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Broad bean (*Vicia faba* L.) pods: a rich source of bioactive ingredients with antimicrobial, antioxidant, enzyme inhibitory, anti-diabetic and health-promoting properties†

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This study was aimed at investigating the chemical composition (proximate, minerals, fatty acids and phenolic compounds) and the *in vitro* (antimicrobial, radical scavenging, anti-acetylcholinesterase and protein denaturing activities) and *in vivo* (anti-diabetic and histo-protective effects in alloxan-induced diabetic mice) biological activities of broad bean pods (BBPs), a food waste by-product material. The results showed that BBPs have high dietary fiber (57.46%), carbohydrate (18.93%) and protein (13.81%) content *versus* low fat content (<1%) contributing to a low energy value of 139.24 kcal per 100 g. Profiling of fatty acids showed an abundance of the essential polyunsaturated α-linolenic and linoleic acids, exhibiting an excellent nutritional quality as revealed by their low atherogenic and thrombogenic indices and their hypocholesterolemic properties. The methanol extract which exhibited the highest total phenolic, flavonoid and tannin contents was found to be the most active extract in terms of antimicrobial and anti-radical activities. In alloxan-induced diabetic mice, the oral administration of a methanol extract (500 mg per kg bw) attenuated the elevated levels of serum alanine aminotransferase (ALA), aspartate aminotransferase (AST), and alkaline phosphatase activities, and urea, uric acid, and creatinine. It effectively normalized the status of lipid profiles, mitigated oxidative stress through the activation of antioxidant enzymes (CAT, GPx and SOD), and alleviated oxidative stress-mediated histopathological changes in the pancreas, liver, kidney and testis. Compositional analysis by HPLC-PDA-ESI-MS/MS revealed the presence of flavan-3-ols (catechin, epicatechin and their derivatives), flavones (apigenin derivatives) and flavonols (glycosides of quercetin and kaempferol), among others. These findings suggest that BBPs may be an effective functional food for the management of diabetes and its complications.

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1. Introduction

Pulse crops are considered to be the major sources of proteins, dietary fiber, micronutrients, and bioactive phytochemicals. Their low glycemic indexes and fat contents, in addition to their health-promoting properties including anticancer, anti-diabetic, anti-obesity, and cardio-protective effects, make them one of the most important components of the human diet.[1,2] They are commonly used as dry grains or seeds, but they are also consumed as green vegetables.[3] Among the numerous pulse crops, broad beans (*Vicia faba* L.) have received particular attention due to their nutraceutical, functional and economic importance. This rustic species represents a source of income for many people in developed and underdeveloped countries, where broad beans are considered as a potential source of affordable alternative proteins. Along with proteins (about 30% of lysine-rich proteins), broad bean seeds are rich in dietary fiber, vitamins, minerals, γ-aminobutyric acid and phenolic compounds to which the antioxidant and numerous food-related biological activities are attributed.[4,5] In this context, epidemiological and randomized controlled trial studies have shown that the consumption of

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broad beans (processed or not) or broad bean-based products such as pasta, fortified bread and biscuits is associated with a low incidence of degenerative diseases such as cardiovascular and inflammatory diseases, cancer and diabetes.[6–8]

Broad bean pods (BBPs) are the primary by-product of the domestic and industrial processing of broad beans. They are an attractive source of valuable ingredients (namely dietary fiber) which might offer many advantages for human consumption and enhance the profitability of this underutilized by-product. To meet this goal, a comprehensive chemical analysis and evaluation of the biological properties of BBPs is mandatory. The few publications concerning the chemistry of BBPs report on their dietary fibre content, soluble sugars, fatty acids and mineral composition.[9] However, little is known about their phenolic profile and biological activity.

Therefore, the present study was designed to comprehensively investigate the BBPs for their mineral contents, lipid content, fatty acids and their nutritional quality, as well as their phenolic composition. The *in vitro* antioxidant, antimicrobial, anti-acetylcholinesterase, and anti-inflammatory and *in vivo* anti-diabetic and hepato-, reno- and repro-protective effects in alloxan-induced diabetic mice were also evaluated.

It is anticipated that the data obtained from this study will be useful (i) to provide basic support for the functional uses of BBPs, (ii) for the conception of new functional and health-promoting bio-products and (iii) for the development of a potential market for bioactive ingredients from residual sources.

2. Materials and methods

2.1. Chemicals

Folin–Ciocalteu reagent, gallic acid, quercetin, catechin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-[3-ethylbenzothiozoline-6-sulphonic acid di-ammonium salt] (ABTS), ferric chloride, 2,4,6-tripyridyl-*s*-triazine (TPTZ), sodium phosphate, ammonium molybdate, acetic acid, bovine serum albumin (BSA), Tris-HCl, trichloroacetic acid (TCA), acetyl salicylic acid, 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), acetylthiocholine iodide, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), standard 37 FAMEs and alloxan monohydrate were procured from Sigma-Aldrich (Steinheim, Germany). Solvents of analytical and HPLC-grade were purchased from Carlo Erba Reactif-CDS (Val de Reuil, France).

2.2. Plant materials

Mature broad beans (*Vicia faba* cv. Major) were purchased from a local market. The biological materials were harvested from field grown mature plants in 2016 at a locality of Bizerte (Northern Tunisia). After the removal of seeds, the green pods were oven-dried at 40 °C until constant weight, ground to a fine powder and stored at −20 °C.

2.3. Chemical composition and nutritional compounds

2.3.1. Proximate and mineral composition. Moisture and ash were determined according to the standard methods of AOAC (1999).[10] The moisture content was determined by drying the sample in an oven at 105 °C until constant weight. The crude protein (N × 6.25) content was evaluated after the determination of nitrogen using the Kjeldahl method. The ash content was determined after the incineration of the samples at 500 °C in a muffle furnace. Crude fat was determined using a Soxhlet apparatus with hexane to extract the lipid.

