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PRECLINICAL ASSESSMENT OF THE NUTRACEUTICAL BENEFITS OF PRUNUS DOMESTICA L. REGARDING THE PREVENTION OF METABOLIC SYNDROME RISK FACTORS

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Abstract

High consumption of fruit and vegetables has an inverse association with cardiometabolic risk factors. Plums (*Prunus domestica* L.) are fruits with low glycemic index, with high concentration of polyphenols and dietary fibers, thus their consumption in adequate amount and on a regular basis could be a potential preventive strategy against metabolic syndrome (MetS). The present study aimed to (1) characterize the metabolite profile of a hydroethanolic extract of *P. domestica*, using a multimethodological approach involving two analytical techniques i.e., targeted ultra-high-performance liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) and untargeted nuclear magnetic resonance (NMR) spectroscopy, and (2) study the biological effects of hydroethanolic extract of *P. domestica* involving *in vitro* and *in vivo* experimental models of MetS risk factors. Initially, two varieties of *P. domestica* fruit (*P. domestica* subsp. domestica and *P. domestica* subsp. syriaca) were selected, the skin and pulp of which were separately extracted with hydroethanolic solution of various strengths (99%, 70% and 50%), followed by the determination of total phenolic contents (TPC) using Folin-Ciocalteu method and *in vitro* antioxidant activity using ABTS assay. Based on the higher TPC, higher antioxidant activity, and the lower concentration of ethanol used for extraction, making the extraction method more sustainable from an economic and environmental point of view, the *P. domestica* subsp. syriaca fruit pulp extract obtained with 50% hydroethanolic solution was selected for the chemical characterization and subsequent biological activities. The UHPLC-HRMS analysis showed the presence of hydroxycinnamic and quinic acid derivatives (46.7% of total peak area), procyanidins (37%), and flavonol glycosides (7.9%). The NMR analysis showed, among

saccharides, an abundant presence of glucose (106.59 µg/mg dry weight) followed by sucrose (31.59 µg/mg) and xylose (0.56 µg/mg). Considering the fact of being used in MetS risk factors, the sugar contents of fruit pulp extract were precipitated out with absolute ethanol and ultra-freezing temperature, leaving behind the extract (with possible minimal sugar contents) for assessment in biological activities.

Inhibition of the activities of different enzymes associated with the MetS by *P. domestica* fruit extract was assessed and the half minimal inhibitory concentration (IC50) for each enzyme was determined by nonlinear regression analysis. The fruit extract inhibited α-amylase, α-glucosidase, HMG CoA reductase, and pancreatic lipase enzyme activities, with IC50 values of 7.01 mg/mL, 6.4 mg/mL, 2.5 mg/mL, and 6.0 mg/mL, respectively. The *in vitro* anti-inflammatory potential of *P. domestica* fruit extract was evaluated using Murine monocyte/macrophage J774 cell lines. The fruit pulp extract significantly inhibited lipopolysaccharide-induced production of pro-inflammatory mediators (nitrite, IL-1β and PGE_2) in activated J774 macrophages in dose dependent fashion, while no such effect was seen in unstimulated cell lines. *In vivo* studies revealed considerable hypoglycemic and hypoinsulinemic activities of *P. domestica* fruit pulp extract in healthy mice. The BALB/c mice were challenged with oral sugars load (sucrose/glucose, 1 mg/6375 mg for 50 ml of solution) followed by the supplementation of *P. domestica* fruit pulp extract (75 mg/mL, 300 µL/mouse). The blood glucose and insulin levels were measured at time intervals T0, T10 min, T20 min, and T 1 h. *P. domestica* fruit extract showed significant effects at T10 min and T20 min in reducing blood glucose and insulin levels, when compared with control mice.

The *P. domestica* fruit pulp extract (without sugar contents) was analyzed with HPLC-MS/MS technique, to determine the change in metabolic profile of the extract during chemical

precipitation of sugar contents. HPLC-MS/MS analysis revealed the presence of 20 polyphenolic compounds representing the polyphenol classes of phenolic acids, flavan-3-ols, and procyanidins, while the presence of flavonol glycosides was not detected, that might be precipitated out with sugar precipitation. The *in vitro* bioaccessibility of *P. domestica* fruit pulp extract using *in vitro* static digestion model revealed the significant decrease in TPC in the extract (in particular at duodenal stage of digestion), where hydroxycinnamic acids turned out to be the only bioaccessible polyphenols class following oro-gastro-duodenal digestion.

The present study investigated the nutraceutical benefits of *P. domestica* fruit pulp extract regards the MetS risk factors reduction. It resulted in the *in vitro* modulation of the activities of metabolic enzymes and significant reduction of pro-inflammatory mediators. The *in vitro* results were strongly supported by *in vivo* experiments, where the fruit pulp extract significantly reduced the blood glucose and insulin levels in healthy mice challenged with oral sugars load. In view of a possible application of this extract as food supplement ingredient, as the *in vitro* bioaccessibility study revealed only the presence of hydroxycinnamic acid derivatives following oro-gastric-duodenal digestion, the use of gastro resistant oral dosage forms is suggested to partially prevent the degradation of the bioactive extract and to maintain the phytocomplex. In conclusion, this extract could be used to reduce MetS risk factors, and in turn to prevent the cardio-metabolic disorders.

Contents

1. Introduction

1.1. *Prunus domestica* L.

Genus *Prunus* belongs to family Rosaceae (subfamily Amygdaloideae or Prunaceae), comprised of around 400 – 430 species of trees and shrubs that are distributed around the World (across African, Russian, European, Asian, and North American countries) [1]. *Prunus* includes some of the important ornamental and fruit plants i.e., *P. domestica* L. (common plum), *P. persica* (L.) Batsch (peach), *P. lanata* (Sudw.) Mack. & Bush (American plum or wild plum), *P. cerasifera* Ehrh. (cherry plum), *P. amygdalus* Batsch (almonds), *P. armeniaca* L. (apricots), *P. avium* (L.) (wild cherry or sweet cherry), *Cerasus pseudocerasus* (Lindl.) Loudon (Chinese sour cherry), and *Cerasus serrulata* var. *serrulate* (Japanese cherry or oriental cherry) [2].

As per earliest data, the origin of plums is China, around 470 BC. Plums are known to Europe for 6000 years as their history dated back to Greek and Roman times [3]. Around 300 European plum varieties have been mentioned in ancient Roman times. Interestingly, European plums introduced to United States by pilgrims. Japanese plums are actually originated in China and were introduced to Japan 200 – 400 years ago. Plums are one of the fruits, first domesticated by Human beings, the remains of which have been found in Neolithic age along with grapes, figs, and olives. In modern world, plums are cultivated in temperate climate countries around the world [4].

1.1.1. Botanical features and taxonomy

Most of the *Prunus* species are evergreen but some are deciduous too. Trees are usually medium-sized (maximum 6 meter tall) with straight branches and greyish-brown trunk [5]. Flowers are hermaphroditic and are usually white to pink/red in color and leaves are simple in nature (with

toothed margins). Some species like *P. spinosa* are blackthorns, having thorns. Plum fruits are best described as fleshy drupes containing a single hard-coated seed [6,7]. **Figure 1** is illustrating *P. domestica* tree.

Fig. 1.*Prunus domestica* L. tree

Plums can be categorized in 5 classes: (a) diploid myrobalane (e.g., *P. cerasifera*) distributed across European and Asian countries, and the fruits of which are soft and sweet with sour skin; (b) tetraploid sloe (e.g., *P. spinosa*) distributed across Europe and Asia like *P. cerasifera*, contained small (6 – 16 g) fruits with dark blue color and bitter fruits skin and pulp; (c) diploid native North American plum species (e.g., *P. americana*, *P. besseyi*, and *P. nigra*) that are cultivated but instead are contributed to the development of the Japanese plums; (d) diploid Japanese plum that is hybrid between North American native diploid plum species and *P. salicina*; (e) European plum (e.g., *P. domestica*) that is typical form of these species and is

widespread across the European countries [3]. Despite of great diversity, only two species (hexaploid European plum and diploid Japanese plum) predominate the modern market [8].

As suggested by Crane and Lawrence (1934), hexaploid *P. domestica* (2n = 6x = 48) is an amphidiploid hybrid of sloe, tetraploid *P. spinosa* (2n = 4x = 32) and diploid *P. cerasifera* (2n = 2x = 16) [9]. In 1994, Salesses and Bonnet demonstrated that *P. spinosa* is itself an amphidiploid hybrid between *P. cerasifera* (genome formula C'C') and unknown diploid type of *P. spinosa* (SS). These findings suggest that *P. domestica* may consist of four *P. cerasifera*- and two *P. spinosa*-derived genomes (SSC'C'CC) [10]. The hybrid nature of *P. domestica* is now widely accepted and the origin of these species is assumed to be the Caucasian region with the fact that both *P. spinosa* and *P. cerasifera* are native there. However, the exact origin of *P. domestica* still remains mysterious, and Damsons and Bullaces are also suggested as the primitive forms of *P. domestica*. Interestingly, as no dark blue genotypes are identified in *P. cerasifera* group, thus *P. spinosa* seems to be involved in the evolution of the European plum [3].

In 1906, Hegi [11] identified European plum as a member of the *Prunophora* subgenus that itself divided into two sections i.e., *Prunocerasus* and *Euprunus*. The North American plum species such as *P. americana*, *P. hortulana*, *P. angustifolia*, *P. maritima*, and *P. munsoniana* are presumed into *Prunocerasus* while the European plum group belongs to the section *Euprunus*, containing plum species distributed in Europe and Asia. Moreover, *P. domestica* species can be categorized in subspecies based on fruit characters. However, subdivision of *P. domestica* species cannot be justified based on genetic studies as concluded by Schmidt (1954) [12] and Johannson and Oldén (1962) [13]. Interestingly, interspecific hybridization between *P. domestica* and *P. armenica* is more successful than *P. domestica* and *P. salicina*, indicating that

P. armenica maybe more closer to *P. domestica* than *P. salicina* [3]. Thus, it can be suggested that *Prunus* species should be reinvestigated for their taxonomic relationship.

Considering fruit characters, all the main groups of plums can easily be distinguished. The stone morphology can be used as the best morphological markers to identify genotypes [14]. Plum fruits **(Fig. 2a)** are round to oval in shape with varying sizes and different colors. They lose their texture when exposed to heating. The pulp is usually clingstone and is soft and juicy. The fruits are usually used for fresh consumption or canning. The soluble solid contents range between 12 – 25% Brix (°Br). Prunes **(Fig. 2b)** in contrast keep their texture when heating; therefore they are commonly used for plum cake. The fruits are usually smaller than plums and are oval to elongated in shape, with high sugar contents (30% °Br). Fruits are mostly in dark blue to purple in color, though some cultivars are also available in pink, red, or yellow color. Pulp color ranges from yellow and green to orange. They are generally used for fresh consumption, baking, cooking and drying [3]. Mirabelle plums (*P. domestica* subsp. syriaca) **(Fig. 2c)** have small round fruits (8 – 12 g) mostly yellow colored with red spots but cultivars with purple and green color are also available. The fruits are very sweet, juicy and freestone. They are used for fresh consumption, canning and brandy industry [3].

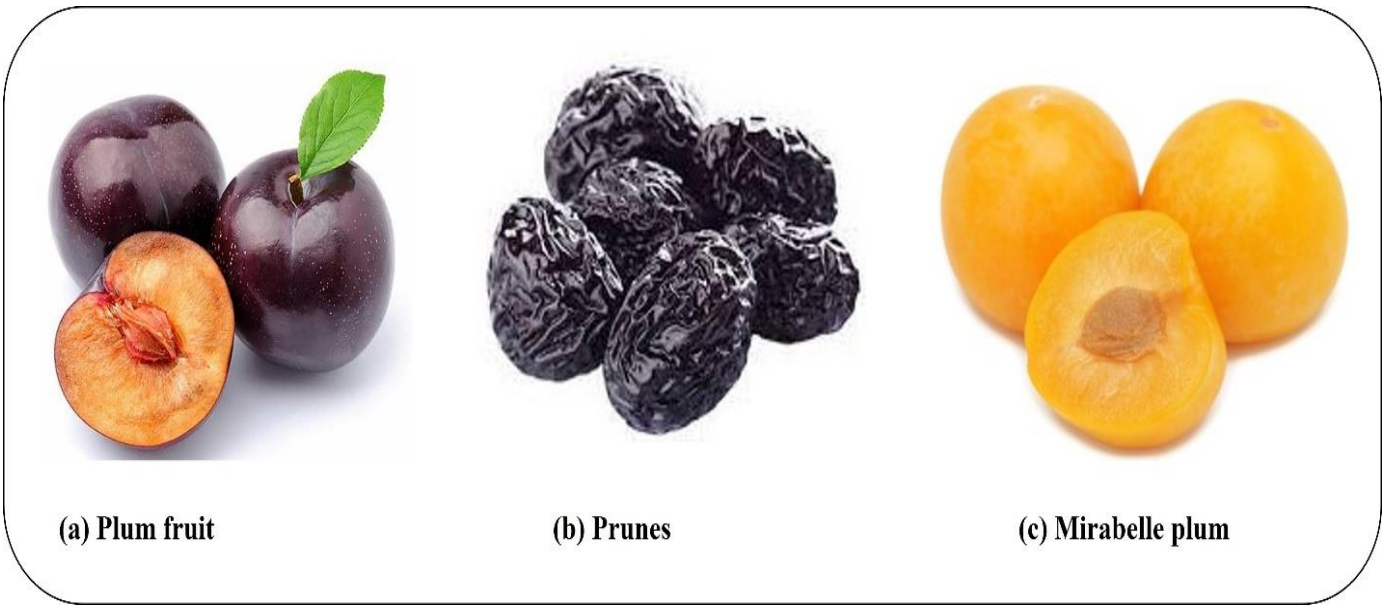

Fig. 2. Plum fruit, prunes and Mirabelle plum

1.1.2. Chemistry

According to the U.S. Department of Agriculture (USDA) National Nutrient Database, *P. domestica* fruits are dense in a number of nutrients including carbohydrates (glucose, fructose, and sucrose), organic acids (malic and citric acid), and micronutrients (vitamins and minerals) [15]. Besides they are also rich in biologically active phytochemicals such as polyphenols (flavonoids and phenolic acids), dietary fibers, phytosterols (stigmasterol and β-sitosterol), and fatty acids (palmitic acid, oleic acid, linoleic acid, and stearic acid) [16], which make daily consumption of plums beneficial in the prevention and control of the numerous chronic degenerative disorders. In addition, presence of about 80% moisture make plums more susceptible to microbial, physical, and chemical spoilage, explaining the reason for their shorter shelf-life [17]. However, methods such as freezing, drying, vacuum packaging, and canning can be utilized in increasing the shelf-life of these fruits [18].

1.1.2.1. Polyphenols

Phenolic acids such as derivatives of chlorogenic acid (5-*O*-caffeicquinic), neochlorogenic acid (3-*O*-caffeicquinic), cryptochlorogenic acid (4-*O*-caffeicquinic) and caffeic acid, along with the smaller amounts of flavan-3-ols, flavonols, and anthocyanins are the predominant polyphenols in plums [19]. Phenolic acids in plums present in varying range depending on the environmental conditions, fruit variety, analytical methods applied i.e., 13 – 430 mg/kg dry weight (DW) for chlorogenic acid, 85 – 1300 mg/kg DW for neochlorogenic acid, and 9 – 56 mg/kg DW for cryptochlorogenic acid [19–21]. Literature data have confirmed the antioxidant activity of chlorogenic acid and isomers (neochlorogenic and cryptochlorogenic acids), that is demonstrated in their beneficial effects on human health such as reducing of reactive oxygen species (ROS), lowering glucose and lipid levels, and anticarcinogenic and antimutagenic effects [22,23]. Catechin, epicatechin, and proanthocyanins (dimers and trimers) among flavan-3-ols and quercetin glycosides (quercetin 3-rutinoside and quercetin 3-glucoside) among flavonols are the major ones present in plums [20,24]. While assessing different fruit species for catechin concentration, plums turned out to contain the highest amount i.e., 49 mg/100 g fresh weight (FW) followed by apples with 10 – 43 mg/100 g FW and berries with 5 – 10 mg/g FW [25]. Cyanidine-3-rutinoside, cyanidine-3-glucoside, and peonidine-3-rutinoside are the major anthocyanins found in plums. Total anthocyanin contents detected was about 18 – 25 mg/100 g FW. In addition, higher concentration of anthocyanins was present in epidermis of the fruits than in pulp. However, in most studies, the total anthocyanin contents were analyzed taking samples from whole fruits [24,26–28]. Polyphenols from different classes found in plums and prunes with their respective quantities are enlisted in **Table 1**.

Table 1. Composition of polyphenols in plums and prunes. Data extracted from Phenol Explorer [29].

Polyphenols	Plums	Prunes
Total phenolic contents	409.79 mg/100 g FW	1195.00 mg/100 g FW
Hydroxycinnamic acids		
3-caffeoylquinic acid	75.88 mg/100 g FW	118.59 mg/100 g FW
3-feruloylquinic acid	1.85 mg/100 g FW	NA
3-*p*-coumaroylquinic acid	1.49 mg/100 g FW	1.32 mg/100 g FW
4-caffeoylquinic acid	1.40 mg/100 g FW	31.25 mg/100 g FW
5-caffeoylquinic acid	8.40 mg/100 g FW	38.79 mg/100 g FW
5-feruloylquinic acid	0.05 mg/100 g FW	NA
Caffeic acid	NA	1.11 mg/100 g FW
p-coumaric acid	NA	1.11 mg/100 g FW
Anthocyanins		
Cyanidin 3-*O*-glucoside	8.63 mg/100 g FW	NA
Cyanidin 3-*O*-rutinoside	33.85 mg/100 g FW	NA

Peonidin 3-*O*-rutinoside	4.85 mg/100 g FW	NA
Peonidin 3-*O*-glucoside	0.46 mg/100 g FW	NA
Flavan-3-ols		
(+)-Catechin	4.60 mg/100 g FW	NA
(-)-Epicatechin	2.22 mg/100 g FW	NA
Procyanidin dimer B1	8.84 mg/100 g FW	NA
Procyanidin dimer B2	5.20 mg/100 g FW	NA
Procyanidin dimer B3	1.00 mg/100 g FW	NA
Procyanidin dimer B4	1.02 mg/100 g FW	NA
Procyanidin dimer B5	1.59 mg/100 g FW	NA
Procyanidin dimer B7	4.69 mg/100 g FW	NA
Procyanidin trimer C1	10.01 mg/100 g FW	NA
Procyanidin trimer EEC	7.73 mg/100 g FW	NA
Flavonols		
Quercetin 3-*O*-galactoside	0.27 mg/100 g FW	NA
Quercetin 3-*O*-glucoside	0.54 mg/100 g FW	NA

Quercetin 3-*O*-rutinoside	5.90 mg/100 g FW	2.50 mg/100 g FW

The higher polyphenolic contents in plums make them fruits with higher antioxidant capacity than bananas, grapes, pears, apples, kiwi, and melons while in dried form (i.e., prunes) it demonstrated greater antioxidant potential than blueberries, black currants, and strawberries [21,30]. The drying process may increase the antioxidant capacity of these fruits due to the formation of Maillard's reaction products. As the drying process showed a decrease in total phenolic contents (TPC) of about 40 – 46 % at 60 ℃ and 31 – 38 % at 85 ℃ temperature, with exponential increase of antioxidant capacity of 0 – 30% at 60 ℃ and 90 – 250 % at 85 ℃ [31]. The increase of antioxidant activity at 85 ℃ is significantly correlated with the contents of hydroxymethylfural (one of the Maillard's reaction products). While assessing TPC and antioxidant capacity in dried fruits, dates showed the highest TPC (1959 mg/100 g FW) followed by cranberries (870 mg/100 g FW), plums (788 mg/100 g FW), grapes (551 g/100 g FW), apricots (402 mg/100 g FW) and figs (320 mg/100 g FW). Regards to antioxidant capacity, prunes and figs showed the highest antioxidant activity (expressed as a phenol concentration) as 4.38 µmol/L and 4.41 µmol/L, respectively. While dates and apricots showed the lowest antioxidant activity as 2.17 µmol/L and 1.93 µmol/L, respectively [32].

