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In vitro antidiabetic and antioxidant activities of lupeol isolated from the methanolic extract of *Andrographis echioides* leaves

S Gurupriya, L Cathrine and J Ramesh

Abstract
In the present study the lupeol isolated from the methanolic extract of leaves of *Andrographis echioides* was studied for alpha amylase and alpha glucosidase inhibition using an in vitro model. The isolated compound lupeol was also examined for its antioxidant activities by using free radical 1, 1-diphenyl-2-picryl hydrazyl (DPPH) scavenging method. The study revealed that the different concentration of lupeol exhibit potent radical scavenging activity using DPPH as substrate. The lupeol exhibited significant α-amylase and α-glucosidase inhibitory activities with an IC50 value 36.2±0.42 and 41.4±0.34 % respectively and well compared with standard acarbose drug. Therefore, it is suggested that lupeol is a potential source for natural antidiabetic and antioxidant compounds and could have potential use in the management of diabetes mellitus.

Keywords: *Andrographis echioides* leaves, lupeol, alpha amylase inhibitory activity, alpha glucosidase inhibitory activity, antioxidant activities

Introduction
Diabetes mellitus is a chronic metabolic disorder that affects the metabolism of carbohydrates, fat and protein. It is characterized by hyperglycemia, in which blood sugar levels are elevated either because the pancreas do not produce enough insulin or cells of the body do not respond properly to the insulin produced (Keerthana *et al*., 2013) [1]. Type 1 diabetes results from inadequate synthesis of insulin by β-cells of the pancreas, while type II diabetes is characterized primarily by insulin resistance (a condition in which peripheral cells do not respond normally to insulin) or β-cell dysfunction (Heise *et al*., 2004) [2]. The treatments for diabetes is reduction of the demand for insulin, stimulation of insulin secretion, enhance the mode of action of insulin at the target tissues and inhibition of degradations of oligo- and disaccharides (Groop *et al*., 1997; Perfetti *et al*., 1998) [3, 4]. The enzymes alpha glucosidase are responsible for the breakdown of oligo- and disaccharides to monosaccharides. The inhibitory action of these enzymes leads to a decrease of blood glucose level. The activity of alpha amylase enzymes which is responsible for the collapse of starch to more simple sugars (dextrin, maltotriose, maltose and glucose). The alpha amylase inhibitors delays the glucose absorption rate thereby maintaining the serum blood glucose in hyperglycemic individuals (Dinesh kumar *et al*., 2010) [5]. Inhibitors of α-amylase and α-glucosidase delay the breaking down of carbohydrates in the small intestine and diminish the postprandial blood glucose excursion (Kwon *et al*., 2010) [6]. Antioxidants such as ascorbic acid, carotenoids and phenolic compounds are more effective which possess free radical chain reaction breaking properties (Duh et al., 1999) [7]. They are known to inhibit lipid peroxidation (by inactivating lipoxygenase), to scavenge free radicals and active oxygen species by propagating a reaction cycle and to chelate heavy metal ions (Sudarajan et al., 2006) [8]. Recently herbal medicines are getting more importance in the treatment of diabetes as they are free from side effects and less expensive when compared to synthetic hypoglycemic agents (Grover *et al*., 2002; Mukherjee *et al*., 2006) [9, 10].

Andrographis echioides belongs to *Acanthaceae* family, used for various medicinal purposes in South Asia particularly India and China. Based on the literature, this plant possess pharmacological properties include antimicrobial activity, anti-inflammatory, diuretic, anthelmintic, analgesic, antipyretic, hepato-protective activities and antioxidant effect. It contains plenty of phytochemical constituents such as flavonoids, flavones, steroids, tannins, carbohydrate, glycosides and alkaloids (Ankita and Handique, 2010; Shanker *et al*., 2008) [13, 12]. The leaf juice of *A. echiodies* is used to cure fevers. Genus of *Andrographis* family plants are used to cure various diseases like goiter, liver diseases, fertility problems, bacterial, malarial and fungal disorders (Zulfkar *et al*., 2009) [14]. *Andrographis echioides* boiled with coconut oil is used to decrease the falling and graying of hair (Kanchana and Rubalakshmi, 2014) [15]. From the leaves extract of *Andrographis echioides* various chemical constituents were isolated dihydro echioidinin, skullcap avone 1 2'-methyl ether, echioidinin, echioidin, skullcap avone 1 and 2'-O-bD-glucopyranoside (Jayaprakasam and Gunasekara, 1999) [16]. Some of the other chemical constituents present in the *A. echioides* are more than 17 compounds such as borneol, cyclohexanol 2,4 dimethyl phenol, 3,4 altroson, ndeconoicacid, Squalene, vitamin E, Methoprene, 2-nonenlol Oxirane,octyl-, 2, 2-cyclopentene-1-undecanoic acid, ketone, 1,5-methylbicyclo [2.1.0] pent-5-ylmethyl and 2,5-cyclohexadiene-1,4- dione, 2, 5- dihydroxy-3-methyl -6- (1-methylethyl) bicycle heptan -3-one (Nirubama and Rubalakshmi, 2014) [17]. Lupeol isolated from the methanolic extract of leaves of *Andrographis echioides* was reported (Gurupriya *et al*., 2018) [18]. Inhibition of alpha amylase and alpha glucosidase enzymes can be important strategy in management of post prandial blood glucose level in type 2 diabetes patient (Sunil *et al*., 2010) [19]. However, no studies have been done to assess the antidiabetic activities of this *Andrographis echioides* (leaves). Therefore, in the present study, the antidiabetic and antioxidant activities of lupeol isolated from the methanolic extract of leaves of *Andrographis echioides* were evaluated employing in vitro assay methods.