The mineral composition was estimated by inductively coupled plasma–atomic emission spectrometry (ICP-AES; Horiba Jobin–Yvon Ultima 2 CE) using optimal instrumental parameters according to Pavlova and Karadjova.[11]

2.3.2. Fatty acid composition and lipid index quality. The fatty acids of the total lipids were converted into their corresponding fatty acid methyl esters (FAMEs) by using sodium methoxide (3%) in methanol.[12] The FAMEs were then analyzed by gas chromatography using a Hewlett-Packard 6890 gas chromatograph series II (Agilent Technologies, Palo Alto, California, USA) equipped with a flame ionisation detector (FID) and an electronic pressure control injector (EPC). The separation of individual FAMEs was performed on a polar TR-FAME capillary column (60 m × 0.25 mm, 0.25 μm film thickness). The oven temperature was initially maintained at 100 °C for 5 min, raised to 240 °C at a rate of 4 °C min^{-1}, and then maintained for 15 min. The temperatures of the injector and FID detector were maintained at 240 °C and 260 °C, respectively. The identification of FAMEs was made by comparing their retention time with those of 37 FAME standards purchased from Sigma-Aldrich (Steinheim, Germany). The percentages of FAMEs were calculated with reference to the total fatty acids.

Lipid quality indices including saturated fatty acids (SFA), unsaturated fatty acids (UFA), UFA/SFA ratio, omega-3/omega-6 ratio, atherogenic index (AI), thrombogenic index (TI), hypocholesterolemic fatty acid/hypercholesterolemic fatty acid ratio (h/H), calculated oxidizability value (Cox) and oxidative susceptibility (OS) were calculated:

$$\mathrm{AI} = [(4 \times \mathrm{C14:0}) + \mathrm{C16:0} + \mathrm{C18:0}] / [\sum \mathrm{UFA} + \sum \omega 6\ \mathrm{PUFA} + \sum \omega 3\ \mathrm{PUFA}]$$

$$\mathrm{TI} = [\mathrm{C14:0} + \mathrm{C16:0} + \mathrm{C18:0}] / [0.5 \times \mathrm{MUFA} + 0.5 \times \omega 6\ \mathrm{PUFA} + 3 \times \omega 3\ \mathrm{PUFA} + \omega 3/\omega 6\ \mathrm{PUFA}]$$ Ulbrich and Southgate[13]

where MUFA is the sum of monounsaturated fatty acids and PUFA is the sum of polyunsaturated fatty acids.

$$\mathrm{h/H} = [\mathrm{C18:1n\text{-}9} + \mathrm{C18:2n\text{-}6} + \mathrm{C20:4n\text{-}6} + \mathrm{C18:3n\text{-}3} + \mathrm{C20:5n\text{-}3} + \mathrm{C22:5n\text{-}3} + \mathrm{C22:6n\text{-}6}] / [\mathrm{C14:0} + \mathrm{C16:0}]$$ Santos-Silva *et al.*[14]

$$\mathrm{Cox} = [\mathrm{C18:1} + 10.3\,\mathrm{C18:2} + 21.6\,\mathrm{C18:3}] / 100$$ Fatemi and Hammond[15]

$$\mathrm{OS} = \mathrm{MUFA} + 45\,\mathrm{C18:2} + 100\,\mathrm{C18:3}$$ Cecchi *et al.*[16]

2.4. Bioactive phytochemicals

The content of bioactive phytochemicals such as phenols, flavonoids and tannins was assessed in four polar extracts, namely methanol, ethanol, butanol and ethyl acetate. After being screened for their *in vitro* biological activities, the most efficient and active solvent extract was retained for the *in vivo* assays and phenolic profiling using HPLC-PDA-ESI-MS/MS.

2.4.1. Total phenolic, total flavonoid and condensed tannin content. The total phenolic content (TPC) was determined using the colorimetric Folin–Ciocalteu method of Singleton and Rossi.[17] Gallic acid was used as the standard and the results were expressed as mg gallic acid equivalents per g extract (mg GAE per g extract). The total flavonoid content (TFC) was determined using the method of Dehpour *et al.*[18] and was expressed as mg quercetin equivalents per g extract (mg QE per g extract). The condensed tannin content was determined using the vanillin method and the results were expressed as mg catechin equivalents per g extract (mg CE per g extract).[19]

2.4.2. Profiling of phenolic compounds by HPLC-PDA-ESI-MS/MS. An Agilent 1100 series HPLC system equipped with a photodiode array detector (PDA), a triple quadrupole mass spectrometer type Micromass Autospec Ultima Pt (Kelso, UK) and an ESI ion source working in negative mode was used for the identification of phenolic compounds. The mobile phase consisted of A (0.1% acetic acid) and B (acetonitrile) with a flow rate of 0.25 mL min^{-1}. Separation was achieved using a Superspher®100 column (12.5 cm × 2 mm i.d., 4 μm, Agilent Technologies, Rising Sun, MD) at 45 °C with a multi-step linear gradient elution program in which phase B changed from 0 to 2% in 5 min, from 2 to 88% in 75 min, and from 88 to 2% in 90 min. The UV-Vis spectra were recorded from 200 to 800 nm, and the ions in the *m*/*z* range of 100–1000 were detected using a scan time of 1 s. The ESI source was conducted under the following operating conditions: capillary voltage, 3.2 kV; cone voltage, 115 V; probe temperature, 350 °C and ion source temperature, 110 °C. Data acquisition was achieved with the Masslynx software version 4.0. The tentative identification of phenolics was carried out considering their UV and mass spectra, as well as by comparison of their retention time and fragmentation pattern with those of authentic standards when available and/or the literature data.[20–23]

2.5. Evaluation of extract bioactivity

2.5.1. *In vitro* assays

2.5.1.1. Antimicrobial activity. A total of six microbial strains obtained from the culture collection center of the Institut Pasteur de Tunis, Tunisia and from our culture collection centre of the Institut National de Recherche et d'Analyse Physico-chimique, Ariana, Tunisia were used for the evaluation of the antimicrobial activity. They included three Gram-negative bacteria (*Enterococcus faecium* (ATCC19434), *Salmonella typhimurium* (ATCC14028) and *Escherichia coli* (ATCC8739)), two Gram-positive bacteria (*Staphylococcus aureus* (ATCC8739) and *Streptococcus agalactiae*) and the yeast *Candida albicans* (ATCC10231).

