Original Research Article

A Comparative Analysis of Phytoconstituents, Cytotoxicity and Antidiabetic Activity of Three Regional *Piper betle* Varieties: First Report From Bangladesh

Abstract

Aim: *Piper betle* is one of the significant species of Piperaceae family that exhibit remarkable medicinal potentials including anticancer and antidiabetic activity. The present study aimed to conduct a comparative study on different regional betel leaves focusing on identification of active phytochemicals and evaluating cytotoxic and antidiabetic activity of leaf extracts.

Methodology: The *P. betle* plant leaves of Moheshkhali, Shaplapur, and Rajshahi regions were included in the study. Active phytochemicals in leaves of different varieties were screened. In addition, brine shrimp lethality assay and α-glucosidase inhibitory assay were conducted for investigating cytotoxicity and antidiabetic activity respectively of the leaf extracts.

Results: We found alkaloid, saponin, tannin, glycoside, phenol, steroid, terpenoid, and flavonoid except anthocyanin in phytochemical screening of three *P. betle* varieties. In cytotoxicity assay, the dichloromethane extract of Rajshahi variety showed the highest cytotoxic effect (100% mortality) with LC50 at 2.91 µg/mL in comparison with Moheshkhali (LC50 at 30.47 µg/mL) and Shahplapur variety (LC50 at 79.98 µg/mL). In antidiabetic assay, the highest (100%) α-

glucosidase inhibition activity was observed by the n-hexane extract of Rajshahi variety with IC50 at 0.001 µg/mL followed by 0.004 µg/mL and 0.015 µg/mL of Shaplapur and Moheshkhai variety respectively.

Conclusion: Collectively, our study ~~identified~~ showed the possible presence of a number of active phytochemical constituents among the three varieties of *P. betle* leaf extracts as well as potent ability of different leaf extracts in cytotoxicity and antidiabetic activity. We hope the *P. betle* leaf extracts of Moheshkhali variety can play significant role as potential medicinal use in treating cancer and diabetes.

Comment [AD1]: ???

Keywords *P. betle*; Phytoconstituents; Cytotoxicity; Antidiabetic activity; Moheshkhali variety

1. Introduction

Plants are a valuable source of natural ~~products which~~products, which are used as pharmaceuticals, flavors, fragrances, colors, bio-pesticides and food additives [1]. Plant-based natural products can be isolated from any part of the plant like bark, leaves, roots, fruits, seeds, fruit rind, etc. Any part may contain active component [2]. The use of plants as medicine in treating diseases is as old as human being. Medicinal plants have been used for centuries with a strong belief in their effectiveness in curing diseases. Although modern medicine is well developed, large sections of the populations in developing countries still rely on medicinal plants and herbal medicines in primary health care [3]. More than 80% of the world's population relies on plant-based medicine for primary healthcare, a system that developed over time by dynamic

interactions between people and their environment [4]. Several modern drugs inspired by the traditional plant medicine system are now in use, prescribed by the formal doctrines, in the treatment of various diseases. Since ancient times researchers are trying to find solutions to various diseases using the effective role of alkaloids, i.e., phytochemicals from medicinal plants which can also be used as candidates for the viral vaccine [5,6].

Piper betle is one of the significant species of Piperaceae family that exhibit remarkable medicinal potentials. They are dioecious, semi-woody, and perennial climbers with strongly flavored leaves which are commonly used as mouth fresheners [7,8,9]. The betel chewing criteria is famous specially in south-east Asia and the Indian subcontinent and its popularity includes Melanesia to Tikopia in the east, Africa to Madagascar in the west, Papua New Guinea in the south and southern China in the north [10]. However, *P. betle* contains a wide variety of phytoconstituents. Terpene-like bodies and phenols are the reason behind the strong pungent aromatic flavors of betel leaves [11]. Betel leaves are found to contain sugars, diastases, starch, and an essential oil composed of allyl pyrocatechol monoacetate, safrole, terpinen-4-ol, eugenol, eugenyl acetate, etc. [12,13]. The terpenoids include 1,8-cineole, camphene, cadinene, caryophyllene, pinene, limonene, chavicol, carvacrol, allyl pyrocatechol, chavibetol, eugenol, and safrole [14-16].

