Nepal Journal of Biotechnology
Publisher: Biotechnology Society of Nepal
Journal Homepage: www.nepjol.info/index.php/njb
ISSN (Online): 2467-9313
ISSN (Print): 2091-1130

Phytochemical Analysis and α-Amylase Inhibitory Activity of Young and Mature Leaves of *Cinnamomum tamala*

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Received: 5th Nov 2020; Revised: 22nd Sep 2021; Accepted: 6th Dec 2021; Published online: 31st Dec 2021

Abstract
The bioactive chemical components of the plant's origin have been used as primary remedies for a wide array of human diseases including diabetes. The present research deal to evaluate and compare anti-diabetic potential of ethanolic and methanolic, young and mature leaves of medicinally valuable *Cinnamomum tamala*. Total phenolic and flavonoid contents of young and mature leaves were determined. *In vitro* *a*-amylase inhibition was carried out using 2-chloro-4-nitrophenyl-*a*-D-maltotrioside (CNPG3) as substrate. Phytochemical screening revealed the presence of polyphenols, flavonoids, terpenoids, quinones, carbohydrates, glycosides, diterpenes, tannins, and reducing sugars. The highest total phenolic content and flavonoid content were observed in methanolic extract of mature leaves (13.725 ± 0.54 mg GAE/g) and young leaves (12.591 ± 0.71 mg QE/g) respectively. Methanolic young leaves extract showed *a*-amylase inhibition with IC_{50} value 224.6 ± 2.76 µg/mL as compared to acarbose with IC_{50} value 5.93 ± 0.14 µg/mL. The result suggests that young leaves of *C. tamala* had anti-diabetic activity so further work should be carried out.

Keywords: *a*-Amylase, Anti-diabetic, *Cinnamomum tamala*, Phytochemicals

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Introduction
Diabetes mellitus is one of the most common health issues among the serious health concerns around the world. It is a chronic multifactorial endocrine disorder of glucose intolerance. Approximately 463 million adults (20-79 years) suffered from diabetes worldwide in 2019 and may escalate to 700 million by 2045. In Nepal, about 696,900 adults are living with diabetes [1]. Among three type of diabetes, Type 1 diabetes occur due to insulin deficiency, type 2 diabetes occur due to inability to utilize insulin and gestational diabetes occur during pregnancy condition [2]. Among them, diabetes of type 2 is the most severe and fast-growing in most countries majorly due to rapid urbanization, diet, lifestyles [3,4]. In hyperglycemia, the rise in blood glucose levels generates excessive superoxide anions which produce hydroxyl radicals through Haber Weiss reaction that can lead to the peroxidation of membrane lipids, oxidative protein damage to cell membranes, and also affects other biomolecules including carbohydrates, proteins, and DNA [5]. Consequently, long-term diabetes leads to stroke, blindness, heart attack, kidney failure, amputation, and the menace of dying prematurely [2]. In recent years, the therapeutic approach for diabetes patients is diet control, exercise, and hypoglycemic drugs such as sulphonylureas, acarbose, and insulin with undesirable side effects such as liver toxicity, lactic acidosis, and diarrhea [6].

In this regard, inhibition of key carbohydrate digestive enzymes (*a*-amylase) is considered one of the best therapeutic techniques for the treatment of diabetes and its associated diseases. Pancreatic *a*-amylase is also known as 1, 4-*a*-D-glucan glucanohydrolase EC (3.2.1.1) plays a vital role in the digestion of starch molecules in the human body. At the first stage, there is partial digestion of starch by salivary amylase enzyme. The enzyme cleaves polymeric substrate (Starch) into shorter oligomers. At the second stage, these shorter oligomers are further split into maltose, maltotriose, and small malto-oligosaccharides with the help of pancreatic *a*-amylase in the gut [7]. In this present study, *a*-amylase catalyzes the endohydrolysis of *a*-1, 4 glycosidic linkages of 2-chloro-4-nitrophenyl-*a*-D-maltotrioside (CNPG3) to yield free chromophore, 2-chloro-nitrophenol (CNP) and release 2-chloro-4-nitrophenyl-*a*-D-maltoside (CNPG2), maltotriose (G3), and glucose (G). The rate of increase in chromophore absorbance is related to the *a*-amylase activity of the sample [8,9]. In recent years, the scientist has been so much attracted on the exploration of the secondary metabolites of plants as inhibitors for most effective formulation aspect of drugs with few or hardly negative effects. The medicinal plant contains various natural antioxidants such as tocopherols, vitamin C, and