The disc diffusion assay was used for the qualitative evaluation of the anti-microbial potential of different extracts according to the National Committee for Clinical Laboratory Standards (NCCLS),[24] whereas quantitative determination in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (CMB) was carried out using the broth micro-dilution method.[25]

2.5.1.2. Antioxidant activity. The radical scavenging activity against the DPPH radical of different BBP extracts was evaluated according to the method of Binsan *et al.*[26] The procedure of Re *et al.*[27] was adopted to evaluate the anti-radical activity against the ABTS radicals. In both assays, the results were expressed as IC_{50} which represents the sample concentration required to achieve half radical (DPPH or ABTS) scavenging activity. The ferric reducing antioxidant power (FRAP) was evaluated according to Benzie and Strain, by using the complex ferrous tripyridyltriazine method, and the results were expressed as μmol Trolox equivalent per g of the extract (μmol TE per g extract).[28] The total antioxidant capacity (TAA) was evaluated by the method described by Prieto *et al.* The results were expressed as equivalents of ascorbic acid per g extract (mg AsA per g extract).[29]

2.5.1.3. Protein denaturing activity. The inhibition of protein denaturation was determined by the method of Sakat *et al.*[30] Acetyl salicylic acid (ASA) was used as a positive control and the results were expressed as a percentage inhibition of protein denaturation (%).

2.5.1.4. Enzyme inhibitory activity. The *in vitro* inhibition of acetylcholinesterase (AChE) was performed according to the method of Falé *et al.*[31] Enzyme activity was estimated as a percentage of the velocities compared to that of the blank sample.

2.5.2. *In vivo* assays

2.5.2.1. Animals and treatment. The assays were conducted on 6-week-old male Swiss albino mice (weight 30 ± 5 g), housed under controlled conditions: temperature of 20 ± 2 °C, photoperiod of 12 h light and 12 h dark with food (standard pellet diet, Badr Utique-TN) and water *ad libitum*. The animals were carefully maintained and treated in accordance with the international and national ethical guidelines, as per the European Directive on Protection of Animals Used for Scientific Purposes (2010/63/EU), the Canadian Council on Animal Care (CCAC), and the Guide for the Care and the Use of Laboratory Animals of the National Institute of Health (NIH). The protocol was approved by the Comité d'Ethique Bio-medicale (CEBM) (JORT 472001) of the Institut Pasteur de Tunis.

The mice were randomized into 4 groups of 12 each. The first group consisted of non-diabetic control mice. The second group of diabetic control mice was subjected to a single intraperitoneal injection of alloxan monohydrate at a dose of 160 mg per kg body weight (b.w.). After 48 h, the alloxan-treated animals were provided with 5% glucose overnight to avoid drug-induced hypoglycemic shock as a result of massive insulin release. Ten days after the injection, blood was col-

lected by puncturing the tail tip with a syringe needle and glucose levels were measured using a glucometer. The animals with blood glucose levels higher than 13 mM L^{-1} were considered diabetic. The treatment began 10 days after alloxan injection for 15 days. The third group consisted of non-diabetic mice treated with a BBP extract (500 mg per kg b.w.) and given normal water and fed *ad libitum*. The fourth group consisted of diabetic mice treated with a BBP extract (500 mg per kg b.w.) and given normal water and fed *ad libitum*.

2.5.2.2. Sample collection. At the end of the experiment (15 days of treatment), overnight fasting mice were sacrificed by cervical dislocation after mild anaesthesia. The post-mortem collection of trunk blood was made on ice-chilled heparinized tubes and centrifuged at 3000*g*. Plasma was immediately recovered and preserved at −80 °C for serum biochemical parameters. Tissues from the pancreas, liver, kidneys and testes were dissected out immediately, washed with ice-cold saline solution and kept at −80 °C for enzyme analysis. At the same time, the pancreas, liver, kidneys and testes were excised and immediately fixed in 10% neutral buffered formalin for histological examination. To evaluate the male mice reproductive performance, the testis and epididymis were rapidly excised and homogenized in saline phosphate buffer and the sperm was collected in 5 cc tubes.

2.5.2.3. Biochemical parameters

Serum biochemical parameters. Serum markers such as glucose, aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase, lactate dehydrogenase (LDH), albumin, urea, uric acid, creatinine, triglycerides, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were determined spectrophotometrically, using enzymatic colorimetric kits (Biomaghreb, Ariana, Tunisia).

Biochemical parameters in tissue homogenates

Tissues from the pancreas, liver, kidneys, and testes were homogenized in phosphate buffer saline (KH_2PO_4/K_2HPO_4, 50 mM, pH 7.4) using a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 10 000*g* for 10 min (4 °C) and the resulting supernatants were then used for the estimation of MDA, H_2O_2, sulfhydryl group (–SH), and antioxidant enzyme activity.

