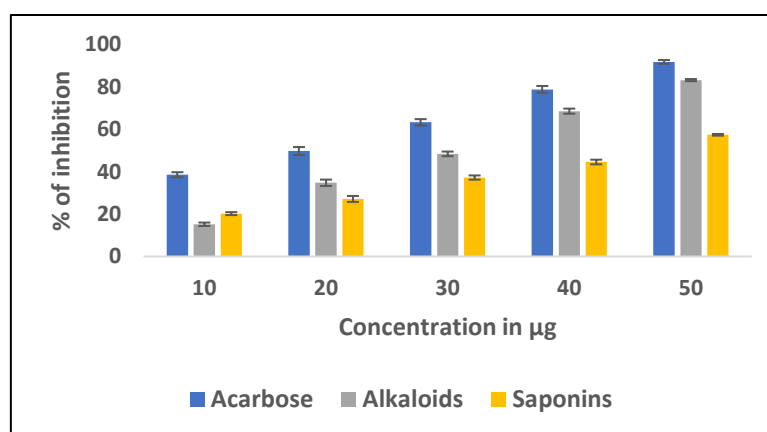
The *in vitro* antioxidant activity of the LAF and LSF was evaluated, the IC<sub>50</sub> values are depicted in Table 2. The suppression of DPPH and nitric oxide scavenging activity are shown (Fig. 3 and 4). By suppressing the free radicals, LSF demonstrated the maximum inhibitory activity of 74% for DPPH and 40% for nitric oxide when compared to LAF, in contrast to standard ascorbic acid antioxidant activity.

**Table 1:** Antidiabetic efficacy of *M. malabaricum* LAF and LSF: IC<sub>50</sub> values of  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition

Sample	$\alpha$ -amylase	$\alpha$ -glucosidase
Ascorbic acid	57.31	19.379
LAF	62.117	30.008
LSF	151.058	43.872



**Fig. 1:**  $\alpha$ -Amylase activity inhibition by *M. malabaricum* LAF and LSF



**Fig. 2:**  $\alpha$ -Glucosidase activity inhibition by *M. malabaricum* LAF and LSF

**Table 2:** Antioxidant efficacy of *M. malabaricum* LAF and LSF: IC<sub>50</sub> values of DPPH radical and nitric oxide radical scavenging

Sample name	DPPH ( $\mu$ g/ml)	Nitric oxide ( $\mu$ g/ml)
Ascorbic acid	10.242	59.439
LAF	110.99	182.877
LSF	17.97	126.837