phenolic compounds which are capable to neutralize oxidative damage and protecting from various diseases including diabetes [10].

Plants have been the primary medicinal source for centuries to cure a wide array of human diseases, particularly in developing countries like Nepal due to scarce resources, affordability, and insufficient access to conventional treatment. Thus, considering the extraordinary biodiversity of Nepal, there is a need for scientific investigations of plant species. Among the various medicinal plants, *C. tamala* is the focus of this research. *C. tamala* (Buch.-Ham.) T. Nees and Eberm (Luraceace) is one of several traditional remedies used under the Ayurvedic system in Nepal. Its leaves are commonly called tejpat and are used as a common ingredient in cooking. This species is a perennial or evergreen tree up to 7.5 m in height and a girth of about 1.4 cm [11]. Young leaves red-brown, smooth and mature leaves sericeous, glabrescent, and rarely glaucous [12]. Leaves are natural food preservatives for pineapple juice [13]. Besides, tejpat is also a traditional dye-yielding plant [14]. It has been traditionally used as an astringent, stimulant, and carminative [15]. *C. tamala* leaves exhibited significant biological properties with scientific validation including antioxidant [16,17], antimicrobial [18,19,20], anti-diabetic [21,22,23], anticancer [24], and anti-inflammatory [25].

To the best of the authors' knowledge, no scientific literature was published regarding the comparison of young and mature leaves extracts of *C. tamala* to evaluate their bioactivities. The aim of this research was therefore to estimate total phenolic and total flavonoid contents along with the evaluation of *in vitro* anti-diabetic activity of the extracts of *C. tamala* by the microplate-based method.

Materials and Methods

Chemicals and reagent

Methanol, ethanol, folin-ciocalteu (FC) reagent were purchased from Qualigens, sodium carbonate, dimethyl sulphoxide (DMSO), potassium acetate, disodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate dihydrate, and sodium chloride were obtained from Fisher Scientific, gallic acid, and quercetin from Hi-media, aluminum chloride from Merck and acarbose, α-amylase from porcine pancreas, and 2-chloro-4-nitrophenyl-α-D-maltotrioside were purchased from Sigma.

Collection and authentication of plant materials

Fresh young and mature leaves of C. tamala were collected separately from Machhegaun, Kathmandu in July 2019. A herbarium specimen was deposited at the Central Department of Botany, Tribhuvan University, and voucher code RM001 was provided.

Extraction

The collected young and mature leaves were washed properly with water, shade dried, and ground into powder. The extraction was done by cold percolation method. In short, the powder of young and mature leaves was soaked in methanol and ethanol for 72 hrs with occasional shaking at room temperature. After 72 hrs, the solvent was filtered and the filtrate was evaporated using a rotary evaporator under vacuum at 40^0 C [26]. The percentage yield was calculated by the given formula:

$$\% \text{ Yield} = \frac{\text{Dry weight of extract}}{\text{Dry weight of a plant}} \times 100$$

Phytochemical screening

The presence of phytochemicals in the young and mature leaves of *C. tamala* was analyzed through the following standard protocol [27, 28, 29].