Materials and Methods

Collection of plant material

The leaves of *Andrographis echioides* were collected in the month of May from the mullipatti, pudukkottai, Tamil Nadu, India. The plant was identified and leaves of *Andrographis echioides* were authenticated and confirmed from Dr. S. John Britto, Director, Rapinat herbarium, St. Joseph College, Tiruchirapalli, and Tamil Nadu for identifying the plants. The voucher specimen number SGP001 (7.06.2017).

Chemicals and reagents

Alpha (α)-Glucosidase, porcine pancreas alpha (α)-amylase, *p*-nitrophenyl-α-D-glucopyranose (*p*-NPG), 3,5-dinitrosalicylic acid (DNS), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid and acarbose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Soluble starch, sodium potassium tartarate, sodium dihydrogen phosphate (NaH_2PO_4), Di-sodium hydrogen phosphate ($Na2HPO_4$) sodium chloride, sodium hydroxide, potassium ferricyanide, ferric chloride ($FeCl_3$) were from Merck Chemical Supplies (Damstadt, Germany). All the chemicals used including the solvents, were of analytical grade.

Preparation of methanol extracts

The leaves of *Andrographis echioides* were washed in running water, cut into small pieces and then shade dried for a week at 35-40ºC, after which it was grinded to a uniform powder of 40 mesh size. The methanol extracts were prepared by soaking 100 g each of the dried powder plant materials in 1 L of methanol using a soxhlet extractor continuously for 10 hr. The extracts were filtered through whatmann filter paper No. 42 (125mm) to remove all unextractable matter, including cellular materials and other constitutions that are insoluble in the extraction solvent. The entire extracts were concentrated to dryness using a rotary evaporator under reduced pressure. The final dried samples were stored in labeled sterile bottles and kept at -20ºC. The filtrate obtained was used as sample solution for the further isolation (Deepti *et al*., 2015) [20].

Isolation of Lupeol by column chromatography

The condensed methanol extract of leaves (986 g) of sample was subjected to column chromatography over TLC grade silica gel. Elution of the column first with n-hexane, increasing amount of ethyl acetate in n-hexane and finally with methanol yielded a number of fractions. The preparation of solvent systems used to obtain Lupeol (104 mg/786g) were n-hexane-ethyl acetate (30:70) from fraction 5. The compounds were detected on TLC plates by spraying with Libermann Burchard reagent and heated at 100°C for 10 minutes (Jain and Bari, 2010) [21].

Purification of isolated compounds by High performance liquid chromatography

The analytical HPLC system (Shimadzu) was equipped with a diode array detector, a 20µl loop, 200 x 4.6 mm C18 column, methanol (HPLC grade, 0.2mm filtered) used as a mobile phase. The isolated Lupeol compounds were separated using a mobile phase of methanol: water (75:25 v/v) at a flow rate of 1.0 ml/min, column temperature 30 °C. Injection volume was 40 µl and detection was carried out at 346 nm (Suthar *et al*., 2001) [22].

Structural elucidation study of isolated compound

Different spectroscopic methods including ^{1}H NMR and ^{13}C NMR were used to elucidate the structure of isolated compounds. ^{1}H and ^{13}C NMR spectra were acquired on Bruker WP 200 SY and AM 200 SY instruments (^{1}H, 200.13 MHz; ^{13}C, 50.32 MHz) using TMS as internal standard and $CDCL_3$ as solvent (Jain and Bari, 2010; Sarfaraj *et al*., 2014; Suthar *et al*., 2001) [21, 22].

