









# Investigation on the Potential of Ethanol Extract (EE) and Lyophilized Juice (LJ) of *Malus pumila* Mill. in the Management of Diabetes Mellitus Type II and its Complications: Dyslipidemia and Oxidative Stress

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**Abstract:** Investigation on the potential of *Malus pumila* Mill in the management of Diabetes Mellitus Type II and its complications. Diabetes mellitus Type II (DM II) is a major public health ailment worldwide. The plants play an important role in the management of DM. *Malus pumila* Mill (*M. pumila*) is rich in alkaloids, terpenoids, flavonoids, and phenolic compounds. The current research aims to investigate the potential of the ethanol extract and lyophilized juice of *Malus pumila* Mill. in managing type II diabetes mellitus and its complications, such as dyslipidemia and oxidative stress. The ethanol extract and lyophilized juice extracted from the fruits of this plant were investigated against rats induced with diabetes mellitus type II (T2DM). EE and LJ were administered for 28 days by oral route to rats. The effects on glucose, lipids, and free radical levels were assessed *in vitro* and *in vivo* using various parameters. Phytochemical screening of extracts confirmed the presence of alkaloids, carbohydrates, sterols, terpenoids, and phenolic compounds. The extract and juice inhibited key enzymes in carbohydrate metabolism, i.e.,  $\alpha$ -amylase and  $\alpha$ -glucosidase, lowered blood glucose levels, and exerted antioxidant effects. These extracts were also found to be effective in controlling oxidative stress and dyslipidemia, which are complications of T2DM. Complications of DM II, such as dyslipidemia and oxidative stress, may also be controlled with the fruits of this plant. The extracts and juices used are of natural origin and have the potential to control blood glucose levels and maintain free radical levels within a normal range. The dyslipidemia was also reversed by the extract of this plant.

**Keywords:** diabetes mellitus; rats; streptozotocin; nicotinamide; *Malus pumila*.

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## 1. Introduction

The constituents of plants have the potential to treat various ailments. Constituents such as alkaloids, terpenoids, fatty acids, and flavonoids are reported to have various therapeutic effects [1-3]. Fruits from various plants have been reported to have antidiabetic, antioxidant, and antihyperlipidemic properties [4,5]. Over the last few years, natural antioxidants have been widely used all over the world instead of synthetic antioxidants [6,7].

Diabetes mellitus is a chronic metabolic disorder that is categorised into two types. In type I, insulin-secreting cells decrease, while in type II, the body becomes unable to utilize insulin circulating in the blood. Diabetes mellitus type II may progress with time, and it may become type 1. A lot of drugs are available to treat diabetes mellitus, but the progression of the disease cannot be controlled for a long time. Moreover, the adverse effects of these drugs pose serious problems. Alternatives to synthetic medicine are still not available. Therefore, plants are used in research continuously to overcome these problems [8-10].

*Malus pumila* Mill., from the family Rosaceae, is a rich fruit-bearing tree. The fruit, commonly known as the golden apple, is widely consumed worldwide. The fruit of this plant is nutrient-dense. It is a good source of lipids, protein, fibres, minerals, and vitamins [11,12]. It contains a variety of constituents, including flavanones, anthocyanins, procyanidins, phenolic acids, gallic acid, and quercetin [13-15]. Extracted constituents from fresh fruit can be more effective in T2DM than dietary supplements [16,17].

This plant has been explored for numerous therapeutic effects. Some of these effects include reduced arterial plaque and antiasthmatic effects [18]. Controlling lung cancer [19], Alzheimer's disease [20], and cardiovascular disease [21]. It showed antiviral [22] and cholesterol-lowering activities [23,24].

The constituents from natural sources, such as plants, have many beneficial effects on our bodies, helping control various ailments [25,26]. The fruits from this plant are reported to have antioxidant activity. Free radicals in the body are also responsible for complications of diabetes mellitus, like cardiovascular toxicity, nephrotoxicity, and neurotoxicity [27]. Therefore, this research aimed to investigate the beneficial effects of *Malus pumila* Mill. fruit in controlling diabetes mellitus, dyslipidemia, and oxidative stress, an early complication of T2DM.

## 2. Materials and Methods

### 2.1. Chemicals.

All the chemicals used in this research were of analytical grade.