Considering the fact polyphenols are protecting against UV radiation and microbial contamination, they are present in epidermis in higher concentrations as compared to fruit pulp. In case of plums, concentration of phenolic contents is 3 – 4 times higher in epidermis than in pulp while the anthocyanin contents are 3 – 30 times higher in fruit skin than in fruit pulp. Since epidermis represents only about 8% of fruit weight, fruit pulp constitutes about 70% of total

polyphenols present in fruits [28]. Interestingly, studies on European plums also demonstrated a considerable correlation between TPC and color parameter L (lightness), where darker color of fruits (i.e., decrease in L values) resulted in increase in TPC [33]. For plums variant V95141, the L value was 49.8 and TPC was 100 mg/100 g FW while for variant V72511 (with dark skin), the L value was 23.0 and TPC was 369 mg/100 g FW.

1.1.2.2. Phytosterols

Phytosterols (plant sterols and stanols) are steroid compounds with considerable impact on human health, particularly they are known for their potential to reduce blood cholesterol levels. The most common phytosterols distributed in plant kingdom are β-sitosterol, campesterol, and stigmasterol [34,35]. Research study showed that overweight individuals do not include phytosterols rich dietary sources in their daily diet [36]. Górnaś *et al*. 2016 [37] recorded 297.2−1569.6 mg/100 g total phytosterol concentration in plum kernel oil samples, with β-Sitosterol being present in highest concentration (i.e., 60 – 80 % of total identified phytosterols). Other phytosterols detected were Δ5-avenasterol (4 – 8 %) and campesterol (3 – 6 %).

1.1.2.3. Carotenoids

Carotenoids are the natural fat pigments found in plants and photosynthetic bacteria, playing vital role in photosynthesis. They are responsible for the yellow, orange, and red color of leaves, flowers, and fruits. Carotenoids mainly possess antioxidant activity in human body [38]. Plums contain relatively a high concentration of carotenoids. Kaulmann *et al*. [39] assessed the carotenoids profile of different plum varieties, and the results indicated Cherry plum, Kirks plum as the richest sources containing 1.96 mg/100 g and 1.95 mg/100 g total carotenoids, respectively followed by Italian plum (1.90 mg/100 g) and Graf Althans plum (1.24 mg/100 g). Mirabelle

plum contained 0.884 mg/ 100 g total carotenoids. The main carotenoids found were α-carotene, β-carotene, lutein, zeaxanthin, phytoene, and betacryptoxanthin.

1.1.2.4. Dietary fibers

Among fruits, plums are one of the richest sources of soluble dietary fibers [40,41]. Dietary fibers particularly soluble fibers exert a range of beneficial effects on human body such as reduction of the risk of metabolic and cardiovascular disorders, gastrointestinal diseases, and malignancies and improvement of neuropsychiatric health. The beneficial effects maybe directly through the reduction of glucose and lipid absorption or indirectly through the prebiotic effects (improving intestinal permeability, insulin sensitivity, and relieving glucose intolerance and inflammation) [42]. While decreasing the intestinal pH via increased production of short chain fatty acids, dietary fibers (both soluble and insoluble fractions) may reduce the absorption of toxic amines [43]. A study by Dikeman *et al*. [44] exhibited the presence of 6% total dietary fibers in dry matter in plums fruits and juices while 65% total dietary fibers in dried waste by-products. Fatimi *et al*. [41] concluded that prunes contained relatively high concentration of dietary fibers i.e., 6 – 7 % of fresh fruit, with 43% insoluble fibers and 57% soluble fibers. Depending on plum varieties and pomace drying, plum pomaces are characterized by 38 – 49 % total dietary fibers with 7 – 13 % soluble fibers in dry matter [45]. A study conducted by Kosmala *et al*. [46] demonstrated the presence of 58 – 82 % polysaccharides in alcohol insoluble solids depending on plum varieties, with galacturonic acid (335–393 mg/g for pomaces and 222–387 mg/g for fruits) and cellulose expressed as glucose (129–172mg/g for pomace and 108–126 mg/g for fruits) being most abundant ones. Other polysaccharides include galactose, arabinose, Xylose, rhamnose, mannose. Other studies also indicted pectin as the one of the abundant polysaccharides in plum fruits [40,47,48].

1.1.2.5. Nutritional importance

Plums and prunes are importance sources of dietary and nutritional components including carbohydrates (glucose, fructose, sucrose, galactose, and maltose), dietary fibers, organic acids (malic and citric acids), vitamins (vitamin A, vitamin E, vitamin K, ascorbic acid, and B complex vitamins), and minerals (calcium, iron, magnesium, phosphorus, potassium, sodium, zinc, copper, mangansese, and selenium) [15,49]. Numerous volatile compounds including benzaldehyde, ethyl nonanoate, linalool, γ-decalactone, and methyl cinnamate have also been isolated from plum fruits, contributing to plum aroma [49]. Nutritional components of plums and prunes along with their respective quantities are enlisted in **Table 2**.

Table 2. Nutritional composition of plums and prunes. Data extracted from USDA Food Composition Databases (amount per 100 g) [15].

Component	Unit	Plums	Prunes
Macronutrients			
Water	g	87.23	30.92
Energy	kJ	192	1006
Carbohydrate	g	11.42	63.88
Protein	g	0.7	2.18
Fat	g	0.28	0.38

Total dietary fibers	g	1.4	7.1
Vitamins			
Vitamin A	IU	345	781
Vitamin E (α-tocopherol)	mg	0.26	0.43
Vitamin K (phylloquinone)	µg	6.4	59.5
Ascorbic acid	mg	9.5	0.6
Thiamine	mg	0.028	0.051
Riboflavin	mg	0.026	0.186
Niacin	mg	0.417	1.882
Pantothenic acid	mg	0.135	0.422
Pyridoxine	mg	0.029	0.205
Folate	µg	5	4
Choline	mg	1.9	10.1
Minerals			
Calcium	mg	6	43
Iron	mg	0.17	0.93

Magnesium	mg	7	41
Phosphorus	mg	16	69
Potassium	mg	157	732
Sodium	mg	0	2
Zinc	mg	0.1	0.44
Copper	mg	0.057	0.281
Mangansese	mg	0.052	0.299
Selenium	μg	0	0.3

1.2. Metabolic syndrome

Metabolic syndrome (MetS) also called as Syndrome X, Reaven's syndrome, the insulin resistance syndrome, and the deadly quartet is a cluster of pathologies including hyperglycemia, obesity, hypertension, and hyperlipidemia, that predispose an individual to increasing risk of type 2 diabetes mellitus (DM2) and cardiovascular disorders [50]. It was referred by Kylin in 1920's to as the "syndrome X" to describe the clustering of hypertension, obesity and gout [51]. Gerald Reaven reintroduced the concept in 1988 in the Banting lecture to describe the clustering of risks for cardiovascular disorders [52]. About 10 years later, the concept of MetS took another turn with the introduction of the diagnostic criteria and definitions by World Health Organization (WHO), International Diabetes Federation (IDF), and National Cholesterol Education Program's

Adult Treatment Panel III (NCEP: ATP III) **(Table 3).** The simplest diagnostic tool used by clinicians is the identification of patients at significantly increased risk for the development of DM2 and/or cardiovascular disorders [53]. According to WHO, insulin resistance is the driven force for the syndrome while IDF and NCEP: ATP III described central obesity as a prerequisite for the syndrome [53].

Table 3: Diagnostic criteria of Metabolic syndrome.

World Health Organization (WHO), 1998	Insulin resistance, DM2, Impaired fasting glycemia (IFG); Impaired glucose tolerance Plus two of the following criteria ✓ Obesity: BMI > 30 or waist/hip ratio of > 0.9 (M) or > 0.85 (F) ✓ Hypertension: BP > 140/90 mm Hg ✓ Dyslipidemia: TG ≥ 1.7 mmol/L or HDL-C < 0.9 mmol/L (M) or < 1.0 mmol/L (F) ✓ Microalbuminuria: > 20 μg/min
International Diabetes Federation (IDF), 2005	Central obesity [Waist circumference of > 94 cm (M) or > 80 cm (F)] Plus any two of the following criteria ✓ Hyperglycemia: FPG ≥ 5.6 mmol/L or Rx

	✓ Hypertension: > 130 mmHg (systolic) or > 85 mmHg (diastolic) or Rx ✓ Dyslipidemia: TG > 1.7 mmol/L or Rx ✓ Dyslipidemia: HDL-C < 1.03 mmol/L (M), < 1.29 mmol/L (F) or Rx
National Cholesterol Education Program's Adult Treatment Panel III (NCEP: ATP III), 2001	Any 3 of the following criteria ✓ Obesity: Waist circumference of > 102 cm (M) or > 88 cm (F) ✓ Hyperglycemia: FPG ≥ 6.1 mmol/L or Rx ✓ Hypertension: ≥ 130/85 mmHg or Rx ✓ Dyslipidemia: TG ≥ 1.7 mmol/L or Rx ✓ Dyslipidemia: HDL-C < 1.0 mmol/L (M), < 1.3 mmol/L (F) or Rx

Male (M); Female (F); Type 2 Diabetes mellitus (DM2); Body mass index (BMI); Triglycerides (TG); High density lipoprotein cholesterol (HDL-C); Blood pressure (BP); Medications already taken for specific disease or condition (Rx); Fasting plasma glucose (FPG).

1.2.1. Epidemiology

The worldwide incidence and prevalence of MetS varies depending on sociodemographic and geographic factors, in addition to the diagnostic criteria used. The incidence of MetS often parallels with the incidence of obesity and DM2. According to the National Health and Nutrition Examination Survey data, average body mass index (BMI) increased by 0.37% in both men and

women in United States during 1980 – 2010. Centers for Disease Control and Prevention (CDC) data published in 2017 estimated that 12.2% adults (30.2 million) in United States had DM2, where one quarter (23.8%) of these people were not aware of having diabetes. Incidence of DM2 increased with age i.e., 25.2% in United States geriatric population (≥ 65 years age) [54].

In China, the prevalence of obesity increased from 14.6% to 21.85 between 1992 and 2002, based on WHO criteria. In urban areas the incidence of MetS was 8 – 10.6 % while in rural areas it was 4.9 – 5.3 % [55]. A global survey of obesity done in 2015 in 195 countries estimated around 108 million children and 608 million adults with obesity. Prevalence of obesity doubled in 73 countries since 1980. Increase in the rates of childhood obesity was one of the major concerns [56]. According to this survey, the highest increase in the prevalence of obesity was observed in countries with low socioeconomic index. Global mortality rate because of high BMI was increased by 28.3% between 1990 to 2015. Moreover, obesity contributed to the 120 million disability-adjusted life years, where Bangladesh (one of the countries with low socioeconomic index) was reported to have highest percentage change in age-standardized BMI associated disability-adjusted life years and deaths. On the other hand, Turkey had decreased age-standardized BMI associated morbidity (decrease by 37.2%) and mortality (decrease by 43.7%).

However, obesity is not always parallel to the MetS and there could be a significant percentage of metabolically healthy obese individuals with enough insulin sensitivity and absence of hyperlipidemia and hypertension [57]. A single center cross-sectional study (CoLaus study), among 6188 randomly selected Caucasian subjects from Lausanne, Switzerland (aged 35–70 years), the prevalence of overweight, obesity, diabetes, hyperlipidemia, and hypertension was around 36.6%, 15.7%, 6.6%, 34.2%, and 36.7%, respectively [58].

Global prevalence of diabetes according the IDF diabetes atlas is around 415 million of world population (8.8%) as of 2015 and is expected to increase to 642 million world population (10.4%) by 2040. American and Caribbean regions were noted with the highest prevalence rates of diabetes (11.5%). More than half of all subjects with diabetes were living in the Western Pacific and Southeast Asian regions. In African region the prevalence of diabetes is still very low but in next 25 years the highest growth in the prevalence rate in Middle East/North Africa (104%) and sub-Saharan Africa (141%) is expected [59].

Though we do not have similar global epidemiological data on the MetS and since it is three times more common than diabetes, thus the global prevalence of MetS can be estimated around one quarter of the world population (i.e., over one billion people).

1.2.2. Risk factors

The potential risk factors for MetS may include positive family history, smoking, advancing age (≥ 60 years of age), obesity, low socioeconomic status, Mexican American ethnicity, postmenopausal status, physical inactivity, sugar-sweetened beverages and soft drink consumption, increased alcohol consumption, Western dietary patterns, some medications like antiretroviral drugs or atypical antipsychotic drug use e.g. clozapine [50]. The National Heart, Lung, and Blood Institute (NHLBI) differentiated the risk factors of MetS that subject can be control (lifestyle habits and occupation) and those factors that cannot be control (age, environment, family history and genetics, sex, and other medical conditions like obesity, polycystic ovary syndrome (PCOS), psoriasis, sleep problems and use of medications to treat other ailments) [60]. Regarding lifestyle habits, being sedentary, eating unhealthy diet, not getting enough sleep, smoking and excessive consumption of alcohol put an individual at higher

risk for MetS. Moreover, individuals working in shifts possess higher risk for MetS because of circadian clocks not aligned with the environment [60]. Melatonin is a neuroendocrine hormone produced by pineal gland, acting as free radical scavenger and circadian rhythm regulator. Studies have demonstrated beneficial effects of melatonin in preventing cardiovascular risk factors and MetS [61]. Low socioeconomic status may lead to inactive lifestyle, unhealthy diet, and sleep problems [60].

Overweight and obesity are one of the key risk factors increasing the chances of MetS because of the increase in low density lipoprotein (LDL-C), triglycerides and blood pressure with a decrease in high density lipoprotein (HDL-C). Maternal obesity in the pregnancy may increase the chances of MetS in children. Moreover, low birth weight in infants and rapid weight gain post birth can increase the risk for MetS in later life [60,62]. The hormone changes associated with PCOS can also increase blood sugar and triglyceride levels, with a decrease in HDL-C levels and larger waistline [63]. Several treatments using to treat immune conditions such as psoriasis may also increase the risk for metabolic dysregulations and MetS [60]. In advanced age, women generally have higher risk of metabolic disorders as compared to men because of the fact that hormonal changes associated with menopause may increase blood sugar levels with larger waistline and low HDL-C levels [64].

1.2.3. Pathobiology

Insulin is produced by β-cells of the pancreas and is responsible for glucose uptake and consumption in various tissues (i.e., skeletal muscles, liver, and adipose tissues). It stimulates glycogenesis utilizing glucose molecules while decreasing gluconeogenesis in the liver. It also inhibits lipolysis in adipose tissues, thus lowering the release of free fatty acids (FFAs). The net

result is increase in glucose uptake, conversion of glucose into glycogen or fat molecules and decrease in blood glucose level and FFA concentration [65]. In case of insulin resistance skeletal muscles, liver and adipose tissues do not respond effectively to insulin resulting in hyperglycemia, and deregulated feedback mechanisms further exacerbated this phenomenon [66]. **Figure 3** has shown the pathophysiological mechanism of MetS.

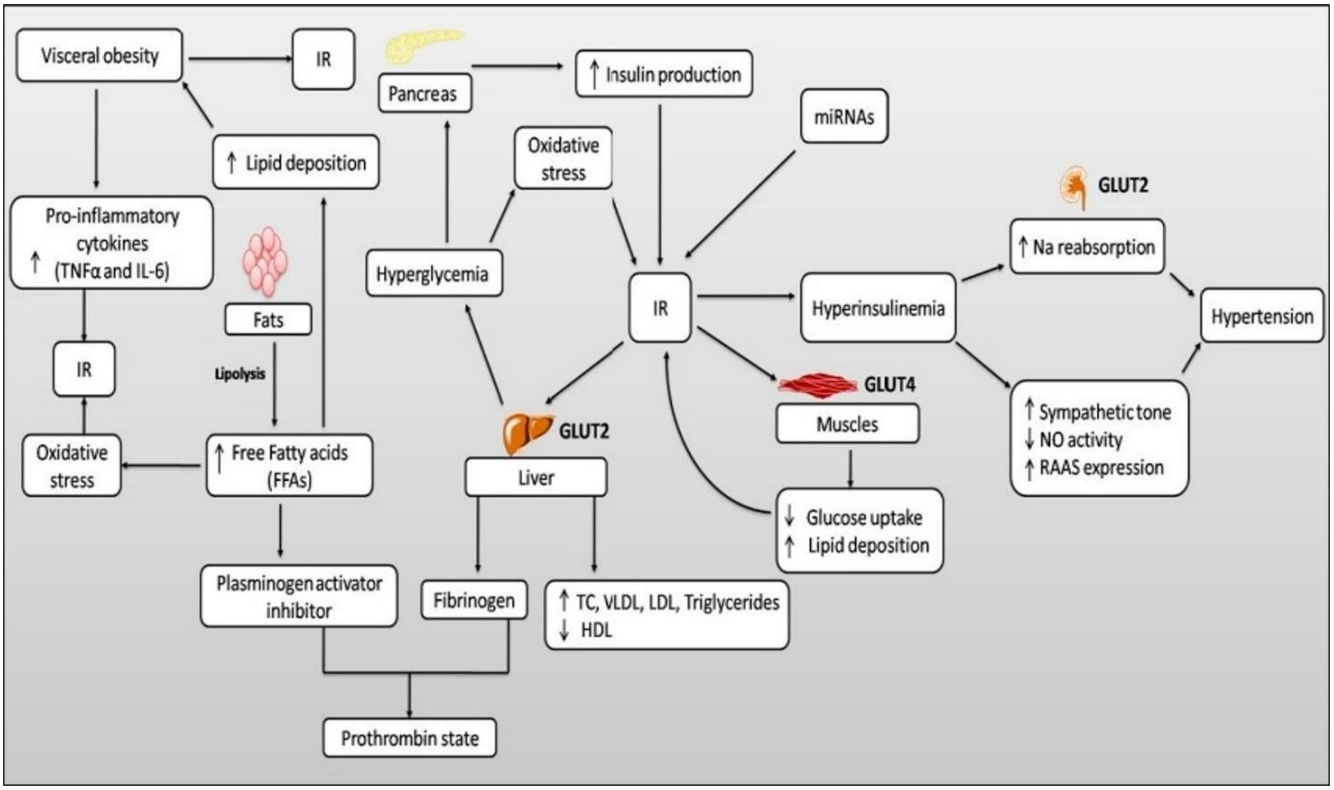

Fig. 3. Pathophysiological mechanisms of metabolic syndrome. Insulin resistance is the central part of MetS which may contribute to individual component of the syndrome. Insulin resistance can be increased by several factors such as miRNAs, oxidative stress and enhanced production or expression of pro-inflammatory cytokines. The primary sites of insulin resistance are skeletal muscles and liver and as a result, dysregulation in the metabolism of glucose and lipids occurs. This phenomenon leads to hyperglycemia, hypercholesterolemia and increased lipid deposition in skeletal muscles, which may further trigger insulin resistance. Hyperglycemia may signal β-

cells of pancreas to produce more insulin but as a result of insulin resistance it eventually leads to hyperinsulinemia, which contributes mainly to produce hypertension by increasing renal sodium reabsorption, increasing sympathetic tone and RAAS expression and decreasing NO activity. Lipolysis can result in increased fatty acids production, which contributes mainly to visceral obesity. Increased production of fibrinogen by liver and increased expression of Plasminogen activator inhibitor leads to prothrombin hypercoagulable state. Increase (↑); decrease (↓); insulin resistance (IR); microRNAs (miRNAs); glucose transporter-2 (GLUT2); glucose transporter-4 (GLUT4); nitric oxide (NO); renin-angiotensin-aldosterone system (RAAS); total cholesterol (TC); very density lipoprotein (VLDL); low density lipoprotein (LDL-C); high density lipoprotein (HDL-C); tumor necrosis factor-alpha (TNFα); interleukin-6 (IL-6).