The betel leaf extracts have remarkable hypoglycemic activity. The suspension of leaf can significantly lower the blood glucose level and glycosylated hemoglobin and decrease the activity of liver fructose-1,6-bisphosphatase and glucose-6- phosphatase, but in Streptozocin (STZ) diabetic rats, liver hexokinase increased in comparison with the untreated diabetic rats [17-19]. Besides, studies evaluated that *P. betle* leaf extracts act as potent cytotoxic and possible anticancer property. The cytotoxic compounds in leaf extract also show potential antitumor or

anticancer property [20]. The brine shrimp cytotoxicity assay is considered as a convenient probe for preliminary assessment of toxicity [21].

P. betle is a locally available and widely used plant in Bangladesh. From early times, this plant has been used as a mouth freshener and a chewing agent. Without knowing the benefits of the plant, many local practitioners used to give the sap of betel leaves as remedies in treating several diseases. This point of view has attracted the researchers and made them inquisitive to study the bioactive compounds of betel leaves that are playing the role in the treatment. Though studies have been done on medicinal properties of *P. betle* in Bangladesh, comparative study using different varieties of this potential medicinal plant has not been done yet in our country. So, the present study aimed to conduct a comparative study for the first time on different regional betel leaf plants of Bangladesh focusing on identification of active phytochemicals and evaluating cytotoxic activity antidiabetic and of leaf extracts.

2. Materials and Methods

2.1 Sample collection and processing

The *Piper betle* plant leaves were collected from three specific regions of Bangladesh, i.e, Moheshkhali, Shaplapur, and Rajshahi. After collection, plant leaves were washed followed by air-dried for 3 days, and ground into fine powder. The powder was packed and stored at -20°C for analysis.

2.2 Preparation of plant extract

P. betle leaf extracts were prepared with n-hexane, dichloromethane ethyl acetate and ethanol for increasing polarity. Approximately, 180 gm of powdered leaves of each variety was soaked in 850 ml each organic solvents, followed by filtering. Each filtrate was collected and stored at 4°C till analysis.

2.3 Phytochemical screening

Screening of pharmacologically active substances in the plant extracts was done by applying following tests:

Test for Tannin: 20 ml of distilled water was added to 0.5 g of dried leaf powder, followed by boiling the mixture. Few drops of 0.1% ferric chloride were added to the mixture and a brownish-green color indicated the presence of tannin in the plant extract [22].

Test for Saponin: 2 g of dry leaf powder was soaked in 20 ml of distilled water. The solution was boiled and filtered. 3 drops of olive oil were added to 10 ml of the filtrate and formation of emulsion indicated the presence of saponin [22].

Test for Flavonoid: Leaf powder was soaked in 10 ml of ethyl acetate. The mixture was kept in a steam bath for 3 minutes and then filtered. 1 ml of dilute ammonia was added to 4 ml of filtrate and formation of yellow color indicated the presence of the flavonoid [22].

Test for Steroid: 0.5 g of ethanol extract was dissolved into 2 ml of acetic anhydride. 2 ml of H_2SO_4 was added to the mixture and formation of green color indicated the presence of steroids [22].

Test for Terpenoid: 2 mg of ethanol extract was dissolved in 1 ml of chloroform. 1.5 ml of concentrated H_2SO_4 was added to the mixture and formation of red-brown color at the interface indicated the presence of terpenoid [23].

Test for Anthocyanin: 2 ml of 2 N HCl and ammonium solution was added to 2 ml of aqueous plant extract. The pink-red to blue-violet color indicated the presence of anthocyanin [24].

Test for Alkaloid: In two separate test tubes, a small portion of ethanolic crude extract was acidified with 2 ml of 1% HCl. Each of the solutions was heated. Then few drops of Mayer's reagent were added to one test tube and few drops of Wagner's reagent were added to the other and formation of turbidity indicated the presence of alkaloids [25].