Antioxidant activity (DPPH free radical scavenging activity) determination

The antioxidant activity of the isolated compound lupeol was examined on the basis of the scavenging effect on the stable DPPH free radical activity (Braca *et al*., 2002) [24]. Ethanolic solution of DPPH (0.05 mM) (300 µl) was added to 40 µl of isolated compound lupeol with different concentrations (20 - 100 µg/ml). DPPH solution was freshly prepared and kept in the dark at 4°C. Ethanol 96% (2.7 ml) was added and the mixture was shaken vigorously. The mixture was left to stand for 5 min and absorbance was measured spectrophotometrically at 517 nm. Ethanol was used to set the absorbance zero. A blank sample containing the same amount of ethanol and DPPH was also prepared. All determinations were performed in triplicate. The radical scavenging activities of the tested samples, expressed as percentage of inhibition were calculated according to the following equation (Yen and Duh, 1994) [25].

Percent (%) inhibition of DPPH activity = [(A – B) / A] x 100

Where B and A are the absorbance values of the test and of the blank sample, respectively. A percent inhibition versus concentration curve was plotted and the concentration of sample required for 50% inhibition was determined and represented as IC50 value for each of the test solutions.

Alpha-Amylase Inhibitory Assay.

This assay was carried out using a modified procedure of McCue and Shetty, 2004. A total of 250μL of isolated compound lupeol (20-100 µg/ml) was placed in a tube and

250 μL of 0.02M sodium phosphate buffer (pH 6.9) containing α-amylase solution (0.5mg/mL) was added. This solution was preincubated at 25ºC for 10 min, after which 250 μL of 1% starch solution in 0.02M sodium phosphate buffer (pH 6.9) was added at timed intervals and then further incubated at for 25ºC for 10min. The reaction was terminated by adding 500 μL of dinitrosalicylic acid (DNS) reagent. The tubes were then incubated in boiling water for 5min and cooled to room temperature. The reaction mixture was diluted with 5mL distilled water and the absorbance was measured at 540 nm using spectrophotometer. A control was prepared using the same procedure replacing the extract with distilled water.
The α-amylase inhibitory activity was calculated as percentage inhibition:

%Inhibition = [(Abs control – Abs lupeol) / Abs control] x 100

Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC50) were determined graphically.

Alpha-glucosidase inhibitory assay

The effect of the isolated compound lupeol on α-glucosidase activity was determined according to the method described by Kim *et al*., 2005 using α-glucosidase from *Saccharomyces cerevisiae*. The substrate solution p-nitrophenyl glucopyranoside (p-NPG) was prepared in 20mM phosphate buffer, and pH 6.9. 100 μL of α- glucosidase (1.0 U/mL) was preincubated with 50 μL of the different concentrations (20-100 µg/ml) of the isolated compound lupeol for 10min. Then 50 μL of 3.0mM (pNPG) as a substrate dissolved in 20mM phosphate buffer (pH 6.9) was then added to start the reaction. The reaction mixture was incubated at 37ºC for 20min and stopped by adding 2mL of 0.1M Na_2CO_3.The α-glucosidase activity was determined by measuring the yellow-colored paranitrophenol released from pNPG at 405 nm. The results were expressed as percentage of the blank control.
The α-glucosidase inhibitory activity was calculated as percentage inhibition:

%Inhibition = [(Abs control – Abs lupeol) / Abs control] x 100

Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC50) were determined graphically.

Statistical analysis

All assays were conducted in triplicate. Statistical analyses were performed with SPSS 16.0 for an analysis of variance (ANOVA) followed by Duncan's test. Differences at $P < 0.05$ were considered to be significant.

Results and Discussion

Structural Elucidation of isolated compounds

In the proton ^{1}H NMR spectrum of lupeol (fig. 1) showed 7.19(CDCL3 peak), 4.62, 4.61, 4.5(H-29, d,d, 2H), 3.14-3.09 (H,3, d,d, 1H, 6 Hz, 5Hz), 2.33(H-19, m, 1H), 2.32 (H-21a, m, 1H), 2.26 (H-15A, t, 1H), 2.10 (H-30, s, 3H), 1.61 (H-12A, 1A, d, 2H), 1.58 (H-13, t, 1H), 1.57 (H-2A, d, 1H), 1.54 (H-2B, q, 1H), 1.53 (H-12A, q, 1H), 1.52 (H-23, s, 3H), 1.50 (H-15A, d, 1H), 1.49 (H-23,s, 3H), 1.46 (H-27, s, 3H), 1.45 (H-18, t, 6 Hz, 1H), 1.44 (H-28, s, 3H),1.43(H-24, s, 3H), 1..34 (H-25, s, 3H), 1. 31 (H-5, d, 1H).