Visceral obesity is known to play a central role in insulin resistance by the increased release of non-esterified fatty acids from adipose tissues and dysregulated adipocytokines (increased production of Plasminogen activator inhibitor-1 (PAI-1) and tumor necrosis factor – alpha (TNF-α) and subsequently a decrease in adiponectin and leptin). The increased release of FFAs results in lipotoxicity, as they accumulated in liver and other organs such as pancreas and heart, resulting in organ dysfunction which leads to impaired insulin secretion and sensitivity, and dysregulated glucose and lipid homeostasis as well as impaired cardiac functions [67,68]. Leptin mediates food intake and hepatic glucose production through modification of signal transducer and activator of transcription-3 (STAT-3) signaling [69–71]. However, control of adipose tissues lipogenesis has been linked with the central activation of phosphoinositide 3-kinase (PI3K) pathway and sympathetic nervous system, and with downstream regulation of adipose tissue endocannabinoid system [72,73]. Adiponectin regulates insulin sensitivity and arrests

macrophage infiltration of adipose tissues. Several studies showed an inverse relation of adiponectin plasma levels with the insulin resistance [74,75].

In addition, incretin hormones (which augment insulin secretion in response to increase in blood glucose levels) are impaired in DM2 patients, and it has been suggested that treatment with incretin mimetics can improve all components of MetS including hyperglycemia, dyslipidemia, hypertension, and systemic inflammation [76]. The circulating FFAs in the presence of insulin resistance and hyperinsulinemia result in the formation of triglycerides, increased production of very low-density lipoprotein (VLDL) and decreased clearance of VLDL. Deregulation of the activity of lipoprotein lipase also occurs in insulin resistant individuals as it is normally regulated by insulin, which results in impaired metabolism of lipids. VLDL is metabolized into LDL-C and remnant lipoproteins, both of which promote atheroma formation. Cholesterol ester transport protein transfers triglycerides in VLDL to HDL-C in exchange for cholesteryl results to form triglycerides enriched HDL-C, which is cleared by hepatic lipase leaving fewer HDL-C particles [77].

Hypertension is a key and silent symptom of MetS and is frequently associated with obesity and impaired glucose and lipids homeostasis [78]. Insulin resistance, hyperinsulinemia and hyperglycemia activate the renin-angiotensin-aldosterone system (RAAS) by upregulating the expression of angiotensinogen and angiotensin receptors (AT I and AT II) [79,80]. The activated RAAS expression promotes oxidative stress, which may induce islet cell dysfunction in the pancreas and insulin resistance in the peripheral tissues [81]. The link between obesity and hypertension has been explained, involving insulin, leptin and sympathetic nervous system. Insulin and leptin are compensatory components required to restore energy balance with the activation of sympathetic nervous system [82].

Low grade chronic inflammation and prothrombin states are essential hallmarks of MetS that increase the risk of cardiovascular disease and may lower life expectancy [83]. Elevation of plasma levels of CRP, TNF-α and interleukin-6 (IL-6) are markers of chronic inflammation that may be seen in patients with dysregulated metabolism [84,85]. Adipose tissue resident macrophages when get activated, cause an upstream regulation of the expression of TNF-α and IL-6. The combination of adipocytes and macrophages with hyperglycemia, hyperinsulinemia and dyslipidemia in the vasculature may lead to endothelial dysfunction and accelerate atherogenic state. The common most feature of endothelial dysfunction is the reduced bioavailability of nitric oxide (NO) in the vasculature; may be due to reduce NO synthesis due to co-factor deficiency and increased NO breakdown due to chemical reaction with oxygen free radicals [86]. These mechanisms are basically responsible for high rate of cardiovascular morbidity and mortality in patients with MetS [87].

1.2.4. Pharmacological therapy and limitations

In addition to the lifestyle interventions targeted pharmacological approach to treat the individual component of MetS is often essential, however therapy must be individualized. Anti-obesity drugs (such as phentermine, phentermine/topiratmate, and orlistat) may be considered because of the direct relation of weight loss and insulin sensitivity [88]. Metformin, pioglitazone, and rosiglitazone are insulin sensitizers and are recommended in patients with insulin resistance. They are also used in the management of DM2 in patients with or without MetS [89–91]. Lipid-lowering agents (such as statins and fibrates) are used as first line agents in atherogenic dyslipidemia [92]. RAAS blockers are recommended in patients with hypertension and cardiovascular risk factors [93]. More often, multiple pharmacological entities are required to correct individual risk factors and to prevent dysmetabolism associated complications [94].

Nevertheless, there are certain limitations to the pharmacological therapy, the most common of which is the occurrence of undesired side effects, the likelihood of which is more common in patients with MetS where drugs from different pharmacological classes are usually used. Gastrointestinal disturbances and lactic acidosis with metformin, muscle weakness with statins and fibrates, and dry cough with anti-hypertensives are some of the commonly occurring adverse events reported with these medications [95]. Alternatively, modifications in diet and lifestyle are necessary in preventing dysmetabolism and MetS risk factors. The use of nutraceuticals and dietary supplements (with solid scientific evidence) may not only reduce the burden of medications in managing MetS but can also decrease the incidence of drug-induced adverse events [96].

1.3. Nutraceuticals as preventive approach

As shown by epidemiological studies, dietary habits possess direct effects on individual's disease risk factors. The beneficial effects of plant derived foods (fruits, vegetables, spices, wine, grains, nuts, and legumes) and bioactive components on human health, in particular age-related pathologies have been widely accepted. The spectrum of age-related diseases (such as metabolic dysregulations, cardiovascular diseases, neurological ailments, and cancers) tends to be wider as the human lives longer. Thus, science has encouraged the intake of healthy foods (especially those derived from plants) to improve the health status and to delay the development of aging and associated diseases [97,98]. However, the health friendly and disease preventing effects of these plant derived foods mainly attributed to the occurrence of non-nutrients components i.e., phytochemicals [97]. Considering these facts, Dr. Stephen Defelice in 1989 coined the term Nutraceuticals for the first time by combining the words "*nutrients*" and "*pharmaceuticals*". Nutraceuticals are bioactive components present in food having properties of both nutrients and

pharmaceuticals, hence they possess scientifically proven medical benefits. According to The Association of American Feed Control Officials (AAFCO), nutrient mean food constituents supporting human or animal life while nutraceuticals are non-toxic food constituents with health benefits (i.e., prevention and treatment of diseases) [99]. Such compounds may range from diets, isolated nutrients, dietary supplements to genetically engineered processed foods and herbal products. "*Pharmaceuticals*" and "*nutraceuticals*" have clear distinction, as the former refer as drugs and is dealing with the treatment of diseases while the latter is intended for the prevention of diseases. However, confusion exists in terms like "*nutraceuticals*", "*functional foods*", "*medical foods*" and "*dietary supplements*", and most of the time people use these terminologies interchangeably. *Functional foods* refer to foods when they cook or prepare applying scientific intelligence with the aim of providing the body with the required amount essential components such as carbohydrates, proteins, fats, and vitamins. When functional foods are aimed of preventing or treating diseases then they are called as *Nutraceuticals* [100]. In other words, functional foods for one subject may act as nutraceuticals for another. *Dietary supplements* refer to products aimed to supplement a diet containing one or more dietary ingredients like vitamins, minerals, amino acids, and herbal extract or bioactive components [101]. *Medical foods* are formulated for the dietary management of specific diseases or conditions [102].

Nutraceuticals and functional foods get considerable interest worldwide over the past few years from scientific community and consumers, owing to their wide range of health friendly effects. Broadly nutraceuticals can be classified into potential and established nutraceuticals, where potential nutraceuticals become an established one after sufficient clinical data [103]. Based on food sources, nutraceuticals can be classified as dietary fibers, probiotics, prebiotics, polyunsaturated fatty acids, antioxidant vitamins, polyphenols, and spices **(Fig.4)** [104]. They

have claimed to have positive effects in oxidative stress, chronic inflammatory diseases, obesity and DM2, cardiovascular disorders, autoimmune reactions, degenerative disorders, and several types of cancers [105].

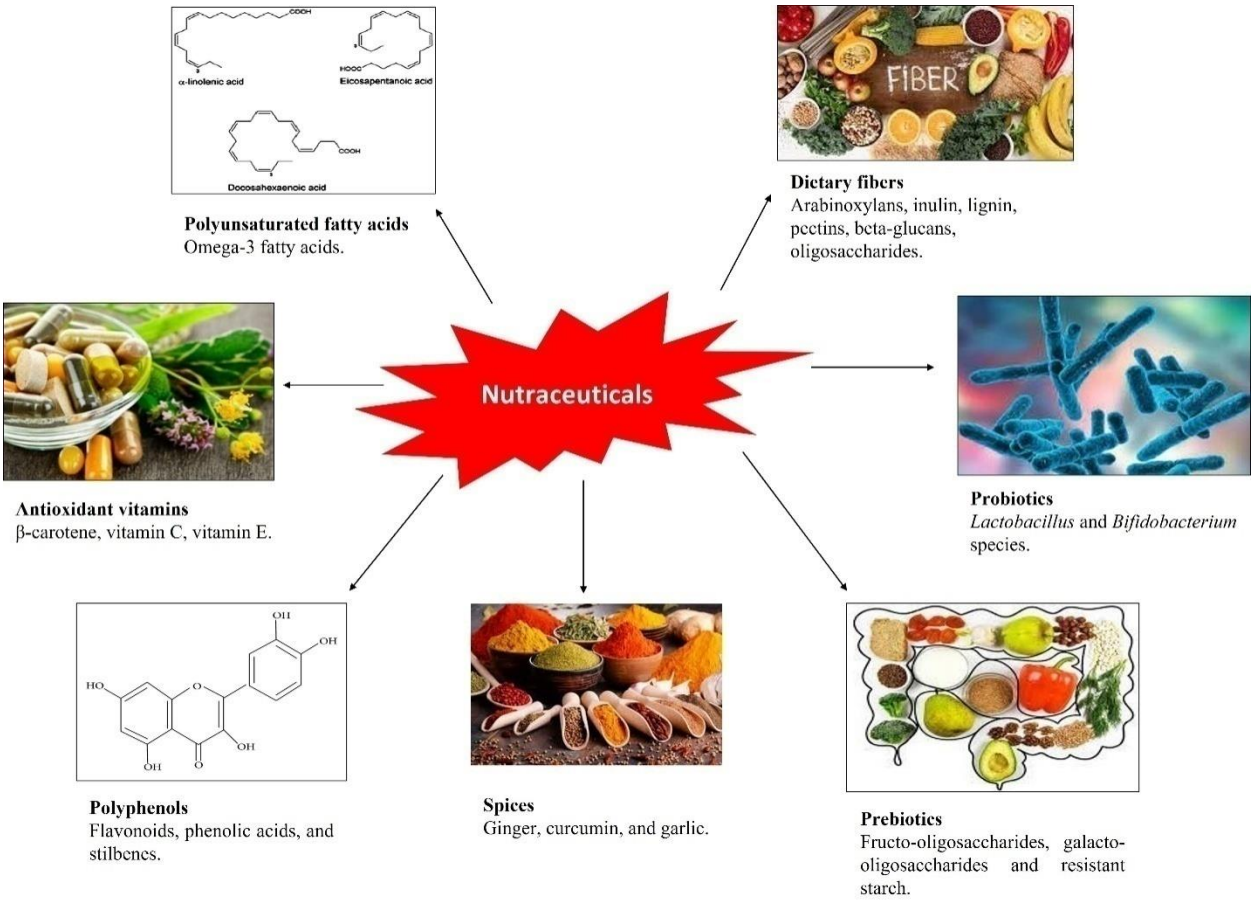

Fig. 4. Classes of nutraceuticals based on food sources.

1.3.1. Dietary Polyphenols

Polyphenols are the largest group of plant secondary metabolites with > 8000 polyphenols being identified to date, containing a benzene ring with hydroxyl (OH) moieties, and categorized mainly into flavonoids and nonflavonoids. Flavonoids **(Fig. 5)** share common carbon skeleton of diphenyl propanes, two benzene rings (ring A and B) joined by a linear three-carbon chain,

where the central three carbon chain form a closed pyran ring with benzene ring A. Based on the substitution patterns and oxidation state of the central pyran ring, flavonoids can further be subdivided into flavonols, flavan-3-ols, flavones, isoflavones, flavanones, and anthocyanidins **(Fig. 6)**. Non-flavonoid polyphenols **(Fig. 6)** can be subclassified into cinnamic acid derivatives, benzoic acid derivatives, coumarins, xanthones, stilbenes, and lignans [106,107]. Phenolic acids account for one third of total polyphenols consumption while flavonoids account for the remaining two third. Anthocyanins, flavan-3-ols (catechins and proanthocyanidins), and their oxidation products are one of the most abundantly flavonoids found in human diet [108]. The most common dietary sources of polyphenols include fruits or fruit juices (apple, pineapple, orange, grapefruit, and *Prunus*), vegetables (cabbage, broccoli, carrot, mint, cucumber, spinach, yellow onion and tomato), beverages (coffee and tea), and wine [109].

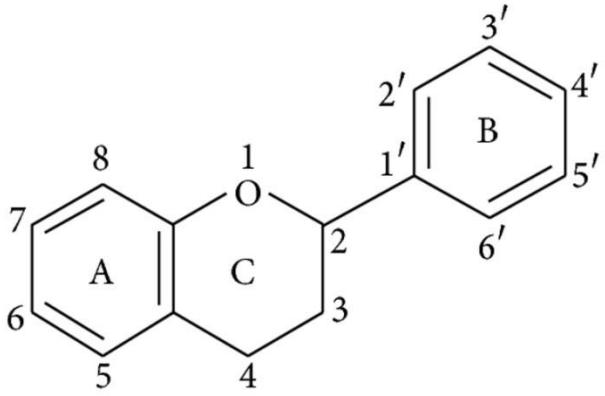

Fig. 5. Basic chemical structure of flavonoid.

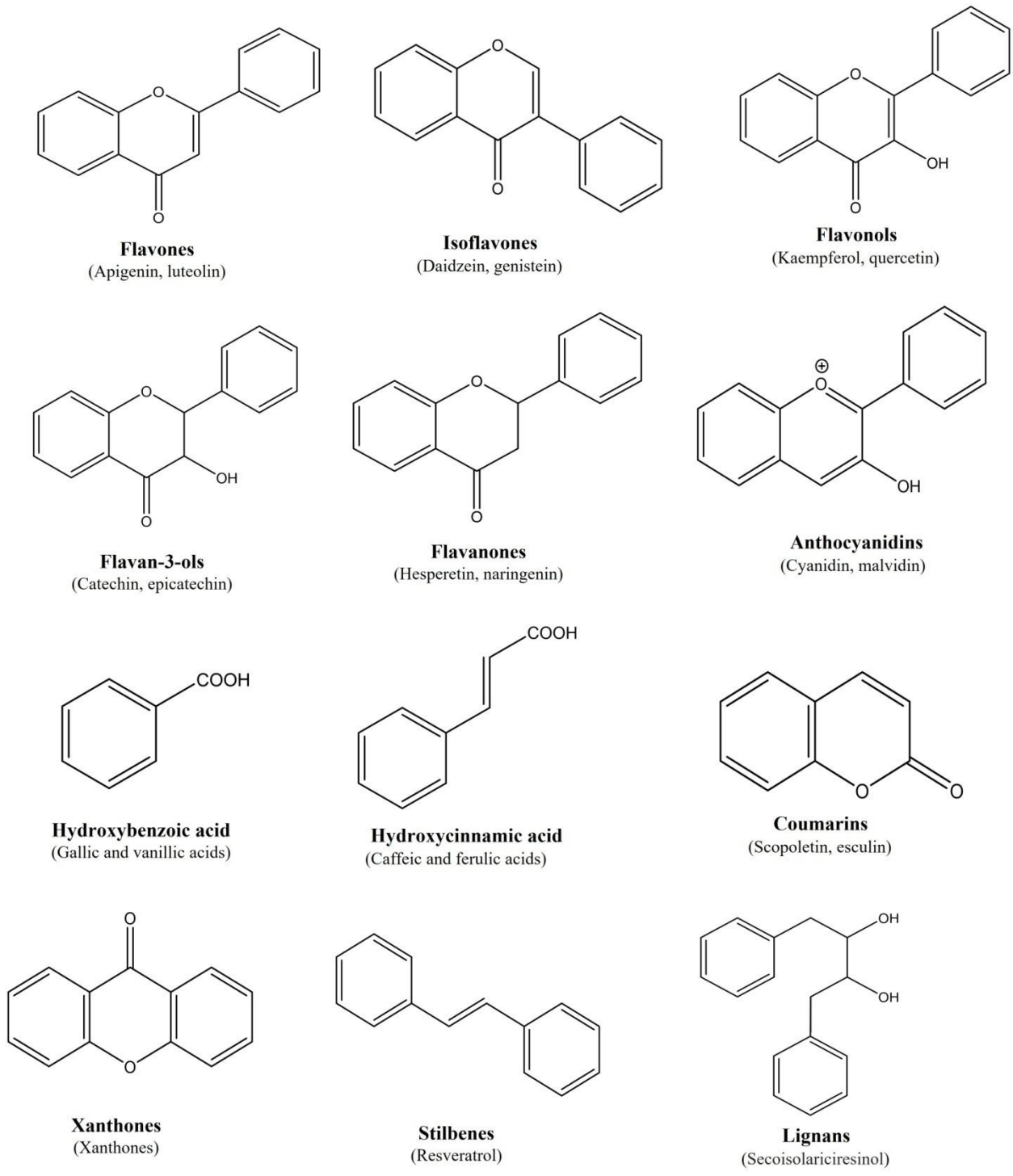

Fig. 6. Basic chemical skeletons of different classes of flavonoids and non-flavonoids.

Dietary polyphenols are potent antioxidants, and they may modulate several signaling pathways at cellular and molecular level. They possess dynamic role in protecting against chronic disorders associated with oxidative and nitrosative stresses such as DM2, cardiovascular disorders, neurodegenerative illnesses, and cancer [110–113]. Extensive literature data suggests that regular intake of dietary polyphenols in adequate concentration may prevent or delay the onset of MetS, via arresting the pathological mechanisms of individual risk factors. They may benefit the host by decreasing body weight, improving insulin sensitivity, reducing blood glucose, maintaining normal blood pressure, improving blood cholesterol profile, and decreasing systemic inflammation [114].

1.3.1.1. Insulin resistance and hyperglycemia

Dietary polyphenols can lower the risk for the development of DM2 through decreasing intestinal carbohydrates absorption, alteration of the activity of glucose metabolizing enzymes, improving pancreatic β-cells function and thus insulin secretion as well as insulin sensitivity [115,116]. Flavonoids and tannins are major phenols responsible for the inhibition of key enzymes involved on metabolism of carbohydrates i.e., α-amylase and α-glucosidase [117,118]. Several polyphenols like naringenin and green tea polyphenols tends to inhibit Na^+-dependent glucose transporters (sodium/glucose co-transporter (SGLT) 1 and 2) [119,120]. Many polyphenols improve insulin sensitivity and inhibit the release of glucagon-like polypeptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) may result in the regulation of postprandial glycemia with the inhibition of glucose intolerance [121,122]. Some polyphenols (ferulic acid, hesperidin and naringenin) are reported to regulate major pathways of carbohydrate metabolism (usually impaired in DM2) like gluconeogenesis, glycogenesis, and glycolysis [123,124]. *In vitro* studies suggest that dietary polyphenols such as quercetin, epigallocatechin

gallate (EGCG), and resveratrol increase glucose uptake by muscles and adipocytes via the upstream regulation of AMP-activated protein kinase (AMPK) signaling pathway [125]. AMPK is one of the crucial factors, sensing cellular energy and possess protective effects against obesity and DM2 [126].