Test for Glycosides: 4 ml of glacial acetic acid and 1 drop of 2% $FeCl_3$ were added to 10 ml of aqueous plant extract. 1 ml of concentrated H_2SO_4 was then added to the mixture and formation of a brown ring between layers indicated the presence of glycoside [26].

2.4 Cytotoxic activity

The cytotoxicity of the plant extracts was measured by using brine shrimp lethality bioassay [27]. A stock concentration of 400 mg/ml in DMSO was prepared for each extract and subsequently used to make dilutions of 200 mg/ml, 100 mg/ml, 50 mg/ml and 25 mg/ml. Brine shrimp eggs were kept over 24 h to hatch in sea water inside a covered chamber of a duo compartment plastic container. Active nauplii were separated from the eggs and used for the cytotoxicity assay. Nauplii viability was measured in each extract by adding 10 ml of each dilution to bikers containing 10 ml of ~~sea water~~seawater and 20 nauplii. After 24 h, the surviving nauplii in each tube were counted, and the lethality percentage was calculated for each dilution of each extract by using the following formula

Mortality (%) = [(Total nauplii – Alive nauplii) / Total nauplii] ×100

2.5 Antidiabetic activity

α-glucosidase inhibitory assay was carried out in 96-well plate followed by Shai et al., 2011 [28]. with some modifications. A reaction mixture of 50 µL of phosphate buffer, 10 µL of an enzyme, and 20 µL of varying concentrations of extract of three different cultivars (400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml and 12.5 µg/ml) were incubated at 37°C for 15 min. Then, 20 µl of 5 mM substrate (p-nitrophenyl alpha-D-glucopyranoside) was added and incubated at 37 ◦C for 20 min. Finally, 0.1 M of Na2CO3 (50 µl) was added to stop the reaction. The released p-nitrophenol absorbance was measured (405 nm) by a microplate reader. DMSO was used as a control [29].

The inhibition percentage was assessed by the following formula:

% of Inhibition= [1- {(As-As_o) / (Ac-Ac_o)}] × 100

Where,

As: absorbance of plant extract + enzyme + phosphate buffer + substrate

As_o: absorbance of plant extract + sample + phosphate buffer+ substrate

Ac: absorbance of phosphate buffer + enzyme + substrate + 1% DMSO

Ac_o: absorbance of phosphate buffer + substrate + 1% DMSO

3. Results

3.1 Screening of phytochemical compounds

The phytochemical screening of leaf powder and ethanol extracts of *P. betle* revealed the presence of some secondary metabolites shown in Table 1.

Table 1 Phytochemical constituents of *P. betle* varieties

Tests	*P. betle* varieties		
	Moheshkhali	**Rajshahi**	**Shaplapur**
Steroid	+++	+++	+++
Terpenoid	+++	+++	+++
Alkaloid (Wagner's)	++	+	+++
Alkaloid (Mayer's)	+++	+++	+++
Flavonoid	+++	+++	+++
Saponin	++	-	+++
Tannin	+++	+++	+++
Glycosides	++	+++	+
Anthocyanin	-	-	-

Here, (-) means Absence; (+) means Present; (++) means Moderately present; (+++) means Appreciable amount

3.2 Cytotoxicity of *P. betle* extracts

The cytotoxicity analysis of three varieties of *P. betle* extracts is summarized in Fig. 1. *P. betle* from Moheshkhali region with dichloromethane extract showed an appreciative cytotoxic effect with an LC_{50} value at 30.47 µg/mL compared to the other three extracts. The LC_{50} values of n-hexane, ethyl acetate, and ethanol extracts were 82.70 µg/mL, 43.06 µg/mL, and 95.33 µg/mL respectively. The plant extracts showed comparatively lower cytotoxic effect than the positive

control (Vincristine sulphate: LC_{50} was 10.96 µg/mL). No mortality was found in negative control (DMSO) group.