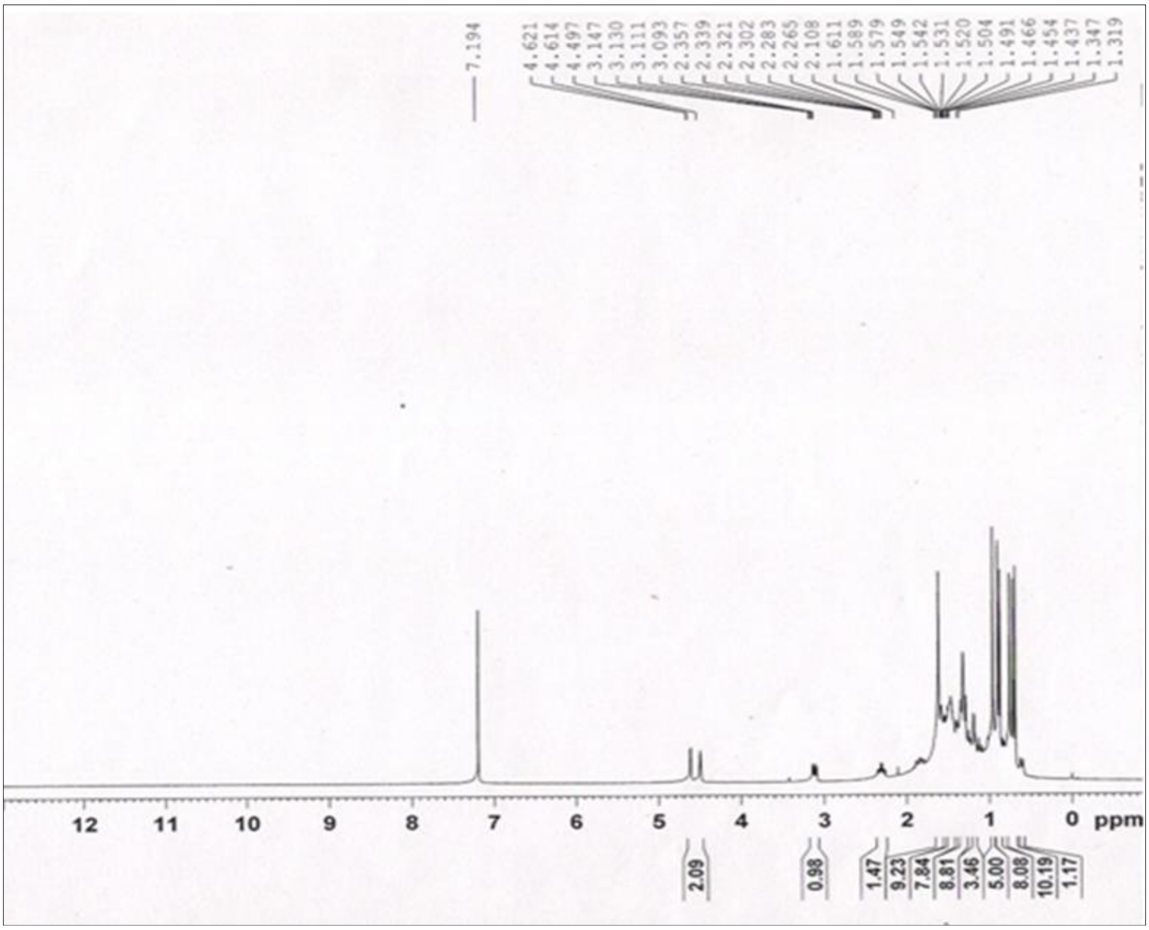

Fig. 1: ^{1}H NMR spectra of the isolated compound

In the [13]C NMR spectrum of lupeol (fig. 2) showed δ_C: δ 37.17 (C-1), δ 20.93 (C-2), δ 79.02 (C-3), δ 38.05 (C-4), δ 55.2 (C-5), δ 18.31 (C-6), δ 27.99 (C-7), δ 38.87 (C-8), δ 50.43 (C-9), δ 34.29 (C-10), δ 19.31 (C-11), δ 20.93 (C-12), δ 35.56 (C-13), δ 40.01 (C-14), δ 25.1 (C-15), δ 29.83 (C-16), δ 40.86 (C-17), δ 48.28 (C-18), δ 48 (C-19), δ 151.13 (C-20), δ 27.96(C-21), δ 38.87 (C-22), δ 25.1 (C-23), δ 15.38 (C-24), δ 15.38 (C-25), δ 15.38 (C-26), δ 14.5 (C-27), δ 17.96 (C-28), δ 109.42 (C-29) and δ 18.31 (C-30).