The extent of lipid peroxidation in different organs was evaluated in terms of malondialdehyde (MDA) and measured according to the preconised method of Draper and Hadley.[32] Briefly, aliquots from different homogenates were mixed with a TCA solution containing 1% BHT (w/v) and centrifuged at 1000*g* for 5 min (4 °C). The supernatant was mixed with a solution containing 0.5 N HCl and 120 mM TBA in 26 mM Tris and then heated at 80 °C for 10 min. After cooling, the absorbance of the pink coloured MDA–TBA chromophore was read spectrophotometrically at 532 nm and the MDA level was determined using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

The H_2O_2 content was determined following the method of Dingeon *et al.*[33] consisting of the determination of the absorbance at 505 nm of the quinoneimine chromophore produced following the reaction between hydrogen peroxide with *p*-hydroxybenzoic acid and 4-aminoantipyrine in the presence of peroxidase.

For the sulfhydryl (thiol) group (–SH), the method of Ellman's was used.[34] Briefly, aliquots from different homogenates were mixed with 100 μL of 10% SDS and 800 μL of 10 mM phosphate buffer (pH 8) and the absorbance (A_0) was then measured at 412 nm. Thereafter, 100 μL of DTNB was added and heated at 37 °C. After a 60 min incubation, a second measure of absorbance (A_1) was performed. The concentration of the sulfhydryl group was determined from the difference between A_1 and A_0 using a molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}. The results were expressed as nmol of thiol groups per mg protein.

Regarding the activity of antioxidant enzymes, the following procedures were used. For catalase (CAT; E.C.: 1.11.1.6.) activity determination, Abei's method based on H_2O_2 decomposition estimation at 240 nm was adopted.[35] Glutathione peroxidase (GPx; E.C.: 1.11.1.9) activity was determined spectrophotometrically at 412 nm according to the method of Flohé and Günzler.[36] Superoxide dismutase (SOD, E.C.: 1.15.1.1) activity was determined using the method of Misra and Fridovich[37] which is based on the spectrophotometric measurement at 480 nm of the SOD ability to inhibit the radical-mediated chain-propagating autoxidation of epinephrine in an alkaline pH medium.

The characterization of SOD isoforms was performed using KCN (2 mM), which inhibits Cu/Zn-SOD or H_2O_2 (5 mM), affecting both Cu/Zn-SOD and Fe-SOD whereas Mn-SOD was insensitive to both inhibitors.[38] Protein concentration was determined according to the Bradford method using bovine serum albumin (BSA) as the standard.[39]

Evaluation of the reproductive performance

The reproductive performance in terms of sperm characteristics was evaluated in all mice groups. For sperm collection, epididymis was excised and minced in 1 mL of RPMI to obtain a spermatozoa suspension.

The sperm count was determined in the cauda epididymis according to the method of Vega *et al.*[40] Sperm motility was assayed following the procedure of Kvist and Bjorndahl.[41] Briefly, 10 μL of the sperm suspension (prepared from excised epididymis) was layered onto a warmed microscope slide. Sperm motility was assessed by counting all progressive motile, non-progressive motile and immotile spermatozoa. The number of motile spermatozoa in each field was divided by the total number, and the average of the fields was assayed. The percentage of motile spermatozoa was thereafter determined. Sperm viability was assessed using eosin stain following the procedure of Tardif *et al.*[42] Morphological sperm abnormality was microscopically examined in an air-dried smeared sperm suspension stained with eosin.[43] The classification of the morphological abnormalities including head, tail and tail–head abnormalities was based on the criteria of Filler.[44]

2.5.3. Histological observations. Pancreas, liver, kidneys and testes from all groups were subjected to a standard tissue

processing procedure that included dehydration in a graded ethanol series (50–100%), clearing in xylene and finally embedding in paraffin. Sections of 5 μm thickness were cut from each block and stained with haematoxylin–eosin for histological examination by light microscopy.

2.6. Statistical analysis

The analyses were made in triplicate and the results are expressed as mean value ± standard deviation. Pearson's correlations between TPC, TFC, condensed tannins, DPPH, ABTS, FRAP and TAA were calculated. One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was used to compare the means. All analyses were performed using the SPSS 18.0. statistical software package.

3. Results and discussion

3.1. Chemical composition, nutritional value and bioactive compounds of BBPs

3.1.1. Proximate and elemental composition and chlorophyll and carotenoid contents. This study was conducted with a major objective to comprehensively investigate the chemical composition and biological activities of BBPs. The results of the proximate composition and mineral elements are presented in Table 1. BBPs had a high moisture content (79.26%, wet weight basis) which makes it highly vulnerable to spoilage and microbial contamination, and this consequently reduces its stability and shelf life. In contrast, such a high moisture content increases its juiciness, palatability and texture making it suitable as a feed for animal nutrition.[45]

The average values of proteins, carbohydrates, lipids and dietary fibre were 13.81, 18.93, 0.92 and 57.46% (dry weight basis), respectively. Concomitantly, the energetic value of BBPs was estimated to be 139.24 kcal per 100 g. This energy value was markedly lower than that found in Spanish specimens (172.5 kcal per 100 g).[9] These differences were mainly due to the high fat (1.3%) and carbohydrate (26.6%) contents in Spanish samples and reflect the effect of origin. In canned broad beans, a value of 125 was observed for the energy value and was mainly attributed to the low fat (0.24%) content.[46] Another point to be considered is that the high fibre content makes broad beans suitable for consumption due to their beneficial effects on the digestive tract, prevention of chronic diseases and the improvement of glucose tolerance in diabetics.[47]

Table 1 Proximate composition, total energetic value, and mineral element (ppm) and pigment contents in BBPs

	Content
Moisture (% wet weight)	79.26 ± 0.48
Protein (g per 100 g d.w.)	13.81 ± 0.51
Carbohydrate (g per 100 g d.w.)	18.93 ± 0.28
Fat (g per 100 g d.w.)	0.92 ± 0.08
Total dietary fibre (g per 100 g d.w.)	57.46 ± 1.23
Total energetic value (kcal per 100 g)	139.24 ± 6.74
Ash (g per 100 g d.w.)	8.87 ± 0.91
Mineral	
Calcium (g per 100 g)	0.39 ± 0.04
Potassium	2.33 ± 0.12
Magnesium	0.18 ± 0.01
Sodium	0.39 ± 0.02
Copper (mg per 100 g)	0.13 ± 0.01
Iron	0.74 ± 0.04
Zinc	0.44 ± 0.05
Pigment (μg g^{-1} fresh weight)	
Chl *a*	37.6 ± 1.24
Chl *b*	11 ± 1.14
Carotenoids	7.3 ± 0.41