### 2.2. Plant.

Fresh, ripe fruit of the tree *Malus pumila* was purchased from the local market in Hisar, Haryana, India, and used after authentication by NISCAIR, New Delhi, India, vide reference number NISCAIR/RHMD/CONSULT/2015/2934-127.

### 2.3. Animals.

Healthy, Albino Wistar rats (150-220g) of either sex were used in the current research. The animals were included in the study only after obtaining approval from the IAEC, and they were kept under standard conditions and fed as per the guidelines of CPCSEA, Guru

Jambheshwar University of Science and Technology, Hisar (Registration no. 0436 dated 23/11/2015).

#### 2.4. Preparation of ethanol extract and lyophilized juice.

The ethanol extract and lyophilized juice were prepared to carry out the study. The fruit was sliced, and the chips were dried in the shade. The dried slices were extracted with 95% v/v ethanol using a Soxhlet apparatus for about 4 days. The extract was subjected to vacuum evaporation to yield a brown-coloured extract. Fresh juice of fruit was prepared by expressing fresh fruit and lyophilizing it. Both the extract and the juice were placed in a desiccator and stored at 2-8°C for further use.

#### 2.5. Coding of plant material.

The ethanol extract was named EE, and the lyophilized juice was named LJ.

#### 2.6. Preliminary phytochemical screening.

Phytochemical screening of the extracts was performed to identify constituents such as alkaloids, steroids, terpenoids, and phenolic compounds (tannins, flavonoids, saponins, etc.), as per the standard procedures described by Amarnath *et al.* [28] and Harborne *et al.* [29].

#### 2.7. In vitro analysis of extract and juice.

*In vitro* analysis of the extract and juice was done by performing  $\alpha$ -amylase and  $\alpha$ -glucosidase assays.

##### 2.7.1. $\alpha$ -amylase inhibition assay.

The activity was calculated using the reported method by Kusano *et al.* [30]. Acarbose was used as a standard. The substrate was prepared by dissolving 500 mg of starch in 25 mL of 0.4 M HCl, heating for 5 min at 100°C, and then cooling in an ice bath. The pH was adjusted to 7.4 with 2M HCl, and the volume was adjusted to 100 mL with distilled water. Various concentrations of test samples were prepared in phosphate buffer (pH 7.4). To each of the microplate wells, 40  $\mu$ L substrate and 20  $\mu$ L sample solutions were added and incubated at 37°C for 15 min. After incubation, 20  $\mu$ L of  $\alpha$ -amylase solution (50  $\mu$ g/mL) was added to each well, and the plate was incubated for an additional 15 min. 80  $\mu$ L of 0.1 M HCl and 200  $\mu$ L of iodine solution (1 mM) were added to terminate the reaction [31]. The absorbance of the mixture was measured at 650 nm, and the % inhibitory activity was calculated as:

$$\text{Inhibition \%} = 1 - \frac{(\text{absorbance of sample and substrates} - \text{absorbance of sample, Substrates and amylase})}{(\text{absorbance of substrates} - \text{absorbance of substrates and amylase})} \times 100 \quad (1)$$

##### 2.7.2. $\alpha$ -glucosidase inhibition assay.

The inhibition of  $\alpha$ -glucosidase was tested on a microplate [32]. 170  $\mu$ L of 0.1M phosphate buffer (pH 7.0), 20  $\mu$ L of enzyme solution (0.2 U/mL  $\alpha$ -glucosidase), and 20  $\mu$ L of concentrates were blended. After an incubation time (15 min, 37°C), the reaction was started by including 20  $\mu$ L of substrate (2.5 mM p-nitrophenyl- $\alpha$ -D-glucopyranoside). After this, the

reaction was stopped by mixing 0.2 M Sodium carbonate (80  $\mu$ L) [32]. The absorbance was measured at 405 nm. The enzyme inhibitory rates of the samples were calculated as:

$$\text{Inhibition \%} = \frac{(\text{absorbance of control} - \text{absorbance of sample})}{(\text{absorbance of control})} \times 100 \quad (2)$$