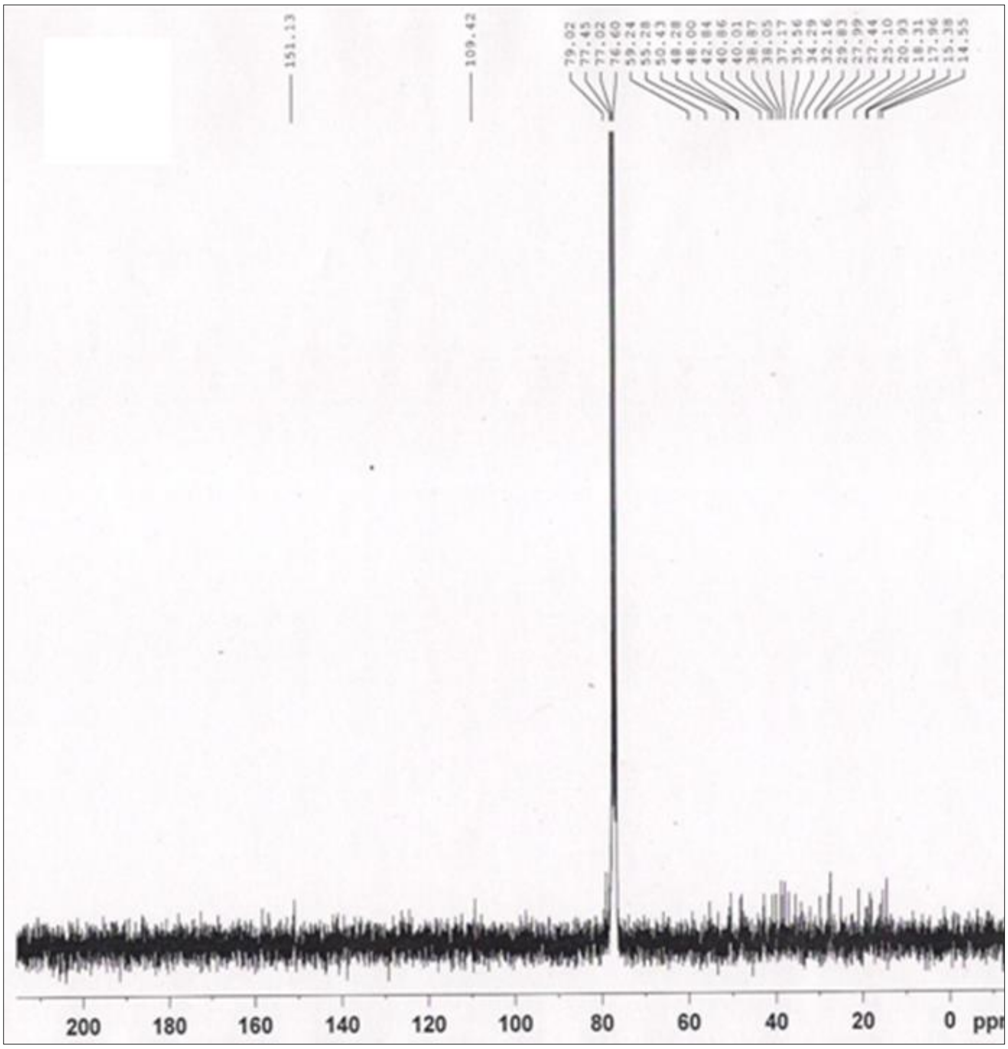

Fig 2: ^{13}C NMR spectra of the isolated compound

In ^{1}H NMR spectrum of lupeol, H-3 proton appeared as a triplet of a double doublet (tdd) at 3.14 (J=4.5 and 1.1 MHZ) and H-29 olefinic proton showed a multiplet at 4.62 and 4.61, respectively. Seven methyl protons also appeared at 1.46, 1.45, 1.44, 1.43, 1.34 and 1.31 (3H each, s, CH_3). Mass spectrum of isolated compound lupeol showed parent molecular ion [M^+] peak at mlz 426 which corresponds to the molecular formula $C_{30}H_{50}O$. These assignments are in good agreement for the structure of lupeol (Vasconcelos *et a*l., 2008; Imam *et al*., 2007; Fernández *et al*., 2001) [27, 28, 29].