The BBP samples contained a high amount of ash (8.87%) which was mainly composed of potassium (K), sodium (Na), magnesium (Mg), calcium (Ca) and some essential trace elements like iron (Fe), copper (Cu) and zinc (Zn). The high concentration of K and low concentration of Na and concomitantly low Na/K ratio <1 make the consumption of BBPs particularly important for human health and for cardiovascular disease prevention.[48] In addition, broad bean consumption may contribute significantly to the daily intake of some essential trace elements which have pivotal biological functions in the human body such as metabolic reactions, energy production, maintenance of acid–base and water balance, and transmission of nerve impulses, among others.

Ash content was nearly the same as that reported in canned broad beans (9.02%) as previously described,[46] but it was remarkably higher than the value reported in Spanish samples (6.3%).[9]

Regarding the chlorophyll content, our value for Chl *a* was 2.22-fold higher than that observed by Belghith-Fendri *et al.*[46] for canned BBPs. The data from the same report indicate that the Chl *b* content was quite similar to that observed herein. The carotenoid content in BBPs was described for the first time and was found to be 7.3 μg g^{-1} fresh weight.

3.1.2. Fatty acid profile and related nutritional indices. Analytical gas chromatography of the total lipid allowed the identification of 11 fatty acids, with unsaturated fatty acids (UFA) being the most abundant fraction (Table 2). The latter was predominantly composed of the essential linoleic (39.74%) and linolenic (24.99%) acids. Palmitic (18.2%) and stearic (6.72%) acids were found to be the main saturated fatty acids (SFA) that account for 30.78% of the total fatty acids.

The evaluation of the nutritional value showed that the lipids of BBPs were of high nutritional quality due to their high UFA/SFA, ω-3/ω-6 and h/H ratios (2.25, 0.63 and 3.67, respectively) as well as their low atherogenic (AI) and thrombogenic (TI) indexes (0.38 and 0.26, respectively). At this point, it can be suggested that lipids from BBPs are of good nutritional quality, and their consumption could be considered to be advantageous by virtue of their low incidence of cardiovascular diseases (low AI and TI indices) and their hypocholesterolemic properties as revealed by their high h/H ratio.

However, their high linolenic acid content makes them particularly vulnerable to oxidation as revealed by their high Cox (9.53) and OS (4291) values. Compared with the few published

Table 2 Composition of fatty acids (% of total fatty acids) and their nutritional quality indices

Fatty acid	
Myristic (C14:0)	0.36 ± 0.01
Pentadecanoic (C15:0)	0.56 ± 0.06
Palmitic (C16:0)	18.22 ± 0.24
Palmitoleic (C16:1)	0.56 ± 0.08
Margaric (C17:0)	1.11 ± 0.18
Stearic (C18:0)	6.72 ± 0.34
Oleic (C18:1)	3.96 ± 0.48
Linoleic (C18:2)	39.74 ± 2.64
Linolenic (C18:3)	24.93 ± 1.78
Arachidic (C20:0)	1.92 ± 0.12
Lignoceric (C24:0)	1.92 ± 0.08
Lipid quality	
SFA	30.78
UFA	69.22
UFA/SFA	2.25
ω-3/ω-6	0.63
AI	0.38
TI	0.26
h/H	3.67
Cox	9.53
OS	4291.21

SFA: saturated fatty acids; UFA: unsaturated fatty acids; AI: atherogenic index; TI: thrombogenic index; h/H: hypocholesterolemic ratio; Cox: calculated oxidizability value; OS: oxidative susceptibility.

data, striking differences owing to genetic and environmental factors can be outlined.[9] The described fatty acid profile was dominated by UFA (78%) with linoleic (44.6%) and linolenic (22.8%) acids as the main components.[9]

Generally, the results of the present study in combination with those of the earlier reports indicate that BBPs could be considered as a consolidated source of nutritional ingredients (carbohydrates, fibre, proteins, essential minerals and essential polyunsaturated fatty acids) and could be utilized for food fortification and functional food development.

In addition to their nutritional properties, the assessment of the biological activities and the determination of the bioactive components of broad beans are of utmost importance. In this direction, we have evaluated the bioactive compounds in terms of total phenolic, total flavonoid and condensed tannin contents using four different solvent systems to ensure the most efficient one.

3.1.3. Total phenolic (TPC), total flavonoid (TFC), and condensed tannin contents. A comparison of the data presented in Table 3 shows that methanol was by far the best extracting solvent. The solvents in decreasing order of yield were: methanol (25.8%) > ethanol (17.5%) > butanol (11.3%) > ethyl acetate (0.81%). The highest recovery of TPC (115.21 mg GAE per g extract) and TFC (47.34 mg QE per g extract) was also observed in methanol extracts, whereas the lowest was observed in ethyl acetate extracts. However, the latter extracts exhibited the highest content of condensed tannins (4.56 mg EC per g extract). These results suggest that most of the phenolic compounds extracted by ethyl acetate were of the catechin or *epi*-catechin type as previously demonstrated.[49] The highest TPC and TFC in the methanol extract indicated that the phenolic and flavonoid compounds in BBPs were of high polarity including flavonoid glycosides and more polar aglycones.[50]

The phenolic contents are not adequately described in the literature and the only published report revealed that TPC ranged from 56.97 to 149.21 mg EGA per g in thirteen genotypes of BBPs cultivated in the same open field and processed under the same conditions.[51] In the same comparative study, the genotypic influence was also evidenced for TFC where values ranging from 10.23 to 45.92 mg RE per g were observed.[51] Our results were within the range reported by these authors.