#### 2.8. DPPH (1,1-diphenyl- $\beta$ -picryl-hydrazyl) assay.

1,1-diphenyl- $\beta$ -picryl-hydrazyl radical scavenging (DPPH) assay was done to estimate the antioxidant effect of the extract and juice. Methanol stock solution (50 mL), in various concentrations, was made in test tubes, and 2 mL of 0.06 mm methanol solution of DPPH was added to each of the test tubes containing the samples. Absorbance of the sample solutions was measured at 517 nm, taking methanol as a blank. Ascorbic acid was taken as a standard solution [33]. % inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{((\text{absorbance of control} - \text{absorbance of test}))}{((\text{absorbance of control}))} \times 100 \quad (3)$$

#### 2.9. Hydrogen peroxide ( $H_2O_2$ ) assay.

The reducing power of the extract and juice was determined by doing the  $H_2O_2$  assay [34]. Different concentrations of the test samples were added to 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide, and incubated at 50°C for 20 min. A portion (2.5 mL) of 10% trichloroacetic acid was added to the mixture. Then, the mixture was centrifuged at 3000 rpm for 10 min. 2.5 mL of the supernatant was added to 2.5 mL of distilled water and 0.5 mL of freshly prepared 0.1%  $FeCl_3$  solution, and the absorbance was measured at 700 nm using a UV-Spectrophotometer. Ascorbic acid was used as a standard. % inhibition was calculated as:

$$\% \text{ Increase in reducing power} = \frac{(\text{absorbance of the test})}{(\text{absorbance of the sample} - 1)} \times 100 \quad (4)$$

#### 2.10. Metal ion chelating activity.

The ferrous/ferric ions chelated by the extract were estimated by the method of Dinis, 1994[35]. Samples of various concentrations were made in DMSO, and the volume was raised to 1ml with methanol. Further, 3.7 mL of methanol followed by 50  $\mu$ L of  $FeCl_3$  (2 mM) was mixed. The reaction was initiated by the addition of 5 mM Ferrozine (0.2 mL), and the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the pink violet solution was then measured at 562 nm. The % of Ferrozine- $Fe^{2+}$  complex formation was calculated as:

$$\text{Inhibition \%} = \frac{(\text{absorbance of control} - \text{absorbance of sample}) \times 100}{(\text{absorbance of control})} \quad (5)$$

#### 2.11. In-vivo studies.

##### 2.11.1. Induction of diabetes mellitus type II.

Diabetes Mellitus Type II was induced in fasting rats (12 h) by one dose of intraperitoneal (i.p.) injection of 50 mg/kg Streptozotocin (STZ) in citrate buffer, pH 4.5; after

15 min of the i.p. injection of nicotinamide (100 mg/kg) in normal saline [36]. Rats with blood glucose levels greater than 200 mg/dl were selected for the study.

### 2.12. Experimental design.

The rats were divided into five groups with six rats in each. Group 1 was the Normal control that received vehicle only (distilled water). Group 2 Diabetic control (DC) was administered with a vehicle. Group 3 standard was administered with Metformin (50 mg/kg; by oral route). Groups 4 and 5 were administered orally with ethanol extract and lyophilized juice at doses of 200 mg/kg and 400 mg/kg, respectively [37].

#### 2.12.1. Treatment.

The ethanol extract and lyophilized juice were administered orally to rats daily for 28 days.

#### 2.12.2. Collection of blood and tissue.

After 28 days, blood samples were collected from all groups of rats, and the serum was separated by centrifugation at 3000-5000 rpm for 5-7 min. The serum obtained was separated and used further for the estimation of different parameters.

#### 2.12.3. Estimation of glucose level and lipid profile.

Parameters such as blood glucose levels and lipid profile were measured in serum using a semi-automatic auto-analyser, Model Chem 5 Plus-V2 (Erba Mannheim, Germany). The Erba-supplied kit was used to measure these parameters.

#### 2.12.4. Estimation of *in vivo* antioxidant parameters.

Liver tissue was collected from all rat groups after sacrifice. The tissue was homogenised using normal saline. The homogenate was used further for estimation of total protein (TP), reduced Glutathione (GSH), Malondialdehyde (MDA), superoxide dismutase (SOD), and catalase by the methods described by Lowry *et al.* [38]; Ellman *et al.* [39]; Okhawa *et al.* [40], Marklund *et al.* [41], and Sinha *et al.* [42], respectively.

### 2.13. Analysis of data.

Analysis of data was done using one-way analysis of variance, followed by Tukey's test. Values  $p < 0.05$  were considered statistically significant.