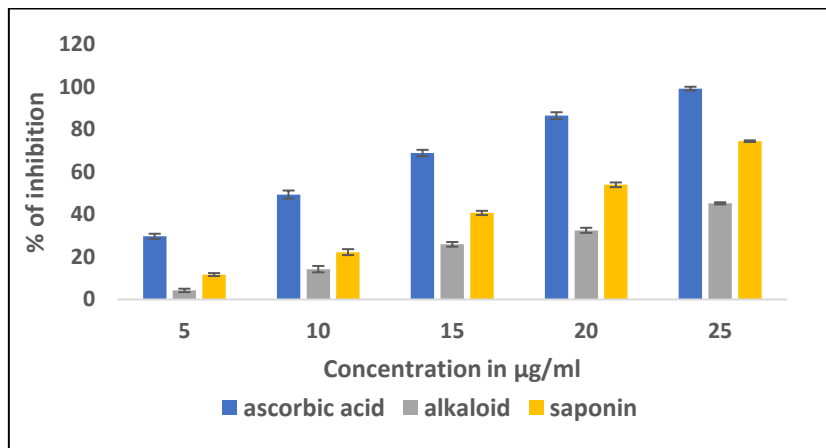


Fig. 3: DPPH radical scavenging activity of *M. malabaricum* LAF and LSF

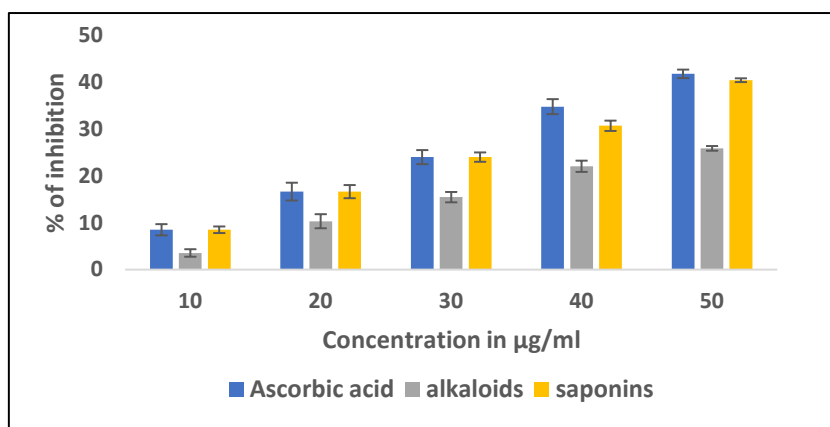


Fig. 4: Nitric oxide radical scavenging activity of *M. malabaricum* LAF and LSF

Table 3: Antibacterial properties of *M. malabaricum* LAF and LSF (inhibition zones)

No.	Sample Name	Conc. (µg/ml)	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>
1	LAF	200	---	---
		400	---	---
		600	6.33 ± 0.57	5.66 ± 0.57
		800	9.66 ± 0.57	8.33 ± 1.15
2	LSF	200	---	---
		400	---	---
		600	10.66 ± 0.57	10.66 ± 0.57
		800	12.33 ± 1.15	13.33 ± 0.57
3	Kanamycin	10	8.33 ± 0.57	8.66 ± 0.57
		20	11.66 ± 0.57	11.33 ± 0.57
		30	13.33 ± 1.15	14.66 ± 0.57
		40	17.66 ± 0.57	17.33 ± 0.57

Table 4: Antibacterial activities of *M. malabaricum* LAF and LSF (MIC)

Sl. No.	Sample name	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>
1	LAF	150	100
2	LSF	200	150
3	Kanamycin (Positive control)	5	5

### Evaluation of antibacterial activity

To determine the antibacterial activity of LAF and LSF against both Gram-positive and Gram-negative bacteria by agar well diffusion method. The outcomes are compiled in Table 3. In this case, LSF exhibited a larger inhibition zone than LAF. Table 4 presents the tabulated

MIC for both LAF and LSF. All the antibacterial activity was compared to standard Kanamycin.

### DISCUSSION

Previous investigations have proved that methanolic extract of *M. malabaricum* plant possesses antidiabetic, antioxidant, anticancer and antimicrobial activities (8). However, till-date there have been no published reports

on specific phytochemical fractions of *M. malabaricum* that are responsible for these biological properties. The results of the present study demonstrated some of the biological activities like antidiabetic, antioxidative, and antibacterial efficacy of alkaloids and saponins present in *M. malabaricum*.

The antidiabetic effect of *M. malabaricum* LAF and LSF was assessed by comparing them to the standard acarbose and measuring their ability to inhibit the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. Compared to acarbose, LAF and LSF were found to have lesser inhibition ability of these two enzymes evaluated for antidiabetic effect. At 100  $\mu$ g/ml the inhibition activity of LAF reached approximately 60% for  $\alpha$ -amylase and 50% for  $\alpha$ -glucosidase inhibition, while at the same concentration LSF reached approximately 35% for  $\alpha$ -amylase and 59% for  $\alpha$ -glucosidase inhibition (Fig 1 & 2). The IC<sub>50</sub> values of LAF and LSF for  $\alpha$ -amylase were determined to be 62.117 and 151.058  $\mu$ g/ml respectively, whereas for acarbose IC<sub>50</sub> value was 51.31  $\mu$ g/ml. The IC<sub>50</sub> values of LAF and LSF for  $\alpha$ -glucosidase were determined to be 30.008 and 43.872  $\mu$ g/ml respectively, whereas for acarbose IC<sub>50</sub> value was 19.379  $\mu$ g/ml. These results confirm that, though *M. malabaricum* LAF and LSF inhibited both  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes, these phytochemical fractions have been found to have lesser efficacy compared to acarbose.

Enzymes like  $\alpha$ -amylase and  $\alpha$ -glucosidase hydrolyze starch into maltose and glucose. Although different prescribed anti-diabetic medicines can help treat diabetes mellitus, they have several negative effects. A naturally occurring selective inhibitor for  $\alpha$ -amylase and  $\alpha$ -glucosidase activity is necessary (19). The antidiabetic effect of LAF and LSF was assessed by comparing them to the standard Acarbose and measuring their ability to inhibit the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. Compared to Acarbose, LAF and LSF were found to have comparatively less antidiabetic effect. At 100 $\mu$ g/ml the inhibition activity of LAF reached approximately 60% for  $\alpha$ -amylase and 50% for  $\alpha$ -glucosidase, whereas LSF reached approximately 35% for  $\alpha$ -amylase and 59% for  $\alpha$ -glucosidase for the same concentration (Figs. 1, 2). When compared to the previous study that was carried out using crude methanolic extract the  $\alpha$ -amylase enzyme has shown more inhibition for the above fractions (20).