Estimation of Total Phenolic Content (TPC)

Crude extract of young and mature leaves of *C. tamala* was estimated for TPC by using FC reagent [30,31,32]. In brief, 20 µL sample was added with 100 µL Folin-ciocalteu (2 N), accompanied by 80 µL Na_2CO_3 (1N). The reaction mixture was incubated in dark at room temperature for 15 minutes until the dark blue color was observed. Finally, the absorbance was taken at 765 nm using a spectrophotometer (Synergy LX, BioTek, Instruments, Inc., USA). A standard calibration curve for gallic acid (10-100 µg/mL) was prepared and phenolic content was expressed as milligrams of gallic acid equivalent per gram of dry weight of the extract (mg GAE/g). All experiments were performed in triplicate and expressed as mean ± standard error of mean.

Estimation of Total Flavonoid Content (TFC)

Crude extract of young and mature leaves of *C. tamala* was estimated for TFC by using the aluminum trichloride ($AlCl_3$) method [32,33]. Briefly, 20 µL sample with 110 µL distilled water was added with 60 µL ethanol, 5 µL $AlCl_3$ (10%), and 5 µL potassium acetate (1 M). The reaction mixture was incubated in dark at room temperature for 30 minutes. After incubation, absorbance was measured at 415 nm using a spectrophotometer (Synergy LX, BioTek, Instruments, Inc., USA). A standard calibration curve for quercetin (10 - 100 µg/mL) was prepared and

flavonoid content was expressed as milligrams of quercetin equivalent per gram of dry weight of the extract (mg QE/g). All experiments were done in triplicate and expressed as mean ± standard error of mean.

In vitro α-amylase inhibition assay

The *in vitro a*-amylase inhibition was performed in a microplate reader as previously described method [34]. Firstly, young and mature leaves extract was prepared using 50% DMSO, and the reaction was carried out using 50 mM sodium phosphate buffer (pH 7) with 0.9% NaCl. Concisely, 20 µL of extract of different concentrations (31.25 - 1000 µg/mL) was mixed with 80 µL of the enzyme at a final concentration of 1.5 U/mL. Then, it was pre-incubated at 37 °C for 10 min and after pre-incubation, 100 µL CNPG3 substrate at a final concentration of 1 mM was added and left for 15 minutes at the same temperature. The absorbance was measured at 405 nm. DMSO (Not more than 5% was taken as final concentration) was taken as negative control and acarbose as a positive control. All experiments were carried out in triplicate.

$$\% \text{ Inhibition} = \frac{\text{A(control)} - \text{A(sample)}}{\text{A(control)}} \times 100$$

A (control) = Absorbance of enzyme-substrate with 50% DMSO

A (sample) = Absorbance of enzyme-substrate with extract as inhibitor.

Statistical analysis

The data were analyzed by using Gen 5 Microplate Data Collection and Analysis Software of microplate reader and then by MS Excel. The IC_{50} value of crude extract was calculated by using Graph Pad Prism version 8 software and the results were expressed as mean ± Standard error mean (SEM).

Results

Phytochemical screening

The percentage yield was found to be higher in methanol as compared to ethanol and are given in **Table 1**. The range of percentage yield of mature and young leaves varies from 7.36 to 18.22%. The young leaf had the highest percentage yield (EEY 10.22 % and MEY 18.22 %) and the mature leaf had the lowest percentage yield (EEM 7.36 % and MEM 12.24 %).

The different phytochemical tests had shown the presence of polyphenols, flavonoids, tannins, quinones, carbohydrates, glycosides, terpenoids, and reducing sugar whereas basic alkaloids are absent in both extracts of *C. tamala* (**Table 2**).