Purification of isolated compound by HPLC

The Retension time of lupeol isolated from the methanolic extract of sample was about 3.750 was shown by HPLC peak (fig 3).

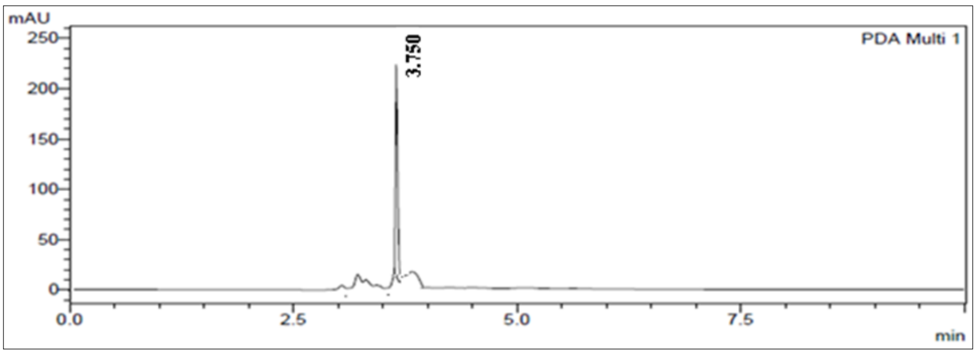

Fig 3: HPLC spectra of purity of the isolated compound

Antioxidant activity of isolated compound lupeol by DPPH method

The result showed that the compound had better percentage antioxidant activities at high concentrations when compared with ascorbic acid (Table 1). The compound showed 95.39 % activity at 100 µg/ml while ascorbic acid gave 95.79 % at the same concentration (fig. 4). The previous study suggested that the lupeol has antioxidant properties by scavenging free radicals, decreasing lipid peroxidation and increasing the endogenous blood antioxidant enzymes levels (Michel *et al*., 2016) [30].

Table 1: Antioxidant activity of lupeol by DPPH activity

S. No	Concentrations	Scavenging Effect (%)	
		Lupeol	Ascorbic acid
1	20 (µg/ml)	29.24±1.56	30.50±1.33
2	40 (µg/ml)	59.37±1.24	64.35±1.37
3	60 (µg/ml)	71.34±1.35	74.73±1.42
4	80 (µg/ml)	83.20±1.42	85.24±1.47
5	100 (µg/ml)	95.39±1.20	95.79±1.50
	IC50	44.3	47.4

Each value was obtained by calculating the average of three experiments and data are presented as mean± SEM