Antioxidants are chemicals that can quench free radicals and mitigate their deleterious effects. Their functioning is dependent on the donation of an electron, which facilitates the stability of unpaired electrons within free radicals while remaining inert themselves. Scavenging is a procedure that neutralizes free radicals (21). The abundance of bioactive constituents such as alkaloids and saponins revealed a diverse range of biological functions and produced significant antioxidant properties. Through comparison with conventional ascorbic acid, the LAF and LSF has

shown the antioxidant activity by scavenging free radicals such as DPPH and nitric oxide. The results showed that LSF, however somewhat below the benchmark, possesses strong scavenging action against both DPPH and Nitric oxide. Approximately 74% of DPPH (Fig. 3) and 40% of nitric oxide (Fig. 4) were scavenged by LSF at a concentration of 100  $\mu$ g/ml. Approximately 45% of DPPH (Fig. 3) and 26% of nitric oxide (Fig. 4) were scavenged by LAF for the same concentration (100  $\mu$ g/ml). Alkaloids from plants can postpone or ameliorate diabetes due to their antioxidant, anti-inflammatory, immunomodulatory, and enzymatic properties (22). In our case the alkaloids are inhibited both the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes.

Saponins are glycosides found in many plants and have been demonstrated to exhibit antimicrobial activity against a range of bacteria and fungi (23). *E. coli*, *S. aureus*, *P. aeruginosa*, *B. subtilis*, and *S. typhi* have shown inhibition for the crude methanolic extract of the plant previously (7,8). The present study describes that compared to alkaloids, saponins have shown superior efficacy against Gram-positive and Gram-negative bacteria. Some alkaloids have been demonstrated to have antibacterial activity however they are often less efficient than saponins (24). Therefore, the saponin content of the *M. malabaricum* leaves can be credited with the exceptional antibacterial activity compared to alkaloids.

## CONCLUSION

*In vitro* tests against the enzymes  $\alpha$ -amylase and  $\alpha$ -glucosidase have demonstrated that alkaloids had the strongest antidiabetic action when compared to saponins. When it comes to antioxidant action, saponins have outperformed alkaloids. The plant's leaves include alkaloids and saponins that may be used to treat the antidiabetic effect. The samples can be utilized as antioxidants as well. When compared to alkaloids, saponins showed more antibacterial action. This is the first research that we are aware of regarding the antidiabetic, antioxidant, and antibacterial properties of the alkaloids and saponins found in the leaves of *M. malabaricum*. Purified alkaloids and saponins in the leaves of *M. malabaricum* may offer better biological properties with lesser IC<sub>50</sub> values. Further analysis using techniques like GC-MS, LC-MS, NMR, and FTIR on purified compounds of alkaloids and saponins is necessary to further our understanding of their structure and mechanism of action.

## CONFLICT OF INTEREST

There are no potential conflicts of interest throughout the course of this inquiry, according to the authors.

## REFERENCES

1. Keita, K., Darkoh, C., Okafor, F. Secondary plant metabolites as potent drug candidates against antimicrobial-resistant pathogens. SN Appl Sci. 2022;4:209.