Table 1. Percentage yield of different extract of *C. tamala*

S. No.	Sample	Percentage yield (%)
1	Ethanolic extract of mature leaf (EEM)	7.36
2	Ethanolic extract of young leaf (EEY)	10.22
3	Methanolic extract of mature leaf (MEM)	12.24
4	Methanolic extract of young leaf (MEY)	18.22

Table 2. Preliminary phytochemical screening of different extract of *C. tamala*

S. No.	Phytochemicals	MEM	MEY	EEM	EEY
1	Polyphenols	+	+	+	+
2	Flavonoids	+	+	+	+
3	Tannins	+	+	+	+
4	Quinones	+	+	+	+
5	Carbohydrates	+	+	+	+
6	Glycosides	+	+	+	+
7	Terpenoids	+	+	+	+
8	Diterpenes	+	+	+	+
9	Reducing sugars	+	+	+	+
10	Basic alkaloids	-	-	-	-

"+" indicates presence, "-" indicates absence

Total Phenolic and Flavonoid Content

The TPC value of ethanolic young and mature leaves extract was 9.83 ± 0.30 mg GAE/g and 8.91 ± 0.39 mg GAE/g respectively whereas, the TPC value of methanolic young and mature leaves extract was 11.26 ± 0.17 mg GAE/g and 13.73 ± 0.55 mg GAE/g. The TFC value of ethanolic young and mature leaves extract was 6.82 ± 0.67 mg QE/g and 7.02 ± 0.32 mg QE/g respectively whereas, the TFC value of methanolic young and mature leaves extract was 12.59 ± 0.71 mg QE/g and 11.84 ± 0.66 mg QE/g as shown in **Table 3**.

Table 3. Total phenolic and flavonoid content of different extract of C. tamala

S. No.	Sample	TPC mg GAE/g	TFC mg QE/g
1	MEM	13.73 ± 0.55	11.84 ± 0.66
2	MEY	11.26 ± 0.17	12.59 ± 0.71
3	EEM	8.91 ± 0.39	7.02 ± 0.32
4	EEY	9.83 ± 0.30	6.82 ± 0.67

Mean ± SEM (n = 3)

In vitro α-amylase inhibition assay

Table 4. Screening for *a*-amylase inhibition of different extract

S. No.	Sample	Concentration	% Inhibition
1	MEM	500 µg/mL	< 50
2	MEY	500 µg/mL	70.33 ± 0.47
3	EEM	500 µg/mL	< 50
4	EEY	500 µg/mL	< 50
5	Acarbose	50 µg/mL	96.77± 0.028

Mean ± SEM (n = 3)

The percentage inhibition of young, and mature leaves of *C. tamala* using methnol and ethanol as solvent were screened for *α*-amylase inhibitory activity at 500 µg/mL **(Table 4)**. Young leaf had the highest inhibition of *α*-

amylase enzyme whereas mature leaf showed the lowest inhibition at the same concentration in our study.

Only methanolic extract of young leaves had shown 70.33% inhibition while screening. So, the methanolic extract of young leaves was further assessed for IC_{50} value at different concentrations (**Table 5)** and was found to be 224.6 ± 2.76 µg/mL. However, compared to standard drug acarbose, the methanolic extract of young leaves showed moderate *α*-amylase inhibitory activity (**Table 6)** and was found to be 5.93± 0.14 as shown in **Table 7**.

Table 5. *a*-Amylase inhibition of methanolic extract of young leaves (MEY) at different concentration

Concentration	Inhibition 1	Inhibition 2	Inhibition 3	Mean ± SEM
500 µg/mL	70.15	69.62	71.23	70.33 ± 0.47
250 µg/mL	54.80	58.59	58.38	57.26 ± 1.23
125 µg/mL	33.37	35.45	31.89	33.57 ± 1.03
62.50 µg/mL	14.89	9.46	10.04	11.46 ± 1.72
31.25 µg/mL	2.83	3.78	6.78	4.46 ± 1.19

Mean ± SEM (n = 3)