The supplementation of rat's pancreatic cells (RIN-m5F) form with green tea polyphenols (EGCG and rutin) resulted in the activation of insulin receptor substrate 2 (IRS2), enhanced ATP and upregulation of AMPK signaling pathway [127]. Quercetin, luteolin and apigenin are showed to suppress nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, resulting in indirectly suppression of pancreatic β-cells damage [128]. Quercetin also preserved β-cells by reducing oxidative stress in streptozotocin-induced diabetic mice [129]. Another study showed enhances insulin secretion by 70% with the treatment of islets from rats to quercetin and epicatechin [130]. Some polyphenols possess shielding effects on pancreatic β-cells by targeting the key signaling pathways. For instance, Chinese bayberry extract (rich in anthocyanins) protected β-cells against oxidative damage by upregulation of heme-oxygenase-1, modulation of PI3K/Akt and extracellular signal regulated protein kinase (ERK1/2) pathways and inhibiting β-cells apoptosis [125]. Resveratrol upregulates some key genes associated with β-cells functioning including pancreatic and duodenal homeobox 1 (*Pdx1*), mitochondrial transcription factor (*Tfam*), insulin 1 (*Ins1*), Glut2, and glucokinase (*Gk*) [131].

Studies have shown that polyphenols not only inhibit glucose uptake in the gut but also regulate glucose uptake in tissues [132,133]. In experimental conditions using Caco-2 cells, flavonoid glycosides and non-glycosylated polyphenols inhibited glucose absorption under Na^+ dependent environments while aglycones and non-glycosylated polyphenols inhibited glucose uptake in Na^+ independent environments. From mechanistic point of view, glycosides inhibited active

transport, aglycosides suppressed the facilitated glucose uptake while non-glycosides showed their effects via steric hindrance [120]. Mulberry leaf polyphenols (rich in rutin, chlorogenic acid, and benzoic acid) inhibited glucose transport in time dependent manner, suppressed sucrase from Caco-2 cells and lowered postprandial glucose levels. RT-qPCR analysis revealed the suppression of SGLT1–GLUT2 pathway via alteration of mRNA expression of cAMP-dependent protein kinase A (PKA), phospholipase and protein kinase C (PKC) [134].

A healthy diet comprised of fruits and vegetables (rich in polyphenols) may lower the risk for DM2 by 14% [135]. A European case control study observed 10% lower risk of DM2 in subjects with highest intake of flavonoids especially flavonols and flavan-3-ols, as compared to subjects with lower intake of flavonoids [136]. A meta-analysis report of prospective studies found an inverse association between apples and pears (high in flavonoids) and DM2 [137]. The regular and long-term consumption of coffee has been linked with reduced chances of the development of DM2 with a significant decrease in insulin resistance [138]. A meta-analysis of epidemiological studies revealed the improvement of Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) scores via changes in fasting insulin with anthocyanins-rich foods [139]. Green coffee extract rich in phenolic acids reduced fasting blood glucose levels but only higher doses were found effective [140]. Supplementation of aged men with phenolic acids (found in olive leaf) including oleuropein and hydroxytyrosol improved both insulin secretion from pancreatic β-cells and sensitivity [141].

1.3.1.2. Obesity

Natural polyphenols from different chemical classes (quercetin, rutin, gallic acid, chlorogenic acid, coumaric acid, and resveratrol) have shown potential anti-obesity effects through

preclinical and clinical studies via modulation of oxidative, inflammatory and cell proliferative processes (associated with numerous metabolic disorders including obesity) [142]. Intake of dietary polyphenols may result in appetite suppression, decreased lipogenesis, increased lipolysis, suppressed fatty acid oxidation, prevented adipogenesis, inhibited apoptosis, and gut microbial modulation [142,143]. Administration of pomegranate polyphenols (anthocyanins, flavonols, and flavanones) improved diet induced obesity, insulin resistance and hepatic steatosis in mice with gut microbial modulation [144]. Peach polyphenols alleviated obesity in high fat mice (reduced body weight, decreased levels of total cholesterol, LDL-C, triglycerides, alanine transaminase (ALT), aspartate transaminase (AST), and increased HDL-C levels) by altering gut microbiota [145]. A meta-analysis on randomized clinical trials showed that polyphenol intake in sufficient amount and on regular basis can considerably reduce body weight, BMI, waist circumference, and body fat mass [146]. Clinical studies are pointing mainly towards green tea polyphenols (EGCG, EGC, epicatechin, epigallocatechin-3-O-gallate, and gallocatechin) regards the prevention against central obesity [147]. A cohort trial designed by Marranzano *et al*. revealed a negative correlation of higher intake of flavonoids and BMI of ≥ 25 [148]. In a double-blind controlled randomized clinical trial, citrus polyphenols (mainly catechin and naringin) significantly decreased hip circumference, and abdominal and waist fats [149].

1.3.1.3. Dyslipidemia

Growing evidence suggests that regular consumption of diet rich in polyphenols may prevent the development of cardiovascular pathogenesis through attenuating oxidative damage, regulating lipid metabolism, improving endothelial dysfunction, inhibiting vasoconstricting factors (i.e., endothelin-1) and enhancing intrinsic vasodilators (i.e., NO) [150–152]. Polyphenols are capable of decreasing digestion as well as absorption of dietary lipids (mainly targeting pancreatic

lipase), thus improving dyslipidemia. Green tea polyphenols have shown to inhibit pancreatic lipase *in vitro*, where galloyl ester reported to be the essential factor in the efficacy of phenols in enzyme inhibition. EGCG inhibited pancreatic lipase in non-competitive manner while epigallocatechin (EGC) was ineffective [153,154]. Galloyl ester-containing polyphenols from black tea including theaflavin-3-gallate, theaflavin-30 -gallate, and theaflavin-3,3'-digallate showed considerable pancreatic lipase inhibition activities while theaflavin (with no galloyl ester) weak enzyme inhibition activity [155]. Furthermore, Shimura *et al*. reported that glycosylation of flavonoids may decrease the lipase inhibition activity of flavonoids [156]. A study showed the inhibition of pancreatic lipase and absorption of triglycerides with apple procyanidins [157]. In another study, apple procyanidins decreased apolipoprotein B synthesis and secretion, inhibited cholesterol esterification, and production of intestinal lipoprotein, resulting in induction of hypolipidemic effects [158]. Hydroxycinnamic acids including caffeic acid, chlorogenic acid, caftaric acid, ferulic acid, *P*-coumaric acid, rosmarinic acid, sinapic acid showed considerable pancreatic lipase inhibition activities [159].

Catechins significantly lowered cholesterol absorption via interacting with proteins involved in the translocation of cholesterol from enterocyte brush border including ATP-binding cassette proteins, B type1-scavenger receptors, multidrug resistance P-glycoprotein 1, and Niemann Pick C-1 like 1 protein [160]. Supplementation of tart cherries (rich in anthocyanins) decreased hyperinsulinemia, hyperlipidemia, and fatty liver through the upstream regulation of hepatic peroxisome proliferator-activated receptor (PPAR)-α and acyl-coenzyme A oxidase [161]. Erlund *et al*. observed a significant increase in HDL-C levels in berry eating group (abundant flavanones, flavonols, and flavan-3-ols) while total cholesterol and triglyceride levels remain unchanged [162]. Another randomized clinical trial showed a marked decrease in circulating

levels of total cholesterol, LDL-C, and non-high-density lipoprotein with green tea consumption for one year. However, HDL-C levels remain unchanged and unexpectedly triglycerides levels were increased to 3.6% [163]. Intake of Goishi tea polyphenols considerably decreased triglyceride and increased HDL-C levels, exhibiting potential benefits in prevention against atherosclerosis [164]. A systematic review of green tea EGCG in decreasing LDL-C levels concluded that intake of EGCG (107 – 856 mg/day) decreased LDL-C levels by 9.29 mg/dL [165]. Aronia extract (containing proanthocyanidins, anthocyanins, and hydroxycinnamic acids) improved microlipid metabolism after 12-weeks of treatment [166].

1.3.1.4. Hypertension

Polyphenols containing foods and beverages (in particular flavan-3-ols, flavonols, isoflavones, anthocyanins, and stilbenes) possess the ability of maintaining normal blood pressure, as evidenced by large number of preclinical and clinical studies. The reported mechanisms are their antioxidant effects, enhancing NO bioavailability, improving endothelial function, regulating of RAAS, and inhibiting endothelin-1 synthesis [167–170]. Quercetin and EGCG reduced oxidative stress as reprogramming mechanism in animal models of hypertension [171,172]. Garlic oil (rich in flavan-3-ols) enhanced NO availability and altered gut microbiota [173]. Resveratrol is one of the most extensively studies polyphenol in this context and it showed to reduce oxidative stress, restore NO, alter gut microbiota, inhibit RAAS, and antagonize Aryl hydrocarbon (AHR) signaling [174–177]. Daily intake of 25-gram chocolate (containing high polyphenol contents) for eight weeks resulted in significant decrease of 5.93 mmHg systolic and 6.4 mmHg diastolic blood pressure [178]. It is also confirmed that habitual intake of dark chocolate (6.3-gram, equivalent with 30 mg polyphenols) may have positive effects on blood pressure in prehypertension or stage 1 hypertension [179]. Importantly, not all polyphenols possess same

effect on systolic and diastolic blood pressure. A meta-analysis report on 24 randomized clinical trials concluded that administered of flavonoid-rich cocoa for 2 weeks resulted in decrease in systolic blood pressure by 1.63 mg whereas diastolic blood pressure remained unchanged [180].

1.3.2. Potential of *Prunus* species in protection against metabolic syndrome risk factors

Consumption of several kinds of fruits and vegetables in sufficient quantity can protect against metabolic syndrome risk factors, however high glycemic index of some fruits might be a problem for individuals with obesity and DM2 [181]. Plums and prunes (dried plums) are fruits with low glycemic index and high fibers content, thus their consumption on regular basis can be a potential protective strategy against individual components of MetS, as evident by preclinical and clinical studies [16]. They can improve glucose and lipid metabolism, reduce inflammatory biomarkers, decrease lipid deposition, and can modulate gut microbiota (*Bacteroidetes*, *Lactobacillus* and *Faecalibacterium* species). Fig 7 illustrated different mechanistic targets that ultimately result in enhanced insulin sensitivity and improvement of impaired energy and glucose and lipids homeostasis.

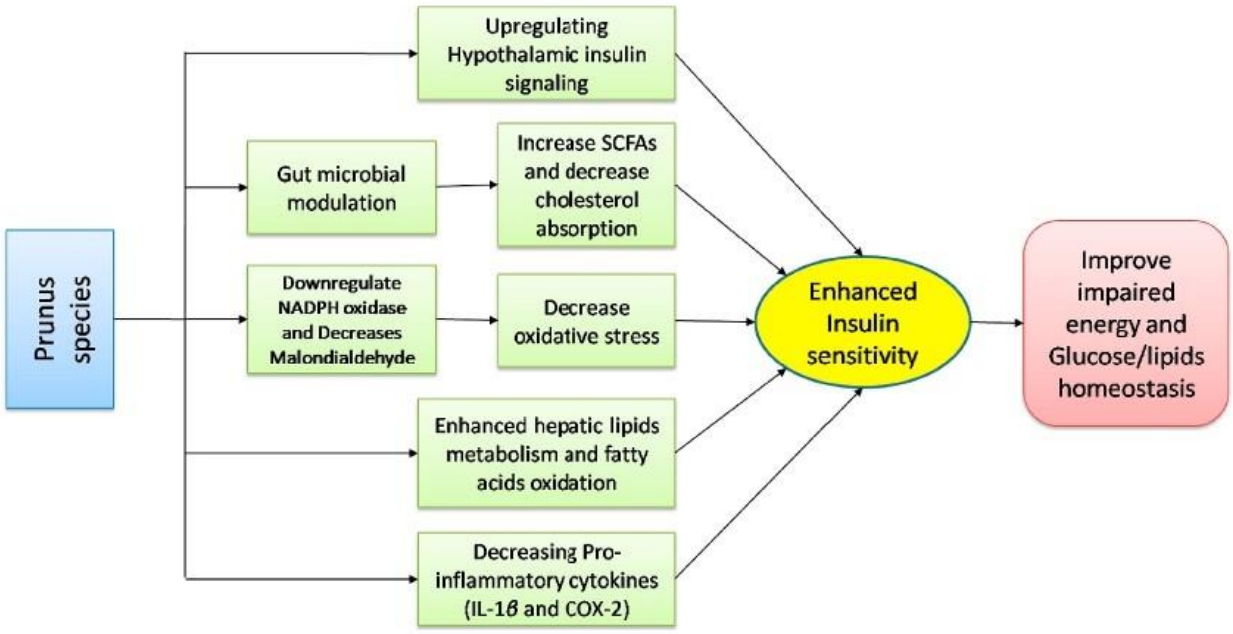

Fig. 7. Mechanistic insights of enhanced insulin sensitivity by *Prunus* species. Enhancing insulin sensitivity can improve impaired energy and glucose/lipids homeostasis. *Prunus* species boost insulin sensitivity via several mechanisms including upregulating hypothalamic insulin signaling, gut microbial modulation, decreasing oxidative stress through down-regulating NADPH oxidase and decreasing malondialdehyde, enhanced hepatic lipids metabolism and decreasing concentration/expression of pro-inflammatory cytokines. Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase); short chain fatty acids (SCFAs); interleukin-1 beta (IL-1β); cyclooxygenase-2 (COX-2).

In a randomized controlled trial, supplementation of recruited subjects with anthocyanin-rich Queen Garnet plum juice significantly reduced the body weight and BMI, with increased adiponectin blood levels and decreased leptin blood levels [182]. Almond skin polyphenolic extract diminished obesity through AMPK activation and attenuated obesity related inflammation in differentiated 3T3-L1 adipocytes [183]. The treatment with the extract inducted

lipolysis via up-regulation of AMPK signaling and suppressed adipogenesis transcription factors. In addition, the extract also inhibited the TNF-α inducing cell inflammatory response through downstream regulation of monocyte chemoattractant protein-1 (MCP-1) and chemokine ligand 5 (CCL-5) secretion.

Prunus species are part of herbal remedies for the treatment of DM2 in Northern Cyprus. Infusion of stalk of *Prunus* species has been used by patients with DM [184]. McCune and Johns [185] selected thirty-five plants including *Prunus serotina* Ehrh. (rum cherry) from literature that were traditionally used by people of North American boreal forest to treat diabetes and associated complications, and investigated their antioxidant activity associated with the symptoms of DM2. Most of the selected plants including *P. serotina* showed greater antioxidant potential that could prevent the progression of diabetes towards complications. The authors further concluded that the use of the traditional plants (rich in bioactive healthy components) has been decreased significantly resulting in a reduced intake of natural antioxidants which may increase the incidence of DM associated complications.

Intake of apple on daily basis possesses a positive impact on cardiovascular risk factors in postmenopausal women, as evidence suggested that it may improve lipid profiles and lower atherogenic risk ratios, and oxidative stress markers [186]. Chai *et al*. [187] studied the comparative analysis of daily consumption of apple and dried plum in postmenopausal women with special focus on cardiovascular disease risk factors. No difference was noted between experimental groups with the exception of TC which was significantly lowered at 6 months for dried apple.

Extracts of *Prunus* species have shown a significant vascular relaxing and hypotensive activities. Considering the evidence that diet rich in polyphenols may reduce the risk of cardiovascular disorders, Luna-Vázquez *et al*. [188] characterized the polyphenolic contents of black cherry fruits (*Prunus serotina*Ehrh.) and determined their antioxidant and antihypertensive effects. As indicated by the results, black cherry fruits contain high phenolic contents being chlorogenic acid, hyperoside and anthocyanins the main components. The aqueous extract displayed antioxidant activity and significant reduction of systolic blood pressure in L-nitro-arginine-methyl ester (L-NAME) induced hypertensive rats.

2. Aim of Research

In the first and second year of PhD, the aim was to select the edible plant with possible potential of prevention against MetS risk factors. Based on extensive literature review, *Prunus domestica* L. fruits (European plum) were selected for the experimental studies. Plums are fruits with low glycemic index, thus their consumption in adequate amount and on a regular basis could be a potential preventive strategy against MetS. In addition, plums are commonly consumed when on a diet, they are commercially available at low cost, and as happens for many fruits, some of the plums produced are discarded as they do not reach the size requirements to be placed on the market.

In the first phase, two subspecies of *P. domestica* (*P. domestica* L. subsp. domestica and *P. domestica* L. subsp. syriaca) were selected and the fruits were separated as skin and pulp, which were later extracted with hydroethanolic solution with different strengths (i.e., 99%, 70%, and 50%). All the extracts were subjected to the determination of total phenolic contents (TPC) and ABTS assays (for determination of Trolox equivalent concentration). The extract with higher TPC was selected for subsequent biological activities.

The *P. domestica* fruit extract was chemically characterized via a multimethodological approach using targeted ultra-high-performance liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) to characterize polyphenols and untargeted nuclear magnetic resonance (NMR) spectroscopy to quantify saccharides and organic acids. The sugar contents were precipitated by the treatment of extract with the absolute ethanol followed by ultra-freezing temperature, before proceeding with the *in vitro* and *in vivo* biological activities.

The *in vitro* enzyme inhibition activities (against α-amylase, α-glucosidase, HMG-CoA reductase, and pancreatic lipase enzymes) and *in vitro* anti-inflammatory activities (against Nitrite, IL-1β, and PGE_2) of the *P. domestica* fruit extract were determined, which gave us insights in the modulation of molecular mechanisms involved in the metabolic disorders.

In the third year of PhD, *in vivo* experiments were performed to assess the hypoglycemic and hypoinsulinemic effects of *P. domestica* fruit extract in healthy mice. *P. domestica* fruit extract was administered orally to mice after the intake of sugars load (glucose/sucrose) and blood samples were taken at baseline (T0), at 10 minutes (T5-10 min), at 20 minutes (T15-20 min), and at one hour (T 1h) to analyse the effects of extract on blood glucose and insulin levels.

In the second half of the third year of PhD course, which took place at the Department of Analytical and Food Chemistry, Universidade de Vigo Spain, *in vitro* bioaccessibility of the *P. domestica* fruit extract was determined using *in vitro* static digestion model, aimed to assess the potential effects of digestion conditions on the stability of polyphenols in fruit extract and to assess the bioaccessible polyphenolic compounds in the extract.

3. Material and Methods

3.1. Preparation of fruit extracts

Two different varieties of *Prunus domestica* L. fruits (*P. domestica* L. subsp. domestica, also known as common plum, and *P. domestica* L. subsp. syriaca, also known as Mirabelle plum) **(Fig.8)** were collected from a local cultivator in the Campania Region (Italy) in October 2020. Eight fruits were sampled for each variety (Common Plum and Mirabelle Plum). All fruits were first washed with water to eliminate every dirt residue and separated into skin and pulp. Both parts were cut into small pieces with a ceramic knife. To control the oxidation during preparation, the samples were cut in an ice bath. The samples were freeze dried and then grounded into fine powder using mortar and pestle. Aliquots of 1 and 2 g of the powdered skin and pulp were added to 20 mL and 40 mL of 50%, 70% and 99% ethanolic solution acidified with 0.1% HCL solution, respectively. The sample pH values were adjusted to 2.0, and the samples were subjected to magnetic stirring for 3 h at room temperature, followed by centrifugation at 6000 rpm for 10 minutes. The precipitate was separated from the supernatant. The same procedure was repeated three times and the supernatants of each sample were collected and filtered through Whatman cellulose filter paper. The filtrate was concentrated in a rotary evaporator at a temperature lower than 30 °C and submitted to freeze drying. The dry extracts were kept at -20 °C for subsequent determination of total polyphenol content, antioxidant activity, untargeted NMR spectroscopy, and targeted UHPLC-HRMS.