Given the recognized biological activities of phenolic compounds, the four extracts were evaluated for their antioxidant, antimicrobial, protein denaturation inhibition and anti-acetylcholinesterase activities.

3.2. *In vitro* bioactivity of BBPs

3.2.1. Antimicrobial activity. The *in vitro* antimicrobial activity of various extracts of BBPs was evaluated against a set of bacterial strains, including the Gram-positive bacteria *Staphylococcus aureus*, *Enterococcus faecium* and *Streptococcus agalactiae*, the Gram-negative bacteria *Salmonella typhimurium* and *Escherichia coli* and the yeast *Candida albicans* (Table 4). The qualitative results based on the disc diffusion assay showed that the methanol extract prevents the growth of the Gram-positive bacteria *E. faecium* and *S. agalactiae* and the Gram-negative bacterium *E. coli* as well as the yeast *C. albicans*. The bacterial strains of *S. aureus* and *S. typhimurium* were found to be the most resistant to all extracts. The antimicrobial potency of the different extracts was further determined by measuring the MIC and the MBC (Table 5). The methanol extract exhibited the lowest MIC (1.5–3 mg mL^{-1}) and MBC (2–4 mg mL^{-1}) values, corroborating thus, the results of the qualitative evaluation by the disc diffusion assay. The anti-

Table 3 The TPC, TFC and condensed tannin content in different extracts of BBPs

Extract	Extract yield (%)	TPC (mg GAE per g extract)	TFC (mg QE per g extract)	Condensed tannins (mg CE per g extract)
Methanol	25.8^{a} ± 1.1	115.21^{a} ± 0.46	47.34^{a} ± 1.22	3.33^{b} ± 0.68
Ethanol	17.5^{b} ± 0.8	56.93^{c} ± 3	39.58^{b} ± 1.77	2.81^{c} ± 0.80
Ethyl acetate	0.81^{d} ± 0.2	20.43^{d} ± 0.82	16.71^{c} ± 0.92	4.56^{a} ± 0.61
Butanol	11.3^{c} ± 1.4	74.22^{b} ± 0.7	44.85^{ab} ± 1.89	3.90^{b} ± 0.93

TPC: total phenol content; TFC: total flavonoid content. Different superscripts within the lines are significantly different at $p < 0.05\%$.

Table 4 Antimicrobial activity of different extracts of BBPs

	Inhibition zone (mm)			
Microbial strain	M	E	B	EA
Staphylococcus aureus (+)	0	0	0	0
Enterococcus faecium (+)	6.5	13	6	16
Streptococcus agalactiae (+)	10	10.5	12.5	13.5
Salmonella typhimurium (−)	0	0	0	0
Escherichia coli (−)	6	0	0	0
Candida albicans	6	8	6	8

M: methanol; E: ethanol; B: butanol; EA: ethyl acetate.

microbial activity of the different extracts is very low in comparison with the synthetic antibiotic ampicillin (MIC 0.78–15.63 μg mL^{-1}; MBC 1.95–31.25 μg mL^{-1}) and nystatin (MIC 3.91 and MFC 7.81 μg mL^{-1}).

In general, Gram-positive bacteria were found to be more sensitive than Gram-negative bacteria. The differential susceptibility between Gram-positive bacteria and Gram-negative bacteria to the different extracts could be attributed to the structural differences in their membrane composition with the occurrence of a very restrictive lipopolysaccharide-containing outer membrane in Gram negative bacteria making them relatively resistant to antibiotics.[52] In their comparative study on the antimicrobial activity of the aqueous hull extracts of three Indian legumes including mung beans (*Vigna radiata*), chickpea (*Cicer arietinum*) and pigeon pea (*Cajanus cajan*), the latter authors pinpointed the efficacy of these legume extracts in inhibiting the growth of *S. aureus* and *E. coli* which was consistent with our findings.[52] The antimicrobial activity of phenolic compounds including flavonols, flavan-3-ols, gallotannins and ellagitannins is well documented and different mechanisms have been proposed.[53,54] In this context, Taguri *et al.*[55] reported the high sensibility of *S. aureus* to catechin, *epi*-gallocatechin, *epi*-gallocatechin-3-*O*-gallate, prodelphinidin oligomer, and procyanidin pointing out the importance of pyrogallol and trihydroxyphenyl groups on the antibacterial activity. The antimicrobial activity of pure phenolic compounds such as gallic acid, vanillic acid, procatechuic acid, caffeic acid, rutin and quercetin against *E. coli* and *Flavobacterium* sp. was observed and such activity was mediated through the adsorption of phenolic compounds to cell membranes and their interaction with enzymes and substrates and metal ion deprivation.[56] The aggregatory effect and their capacity to penetrate the cell phospholipid membrane are proposed as the main antibacterial mechanisms of some flavonols such as morin, rhamnetin and quercetin.[53] The ability of flavan-3-ols, flavonols, and isoflavones to induce damage in the cytoplasmic membrane (through the generation of hydrogen peroxide) inhibits the synthesis of nucleic acids (through the inhibition of topoisomerase and dihydrofolate reductase) and cell wall compounds and disrupts energy metabolism through the inhibition of ATP synthase as the main antimicrobial mechanisms.[54] The antimicrobial activity observed herein could be linked to one or more of these mechanisms.

In general, the present results have extended our knowledge on the antimicrobial activity of BBP extracts against *E. faecium*, *S. agalactiae* and *S. typhimurium* which was reported for the first time.