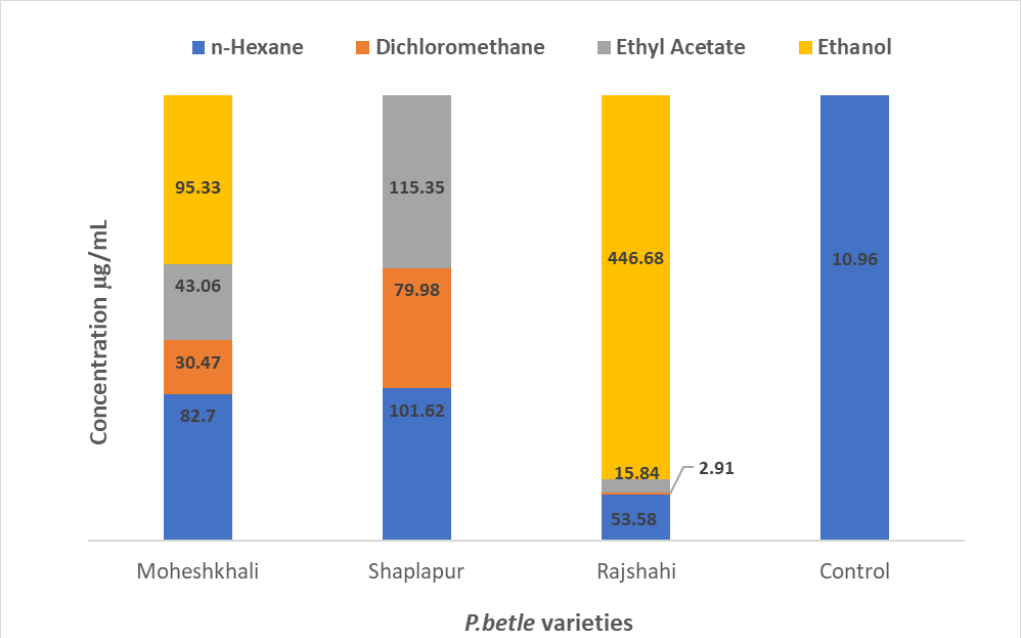

Fig. 1: Cytotoxic effect of different *P. betle* extracts from three regions

Of Shaplapur variety, the dichloromethane extract showed a better cytotoxic effect than n-hexane and ethyl acetate extract with LC_{50} at 79.98 µg/mL, 101.62 µg/mL and 115.35 µg/mL respectively and each extract showed lower cytotoxic activity than the positive control. The ethanol extract didn't show cytotoxic effect. No mortality was found in the negative control group (Fig. 1).

The dichloromethane and ethyl acetate extract of Rajshahi region showed outstanding cytotoxic effect with LC_{50} value at 2.91 µg/mL and 15.84 µg/mL respectively compared to n-hexane and ethanol extract. In addition, the dichloromethane extract showed better cytotoxic effect compared to the positive control. No mortality was found in the negative control group (Fig. 1).

3.3 Antidiabetic activity *(*α-glucosidase inhibitory assay)

The analysis of antidiabetic activity of three *P. betle* varieties is summarized in Fig. 2. The IC_{50} values of n-hexane, dichloromethane, ethyl acetate, and ethanol extracts of Moheshkhali variety were 0.015 µg/mL, 1.04 µg/mL, 9.49 µg/mL, 9.10 µg/mL respectively. In the contrary, the IC_{50} of positive control (acarbose) was 26.44 µg/mL. No inhibition was found in the negative control.

The Shaplapur variety also exhibited good inhibition of the α-glucosidase enzyme. The IC_{50} values of n-hexane, ethyl acetate, ethanol, and dichloromethane extracts were 0.004 µg/mL, 4.69 µg/mL, 10.54 µg/mL, 14.92 µg/mL respectively and all extracts showed better inhibition than the positive control. No inhibition was found in negative control (Fig. 2).