Based on the assessment of total polyphenol content and antioxidant activity, the fruit pulp extract of *P. domestica* subsp. syriaca obtained with 50% hydroethanolic solution was selected, and in view of the high content of glucose and sucrose determined via NMR, it was subjected to

the chemical precipitation of sugars by treatment with absolute ethanol, followed by ultra-freezing temperature. The organic solvent was removed under reduced pressure by rotary evaporator and the dry extract obtained from the fruit pulp extract of *P. domestica* subsp. syriaca without sugars was kept at -20 °C for subsequent biological assays.

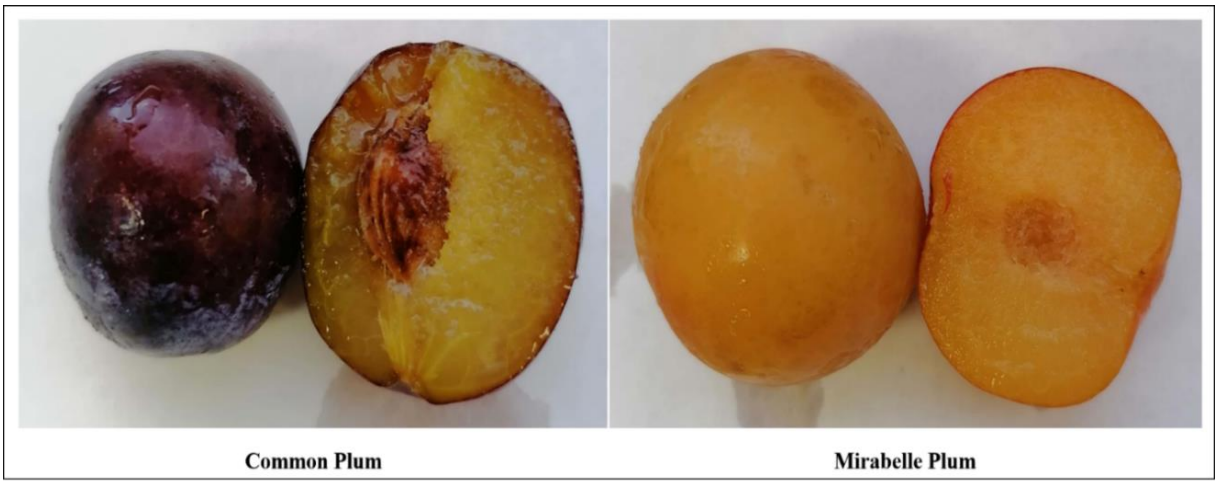

Fig.8. Plum varieties, collected from a local cultivator in the Campania Region (Italy). *P. domestica* L. subsp. domestica (Common plum) and *P. domestica* L. subsp. syriaca (Mirabelle plum).

3.2. Total phenolic contents

Total phenolic content (TPC) was determined using a colorimetric assay (Folin-Ciocalteu method), following the same protocol as set by Singleton *et al.* with some modifications [189]. An aliquot (10 µl) of the samples (50 mg/mL) or gallic acid standard solutions (200 - 1000 µg/mL) was taken and added to 50 µl of Folin-Ciocalteu reagent. The solutions were cyclomixed for 4 min and added to 200 µl Na_2CO_3 (15%). The final volume was made to 1 mL with distilled water and allowed to incubate for 2 h at room temperature and under dark conditions. The solutions were read spectrophotometrically at 750 nm. Gallic acid was used as standard

compound, with serial dilutions being prepared with known concentrations ranging from 200 to 1000 µg/mL. The results were expressed as mg equivalent to gallic acid/ g of extract on dry weight basis.

3.3. Antioxidant assay

ABTS (2, 2'-azino-bis3-ethylbenzthiazoline-6- sulfonic) assay was performed to evaluate the antioxidant potential of the fruit extracts following protocols set by Kok *et al*. with slight modifications [190]. The assay was conducted by placing 1 mL ABTS solution in a microtube, to which 10 µL of sample (50 mg/mL) or Trolox (0, 15, 20, 25, 30 and 35 µM) was added. The mixture was allowed to incubate for 2.5 min, and the absorbance was read at 734 nm. Thus, the antioxidant compounds present in the fruit extracts quench the color and produce a decoloration of the solution which is proportional to their antioxidant activity. The results were expressed as Trolox equivalent concentration (µM/g of extract on dry weight basis).

3.4. Metabolic profiling of P. domestica fruit pulp extract

The metabolic profiling of *P. domestica* fruit pulp extract was evaluated using UHPLC-HRMS and NMR analysis.

3.4.1. RP-UHPLC-HRMS analysis

P. domestica fruit pulp extract was solubilized in methanol/water (50:50 v/v). The sample was then filtered through a cellulose acetate/cellulose nitrate mixed esters membrane (0.45 µm; Millipore Corporation, Billerica, MA), and analyzed by RP-UHPLC-HRMS. UHPLC-HRMS analysis was performed on a Shimadzu Nexera UHPLC system, consisting of a CBM-20A controller, two LC-30AD dual-plunger parallel-flow pumps, a DGU-20 AR5 degasser, an SPD-

M20A photo diode array detector (PDA), a CTO-20A column oven, a SIL-30AC autosampler. The system was coupled online to a hybrid Ion trap-Time of Flight Mass spectrometer (LCMS-IT-TOF, Shimadzu) equipped with an electrospray source (ESI). For RP-UHPLC analysis a Kinetex Biphenyl 100 mm × 2.1 mm, 2.6 µm (L × I.D, particle size, Phenomenex®, Bologna, Italy) column was employed at a flow rate of 0.4 mL/min. The mobile phases consisted of A) 0.1% CH_3COOH in H_2O and B) ACN plus 0.1% CH_3COOH. Analysis was performed in gradient as follows: 0-20.0 min, 2-20% B; 20.01-22.0 min, 20.01-99% B; 99% B hold for 1 min; returning to initial conditions in 0.1 min. Column oven was set to 40 °C, 5 µL sample was injected. PDA detection parameters were sampling rate 12 Hz, time constant 0.160 s and chromatograms were extracted at 280 and 330 nm. LC data elaboration was performed by the LCMS solution® software (Version 3.50.346, Shimadzu). MS detection was performed in negative mode ionization as follows: curve desolvation line (CDL), 250°C; Block Heater, 250°C; Nebulizing and Drying gas, 1.5 and 10 L/min; ESI^- Capillary Voltage, -3.5 kV; MS range, m/z 150-1500; ion accumulation time, 30 ms; ion trap repeat, 3. MS/MS was performed in a data dependent acquisition (DDA), precursor ions selection was based on the base peak chromatogram (BPC) intensity of 700.000. Collision induced dissociation (CID), 50%, ion trap repeat. For analysis, the instrument was tuned using Sodium trifluoroacetate (NaTFA). Metabolite annotation was based on accurate mass measurement, MS/MS fragmentation pattern and comparison within silico spectra with MS database searching [191,192]. "Formula Predictor" software (Shimadzu) was used for the prediction of the molecular formula, using the following settings: maximum deviation from mass accuracy: 5 ppm, fragment ion information, and nitrogen rule.

3.4.2. NMR analysis

P. domestica fruit pulp extract (500 mg) was solubilized in 10 mL of 400 mM phosphate buffer/D2O containing 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSPA), used as internal standard for quantitative measurements, and EDTA, used as a complexing agent for metal ions. Then, an aliquot of 0.7 mL was transferred in a 5 mm NMR tube. The NMR spectra were recorded at 25 °C on a JNM-ECZ 600R (JEOL Ltd, Tokyo, Japan) spectrometer operating at the proton frequency of 600.17 MHz equipped with an autosampler and the SuperCOOL cryogenic probe (JEOL Ltd, Tokyo, Japan). The ^{1}H spectrum was acquired using a presaturation pulse sequence to suppress water signal, a 90° pulse of 12.8 μs and 65 K data points. All the NMR spectra were processed using the JEOL Delta v5.3.1. software (JEOL Ltd, Tokyo, Japan). The ^{1}H spectrum after Fourier transformation was manually phased, automatically base corrected and referred to the β-glucose CH-1 signal set at 4.66 ppm. 2D NMR experiments namely ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY,^{1}H-^{13}C HSQC and ^{1}H-13C HMBC were performed using the following experimental conditions: ^{1}H-^{1}H COSY and ^{1}H-^{1}H TOCSY experiments were carried out with water presaturation during relaxation delay and 9 kHz of spectral width in both dimensions. ^{1}H-^{1}H COSY was acquired using 4k x 256 points in F1 and F2 respectively, a relaxation delay of 2.5 s and 44 scans whereas in the case of ^{1}H-^{1}H TOCSY experiment, 8k x 256 points in F1 and F2 dimensions respectively, a mixing time of 80ms, a relaxation delay of 2s and 52 scans were used. ^{1}H-^{13}C HSQC experiment was carried out using a 90° ^{1}H pulse of 12.8 μs and 90° ^{13}C pulse of 14.0 μs, a spectral width of 9 kHz and 33 kHz for the ^{1}H and ^{13}C dimensions respectively, 8k x 256 points, a relaxation delay of 2 s, 80 scans and a coupling constant 1JC–H of 150 Hz. ^{13}C spectra were referenced to the CH-1 resonance of β-glucose at 97.00 ppm. ^{1}H-13C HMBC experiment was carried out with 12.8 μs for ^{1}H and 14.0 μs for ^{13}C 90° pulse, a spectral width of 9 kHz and 38 kHz for the ^{1}H and ^{13}C dimensions respectively, 8k x 256 points in F1 and F2

dimensions, a relaxation delay of 2s, a delay for the evolution of long-range couplings of 50 ms and 76 scans. In order to quantify the assigned compounds, the integral of the corresponding selected ^{1}H resonances were measured with respect to the integral of TSP methyl group signal normalized to 100. Quantitative results were expressed in µg/mg of dry weight.

3.5. Enzyme inhibition assays

Inhibition assays of different enzymes associated with the MS were performed as described below. The selected fruit extract (*P. domestica* subsp. syriaca fruit pulp), dissolved in 1% DMSO (SERVA Electrophoresis GmbH, Aurogene, Rome, Italy) and respective positive controls were tested using different concentrations to obtain half minimal inhibitory concentration (IC_{50}) for each enzyme, by nonlinear regression analysis. The absorbance of the sample blank (buffer in place of enzyme solution) and control (buffer in place of extract) was recorded as well. The inhibition of enzyme activity was calculated using following equation:

$$\% \text{ inhibition} = [(A_{\text{control}} - A_{\text{extract}})/A_{\text{control}}] \times 100$$

3.5.1. α-amylase inhibition assay

The α-amylase from porcine pancreas inhibition assay was performed according to the protocol set up by Cicolari *et al*. with slight modifications [193]. The reaction mixture contained 20 µl fruit extract solution (concentration range: 0.0625 – 25 mg/mL) or acarbose (concentration range: 15.56 – 400 µg/mL), and 20 µl enzyme solution (0.5 mg/mL) in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl), which was pre-incubated for 10 minutes at 25 °C. Then 20 µl of 1% starch solution was added to each tube at timed intervals and allowed to incubate for 10 minutes at 25 °C. The reaction was stopped by the addition of 40 µl color reagent (DNSA). The

test tubes were incubated in a boiling water bath for 10 minutes, and then cooled to room temperature. Finally, 600 µl of bidistilled water was added to dilute the reaction mixture and the absorbance was read at 540 nm using a microplate reader.

3.5.2. α-glucosidase inhibition assay

The α-glucosidase from *Saccharomyces cerevisiae* inhibition assay was performed according to the protocols set by Cicolari *et al*. with slight modifications [193]. The reaction mixture containing 50 µl fruit extract solution (concentration range: 0.0313 – 25 mg/mL) or acarbose (concentration range: 15.56 – 900 µg/mL), and 100 µl enzyme solution (1 unit/mL) in 0.1 M phosphate buffer (pH 6.9), was incubated in a 96-well plate for 10 minutes at 25 °C. After pre-incubation, 50 µl of 0.1 M phosphate buffer (pH 6.9) solution containing 5 mM p-nitrophenyl-α-D-glucopyranoside was added to each well at timed interval and was incubated for 5 minutes at 25 °C. The absorbance was read at 405 nm using a microplate reader.

3.5.3. HMG-CoA reductase inhibition assay

The assay was conducted according to the manufacturer's protocol (Sigma-Aldrich). The assay was conducted by placing 910 µL phosphate buffer with 5 µL fruit extract (concentration range: 0.0625 – 30 mg/mL) or 5 µL pravastatin (concentration range: 18.75 – 300 µM) into microtubes; 20 µL of NADPH and 60 µL of HMG-CoA reductase substrate were then added. The analysis was initiated (time 0) by the addition of 5 µL of HMG-CoA reductase, incubated at 37 °C. The rate of NADPH consumed was monitored every 15 seconds for up to 5 minutes by reading the decrease in absorbance at 340 nm, using the microplate reader.

3.5.4. Pancreatic lipase inhibition assay

Porcine Pancreatic Lipase (PPL) inhibition assay was conducted according to the protocols reported by Nwakiban *et al*. (2019) [194]. The assay was conducted by mixing 30 µL PPL (2.5 mg/mL in 10 mM MOPS and 1 mM EDTA, pH 6.8) with 850 µl Tris buffer (100 mM Tris-HCl and 5 mM CaCl2, pH 7.0). Then, either 100 µL of fruit extract (concentration range: 1.30 – 12.5 mg/mL) or orlistat (concentration range: 1 – 500 µg/mL) was added to the mixture and incubated for at 37 °C for 15 minutes, followed by the addition of 10 µL substrate (10 mM p-NPB in dimethyl formamide). The mixtures were incubated again at 37 °C for 30 minutes. The absorbance was read at 405 nm using a microplate reader, to determine the lipase activity by quantifying the hydrolysis of p-NPB to p-nitrophenol.

3.6. In vitro anti-inflammatory assays

3.6.1. Cell culture

Murine monocyte/macrophage J774 cell line was obtained from the American Type Culture Collection (ATTC TIB 67). The cell line was grown in adhesion in Dulbecco's modified Eagles medium (DMEM) supplemented with glutamine (2 mM, Aurogene Rome, Italy) Hepes (25 mM, Aurogene Rome, Italy) penicillin (100 U/mL, Aurogene Rome, Italy), streptomycin (100 µg/mL, Aurogene Rome, Italy), fetal bovine serum (FBS, 10%, Aurogene Rome, Italy) and sodium pyruvate (1.2%, Aurogene Rome, Italy) (DMEM completed). The cells were plated at density of ~1 x 10^6 cells in 75 cm^2 culture flasks and maintained at 37 C under 5% CO_2 in a humidified incubator until 90% confluence. The culture medium was changed every 2 days. Before a confluent monolayer appeared, sub-culturing cell process was carried out. *P. domestica* subsp. syriaca fruit pulp extract was solubilized in DMSO at the concentration of 200 mg/mL (stock solution). Then it was diluted in DMSO to have solutions at the concentrations of 150 mg/mL,

100 mg/mL, 20 mg/mL and 2 mg/mL. Cells were plated to a seeding density of 5.0 x 10^5 in 24 multiwell. After 2 h of adhesion, cells were pre-treated (for 2 h) with increasing concentration of *P. domestica* subsp. syriaca fruit pulp extract (5 µL of 2, 20, 100, 150 and 200 mg/mL, which correspond to a final concentration in the well (1 mL) of 0.01, 0.1, 0.5, 0.75 and 1 mg/mL). After the pre-incubation, macrophages were stimulated with or without LPS from *Escherichia coli*, Serotype 0111:B4, (10 µg/mL; 100 µL of solution 100 µg/mL in DMEM completed with FBS, Sigma Aldrich, Milan, Italy) for 24h [195].

3.6.2. Nitrite, IL-1β and PGE_2 determination

After 24 h of the incubation, the supernatants were collected for the nitrite, IL-1β and PGE_2 measurement. The nitrite concentration in the samples was measured by the Griess reaction, by adding 100 µL of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_2PO_4; vol. 1:1; Sigma Aldrich, Milan, Italy) to 100 µl samples. The optical density at 540 nm (OD_{540}) was measured immediately after Griess reagent addition, using ELISA microplate reader (Thermo Scientific, Multiskan GO). Nitrite concentration was calculated by comparison with OD_{540} of standard solutions of sodium nitrite prepared in culture medium. IL-1β (R&D Systems, Aurogene, Rome, Italy) and PGE_2 (Cayman Chemical, Bertin Pharma, Montigny Le Bretonneux, France) levels were measured with commercially available ELISA kits according to the manufacturer's instructions.

3.6.3. Cell viability

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Aldrich, Milan, Italy) to formazan. Cells were plated to a seeding density of 1.0 x 10^5 in 96

multiwell. After stimulation with LPS in the absence or presence of test compounds for 24 h, cells were incubated in 96-well plates with MTT (0.2 mg/mL), for 1 h. Culture medium was removed by aspiration and the cells were lysed in DMSO (0.1 mL). The extent of reduction of MTT to formazan within cells was quantified by the measurement of OD_{550}.

3.7. In vivo hypoglycemic and hypoinsulinemic effects of P. domestica fruit pulp extract

3.7.1. Experimental animals

The animal study was performed in compliance with the recommendations made by EU Directive 2010/63/EU for animal experiments and the Basel declaration including the 3Rs concept [196,197]. BALB/c male mice (10–14 weeks of age, 25–30 g of weight), were purchased from Charles River (Milan, Italy) and kept in an animal care facility under controlled temperature, humidity, and on a light:dark cycle, with *ad libitum* access to water and standard laboratory chow diet. All procedures were carried out to minimize the number of animals used (*n* = 4-5 per group) and their suffering. Experimental study groups were randomized, and their assessments were carried out by researchers blinded to the treatment groups.

3.7.2. Experimental design

To investigate the potential hypoglycemic and hypoinsulinemic effects of *P. domestica* fruit pulp extract, fasted mice were subjected to the oral sugars load (300 μL/mouse; sucrose/glucose, 1 mg/ 6375 mg for 50 ml of solution) and divided into six groups, where groups 1, 2, and 3 received only sugars by gavage and blood samples were collected by cardiac puncture after 10 minutes, 20 minutes, and 60 minutes, respectively, while groups 4, 5, and 6 were supplemented with *P. domestica* fruit pulp extract (750 mg/kg of mouse body weight, corresponds to an adult

portion of fresh *P. domestica* fruit i.e., 100 g, which corresponds to 20.5 g extract) and blood samples were collected by cardiac puncture after 10 minutes, 20 minutes, and 60 minutes, respectively. Blood plasma was obtained to measure blood glucose and insulin levels [198].

3.7.3. Blood biochemical analysis

Blood glucose levels were quantified with a colorimetric method using a Glucose oxidase/peroxidase (GOD-POD) immunoenzymatic kit (Sphera instrument) at a wavelength of 510 nm at 37°C optical path 1 cm. Blood insulin levels were quantified using a Elabscience mouse INS (insulin) ELISA kit. The absorbance was read at 450 nm using the microplate reader and both the concentrations were determined following manufacturer's instructions.

3.8. Determination of in vitro bioaccessibility of polyphenols identified in the fruit pulp extract

3.8.1. Simulated in vitro digestion

In vitro digestion of *P. domestica* extract was performed by simulated oro-gastric-duodenal digestion processes following protocols set by Minekus *et al*. with slight modifications [199]. In brief, 3.5 g of extract was added to 3. 5 mL of previously prepared simulated salivary fluid (SSF), followed by the addition of 0.5 mL fresh α-amylase solution (1500 U/mL) prepared in SSF. Final volume was made to 10 mL with the addition of water and the mixtures were incubated for 2 minutes at 37 ℃. The bolus obtained was mixed with 7. 5 mL simulated gastric fluid (SGF) and 1.6 mL fresh pepsin (25,000 U/mL), the pH of which was adjusted to 2.00 ± 0.02 using 1 M HCl. The mixture was set again to oscillation for 2 hours at 37 ℃. The samples were brought up to 20 mL and were allowed to incubate for 2 hours at 37 ℃ in a shaking water bath. Subsequently, gastric chyme was mixed with 5 mL of freshly pancreatin (800 U/mL) and

2.5 mL of fresh bile mixture (160 mM), and final volume was made up to 40 mL by the addition of water, the pH of which was adjusted to 7.00 ± 0.02 using 1 M NaOH and the mixture was allowed to incubate for 2 hours at 37℃ in a shaking water bath. At the end of each step, enzyme activity was stopped by immersion of tubes in ice for 10 minutes. Afterwards, tubes were centrifuged at 6000 rpm for 10 minutes and the supernatant was collected and freeze dried for subsequent TPC determination and HPLC-MS analysis.