3.2.2. Antioxidant, protein denaturing and anti-acetylcholinesterase (anti-AChE) activities. The data from Table 6 show

Table 5 MIC and MBC values of different extracts of BBPs

	M		E		B		EA	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Staphylococcus aureus (+)	3	4	3	4	3	4	3	4
Enterococcus faecium (+)	2	2	2	3	2	3	1.5	2
Streptococcus agalactiae (+)	2	3	2	2	2	3	1.5	2
Salmonella typhimurium (−)	2	3	2	3	2	3	1.5	2
Escherichia coli (−)	2	2	1.5	2	2	3	1.5	2
Candida albicans	2	2	1.5	2	2	3	1.5	2

M: methanol; E: ethanol; B: butanol; EA: ethyl acetate; MIC: minimum inhibitory concentration (mg mL^{-1}); MBC: minimum bactericidal concentration (mg mL^{-1}).

Table 6 Anti-radical, reducing power and total antioxidant activities of various extracts of BBPs

Extracts	DPPH[a]	ABTS[a]	FRAP[b] (mM TE per g extract)	TAA[c] (mg AsA E per g extract)
Methanol	$157.94^{d} \pm 2.09$	$610.61^{c} \pm 0.56$	$410.21^{b} \pm 4.43$	$60.72^{a} \pm 2.27$
Ethanol	$290.82^{c} \pm 11.38$	$908.99^{b} \pm 19.77$	$462.01^{a} \pm 9.12$	$49.26^{b} \pm 2.17$
Butanol	$609.77^{b} \pm 6.42$	$597.97^{c} \pm 3.81$	$203.23^{c} \pm 4.78$	$42.73^{c} \pm 1.04$
Ethyl acetate	$1098.68^{a} \pm 14.22$	$2026.95^{a} \pm 24.56$	$132.51^{d} \pm 6.21$	$63.26^{a} \pm 4.64$

[a] IC_{50} values for DPPH and ABTS (μg mL^{-1}). [b] TE: Trolox equivalents (mM TE per g extract). [c] AA: ascorbic acid equivalent (mM AAE per g extract). Different superscripts within the lines are significantly different at $p < 0.05\%$.

that the methanol extract exhibited virtually the highest total antioxidant activity (TAA) (60.72 mg AsA E per g extract). It also showed the highest free radical scavenging activity as revealed by its low IC_{50} for both DPPH and ABTS assays. In the FRAP assay, the ethanol extract was found to be the most effective extract. In general, the DPPH and ABTS radical scavenging activity showed a similar trend to that of TPC (r = 0.834 and 0.857 for TPC *vs.* DPPH and TPC *vs.* ABTS, respectively) and TFC (r = 0.878 and 0.995 for TPC *vs.* DPPH and TPC *vs.* ABTS, respectively) suggesting that phenolic compounds are the main contributors in the observed activity (Table S1†). These results were consistent with the findings of Chaieb *et al.*[51] where a strong positive correlation between DPPH and TPC was observed in 13 genotypes of broad beans. These observations were later confirmed by Siah *et al.*[8] who found high positive correlations between TPC, TFC, DPPH and FRAP in faba beans differing in seed coat colours. At this point, the hypothesis that TPC and TFC could be considered as excellent indicators of the DPPH radical scavenging potential of BBP extracts could make sense.

A significant (r = 0.633; p = 0.027) correlation between FRAP and total flavonoids was also outlined suggesting the contribution of flavonoids to the ferric reducing ability of the methanol extract of BBPs. There were also highly significant relationships between DPPH, ABTS and FRAP which imply that these assays involve the same mechanisms and/or are mediated through the same antioxidant compounds. A weak but significant correlation between TAA and ABTS (r = 0.619; p < 0.032) was observed. In contrast, the correlations between TAA, TPC, TFC and condensed tannin content were less evident and imply the role of other compounds such as pigments, tocopherols, organic acids, alkaloids and iridoids in the observed TAA (Table S1†). These results support the earlier findings that condensed tannins were not implied in the antioxidant activity of faba beans.[8]

The lowest anti-radical and reducing activities observed for the ethyl acetate extracts may be associated with their high tannin content as previously described in tannin-rich extracts.[57,58] These findings were recently confirmed in broad bean seeds where tannin-rich extracts have revealed the lowest reducing activity using the FRAP assay.[22] To the best of our knowledge, this is the first report describing the anti-radical activity using ABTS radicals and TAA.

The hydrogen-donating ability of polar solvents like hydro-alcoholic solvent (*i.e.* 70% ethanol) has already been reported in BBPs where a strong antiradical activity had been observed.[51] Consistent with our results, the highest reducing power using the FRAP assay and the strongest DPPH-radical scavenging activity were found in TPC- and TFC-rich extracts of BBPs.[51] Recently, it has been found that extracts from broad bean seeds having the highest TPC and TFC exhibit the highest TAA, the strongest DPPH-radical scavenging activity and the most effective reducing power.[22] Collectively, these results confirm that BBPs could serve as a potential source of natural antioxidants.

In addition to their radical scavenging activities and reducing power, the four extracts were screened for their potential to inhibit protein denaturation as an indication for a possible *in vitro* anti-inflammatory activity.

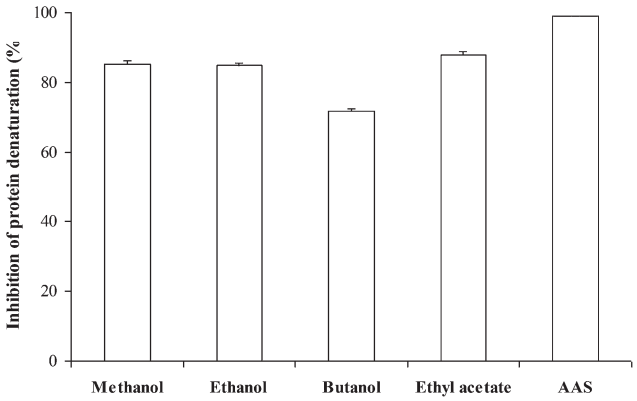

Fig. 1 Percentage inhibition of protein (BSA) denaturation by different solvent extracts of BBPs.