Of Rajshahi variety, the leaf extracts highest inhibition of the α-glucosidase enzyme. n-hexane extract exhibited the best inhibitory effect with IC_{50} at 0.001 µg/mL. The ethanol and ethyl acetate had almost similar IC_{50} at 5.02 µg/mL and 5.06 µg/mL respectively. But the dichloromethane extract showed the lowest activity with LC_{50} of 20.72 µg/mL. No inhibition was found in the negative control (Fig. 2).

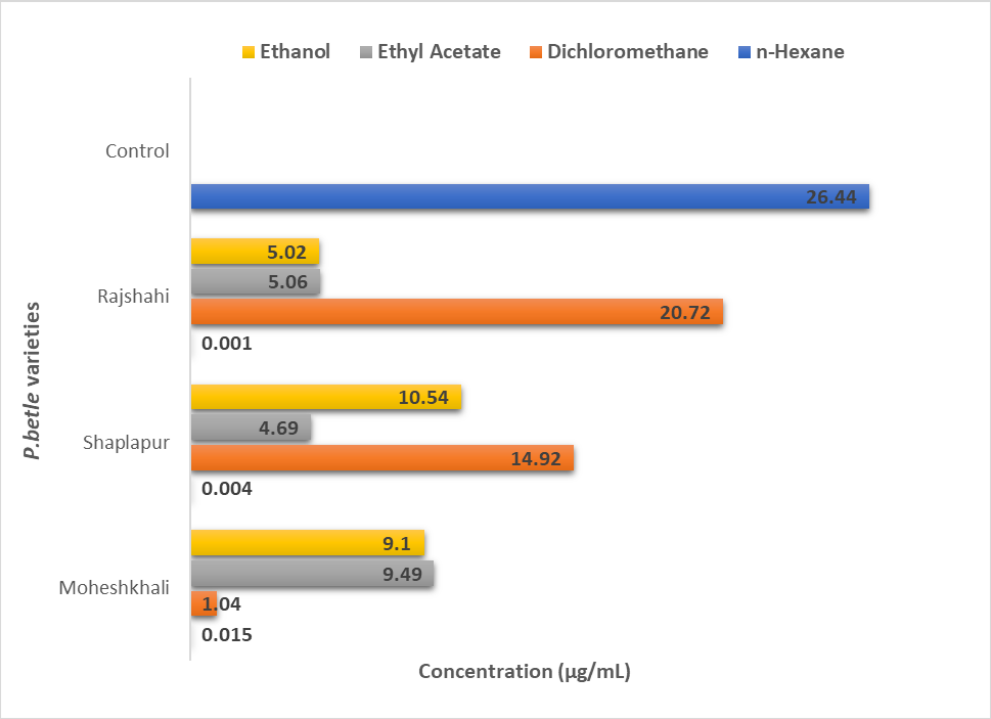

Fig. 2 Anti-diabetic activity of different *P.betle* extracts from three regions

Comment [AD2]: This figure is Vague, it need be described well – the key for n-hexane is representing the value for acarbose?

4. Discussion

4.1 Phytochemical analysis

Many polyphenolic compounds, triterpenoids and other chemical compounds exist in leaf extract of plants may account for the observed anti-diabetic effect. *P. betle* leaves are used in much traditional medicines, the anti-tumor property of phytochemicals like polyphenols and alkaloids [30]. The present study identified a number of biologically active phytochemical compounds in the studied three varieties of *P.betle* leaves collected from Moheshkhali, Rajshahi and Shaplapur.

Steroid, terpenoid, alkaloid, flavonoid, saponin, tannin, glycosides were identified among the three varieties except anthocyanin. Among them, steroid, terpenoid, alkaloid, flavonoid, saponin and tannin were tested positive with more intensity in Shaplapur variety. On the other hand, glycosides were higher but saponin was absent in Rajshahi variety (Table 1). In general, the bioactive properties of phenolic compounds are considered as anticarcinogenic, antimutagenic, antioxidant, anti-inflammatory and inducible for apoptosis by cell cycle arrest [31]. –A study showed that terpenoid decreases triglycerides and plasma cholesterol in hyperlipidemic rats, and hyperlipidemia plays significant role in developing heart disease [32]. Flavonoids and tannins are phenolic compounds and plant phenolics are a major group of compounds that act as primary antioxidants [33]. In addition, alkaloids exhibit a variety of medicinal properties including anti-hyperglycemic and anti-hyperglycemic activities extracted from piperaceae family [34]. The present study showed that the Shaplapur variety of *P.betle* leaf extract could be considered as an effective source of biologically active phytochemical compounds for designing any drugs for treating diseases.