3.8.2. HPLC-MS/MS analysis

Chromatographic analysis was performed on the HPLC Accela (Thermo Fischer Scientific, Bremen, Germany) using an Agilent Poroshell 120, C18 column with 2.7µm particle size and dimensions of 250×4.6 mm. The mobile phases used were A: 0.1% formic acid in water and B, 0.1% formic acid in acetonitrile; linear gradient (0 min, 10% eluent B to 50 min, 28% eluent B).

Mass spectrometry analysis was done on an Orbitrap TM Exploris 120 (Thermo Fischer Scientific, Bremen, Germany) controlled by Orbitrap Exploris 120 Tune Application 2.0.182.35 and Xcalibur 4.4.16.14. The capillary voltage of the electrospray ionization source (ESI) was set to 3.5 kV in positive and 2.8 kV in negative mode. The capillary temperature was 300ºC. The sheath gas and auxiliary gas flow rate were at 50 and 10 (arbitrary unit as provided by the software settings). The MS scan resolution was set at 60,000. Data dependent MS/MS was performed on HCD using nitrogen as gas with collision energy settings of 35 V.

MS data handling software (Xcalibur Quall Browser software, Thermo Fischer Scientific) to control the equipment and analyse the spectral information. Samples were analysed in full scan mode in positive and negative ionization mode.

3.9. Statistical analysis

For *in vitro* enzyme inhibition, Student t test was used to determine the level of significance and statistical differences among variables using GraphPad prism, version 5 (GraphPad, San Diego, CA, USA). The results were expressed as mean ± standard deviation (SD). For *in vitro* anti-inflammatory studies, the results were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. The results were expressed as mean ± standard error (SE). Triplicate wells were used for the various treatment conditions. A *p*-value less than 0.05 was considered significant. Regarding the *in vivo* experiments *p*-value was calculated with MedCalc Software Ltd. Comparison of means calculator (https://www.medcalc.org/calc/comparison_of_means.php, Version 20.215; accessed January 28, 2023).

4. Results

4.1. Description of P. domestica extracts

Two different varieties of *P. domestica* fruits (*P. domestica* subsp. domestica and *P. domestica* subsp. syriaca), which were separated into pulp and skin, were submitted to three different hydroethanolic extractions to evaluate the effects of ethanol percentage in the extraction solvent on the total polyphenol content and antioxidant activity. The extraction yield calculated for the freeze-dried fruit skin ranged from 37 to 43% regardless with the *Prunus* variety. On the contrary, the extraction yield calculated for the freeze-dried fruit pulp, ranged from 59.5 to 67% and from 45 to 49%, for subsp. domestica and subsp. syriaca, respectively **(Table 4)**.

Table 4. Fruit samples and yield of extracts.

***Prunus* variety**	**Common name**	**Skin color**	**Fruit part extracted**	**Ethanol %**	**Dry extract (g/g)[1]**	**Extraction yield (%)**
P. domestica subsp. domestica	Common plum	Purple	Skin	99	0.42	42.0
				70	0.39	39.0
				50	0.38	38.0
			Pulp	99	0.60	59.5
				70	0.63	63.0
				50	0.67	67.0

P. domestica subsp. syriaca	Mirabelle plum	Yellow	Skin	99	0.37	37.0
				70	0.41	41.0
				50	0.43	43.0
			Pulp	99	0.45	45.0
				70	0.46	46.0
				50	0.49	49.0

[1]The weight of dry extract obtained in grams per gram of sample used for extraction

4.2. Total phenolic contents and in vitro antioxidant activity

A total of 12 hydroethanolic extracts were evaluated for their total phenolic content (TPC) and *in vitro* antioxidant activity **(Table 5)**. The pulp of *P. domestica* subsp. syriaca extracted with 50% ethanol showed the highest TPC (12.9 mg GAE/g on dry weight basis), followed by fruit skin of *P. domestica* L. subsp. domestica extracted with 70% ethanol containing 12.8 mg GAE/g on dry weight basis. Overall, the fruit extracts extracted with 99% of ethanol exhibited relatively less phenolics, with fruit pulp of *P. domestica* subsp. syriaca showing the lowest content of total phenolics (6.5 mg GAE/g on dry weight basis). In general, fruit skin showed the highest antioxidant activity with fruit skin of *P. domestica* L. subsp. domestica (extracted with 50% ethanol) showing a Trolox equivalent concentration of 1944.1 µM/g, while fruit pulp of *P. domestica* subsp. syriaca (extracted with 99% ethanol) showed the lowest Trolox equivalent concentration of 585.5 µM/g. On the basis of the higher polyphenol content, to which the

inhibitory activity of the enzymes involved in MetS is generally ascribed [29], the good antioxidant activity, the higher yield (49 %), and the lower percentage of ethanol used as extraction solvent (50 %), the *P. domestica* subsp. syriaca fruit pulp extract obtained with 50% hydroethanolic solution was selected for the subsequent chemical characterization.

Table 5. Total phenolic content and Trolox equivalent concentration of the extracts obtained from the two varieties of *P. domestica*.

***Prunus* variety**	**Fruit part extracted (ethanol %)**	**TPC (GAE/g on dry weight basis)**	**Trolox equivalent concentration (µM/g on dry weight basis)**
P. domestica subsp. domestica	skin (99 %)	9.1 ± 1.0	1282.4 ± 84.1
	skin (70 %)	12.8 ± 0.9	1826.2 ± 216.4
	skin (50 %)	11.0 ± 0.6	1944.1 ± 138.1
	pulp (99 %)	7.2 ± 1.0	630.5 ± 44.1
	pulp (70 %)	11.3 ± 0.2	1611.9 ± 289.5
	pulp (50 %)	9.7 ± 0.2	1290.7 ± 155.5
P. domestica subsp. syriaca	skin (99 %)	7.0 ± 0.2	708.0 ± 25.1
	skin (70 %)	11.2 ± 1.4	1597.4 ± 88.2
	skin (50 %)	7.9 ± 0.8	1602.1 ± 368.1

	pulp (99 %)	6.5 ± 0.4	578.5 ± 53.5
	pulp (70 %)	10.0 ± 0.9	727.7 ± 43.9
	pulp (50 %)	12.9 ± 1.7	1119.4 ± 93.1

Data are expressed as mean ± SD (n = 3).

Based on the assessment of total polyphenol content and antioxidant activity, on the higher yield (49 %), and the lower percentage of ethanol used as extraction solvent (50 %), the fruit pulp extract of *P. domestica* subsp. syriaca obtained with 50% hydroethanolic solution was selected, and in view of the high content of glucose and sucrose determined via NMR, it was subjected to the chemical precipitation of sugars by treatment with absolute ethanol, followed by ultra-freezing temperature. The organic solvent was removed under reduced pressure by rotary evaporator and the dry extract obtained from the fruit pulp extract of *P. domestica* subsp. syriaca without sugars was kept at -20 °C for subsequent biological assays.

4.3. UHPLC-HRMS profile

The list of the metabolites occurring in the hydroethanolic (50%) extract obtained from *P. domestica* subsp. syriaca fruit pulp, with tentative identification based on accurate mass and fragmentation pattern compared against reference MS/MS spectra reported *in silico* and in previous literature, is reported in **Table 6**. In particular, 23 compounds belonging to different classes (organic and hydroxycinnamic acids and flavonoids, both aglycone and glycosylated) were identified in the extract (**Fig.9).** Hydroxycinnamic and quinic acid derivatives were the most abundant compounds retaining the 46.7% of total peak area, followed by procyanidins, in

particular dimer (17%), monomers (13.10%) and trimers (6.9%). Lastly, flavonol glycosides represented the remaining 7.9%. (**Table 7**)

Table 6. Identified compounds in *P. domestica* subsp. syriaca fruit pulp extract according to the retention time (RT), compound, *m/z* and MS/MS and molecular formula.

Peak	**RT (min)**	**Compound**	**[M-H]$^-$**	**MS/MS**	**Molecular Formula**	**Error (ppm)**
1	0.60	Citric acid	191.0227	111.0103; 173.0103	$C_6H_8O_7$	1.57
2	3.12	Chlorogenic acid	353.0874	173.0489; 191.0576	$C_{16}H_{18}O_9$	-1.13
3	4.68	Coumaroylquinic acid Isomer	337.0945	163.0417 119.0558	$C_{16}H_{18}O_8$	4.75
4	5.45	Catechin	289.0729	245.0816	$C_{15}H_{14}O_6$	3.81
5	6.08	(+) Epicatechin dimer B type	577.1328	407.0787; 289.0728	$C_{30}H_{26}O_{12}$	-4.16
6	6.50	Feruloylquinic acid	367.1053	193.0531; 134.0390	$C_{17}H_{20}O_9$	4.90
7	6.70	Coumaroylquinic acid isomer	337.0928	163.0447; 191.0594	$C_{16}H_{18}O_8$	1.19
8	7.20	Coumaroylquinic acid isomer	337.0952	173.0458; 163.0418	$C_{16}H_{18}O_8$	2.30

9	8.12	(+) Epicatechin	289.0735	245.0816	$C_{15}H_{14}O_6$	5.88
10	8.48	(+) Epicatechin trimer B type	865.1979	407.0790; 287.0569; 577.1344	$C_{45}H_{38}O_{18}$	3.40
11	8.86	(+) Epicatechin dimer B type isomer	577.1344	407.0790; 289.0732	$C_{30}H_{26}O_{12}$	-1.39
12	9.70	Quinic acid derivative	393.1777	149.0465; 191.0561	$C_{17}H_{30}O_{10}$	2.80
13	10.50	Feruloyl-coumaroylquinic acid derivative	559.1665	337.0947; 193.0514	$C_{24}H_{32}O_{15}$	-0.54
14	11.29	Feruloyl-coumaroylquinic acid derivative	559.1670	337.0949; 193.0510	$C_{24}H_{32}O_{15}$	-0.50
15	12.19	Feruloyl-coumaroylquinic acid derivative	559.1677	337.0946; 193.0514	$C_{24}H_{32}O_{15}$	1.61
16	12.32	(+) Epicatechin dimer B type isomer	577.1358	407.0831; 289.0742	$C_{30}H_{26}O_{12}$	1.04
17	12.74	(+) Epicatechin B type trimer isomer	865.2015	407.0778; 287.0569; 577.1344; 543.0905	$C_{45}H_{38}O_{18}$	3.47
18	13.20	Quercetin-rutinoside	609.1477	301.0351; 271.0254; 255.0320	$C_{27}H_{30}O_{16}$	3.47

19	13.48	(+) Epicatechin A type trimer	863.1823	575.1180; 423.0711; 285.0393	$C_{45}H_{36}O_{18}$	-0.20
20	14.04	(+) Epicatechin A type trimer isomer	863.1828	575.1180; 423.0711; 285.0393	$C_{45}H_{36}O_{18}$	-0.12
21	14.82	(+) Epicatechin A type dimer	575.1197	423.0746; 285.0395	$C_{30}H_{24}O_{12}$	1.22
22	15.75	Quercetin-rhamnoside	447.0924	301.0371; 255.	$C_{21}H_{20}O_{11}$	-0.9
23	16.52	(+) Epicatechin A type dimer isomer	575.1187	423.0716; 285.0398	$C_{30}H_{24}O_{12}$	-1.39

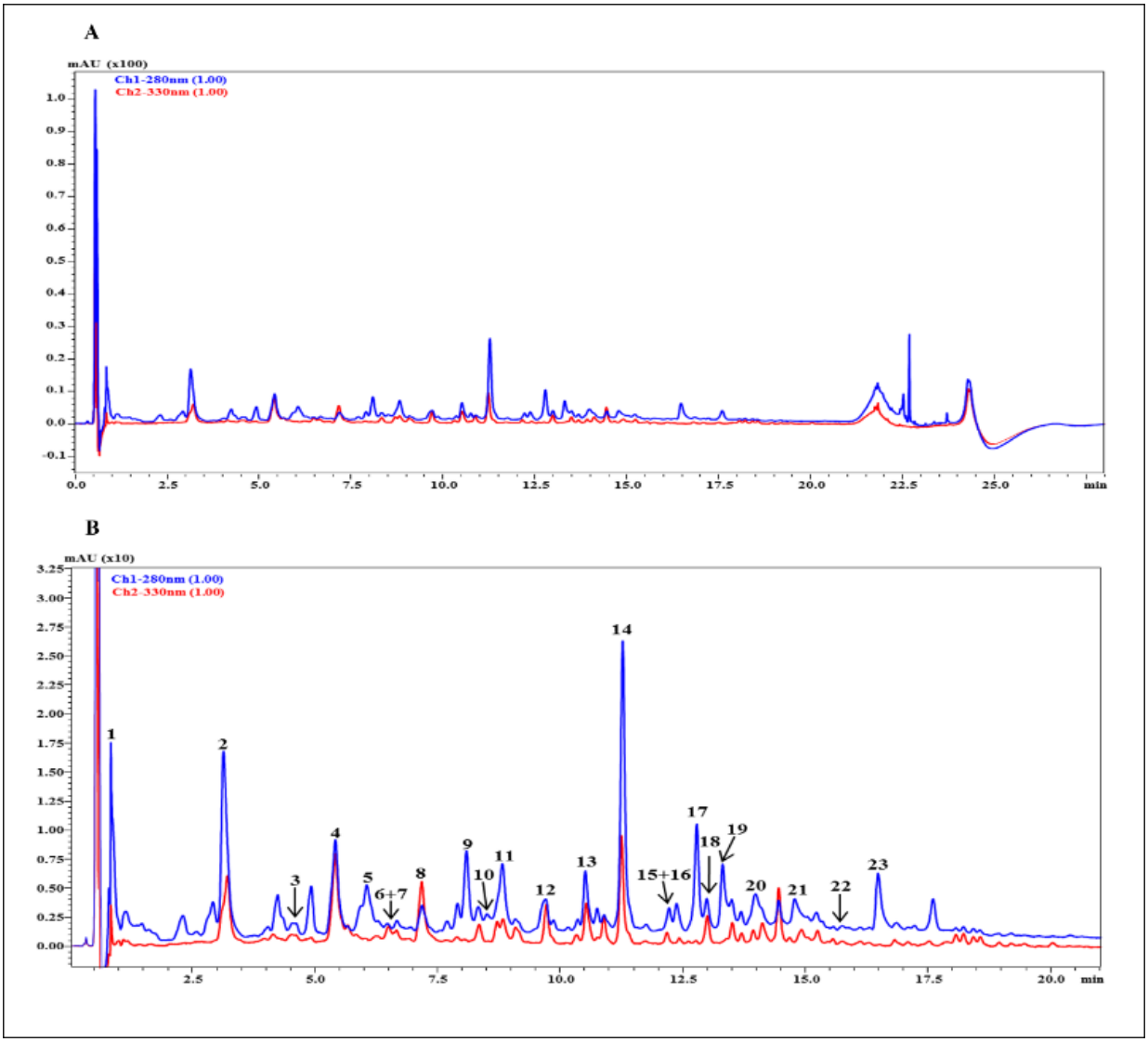

Fig.9. RP-UHPLC chromatograms of *P. domestica* subsp. syriaca fruit pulp extract with UV-detection registered at λ 280 nm and 330 nm (A), and chromatogram expansion with corresponding peak HRMS assignment (B).

Table 7. Retention time (min) and peak area, expressed as percentage of total area of the identified compounds in *P. domestica* subsp. syriaca fruit pulp extract.

Peak#	**Compound**	**Retention Time**	**Area %**
1	Citric acid	0.6	7.92
2	Chlorogenic acid	3.12	15.43
3	Coumaroylquinic acid Isomer	4.68	0.74
4	Catechin	5.45	8.61
5	(+) Epicatechin dimer B type	6.08	5.74
6	Feruloylquinic acid	6.5	0.21
7	Coumaroylquinic acid isomer	6.7	0.39
8	Coumaroylquinic acid isomer	7.2	2.42
9	(+) Epicatechin	8.12	4.54
10	(+) Epicatechin trimer B type	8.48	0.24
11	(+) Epicatechin dimer B type isomer	8.86	5.47
12	Quinic acid derivative	9.7	1.98
13	Feruloyl-coumaroylquinic acid derivative	10.5	3.01

14	Feruloyl-coumaroylquinic acid derivative	11.29	19.55
15	Feruloyl-coumaroylquinic acid derivative	12.19	1.49
16	Feruloyl-coumaroylquinic acid derivative	12.32	1.47
17	(+) Epicatechin dimer B type isomer	12.74	6.27
18	(+) Epicatechin B type trimer isomer	13.2	0.79
19	Quercetin-rutinoside	13.48	3.20
20	(+) Epicatechin A type trimer	14.04	4.05
21	(+) Epicatechin A type trimer isomer	14.82	1.81
22	(+) Epicatechin A type dimer	15.75	0.16
23	Quercetin-rhamnoside	16.52	4.50

4.4. NMR analysis and quantification of sugar and organic acid contents

The ^{1}H spectrum of the *P. domestica* subsp. syriaca fruit pulp extract dissolved in phosphate buffer/D2O shows the presence of glucose, sucrose, xylose and citric, malic and quinic acids. The ^{1}H spectral assignment was obtained by literature data regarding other fruits [200,201] and 2D NMR experiments [202]. The integrals of selected signals due to sugars namely xylose, glucose, and sucrose at 5.20 ppm, 5.25 ppm, and 5.42 ppm respectively and to organic acids namely citric, malic and quinic acids at 1.88 ppm, 2.54 ppm, and 4.30 ppm respectively, were

used for compound quantification (**Table 8**). The ^{1}H NMR spectrum with the selected signals used for the quantification of metabolites (**Fig.10**). In the case of some compounds only a partial assignment was obtained due to compound low concentration. However, the partial assignment included diagnostic signals that allowed the identification of the reported compounds. Glucose and malic acid turned out to be the sugar and the organic acid respectively, present in major amounts.

Table 8. Compounds identified in the ^{1}H NMR spectrum of *P. domestica* subsp. syriaca fruit pulp extract dissolved in phosphate buffer/D2O and the corresponding chemical shift signals (ppm) used in the integration process. The compound amounts in μg/mg of dry weight are also reported.