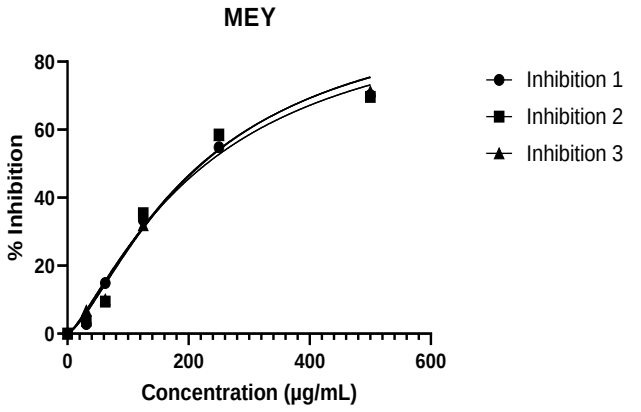

Table 6. *a*-Amylase inhibition of acarbose at different concentration

Concentration	Inhibition 1	Inhibition 2	Inhibition 3	Mean± SEM
50 µg/mL	96.82	96.75	96.72	96.76± 0.02
25 µg/mL	90.08	89.93	89.80	89.94± 0.08
12.5 µg/mL	77.72	77.07	76.88	77.22± 0.25
6.25 µg/mL	55.44	51.92	53.38	53.58± 1.02
3.125 µg/mL	24.27	23.77	33.52	27.19± 3.16
1.563 µg/mL	5.33	6.64	4.01	5.33± 0.75

Mean ± SEM (n = 3)

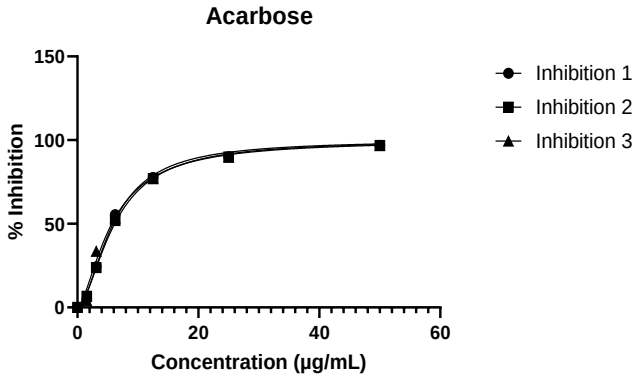

Table 7. IC_{50} values of acarbose and potent sample (MEY)

S. No.	Sample	IC_{50} (µg/mL)
1	Acarbose	5.93± 0.14
2	MEY	224.6 ± 2.76

Mean ± SEM (n = 3)

Discussion

Diabetes has been stated as the primary cause of global morbidity and mortality [35]. Retarding the absorption and digestion of carbohydrates in the intestine through inhibition of carbohydrate digestive enzymes such as *a*-amylase is one of the therapeutic strategies to minimizing postprandial hyperglycemia [36, 37, 38]. This study reported the potential *in vitro* amylase inhibitory properties of *C. tamala* mature and young leaf by identifying its main phytochemical constituents. Extraction of phytochemicals is the preliminary process for recovering and isolating compounds from the pulverization of plant materials [39]. The extraction yield depends upon different parameters such as solvent with varying polarity, pH, temperature, extraction time, and sample composition [40]. In our study methanolic extracts showed (MEM, MEY) higher yield than that of ethanolic extracts (EEY, EEM). The difference might be due to the high polarity of methanol that may cause higher solubility of phenolics, flavonoids, alkaloids, and terpenoids compounds in methanol as compared to other solvents including ethanol [40, 41,42].

The phytochemicals obtained from *C. tamala* are shown in **Table 2** that was compared with the previously reported study and found similar phytochemical profiling [43]. Our findings exhibited that methanolic extracts have the higher TPC and TFC relative to ethanolic extracts as seen in **Table 3**. Aryal *et al.* suggested that numerous phenolic compounds are present in plant extracts that are responsible for enzyme inhibition supported by TPC (mg GAE/g) and TFC (mg QE/g) of MEM (13.73 ± 0.55, 11.84 ± 0.66), MEY (11.26 ± 0.17, 12.59 ± 0.71), EEM (8.91 ± 0.39, 7.02 ± 0.32), and EEY (9.83 ± 0.30, 6.82 ± 0.67) respectively [44]. Phenolics and flavonoids compounds such as quercetin, ferulic acid,