Comment [AD3]: The preliminary phytochemical screening can indicate possible presence of the tested phytochemicals class of compounds – but not identification

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4.2 Cytotoxicity assay

The study examined the cytotoxic effect of three *P. betle* varieties (Moheshkhali, Rajshahi, and Shaplapur) using Brine shrimp lethality assay and found significant results. The dichloromethane extract from Moheshkhali variety showed better cytotoxic effect with LC_{50} at 30.47 µg/mL compared to ethyl acetate, n-hexane, and ethanol extracts. Similar result was found for Shahplapur variety whereas the dichloromethane extract exhibited cytotoxicity with LC_{50} at 79.98 µg/mL in comparison with n-hexane and ethyl acetate extract, but ethanol extract did not show any effect. Compared to Moheshkhali variety and Shahplapur variety, *P. betle* extracts of

Rajshahi region showed outstanding cytotoxic effect. The dichloromethane extract of Rajshahi variety showed the highest cytotoxicity having LC_{50} at 2.91 µg/mL in comparison with Moheshkhali variety (30.47 µg/mL) and Shahplapur variety (79.98 µg/mL). However, the ethanol extract of Rajshahi variety found to be the lowest cytotoxicity (Fig. 1). So, the extracts, in order of most cytotoxic to the least cytotoxic, were dichloromethane>ethyl acetate>n-hexane>ethanol. This comparative analysis revealed the dose-dependent cytotoxic effect Rajshahi variety could be considered as antitumor or anticancer potential.

Similar studies reported that ethyl acetate and hexane extract of *P. betle* leaf exhibited a dose-dependent inhibitory effect on the MCF-7 human breast cancer cells with IC_{50} at 65 µg/mL and 163 µg/mL respectively [30]. In addition, methanol extract showed significant activity toward Epstein-Barr virus (EBV) activation in Raji cells at IC_{50} values of 40 µg/mL [35]. A study showed that dichloromethane extract of *P. betle* demonstrated strong cytotoxic effect on MDA-MB-468 and MDA-MB-231 breast cancer cell lines with IC_{50} value of 11.26 ± 0.01 µg/mL and 19.76 ± 2.87 µg/mL respectively [36]. However, a previous study from the country demonstrated that *Piper betle* ethanol extract at LC_{50} value of 274.6 µg/mL showed promising cytotoxic effect in brine shrimp lethality bioassay [37]. Another study showed that ethanol and methanol extract of betel leaf had 50% mortality at 23.65 µg/mL and 85.50 µg/mL respectively [38]. So, *P. betle* leaf extracts exhibited cytotoxic potential and can be used for the treatment of several diseases. The potential lethality of *P. betle* extract against brine shrimp is a sign of the presence of potent cytotoxic components found in the plant. The cytotoxic potential of a compound does not always mean its direct toxic effect, but it can also suggest its antitumor or anticancer potential [39]. The cytotoxicity result of our study indicates the anticancer property of *P. betle* leaf ~~extract which~~extract, which also corroborates the other studies that betel leaf is not

Comment [AD4]: Solvents show polarity difference, not dose-dependence

Comment [AD5]: Brine shrimp lethality assay is used as preliminary assessment of toxicity (safety), but not anti-tumor or anti-cancer activity. Thus, according to these result, the Rajshahi variety has more safety concern, but does not show anticancer potential

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carcinogenic. Scientific studies have also shown that betel leaf does not have mutagenic and/or carcinogenic effects. A study reported for the very first time that aqueous extract of betel leaf failed to cause any tumor in both C17 mice and Swiss albino mice which proved betel leaf was devoid of any carcinogenic compound [40].