## 3. Results and Discussion

### 3.1. Preliminary phytochemical screening.

The result, as shown in Table 1, reveals the presence of various phyto-constituents in both the ethanol extract (EE) and lyophilized juice (LJ) of the fruit.

**Table 1.** Preliminary phytochemical screening of the fruit of *M. pumila*.

Sr. No.	Plant constituents and test reagents	EE	LJ
1	Alkaloid		
	Mayers reagent	+	-
	Wagners reagent	+	-

Sr. No.	Plant constituents and test reagents	EE	LJ
2	Carbohydrates		
	Fehling's test	+	+
	Benedict's test	+	+
3	Sterols		
	Liebermann-Burchard test	+	-
	Salkowski test	+	-
4	Saponins		
	Faom test	+	+
5	Phenolic compounds and tannins		
	Ferric chloride test	+	-
	Lead test	+	+
	Potassium dichromate test	+	+
	Gelatin solution test	+	+
	Potassium permanganate test	+	+
	Iodine test	+	+
6	Flavonoids		
	Shinoda/Pew test	+	+
	Ammonia test	+	-
7	Resins	-	+
8	Terpenoids	+	+

+: indicates presence of constituents; - : indicates absence of constituents.

## 2.2. *In vitro* analysis of extract and juice.

### 3.2.1. $\alpha$ -amylase and $\alpha$ -glucosidase inhibition activity.

The concentration required to inhibit 50% of the enzyme (IC<sub>50</sub>) values for Acarbose, EE, and LJ were 60.29±1.79, 600.08±10.28, and 400.96±3.50 µg/mL for  $\alpha$ -amylase, and 39.50±1.24, 598.86±2.96, and 397.38±3.45 µg/mL for  $\alpha$ -glucosidase, respectively.

### 3.2.2. DPPH, H<sub>2</sub>O<sub>2</sub>, and metal chelating assay.

The values for DPPH, H<sub>2</sub>O<sub>2</sub>, and the metal chelating assay are depicted in Table 2. The EE extract has shown good antioxidant properties, comparable to those of the standard (ascorbic acid).

**Table 2.** IC<sub>50</sub> Values of DPPH, H<sub>2</sub>O<sub>2</sub>, and metal chelating assays.

Substance	Metal chelating IC <sub>50</sub> (µg/ml)	H <sub>2</sub> O <sub>2</sub> IC <sub>50</sub> (µg/ml)	DPPH IC <sub>50</sub> (µg/ml)
Ascorbic acid	77.06±1.41	44.93 ±0.61	28.28±1.04
Ethanol extract (EE)	87.06±1.38	62.07 ±5.077*	50.72±1.27
Lyophilized Juice (LJ)	172.74±1.36	135.71±1.55	82.21±0.75

Values are expressed as Mean ± SEM; \* p<0.01 vs. ascorbic acid.

## 3.3 *In vivo* studies.

### 3.3.1. Blood glucose.

At the end of the study, blood glucose was estimated, and the results are presented in Table 3. Extract and juice reduced the raised blood glucose level.

**Table 3.** Blood glucose level after 28 days.

Experimental groups	Blood glucose level (mg/dl)
Normal	82.51±1.10
DC	277.5±1.91
Met.	120.5±1.33
EE	131.5±1.25*
LJ	212.66±1.94

Values are expressed as Mean ± SEM; \* p<0.01 vs. metformin

EE was found to be more efficient when compared to LJ in controlling blood glucose levels.

### 3.3.2. *In vivo* antioxidant parameters.

Liver tissue from all groups of rats was analysed to assess the *in vivo* antioxidant potential. The results have been expressed in Table 4. Both EE and LJ have an antioxidant effect.

**Table 4.** *In vivo* antioxidant parameters after 28 days in liver tissue.

Experimental groups	Total protein (mg/dl)	GSH (µg/mg protein)	MDA (nmoles/mg protein)	SOD (µg/mg protein)	Catalase (µmoles/mg protein)
Normal	63.04±0.83	11.68±0.34	2.09±0.030	3.3±0.08	9.06±0.05
DC	28.56±0.43	5.57±0.16	6.16±0.045	0.9±0.02	3.87±0.03
Met.	54.45±0.65	10.16±0.17	2.90±0.030	2.4±0.06	7.87±0.03
EE	51.22±0.29*	9.40±0.055	3.10±0.051**	2.1±0.05***	7.68±0.04****
LJ	39.56±0.58	6.37±0.061	5.61±0.030	1.3±0.06	5.16±0.01

Values are expressed as Mean ± SEM; Total Protein: \* p<0.05 vs metformin; MDA: \*\*p<0.01 vs. metformin; SOD: \*\*\* p<0.05 vs. metformin; Catalase: \*\*\*\*p<0.01 vs metformin.