2. Compean, K.L., Ynalvez, R.A. Antimicrobial activity of plant secondary metabolites: A review. *Res J Med Plants*. 2014; 8: 204-213.
3. Sonkamble, V.V., Wagh, N.S., Pai, S.R. Role of plant secondary metabolites as antidiabetic agents. In: Akhtar, M., Swamy, M., Sinniah, U. (eds) *Natural Bio-active Compounds*. Springer. 2019; p.529-550
4. Tran, N., Pham, B., Le, L. Bioactive compounds in anti-diabetic plants: From herbal medicine to modern drug discovery. *Biology*. 2020; 9:252.
5. Zeeshan Bhatti, M., Ismail, H., Khan Kayani, W. Plant secondary metabolites: Therapeutic potential and pharmacological properties Secondary metabolites - Trends and reviews. IntechOpen, 2022. Available from: <http://dx.doi.org/10.5772/intechopen.103698>
6. Bhandary, M.J., Chandrashekar, K.R. Sacred groves of Dakshina Kannada and Udupi districts of Karnataka. *Current Sci*. 2003; 85 (12): 1655-1656.
7. Hullatti, K.K., Rai, V.R. Antimicrobial activity of *Memecylon malabaricum* leaves. *Fitoterapia*. 2004; 409-411.
8. Namratha, P.H., Basavaraj, S.H. Isolation, identification and *in vitro* biological evaluation of phytochemicals from *Memecylon randerianum*: a medicinal plant endemic to Western Ghats of India. *Nat Prod Res*. 2021;35(23):5334-5338.
9. Edeoga, H.O., Okwu, D.E., Mbaeble, B.O. Phytochemical constituents of some Nigerian medicinal plants. *Afr J Biotechnol*. 2005; 4: 685-688.
10. Makkar, H.P.S., Siddhuraju, P., Becker, K. Plant secondary metabolites: Methods in Molecular Biology, Humana Press Inc., Totowa NJ, 2007; vol. 393.
11. Rao, T. M., Rao, B. G., Rao, Y. V. Antioxidant activity of *Spilanthes acmella* extracts. *Intl J Phytopharmacol*. 2012;3(2): 216-220.
12. Poovitha, S., Parani, M. *In vitro* and *in vivo*  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibiting activities of the protein extracts from two varieties of bitter melon (*Momordica charantia* L.). *BMC Complement, Altern Med*. 2016;16:185.
13. Tsujii, E., Muroi, M., Shiragami, N., Takatsuki, A. Nectrisine is a potent inhibitor of alpha-glucosidases, demonstrating activities similarly at enzyme and cellular levels. *Biochem Biophys Res Commun*. 1996; 220:459-466.
14. Blois, M.S. Antioxidant determinations by the use of a stable free radical. *Nature*. 1958; 181(4617): 1199-1200.
15. Rao, M.N. S. Nitric oxide scavenging by curcuminoids. *J Pharm Pharmacol*. 1997;49:105-107.
16. Nagashree, S., Karthik, C.S., Sudarshan, B.L., Mallesha, L., Spoorthy, H.P., Sanjay, K.R., et al., *In vitro* antimicrobial activity of new 2-amino-4-chloropyridine derivatives: A structure-activity relationship study. *J Pharm Res*. 2015; 9(8): 509-516.
17. Mueller, J.H., Hinton, J.A. Protein-free medium for primary isolation of the *Gonococcus* and *Meningococcus*. *Proc Soc Exp Biol Med*. 1941;48(1):330-333.
18. Mann, C.M., Markham, J.L. A new method for determining the minimum inhibitory concentration of essential oils. *J Appl Microbiol*. 1998;84:538.
19. Piparo, E.L., Scheib, H., Frei, N., Williamson, G., Grigorov, M., Chou, C.J., et al., Flavonoids for controlling starch digestion: Structural requirements for inhibiting human alpha-amylase. *J Med Chem*. 2008;51:3555-3561.
20. Tejavathi, D.H., Sumalatha, B.S., Phytochemical, antioxidant, and anti-diabetic analysis of leaf and stem extracts of *Memecylon malabaricum* (C.B.Clarke) Cogn. *Int J Pharm Sci Drug Res*. 2020; 12(6):606-613.
21. Wong, F.C., Yong, A.L., Ting, E.P., Khoo, S.C., Ong, H.C., Chai, T.T., et al., Antioxidant, metal chelating, anti-glucosidase activities and phytochemical analysis of selected tropical medicinal plants. *Iran J Pharm Res*. 2014;13(4):1409-1415.
22. Rajani, S.A. Mini review on antidiabetic plant derived alkaloids. *Indian J Sci Res*. 2020;10(2):137-141.
23. Ngoufack, T.C., Tamokou, J.D., Kengne, I.C. Antimicrobial activities of saponins from *Melanthera elliptica* and their synergistic effects with antibiotics against pathogenic phenotypes. *BMC Chem*. 2018;12:97.
24. Raji, P., Samrot, A.V., Keerthana, D. Antibacterial Activity of alkaloids, flavonoids, saponins and tannins mediated green synthesized silver nanoparticles against *Pseudomonas aeruginosa* and *Bacillus subtilis*. *J Clust Sci*. 2019;30:881-895.