4.3 Anti-Diabetic assay

In the present study, α-glucosidase inhibition activity was studied to examine the antidiabetic properties of three *P. betle* leaf varieties (Moheshkhali, Rajshahi and Shaplapur). All the four extracts e.g. ethanol, ethyl acetate, dichloromethane and n-hexane of three different varieties showed significant antidiabetic properties. Based on the IC_{50} obtained in the antidiabetic assay of different extracts, the Moheshkhali variety exhibited the best inhibition followed by the Rajshahi and Shaplapur variety. The highest α-glucosidase inhibition activity was observed by the n-hexane extract with the following IC_{50} at 0.001 µg/mL, 0.004 µg/mL and 0.015 µg/mL, in Rajshahi, Shaplapur and Moheshkhai varieties respectively. ~~Followed~~ Following ~~by~~ the n-hexane extract, ethyl acetate extracts of the three leaf varieties had better inhibition activity compared to ethanol and dichloromethane. Interestingly, the dichloromethane extract of Moheshkhali variety exhibited the highest inhibition activity with LC_{50} value of 1.04 µg/mL in comparison with Rajshahi (20.72 µg/mL and Shaplapur (14.92 µg/mL) variety (Fig. 2).

However, several studies have repeatedly shown the antidiabetic property in *P. betle* extracts [41]. In a study, orally administered *P. betle* (75 and 150 mg/kg of body weight) was found to lower blood glucose and improve body weight at a significant level. ~~The~~ This study discussed that the reduction of body weight in STZ (streptozotocin) induced diabetic rats is clear evidence of the degradation of structural proteins caused by diabetes. The structural proteins generally

contribute to the body weight [42]. The protection from huge bodyweight loss seems to be correlated to the ability of *P. betle* to reduce diabetes [43]. Another study reported that blood glucose in rats was significantly reduced by the administration of *P. betle* extract [44]. Similar study on antidiabetic potential of different variety of *P. betle* leaf extracts showed that 50% of α-glucosidase enzyme inhibition by methanolic extracts at 96.8 µg/mL, 157.7 µg/mL, and >200 µg/mL in Sanchi, Kapoori, and Bangla variety respectively [29]. For the treatment of type 2 diabetes mellitus, α-glucosidase inhibitors are administrated orally as anti-diabetic medications [45]. In diabetes, these pharmacological enzyme inhibitors exhibit ~~short term~~short-term effect to decrease high blood glucose levels in blood [46]. The difference in total flavonoid content, phenolic content in different cultivars of *P. betle* can be the reason for different antidiabetic activities [47].

Comment [AD7]: But these constituents are not expected in n-hexane, which showed potent activity

The present study aimed to investigate the inhibitory potentials of different varieties of *P. betle* leaf extracts on α-glucosidase, one of the key enzymes involved in carbohydrate hydrolysis. This study revealed that the n-hexane extract of different *P. betle* leaf extracts exhibited the highest role in α-glucosidase inhibition which can be considered as a potential pharmaceutical therapeutic to treat diabetes in Bangladesh. We hope this comparative study among the three *P. betle* varieties can be beneficial with further explorative study in choosing a natural therapeutic alternative for the treatment of cancer and diabetic patients with minimal side effects.

Comment [AD8]: ???

Conclusion

The present study ~~identified~~ showed the possible presence of a number of active phytochemical constituents among the three varieties of *P. betle* leaf extracts as well as potent ability of different leaf extrats in cytotoxicity and antidiabetic activity. In addition, the *P. betle* leaf extracts of Moheshkhali variety can play a significant role as potential medicinal use in treating

cancer and diabetes. Moreover, the quantification of individual phytoconstituents as well as pharmacological profile based on *in-vivo* studies of Rajshahi, Shaplapur and Moheskhali variety should be further investigated.

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