Compound	**Chemical shift (ppm) of selected resonances used for quantification**	**μg/mg dry weight**
Quinic acid	1.88 (CH2-1)	7.50
Citric acid	2.54 (α,γ- CH)	0.84
Malic acid	4.30 (α- CH)	38.49
Xylose	5.20 (CH-1)	0.56
Glucose	5.25 (CH-1)	106.59

Sucrose	5.42 (CH-1)	31.59

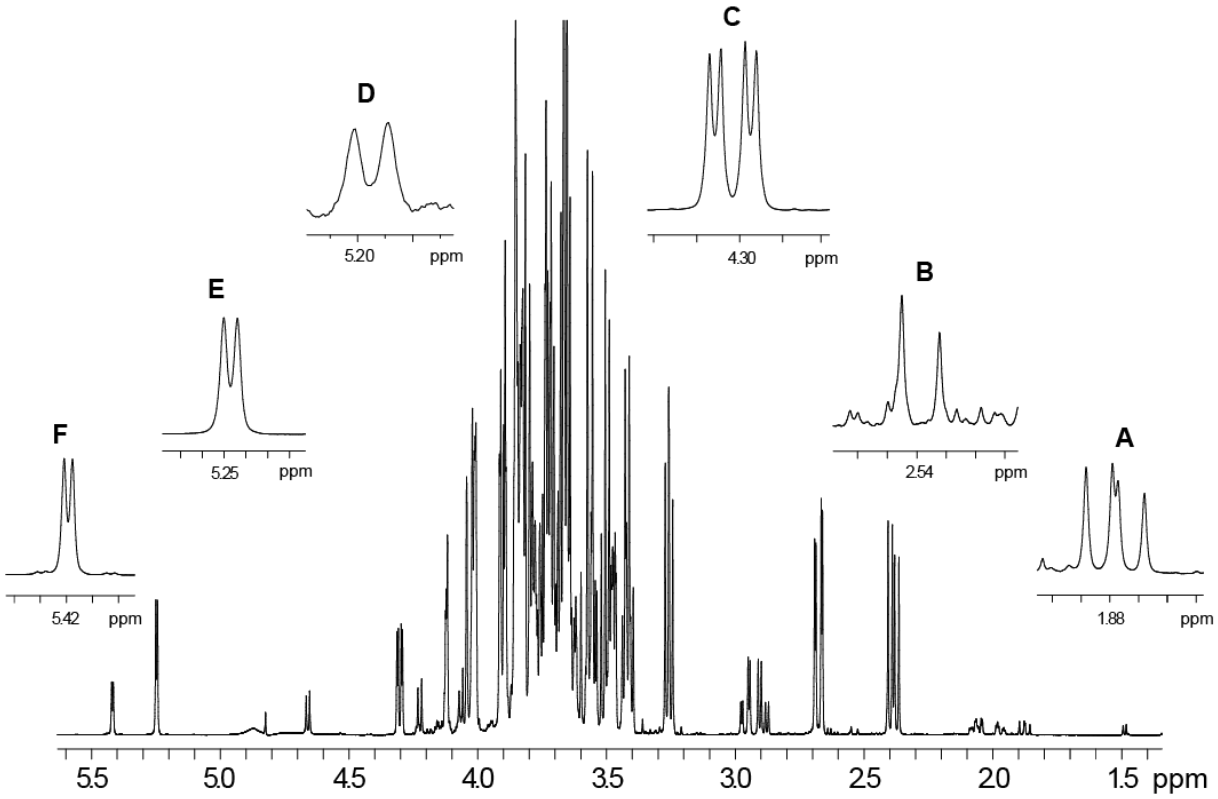

Fig.10. 1H NMR spectrum of *P. domestica* subsp. syriaca fruit pulp extract. Quantified selected NMR signals are reported in expanded regions. **(A)** CH2-1 protons of quinic acid (1.88 ppm), **(B)** α,γ-CH protons of citric acid (2.54 ppm), **(C)** α-CH proton of malic acid (4.30 ppm), **(D)** CH-1 proton of in α-xylose (5.20 ppm), **(E)** CH-1 proton of α-glucose (5.25 ppm), **(F)** CH-1 proton of sucrose (5.42 ppm).

4.5. Preparation of P. domestica fruit extract without sugar

In view of the high sugar content (about 14 % of the whole extract) of *P. domestica* subsp. syriaca fruit pulp crude extract, it was subjected to chemical precipitation of sugar contents by treatment with absolute ethanol supported by ultra-freezing temperature. The percent extraction yield following sugar precipitation was 41.8%.

4.6. Effect of P. domestica fruit pulp extract on enzyme activities

P. domestica subsp. syriaca fruit pulp extract with reduced content of sugars was used for enzyme inhibition activities, with the aim of reducing the substances that may interfere with the enzyme inhibition activities of the vegetable extract. The inhibition of the enzyme activities performed by the extract at increasing concentration and the IC50 values for each enzyme, calculated with the nonlinear regression analysis have been illustrated in **Fig.11**.

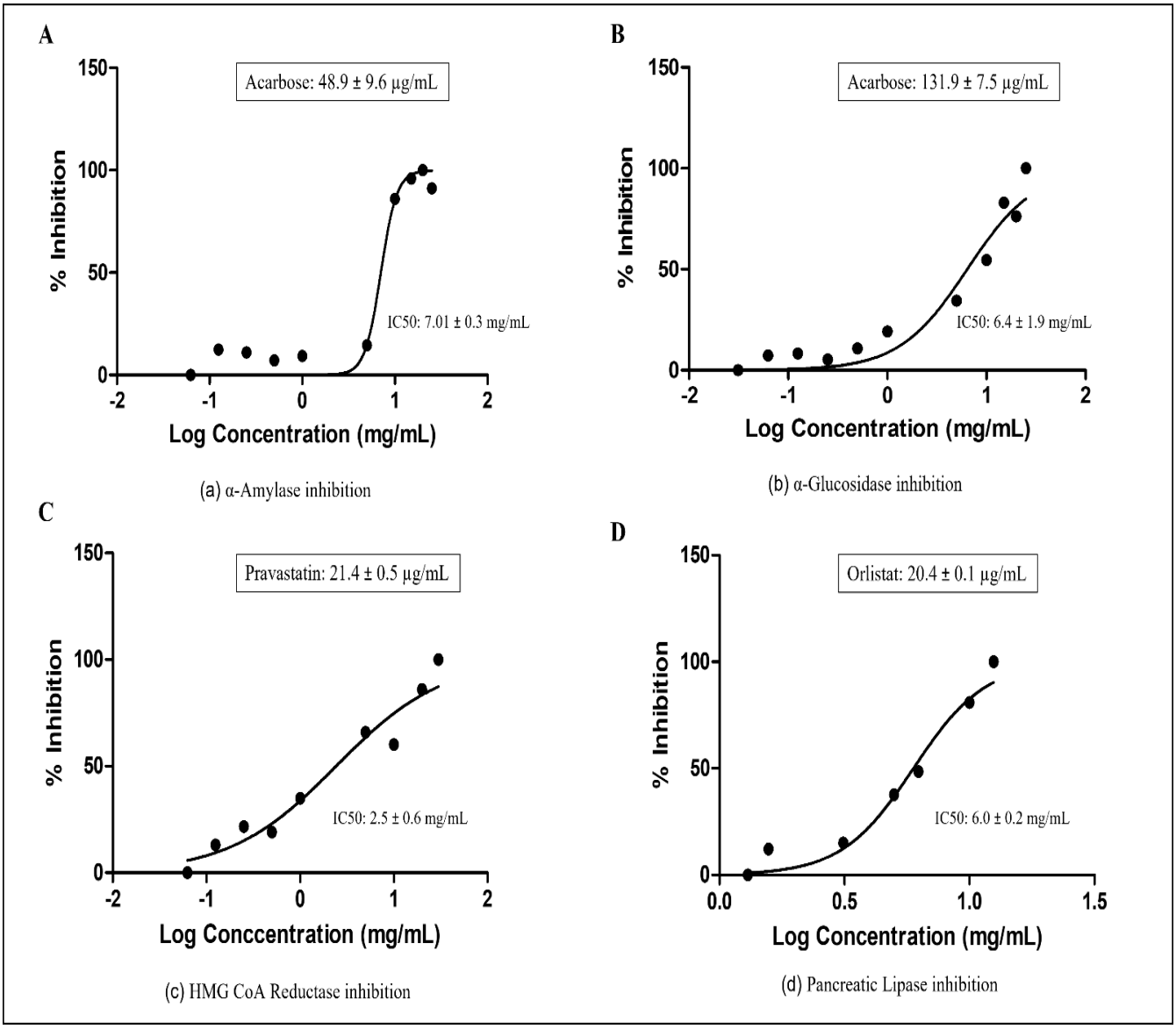

Fig.11. Enzyme inhibition activities and calculated IC50 values of *P. domestica* subsp. syriaca fruit pulp extract. Data expressed as mean ± SD. The IC50 values of the fruit extract against each enzyme were calculated using nonlinear regression analysis.

P. domestica subsp. syriaca fruit pulp extract inhibited α-amylase and α-glucosidase enzymes in a concentration dependent manner, with IC50 value of 7.01 mg/mL as compared to acarbose, used as positive control, (IC50: 48.9 µg/mL) **(Fig.11A)**, and IC50 value of 6.4 mg/mL as

compared to acarbose (IC50: 131.9 µg/mL) **(Fig.11B)**, respectively. As far as HMG-CoA reductase is concerned, *P. domestica* subsp. syriaca fruit extract inhibited HMG-CoA reductase enzyme in a concentration dependent manner with IC50 value of 2.5 mg/mL, while the reference inhibitor pravastatin inhibited HMG-CoA reductase with IC50 value of 21.4 µg/mL **(Fig.11C)**. Finally, as regards pancreatic lipase, the results suggest that *P. domestica* subsp. syriaca fruit pulp extract inhibited this enzyme in a concentration dependent manner with IC50 value of 6.0 mg/mL as compared to reference inhibitor orlistat (IC50 value: 20.4 µg/mL) **(Fig.11D)**. Overall, the mean IC50 values of the fruit extract was found significantly different from the values obtained from the positive controls ($p < 0.05$).

4.7. In vitro anti-inflammatory effects

In order to assess the anti-inflammatory proprieties of *P. domestica* subsp. syriaca fruit pulp extract, murine macrophage cell line J774 stimulated with LPS (10 µg/mL, 24h), a well-known proinflammatory stimulus, were used. The anti-inflammatory activities were assessed by measuring the levels of proinflammatory mediators such as nitrites, PGE_2 and IL-1β. Pre-incubation of J774 macrophages with *P. domestica* subsp. syriaca fruit pulp extract (2 h before LPS treatment) inhibited significantly and in concentration-dependent manner (0.01, 0.1, 0.5, 0.75 and 1 mg/mL) the production of nitrite (IC50 0.46 mg/mL, **Fig.12A**), PGE_2 (IC50 0.56 mg/mL, **Fig.12B**) and IL-1β (IC50 0.18 mg/mL, **Fig.12C**) induced by LPS, starting from the concentration of 0.1 mg/mL. No effects of *P. domestica* subsp. syriaca fruit pulp extract on pro-inflammatory mediator production were observed in unstimulated cells (without LPS) (**Figures12 D-F**). To rule out any alteration of cell viability, MTT assay was performed and did not show any statistical reduction of cell viability after treatment with extract **(Fig.12G)**.

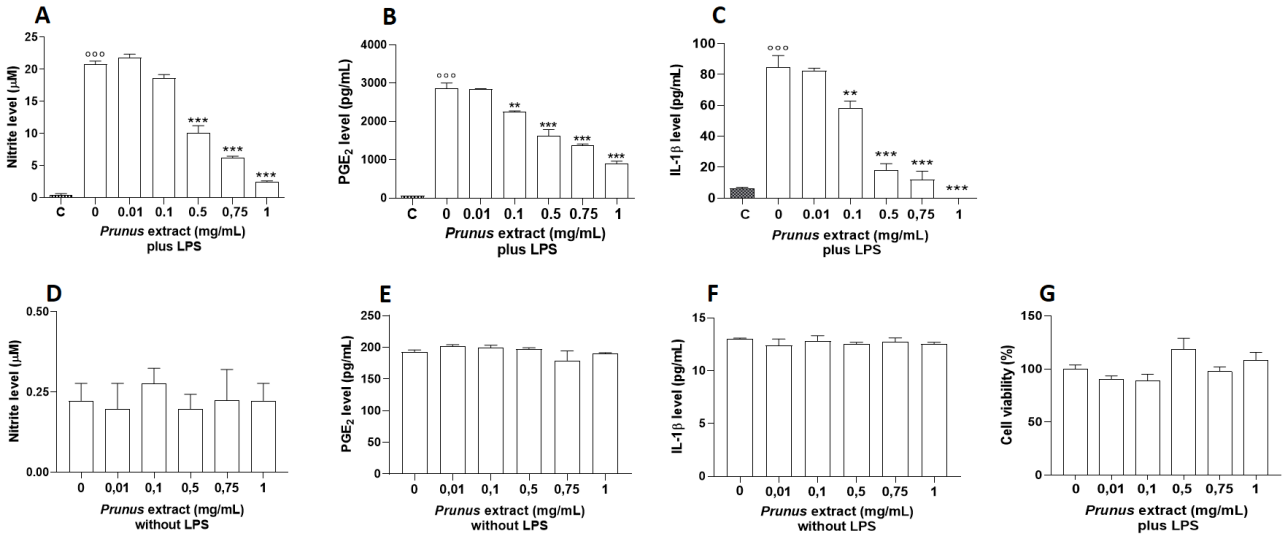

Fig.12. Effect of the *P. domestica* subsp. syriaca fruit pulp extract on LPS-induced nitrite, IL-1β and PGE_2 production. J774 cells were pre-treated for 2 h with increasing concentrations of the extract (0.01, 0.1, 0.5, 0.75 and 1 mg/mL) and then stimulated with LPS (10 µg/mL) for 24 h (plus LPS). Effects of extract were evaluated also in absence of LPS (without LPS). Unstimulated J774 cells acted as a negative control (C). Nitrites (A and D), stable end-products of NO were measured in the supernatants by the Griess reaction, whereas IL-1β (B and E) and PGE_2 (C and F) were measured by ELISA. Cell viability was evaluated by the mitochondrial-dependent reduction of MTT to formazan (G).°°°$P < 0.001$ vs unstimulated cells (C), ***$p < 0.001$ and ** $P < 0.01$ vs LPS alone.

4.8. In vivo hypoglycemic and hypoinsulinemic effects

Our investigation revealed that there was initial increase in the blood glucose and insulin levels (T10 min) after the administration of extract and control, followed by decrease in the glucose and insulin levels (T20 min through T 1h) **(Fig.13)**. The increase in blood glucose and insulin levels

was greater in the mice treated with control as compared to *P. domestica* extract, the mean differences between mice treated with control and extract were not statistically significant. The maximum reduction of both glucose and insulin levels were observed at T20 min, post-dose. The mean blood glucose levels in *P. domestica* vs control mice were 86.0 ± 5.0 vs 86.0 ± 5.8mg/dL at T0, 179.0 ± 22.7 vs 267.7 ± 20.6 mg/dL at T10 min (standard error: 17.981; significance level: p = 0.0081), 137.3 ± 8.1 vs 171,0 ± 6.6 mg/dL at T20 min (standard error:6.137; significance level: p = 0.0052), and 132.3 ± 14.0 vs 143 ± 7,6mg/dL at T 1h (standard error: 9.037; significance level: p = 0.2904). Similarly, the mean blood insulin levels in *P. domestica* vs control mice were 55.3 ± 10.7vs 55.3 ± 10.6 pg/mL at T0, 116.2 ± 4.0 vs 158.7 ± 15.0 pg/mL at T10 min (standard error: 8.963; significance level: p = 0.0094), 82.12 ± 8.7 vs 106.5 ± 10.2 pg/mL at T20 min (standard error: 7.767; significance level: p = 0.036), and 64.0 ± 4.0 vs 76.5 ± 10.7 pg/mL at T 1h (standard error: 6.758; significance level: p = 0.1504).

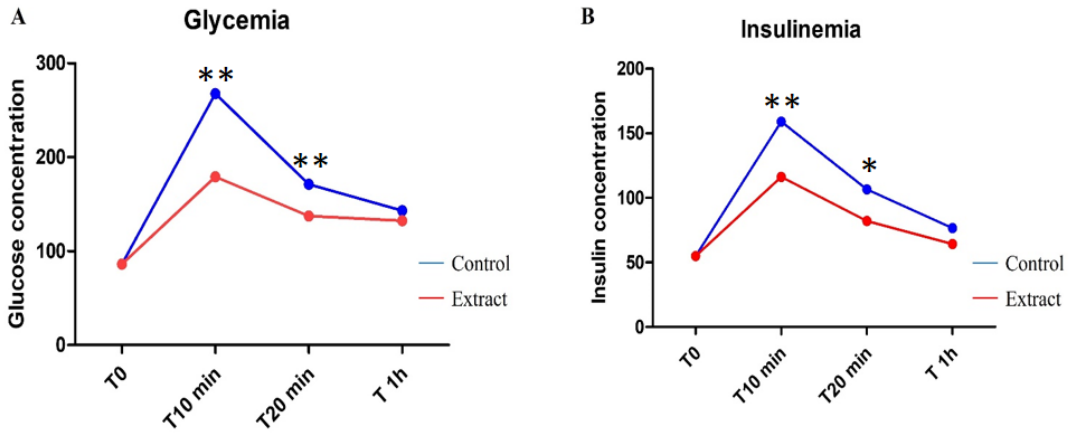

Fig. 13. Effect of the *P. domestica* subsp. syriaca fruit pulp extract on blood glucose **(A)** and insulin **(B)** levels in BALB/c male mice challenged with oral sugars load. *P. domestica* fruit pulp

extract possessed greater effect in reducing blood glucose and insulin levels in mice subjected to oral sugars load (300 µL/mouse; sucrose/glucose, 1 mg/ 6375 mg for 50 ml of solution). A trend of initial increase in blood glucose and insulin levels was observed but that may be ascribable to the presence of a small amount of sugar in tested extract. ** $p< 0.01$ and * $p< 0.05$.

4.9. Effect of digestion on TPC in fruit pulp extract

To assess the influence of digestion on the phenolic profile of *P. domestica* fruit pulp extract, the TPC was determined before and after *in vitro* digestion **(Table 9)**, using Folin-Ciocalteu assay according to the protocols set by Singleton *et al.* with slight modifications [189]. The results were expressed as mg equivalent to gallic acid/ g of extract on dry weight basis. The TPC in *P. domestica* extract before digestion was 6.1 mg (GAE/g) and after digestion were 5.8 mg (GAE/g) after in oral phase, 5.5 mg (GAE/g) in oro-gastric phase, and 5.25 mg (GAE/g) in oro-gastric-intestinal phase. The mean difference between TPC in fruit extract and oral phase ($p = 0.4429$), and TPC in fruit extract and oro-gastric phase ($p = 0.0630$) was not significant, while the mean difference between TPC in fruit extract and oro-gastirc-duodenal phase was significant ($p = 0.0329$).

Table 9. Total phenolic contents in *P. domestica* fruit pulp extract before and after simulated digestion.

Sample		**TPC (GAE/g on dry weight basis)**
P. domestica subsp. syriaca		6.1 ± 0.2^{a}
In vitro digestion	Oral phase	5.8 ± 0.4

	Oro-gastric phase	5.5 ± 0.0
	Oro-gastric-duodenal phase	5.25 ± 0.0[a]

Data presented as mean ± SD (n = 2).The assigned values of different letters in a column are showing significantly difference among the mean values (p< 0.05). p-value was calculated with MedCalc Software Ltd. Comparison of means calculator (https://www.medcalc.org/calc/comparison_of_means.php, Version 20.215; accessed January 29, 2023).

4.10. In vitro bioaccessibility of polyphenols in the fruit pulp extract

Prior to determination of *in vitro* bioaccessibility, *P. domestica* fruit pulp extract was subjected to the qualitative analysis of metabolic profile after the precipitation of sugar contents (with absolute ethanol and ultra-freezing temperature) via LC-MS/MS technique. The list of metabolites found in *P. domestica* fruit pulp extract with tentative identification based on accurate mass and fragmentation pattern compared against reference MS/MS spectra reported in previous literature, is enlisted in **Table10**. In particular, 20 polyphenolic compounds were identified belonging to phenolic acids, flavan-3-ols, and procyanidins, where hydroxycinnamic acids represent the major part. However, flavonol glycosides were not observed and most possibly they may precipitate out with absolute ethanol while precipitating sugar contents from the fruit extract.

A simulated oro-gastric-duodenal digestion showed a change in the polyphenolic contents in *P. domestica* fruit pulp extract throughout the oral, gastric, and intestinal routes of digestion **(Table 11)**. A qualitative analysis of oral, oro-gastric, and oro-gastro-duodenal digested samples of *P.*

domestica fruit pulp extract revealed the presence of hydroxycinnamic acids, while flavan-3-ols and procyanidins were not detected that may digested and metabolized into other metabolites. For instance, polyphenolic compounds detected after oral digestion were cinnamoylquinic acid derivative (m/z 369.14), 5-*O*-caffeoylquinic acid (m/z 354.3), chlorogenic acid (m/z 353.2), coumaroylquinic acid isomer (m/z 338.34), while after oro-gastric and oro-gastric-duodenal digestion the polyphenols detected were 5-*O*-caffeoylquinic acid (m/z 354.3), chlorogenic acid (m/z 353.2), coumaroylquinic acid isomers (m/z 338.34).