anthocyanins, catechin, and resveratrol are the reason behind controlling glycemia via *a*-glucosidase, *a*-amylase, and lipid peroxidation inhibition, insulin secretion, and accelerating glucose uptake [45,46]. The difference in TPC and TFC is due to different nature of extracting solvents [47]. Previously, it was revealed that younger leaves are most active in biosynthesis and accumulation of phenolic, flavonoid, and proanthocyanidins than the mature leaves of *Lantana camara* [48]. Similarly in our study, among young and mature leaf extracts, only methanolic extract of young leaf showed significant inhibition toward the *a*-amylase enzyme. The ethanolic extract of young leaf failed to show significant inhibition that might be due to its less polar nature causing lower yield of phenolics and flavonoids compounds.

Besides that, high flavonoid content of MEY might be responsible for high inhibition as compared to other extracts. Several potential *a*-amylase and *a*-glucosidase inhibitors from medicinal plants belongs to flavonoid class as reported by earlier study [49]. Phenolic compounds including flavonoids and tannins have been reported to play a vital role in controlling diabetes [50, 51, 52]. Phenolic compounds mainly flavonoids interact strongly with proteins and could inhibit their enzymatic activities by making complexes and changing conformation [53]. The IC_{50} value of acarbose as positive control showed around 40 times more potency than MEY as shown in **Table 5**. This might be due to plant extract contains a jumble of multiple compounds as compared to pure inhibitor compound acarbose [34, 54].

Previous studies reported that polyphenol compounds (proanthocyanidins) derived from different plant sources showed potent amylase inhibitors [55, 56]. A-type procyanidin oligomer of cinnamon led to potent antioxidant properties and acts as an insulin sensitizer [57]. Besides that, cinnamtannin D-1 (CD1) isolated from *C. tamala* was reported to shield pancreatic *β*-cells from palmitic acid-induced apoptosis and oral consumption of cinnamaldehyde had hypoglycemic and hypolipidemic activity in STZ-induced diabetic rats respectively [23, 58]. These compounds might be present in the extract of young leaves, so further isolation and molecular characterization are needed.

Conclusion

The present study evaluated phytochemical constituents as well as *a*-amylase inhibition of methanolic and ethanolic extracts from young and mature leaves of *C. tamala*. The *in vitro* results revealed that methanolic extract and young leaves possess higher antidiabetic activity. Thus, it was suggested that further investigation for isolation and characterization of a bioactive compound and its kinetic study from MEY should be carried out that can act as an anti-diabetic agent.

Abbreviations

GAE (Gallic Acid Equivalent), QE (Quercetin Equivalent), CNPG3 (2-chloro-4-nitrophenyl-*a*-D-maltotrioside), MEY (Methanolic Extract of Young leaf), MEM (Methanolic Extract of Mature Leaf), EEY (Ethanolic Extract of Young leaf), EEM (Ethanolic Extract of Mature Leaf), TPC (Total Phenolic Content), TFC (Total Flavonoid Content)

Author's contributions

This research was performed in collaboration with all authors. RM and KK have contributed to the plan of the research work. The first draft was written by RM, responsible for data analysis, who conducted technical work (Lab). SKK supervised the research project, PT revised the manuscript. The final manuscript was read and approved by all authors.

Competing interests

No competing interests.

Funding

The research was completed by financial support from the University Grant Commission (Sanothimi, Bhaktapur, Nepal).

Acknowledgments

The authors extend their appreciation to the Central Department of Chemistry, Tribhuvan University for providing an opportunity to conduct this research and Prof. Dr. Suresh Kumar Ghimire of Central Department of Botany, Tribhuvan University for plant identification

Ethical approval and consent

Not applicable

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