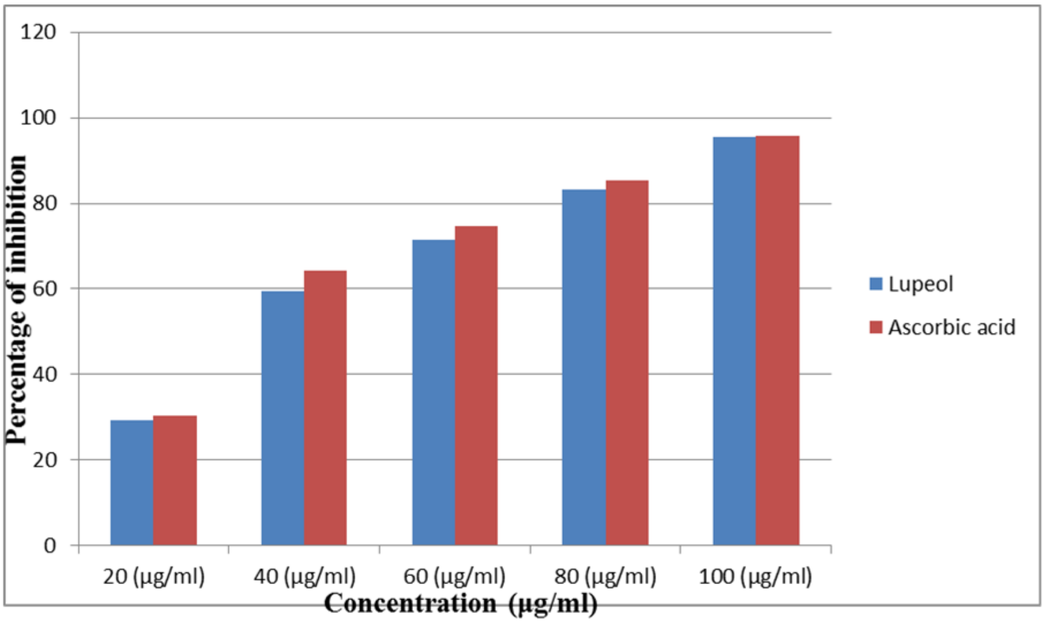

Fig 4: Antioxidant activity of lupeol by DPPH activity

***In vitro* alpha amylase inhibitory assay**

In this study the *in vitro* alpha amylase inhibitory activities of the lupeol isolated from methanolic extract of *Andrographis echioides* leaves was investigated. The result of experiment showed that, there was a dose-dependent increase in percentage inhibitory activity against alpha amylase enzyme. The lupeol (20-100 µg/ml) of the various concentrations exhibited potent α-amylase inhibitory activity in a dose dependent manner. The lupeol showed inhibitory activity from 25.82±0.25 to 68.41±0.37% with an IC50 value of 36.7±0.24 µg/ml (Table 2). Acarbose is a standard drug for α-amylase inhibitor. Acarbose at a concentration of (20-100 µg/ml) showed α-amylase inhibitory activity from 37.85±0.24 to 75.97±0.37% with an IC50 value 41.4±0.34 µg/ml. A comparison of α–amylase inhibitory activity between the standard drug has been depicted in fig. 5. Our results are in accordance with the previous study wherein, there is a positive relationship between the total polyphenol and flavonoid content and the ability to inhibit intestinal α-glucosidase and pancreatic α-amylase (Ramkumar *et al*., 2010; Manikandan *et al*., 2013) [31, 32]. The isolated compounds were tested for their antidiabetic potential in vitro by inhibition of α-amylase enzyme. Total saponins, Lupeol and stigmasterol showed higher alpha amylase inhibitory activity which confirms its antidiabetic potential was reported (Sincy Joseph *et al*., 2016) [33].

Table 2: *In vitro* antidiabetic activity of the lupeol using alpha amylase method and comparison with standard drug acarbose.

S. No	Concentrations	Alpha amylase (%)	
		Lupeol	Acarbose
1	20 (µg/ml)	25.82±0.25	37.85±0.24
2	40 (µg/ml)	37.98±1.24	50.21±1.37
3	60 (µg/ml)	40.56±1.35	61.20±1.42
4	80 (µg/ml)	58.53±1.42	69.25±1.47
5	100 (µg/ml)	68.41±0.37%	75.97±0.37%
	IC50	36.7±0.24	41.4±0.34

Each value was obtained by calculating the average of three experiments and data are presented as mean± SEM

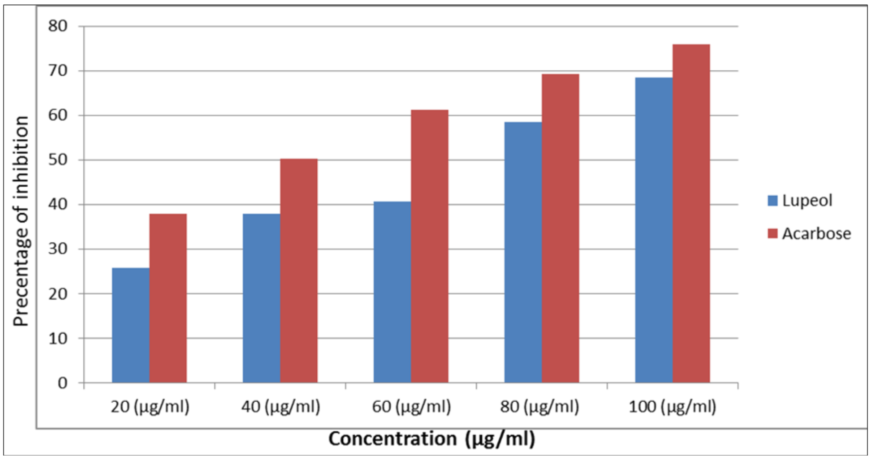

Fig 5: α-Amylase inhibitory activity of acarbose vs lupeol isolated from *Andrographis echioides* leaves

In vitro α-glucosidase inhibitory assay

The results of antidiabetic activity using α- glucosidase inhibitory assay of the lupeol isolated from methanolic extract of *Andrographis echioides* leaves are shown in Table 3. The lupeol revealed a significant inhibitory action of α-glucosidase enzyme. The percentage inhibition at 20-100 µg/ml concentrations of lupeol showed a dose dependent increase in percentage inhibition.