Table10. Identified compounds in *P. domestica* subsp. syriaca fruit pulp extract according to the retention time (RT), compound, ionization mood, m/z ratio (precursor ion) and MS/MS fragmentation.

RT (min)	**Compound**	**Ionization mood**	**MS**	**MS/MS**
0.7	Feruloylquinic acid	$[M + H]^+$	367.12	205.07; 78.61; 90.22
0.82	Cinnamoylquinic acid derivative	[M - H]-	369.14	207.09
0.88	Cinnamoylquinic acid derivative	[M - H]-	369.05	207.09
1.17	(-) Epicatechin derivative	$[M + H]^+$	291.04	268.89
15.2	Procyanidin dimer B type	$[M + H]^+$	576.28	287.19

15.25	Procyanidin dimer B type	$[M + H]^+$	576.28	288.3
21.96	Vanillic acid-glycoside	[M - H]-	329.23	152.35
24.2	(+) Epicatechin	$[M + H]^+$	290.27	242.25; 122.08
24.51	(+) Catechin	$[M + H]^+$	290.27	242.25; 187.91
29.88	5-*O*-Caffeoylquinic acid	$[M + H]^+$	354.3	312.29; 60.04; 163.15
40.19	Chlorogenic acid	[M - H]-	353.2	79.96
40.37	Caffeoylquinic acid	[M - H]-	353.2	135.59
40.55	Caffeoylquinic acid	[M - H]-	353.2	179.97; 191.79
48.09	*p*-coumaroylquinic acid	[M - H]-	337.21	213.31, 219.74
48.19	*p*-coumaroylquinic acid	[M - H]-	337.21	249.54
48.23	*p*-coumaroylquinic acid	[M - H]-	337.21	217.61
48.44	*p*-coumaroylquinic acid	[M - H]-	337.21	138.69

49.31	*p*-coumaroylquinic acid	[M - H]-	337.21	185.33; 165.65
64.14	Coumaroylquinic acid isomer	$[M + H]^+$	338.34	163.15; 191.18
64.2	Coumaroylquinic acid isomer	$[M + H]^+$	338.34	191.18; 303.31

Retention time (rt); precursor ion (MS); fragment ions (MS/MS).

Table 11. Qualitatively analyzed compounds in *P. domestica* subsp. syriaca fruit pulp extract before and after simulated digestion.

S.No.	***P. domestica* extract**	**m/z**	**Ionization mood**	**Oral phase**	**Oro-gastric phase**	**Oro-gastric-duodenal phase**
1	Feruloylquinic acid	367.12	$[M + H]^+$			
2	Cinnamoylquinic acid derivative	369.14	[M - H]-	✓		
3	Cinnamoylquinic acid derivative	369.05	[M - H]-			
4	(-) Epicatechin derivative	291.04	$[M + H]^+$			

5	Procyanidin dimer B type	576.28	$[M + H]^+$			
6	Procyanidin dimer B type	576.28	$[M + H]^+$			
7	Vanillic acid-glycoside	329.23	[M - H]-			
8	(+) Epicatechin	290.27	$[M + H]^+$			
9	(+) Catechin	290.27	$[M + H]^+$			
10	5-*O*-Caffeoylquinic acid	354.3	$[M + H]^+$	✓	✓	✓
11	Chlorogenic acid	353.2	[M - H]-	✓	✓	✓
12	Caffeoylquinic acid	353.2	[M - H]-			
13	Caffeoylquinic acid	353.2	[M - H]-			
14	*p*-coumaroylquinic	337.21	[M - H]-			

	acid					
15	*p*-coumaroylquinic acid	337.21	[M - H]-			
16	*p*-coumaroylquinic acid	337.21	[M - H]-			
17	*p*-coumaroylquinic acid	337.21	[M - H]-			
18	*p*-coumaroylquinic acid	337.21	[M - H]-			
19	Coumaroylquinic acid isomer	338.34	$[M + H]^+$	✓	✓	✓
20	Coumaroylquinic acid isomer	338.34	$[M + H]^+$	✓	✓	✓

5. Discussion

Fruit skin and pulp of two different *P. domestica* varieties (*P. domestica* subsp. domestica and *P. domestica* subsp. syriaca), extracted with different concentrations of ethanolic solution were evaluated for TPC and the Mirabelle plum extract showing the highest TPC was selected for subsequent biological activities. Chemical profiling of *P. domestica* subsp. syriaca fruit pulp extract was evaluated using a multimethodological approach based on the application of new technologies, consisting of untargeted NMR spectroscopy and untargeted UHPLC-HRMS, which favors a holistic approach as opposed to the traditional reductionist methods, allowing to overcome the concept of identifying one compound responsible for the obtained biological effect, and to ascribe the bioactivity to the whole phytocomplex [203]. The results suggested the presence of hydroxycinnamic acids (p-coumaroylquinic acid isomers and feruloylquinic acid derivatives), which resulted to be the most represented polyphenols followed by flavanols (catechin, epicatechin, procyanidins), flavonols (rutinoside and rhamnoside derivatives of quercetin), organic acids (quinic acid, citric acid, and malic acid), and carbohydrates (xylose, glucose, and sucrose). The carbohydrate quantitative analysis showed that glucose (106.6 mg/g dry weight of extract) is the main saccharide found in the fruit extract in agreement with USDA Food Composition Databases, followed by sucrose (31.6 mg/g dry weight of extract) and xylose (0.6 mg/g dry weight of extract). Sugars of the fruit extract were at least in part chemically precipitated with ethanol before proceeding with the *in vitro* and *in vivo* studies to remove the constituents that may interfere with the biological activities of the vegetable extract.

Metabolic disorders remain a prevalent and urgent concern in the healthcare field, which today are remedied through the pharmacological approach prescribed to target some specific pathogenetic mechanisms which, in turn, is often accompanied by adverse effects and poor

compliance by patients. [204]. Therefore, the study of new food supplement ingredients for the reduction of risk factors of MetS is essential and find new agents able to modulate the enzyme activities associated to carbohydrate/lipid digestion and cardio-metabolic diseases is one of the essential targets in the prevention and treatment of cardio-metabolic disorders. As evident by preclinical and clinical trials, *Prunus* species can improve energy homeostasis involving glucose and lipid metabolism, decrease inflammatory mediators, reduce lipid deposition, and modulate gut microbiota, and thus can reverse metabolic dysregulation states [205]. In this study, the ability of a hydroethanolic extract of *P. domestica* subsp. syriaca (Mirabelle plum) fruit pulp to prevent against MetS risk factors was studied, supported by both *in vitro* and *in vivo* experiments.

The inhibition of α-amylase and α-glucosidase enzymes are important to reduce the digestion of complex carbohydrates and in turn the absorption of glucose with the aim to normalize the blood glucose level both in subjects with mild hyperglycemia and in diabetic patients to support glucose-lowering medication [206]. Acarbose is a pharmacologic drug, currently employed in the treatment of subjects with diabetes, due to the inhibition of α-amylase and α-glucosidase enzymes, thus reducing carbohydrate digestion, slowing down the absorption of carbohydrates, and decreasing the post-prandial insulin secretion, in addition to stimulating glucagon-like peptide (GLP-1) release [207]. The extract of *P. domestica* subsp. syriaca fruit pulp showed inhibitory activity against α-amylase and α-glucosidase enzymes with IC50 values of 7.01 mg/mL and 6.4 mg/mL, respectively. While evaluating antidiabetic activity of novel smoothies from selected *Prunus* fruits, Nowicka *et al*. demonstrated that anthocyanin and flavonol content have the highest impact on α-glucosidase enzyme, whereas flavanols may have potential to inhibit α-amylase [208]. Some studies also reported that inhibition of α-glucosidase may be

associated with the content of hydroxycinnamic acid derivatives such as ferulic acid or *p*-coumaric acids [209]. The researchers indicated that flavonols can interact with hydroxycinnamic acids or anthocyanins, which may increase the inhibition of α-glucosidase [210]. Procyanidins-rich fruits are effective α-amylase inhibitors, possibly through the formation of enzyme-tannin complexes resulting in the prevention of enzyme from interaction with starch [210]. Literature showed an inhibition of α-amylase and α-glucosidase enzymes with numerous botanical extracts including *Elateriospermum tapos* Blume [211], *Xylopia parviflora* Spruce, *Monodora myristica* (Gaertn.) Dunal, *Tetrapleura tetraptera* (Schum. &Thonn.) Taub., *Dichrostachys glomerata* (Forssk.) Chiov., *Aframomum melegueta* K.Schum., *Aframomum citratum* (C.Pereira) K.Schum [194] and *Adansonia digitata* L. [193]. Different *Prunus* fruits (including Common European plum, 'vlaškača', damson plum, white damson, purple-leaf cherry plum, white cherry plum, red cherry plum, sweet cherry, sweet cherry - wild type, sour cherry, steppe cherry, mahaleb cheery, blackthorn, and peach)extracted with 50% ethanol exhibited inhibition of α-amylase (IC50 value range: 1.11 – 136.23 mg/mL) and α-glucosidase (IC50 value range: 0.41 – 28.44 mg/mL) [212]. *Prunus* species in general showed a greater affinity towards α-glucosidase enzyme as compared to α-amylase [212]. Altogether, these data suggested that inhibition of the enzymes involved in the digestion of carbohydrates by vegetable extracts may have promising potential in the management of glucose metabolism disorders.

Statins are effective lipid-lowering agents, widely used as a first line therapy in the atherosclerotic CVDs, which are known to competitively inhibit HMG-CoA reductase enzyme (rate limiting enzyme of cholesterol synthesis) [213]. Considering the same approach of decreasing cholesterol synthesis, *P. domestica* subsp. syriaca fruit pulp extract was tested against the HMG-CoA reductase activity, showing an IC50 value of 2.5 mg/mL. The mean IC50 value

calculated for the fruit extract was significantly higher from the reference statin (pravastatin). Susilowati *et al*. (2020) performed *in silico* analysis while evaluating antihyperlipidemic effects of apple peel extract, which showed highest HMG CoA reductase inhibition by catechin, epicatechin, quercetin (aglycosidal form) and chlorogenic acid [214]. Several other vegetable extracts have already shown HMG-CoA reductase inhibitory activities including, but not limited to *Basella alba* L. [215], *Syzygium polyanthum* (Wight) Walp. [216], *Ficus palmata* Forssk. [217], and *Amaranthus viridis* L. [218]. Inhibition of pancreatic lipase is a clinically validated approach in the treatment of obesity, as it reduces the hydrolysis of fats and decreases their absorption [219]. Food and Drug Administration (FDA) approved orlistat in 1999 for the pharmacological management of obesity in conjunction with reduced caloric diet, while in 2007 it was approved as over the counter (OTC) agent for weight loss in overweight adults (18 years or older) [220]. Later, FDA revised the label for orlistat by adding a new warning about severe liver injury that has been reported rarely with this drug [221]. Following concerns about the possible cause of severe hepatic toxicities with orlistat, the European Medicines Agency completed a review for this medicine, where the Agency's Committee for Medicinal Products for Human Use (CHMP) concluded that the benefits of orlistat continue to outweighs the risks, but also recommended the marketing authorizations to ensure the safety information on rarely occurred liver injuries be provided on the product information of all orlistat-containing medicines [222]. As reported in the present study, *P. domestica* subsp. syriaca fruit pulp extract inhibited the pancreatic lipase enzyme with an IC50 value of 6.0 mg/mL, although this value was significantly higher than that determined for Orlistat. Hydroxycinnamic acids and proanthocyanidins have proven efficacy against lipase activity [223,224]. The presence of hydroxyl groups in the molecule (more potent), methoxy groups (less potent) and position of

hydroxyl groups in the phenolic ring could influence the activity of polyphenols in inhibiting lipase enzyme [223]. Moreover, flavan-3-ol esters showed a stronger lipase inhibition as compared to non-esterified flavanols such as catechin and epicatechin [223]. Plant species of different families (*Vitis vinifera* L., *Rhus coriaria* L., *Origanum dayi* Post, *Quercus infectoria* G.Olivier, *Eucalyptus galbie*, *Rosa damascene*, and *Levisticum officinale* W.D.J.Koch) showed considerable inhibition of pancreatic lipase enzyme [225,226]. Nowicka *et al*. observed an inhibition of pancreatic lipase enzyme with *Prunus persica* L. Batsch fruits (different cultivars) with an IC50 value ranging from 0.07 to 2.06 mg/mL [227]. In the whole, although the values of IC50 are much higher than those found for drugs, it must be considered that the extract being derived from a food commonly consumed with the diet and having been deprived of sugars should not show adverse effects unlike medicines and therefore it can be taken in larger quantities and for very long periods. *In vivo* studies are needed to confirm inhibitory activity against the enzymes considered.

Lifestyle modifications (including diet and exercise) and pharmacological agents (such as angiotensin-converting enzyme (ACE) inhibitors, Angiotensin receptor blockers (ARBs), and PPAR-α agonists) all are targeting inflammation in various ways, and thus can reduce MetS-associated complications. Ongoing research studies are uncovering inflammatory pathways (related to obesity, DM2, and MetS), which may be potential targets for novel preventive and treatment strategies with the aim of improving overall patient quality of life and reduced mortality, preferentially by preventing the adverse sequelae from MetS [228]. *P. domestica* subsp. syriaca fruit pulp extract showed promising results against nitrite, IL-1β, and PGE_2 levels in LPS-stimulated macrophages in concentration-dependent manner with an IC50 of 0.46, 0.18 and 0.56 mg/mL, respectively. These results are in agreement with those previously published

[229,230], although our extract was more active. In particular, dried plum polyphenols suppressed significantly the production of NO and COX-2 in LPS-stimulated RAW 264.7 macrophages at the concentration of 100 and 100 mg/mL, respectively [230]. The higher anti-inflammatory effects of *P. domestica* subsp. syriaca fruit pulp extract could be due to the presence of polyphenolic compounds in different concentrations, mainly flavonoids (catechin and (+) epicatechin) and hydroxy cinnamic acids (chlorogenic acid and feruloyl-coumaroylquinic acid derivative). Each compound inhibited the production of pro-inflammatory mediators at different concentrations and whether the anti-inflammatory effects of this *Prunus* extract are due to additively or synergistically polyphenolic action is not known. For example, inhibitory effects of catechin [231] and chlorogenic acid [232] on various inflammatory mediators using LPS-stimulated RAW 264.7 macrophages were reported. In particular, chlorogenic acid inhibited completely NO production in LPS-stimulated macrophage RAW 264.7 cells at the concentration of 40 ug/mL [232]. A medicinal plant *Inonotus (I.) sanghuang* (rich in rutin, chlorogenic acid, isorhamnetin, quercetin, and quercitrin) improved insulin resistance and MetS by reducing inflammation via modulation of the crosstalk between macrophages and adipocytes [233]. Grape powder extract (rich in quercetin-3-glucoside, catechin, epicatechin, rutin, gallic acid and resveratrol) showed to decrease LPS-stimulated inflammation in macrophages by affecting the gene expression of IL-6, IL-8, IL-1β and TNF-α, and in turn decreased insulin resistance [234]. *Sambucus nigra* L. fruit extract alleviated insulin resistance by suppressing the enhanced production of NO, TNF-α, IL-6, and PGE_2, where the presence of cyanidin-based anthocyanins, flavan-3-ols, flavonols, and hydroxycinnamic acids were detected [235].

Considering the results from *in vitro* activities, *in vivo* study was conducted to confirm the antidiabetic potential of *P. domestica* subsp. syriaca fruit pulp in healthy animals. Oral

supplementation of mice with *P. domestica* fruit pulp extract after oral sugars load resulted in decrease in blood glucose and insulin levels through the time period 20 minutes to 1 hour. *P. domestica* extract exerted a significant decrease in blood glucose and insulin levels when compared to the control, yet the blood samples were analyzed after single administration of *P. domestica* fruit pulp extract and the results maybe different and more significant after long time use of *P. domestica* extract. The results obtained are aligned with the data reported in the literature and confirmed the likely benefits of *P. domestica* fruit pulp extract in subjects carrying high risk for the development of DM2. *P. davidiana* [236], *P. laurocerasus* [237], and *P. divaricata* [238] are showed to decrease blood glucose levels in diabetic rats along with the improvement of other biochemical parameters such as body weight, visceral fat mass, total cholesterol, LDL-C, and triglycerides. Treatment of high-fat diet induced obesity in mice with *P. persica* (0.2% or 0.6% flower extract for 8 weeks) significantly decreased body weight, visceral fat mass, serum glucose levels, and ALT and AST [239]. Methanolic extract of *P. domestica* fruits (200 and 400 mg/kg body weight i.p.) resulted in reducing body weight and blood levels of glucose, total cholesterol, and triglycerides in diabetic rats when administered for 14-consecutive days [240].

Bioaccessibility, absorption, and transformation are the main rate limiting steps to the oral bioavailability of food bioactive components, and thus they are essential to determine to have complete picture of the bioaccessible food bioactives, before considering nutraceuticals for clinical use [241]. *In vitro* bioaccessibility is useful tool to assess the possible interactions of food components and/or nutrients as well as the effect of digestion factors (such as pH, digestive enzymes, and salts), and nature of food matrix on bioaccessibility (i.e., potential of food bioactive components to be absorbed). *In vitro* methods are mostly preferred to determine

bioaccessibility because these methods are generally faster, less expensive, and offer better control of experimental variables in comparison to *in vivo* or clinical studies [242].After simulated digestion of *P. domestica* fruit pulp extract, TPC was decreased significantly as compared to the non-digested extract. The current study indicated the presence of only hydroxycinnamic acids in the oro-gastro-duodenal digested fraction, which point out relatively low bioaccessibility of the extract. A literature data has demonstrated the significant decrease of TPC and antioxidant activities of fruits and vegetables following gastrointestinal digestion, mainly due to the degradation of polyphenolic compounds upon exposure to digestion conditions such as varying pH of gastrointestinal tract and digestive enzymes [243–245]. These preliminary results suggest the potential polyphenols from the fruit pulp extract of *P. domestica* subsp. syriaca for human health and that these components could be incorporated into functional foods, aimed for the prevention of MetS risk factors.

6. Conclusions

Nutraceuticals and functional foods are known to play an important role in the maintenance of health and wellbeing of mankind, through the prevention of chronic diseases. A substantial increase in the worldwide usage of vegetable extracts has been observed in last decades, probably due to the increasing trend of consumer propensity towards the preventive care through natural substances. Currently, more than 80% of world population is relying on the use of vegetable products for their primary health concerns [246]. The present investigation studied the nutraceutical benefits of *P. domestica* subsp. syriaca fruit pulp extract in relation to MetS risk factors. It resulted to inhibit key enzymes involved in the metabolism of carbohydrates and lipids as well as in the cholesterol synthesis. These enzymes are one of the potential targets in the management of metabolic dysregulations and modulating these enzymes could be a fruitful strategy in the prevention of individual component of MetS and thus DM2, cardiovascular disorders and cerebrovascular accidents. In addition, *P. domestica* fruit extract attenuated LPS-stimulated release of proinflammatory mediators, reflecting its potential to improve insulin sensitivity, endothelial dysfunction, and prevention against cardiovascular complications secondary to MetS. The mechanisms of *P. domestica* extract in the inhibition of tested enzymes and release of inflammatory mediators are unknown, but the chemical characterization showed the presence of polyphenolic contents, that potentially justified the biological properties observed. Additionally, the hypoglycemic and hypoinsulinemic effects of *P. domestica* fruit pulp extract in healthy mice confirmed the anti-diabetic potential of *P. domestica* extract. Altogether, these results suggest the possible nutraceutical benefits of *P. domestica* fruit pulp extract in subjects with high risk for the development of DM2 or obesity and in the prevention of the associated complications.

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