### 3.3.3. Lipid profile.

The values presented in Table 5 reveal that EE-treated rats exhibit improved levels of all lipid parameters. The effect is significant compared with the standard drug, metformin. LJ-treated rats also show that juice is effective in dyslipidemia, but it is less potent than EE and metformin.

**Table 5.** Effect of ethanol extract and lyophilized juice on lipid profile.

Treatment	Dose(mg/kg)	Cholesterol (mg/dl)	HDL (mg/dl)	LDL(mg/dl)	TG(mg/dl)
Normal	--	93.11±0.88	29.13±0.5	43.9±0.23	116.16±0.6
DC	--	167.48±0.44	11.85±0.27	104±0.53	180.92±0.42
Met	100	120.97±0.22	22.96±0.28	55.9±0.38	151.32±0.28
EE	600	123.45±0.38	20.78±0.28	58.32±0.46	153.54±0.44**
L J	400	153.88±0.33	13.58±0.36*	73.6±0.39	174.28±0.57

Values are expressed as Mean ± SEM; HDL: \* p<0.01 vs DC; TG: \*\*p<0.05 vs. metformin.

### 3.4. Discussion.

The fruit of the plant *Malus pumila* Mill. contain various phyto-constituents like alkaloids, carbohydrates, sterols, saponins, phenolic compounds, flavonoids, resins, and terpenoids. These constituents of EE and LJ may be responsible for various activities, such as antidiabetic, antioxidant, and lipid-lowering, as shown in the results. Alkaloids possess anti-diabetic, anti-inflammatory, antioxidant, anticholinergic, antitumor, diuretic, antiviral, anti-hypertensive, antiulcer, and analgesic-like activities [43-45]. Terpenoids have various therapeutic effects in conditions such as cancer, malaria, inflammation, and many infectious diseases. The positive impact of terpenes on the treatment of diabetes mellitus and its complications has also been reported [46-50]. Phenolic compounds have antibacterial activity. The role of phenols in reducing the risk of cardiovascular disease is also well documented. Polyphenols present in many plants have the potential to manage diabetes mellitus [51-53]. Sterols and saponins have antifungal and antiviral properties. Plant sterols can lower lipid levels in both diabetic and nondiabetic individuals [54]. Flavonoids can combat cancer, stroke, heart disease, and diabetes mellitus [55-57].

## 4. Conclusions

The plant *Malus pumila* Mill. is easily available. Current research has shown the therapeutic effects of this plant in the management of type II diabetes mellitus. Complications of DM II, like dyslipidemia and oxidative stress, may also be controlled with the fruits of this plant. Moreover, the juice is of natural origin and can be consumed directly in the daily diet. One of the major advantages of this fruit is that it has no side effects like synthetic drugs. Further investigation is required at the clinical level to demonstrate these effects in humans. Additional studies are also required to determine the exact mechanism of action of the extract and the juice at the cellular level.

## Author Contributions

Conceptualization, A.K. and S.S.; methodology, A.K.; software, T.H.; validation, S.L., S.K., and N.V.; formal analysis, R.K.; investigation, S.K.; resources, S.S.; data curation, R.K.; writing—original draft preparation, T.H.; writing—review and editing, A.L.; visualization, S.K.; supervision, S.S.; project administration, S.S.; All authors have read and agreed to the published version of the manuscript."

## Institutional Review Board Statement

The animal study protocol was approved by the IAEC, and animals were maintained under standard conditions and fed in accordance with the CPCSEA guidelines, New Delhi, at Guru Jambheshwar University of Science and Technology, Hisar (Registration no. 0436 dated 23/11/2015).

## Informed Consent Statement

Not applicable.

## Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author."

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## Conflicts of Interest

The authors declare that they have no competing interests.

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