The percentage inhibition varied from 31.88±0.49% - 88.72±0.91% for highest concentration to the lowest concentration. Thus the inhibition of the activity of α-glucosidase by lupeol would delay the degradation of carbohydrate, which would in turn cause a decrease in the absorption of glucose, as a result the reduction of postprandial blood glucose level elevation. A comparison of α–glucosidase inhibitory activity between the standard drug has been depicted in fig. 6.

In this study acarbose was also used as a standard drug for α-glycosidase inhibitor. Acarbose at a concentration of (20-100 µg/ml) showed α-glucosidase inhibitory activity from 42.70 ±1.40 to 91.68±1.38 % with an IC50 value 45.03±1.03 µg/ml.

This indicates that the lupeol is very potent α-amylase and α-glucosidase inhibitor in comparison with acarbose (Mai *et al*., 2007) [34] The hypoglycemic activity of crude extracts and isolated compounds (lupeol acetate, cis-p-coumaric acid, lupeol, β-sitosterol, trans-p-coumaric acid, linoleic acid, (+)-catechin, afzelin and quercitrin) was assessed by the ability to inhibit α-amylase and α-glucosidase enzymes (Maria Torres Naranjo *et al*., 2016) [35].

Table 3: *In vitro* antidiabetic activity of the lupeol using alpha glycosidase method and comparison with standard drug acarbose.

S.No	Concentrations	Alpha glycosidase (%)	
		Lupeol	Acarbose
1	20 (µg/ml)	31.88±0.49	42.70 ±1.40
2	40 (µg/ml)	40.57±0.92	52.34±1.37
3	60 (µg/ml)	57.10±0.55	65.48±1.42
4	80 (µg/ml)	66.02±1.90	74.54±1.47
5	100 (µg/ml)	88.72±0.91	91.68±1.38
	IC50	36.2±0.42	45.03±1.03

Each value was obtained by calculating the average of three experiments and data are presented as mean± SEM

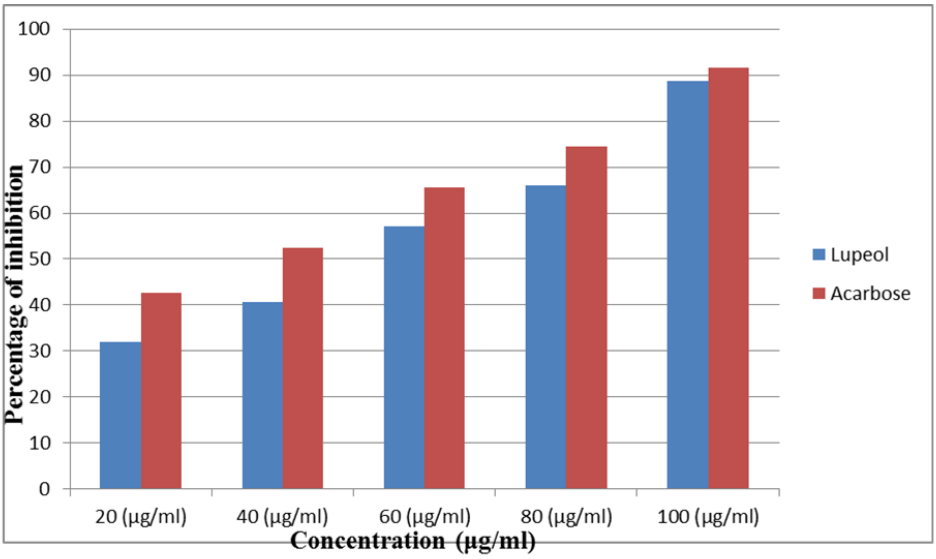

Fig 6: α- glycosidase inhibitory activity of acarbose vs lupeol isolated from *Andrographis echioides* leaves

Conclusion

The plant *Andrographis echioides* showed significant enzyme inhibitory activity, so the compound lupeol isolated and characterized which are responsible for inhibiting activity, have to be done for the usage of antidiabetic agent. To investigate the biological activities of lupeol, the antioxidant and antidiabetic activities of the lupeol isolated from the methanolic extract of *Andrographis echioides* leaves has been analysed. As a result, we found that the lupeol have free radical scavenging activity and inhibitory activity against α-amylase and α-glucosidase and this therapeutic potentiality could be exploited in the management of post prandial hyperglycemia in the treatment of type 2 diabetes mellitus.

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Author contribution
All authors contribute equally to this manuscript.

Conflicts of interests
The authors declare that they have no conflict of interest. It has not been published elsewhere. That it has not been simultaneously submitted for publication elsewhere. All authors agree to the submission to the journal.

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