The data from Fig. 1 show that all extracts at a concentration of 1 mg mL^{-1} were able to inhibit heat-induced BSA denaturation. With more than 87% inhibition, the ethyl acetate extract was found to be the most efficient, while the butanol extract exhibited the lowest inhibitory effect (71.66%). At the same concentration, the polar methanol and ethanol extracts showed similar efficacies (85.35 and 84.75% for methanol and ethanol, respectively).

The inhibitory effect of the BBP extract on heat-induced albumin denaturation has not been reported previously, but the anti-inflammatory effect using other *in vitro* assays has been described in various legume species.[59] For example, it has been found that the aqueous acetone extract (70%) of common bean (*Phaseolus vulgaris* L.) hulls showed a strong inhibitory effect on both cyclooxygenases Cox-1 and Cox-2 and lipoxygenase type 15-Lox.[60] The authors of the study also indicated that the anti-inflammatory activity is deeply influenced by the phenolic content, cultivars and extracting solvent.[60] These results were later contradicted by Boudjou *et al.*[61] and Šibul *et al.*[59] who observed mild and weak anti-inflammatory effects of different legume extracts in the cyclooxygenase and lipoxygenase assays.

From a mechanistic standpoint, the inhibitory effects on pro-inflammatory mediators (inhibition of the arachidonic acid metabolizing enzymes, cyclooxygenase and lipoxygenase) and the scavenging radical potency of phenolic compounds such as flavonoids and condensed tannins could explain the *in vitro* anti-inflammatory activity of BBP extracts.[62] Support for these suggestions has been recently provided by Šibul *et al.*[59] who in their *in vitro* and *ex vivo* experiments reported that legume seed membrane extracts not only dose-dependently inhibit the biosynthesis of eicosanoids, thromboxanes and prostaglandins of the cyclooxygenase and lipoxygenase parts, but also hampered the cytochrome P450 and epoxygenase pathways.

The results of the *in vitro* anti-AChE activity are depicted in Fig. 2. As shown, all extracts at the dose assessed

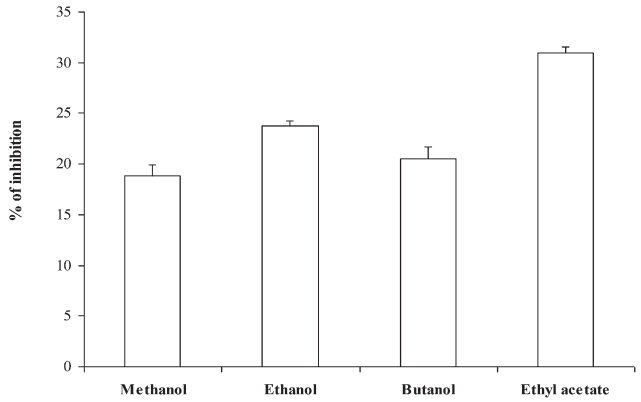

Fig. 2 Anti-AChE activity (expressed as % inhibition of AChE) of different solvent extracts of BBPs.

(100 μg mL^{-1}) exhibited anti-AChE activity (18.8–30.94%) with varying degrees. Once again, the ethyl acetate extract displayed the strongest inhibitory activity, whereas the methanol extract exhibited the least AChE activity.

The *in vitro* anti-AChE activity of BBPs has not been assessed so far, but the efficacy of some Fabaceae species has been described in a few studies. The pioneering study of Orhan *et al.*[63] showed that a chloroform : water extract (1 mg mL^{-1}) of the whole broad bean fresh plant showed similar acetylcholinesterase inhibitory potential as that of the standard galantamine (45.23 and 48.8% for broad beans and galantamine, respectively). Seven years later, Adewusi *et al.*[64] screened the anti-AChE activity of some selected South African medicinal plants and found that water extracts from the roots and barks of *Schotia brachypetala* exhibited strong AChE inhibitory activity (IC_{50} of 3.4 and 0.49 mg mL^{-1} for organic and water extracts for roots and barks, respectively). More recently, a very low anti-AChE activity (6% inhibition at a dose of 100 μg mL^{-1}) has been reported for fenugreek.[65] These results were later confirmed by Kaufmann *et al.*[66] who reported the inefficacy of the polar extracts (water and methanol) of 7 Fabaceae species as AChE inhibitors. In general, BBPs could serve as a valuable natural source for the isolation of AChE inhibitors useful for the treatment of Alzheimer's disease, senile dementia, ataxia and Parkinson's disease.

The compiled data on the *in vitro* experiments suggest that BBPs are endowed with remarkable antioxidant, anti-inflammatory, anti-AChE and moderate antimicrobial activities supporting our previous suppositions about their possible use as a good source of bioactive ingredients with potential functional and health-promoting properties. In view of the potential biological activity of the methanol extract (highest yield and strongest bioactivity) and considering that the antioxidant properties associated with the anti-inflammatory activity could be beneficial for numerous oxidant- and/or inflammatory-related disorders, the anti-diabetic, hepato-protective, reno-protective and repro-protective activities of the methanol extract from BBPs were evaluated *in vivo*.

3.3. *In vivo* bioactivity of the BBP extract

3.3.1. Anti-diabetic, hepato-protective and reno-protective activities in alloxan-induced diabetic mice. The administration of a single intra-peritoneal injection of 160 mg per kg bw of alloxan monohydrate induced hyperglycemia and severe diabetes after 5 days. Serious liver and renal dysfunction as revealed by the remarkable increase of the main serum biochemical parameters including ALA, AST, alkaline phosphatase, urea, uric acid, and creatinine was observed in alloxan-induced diabetic mice. These manifestations were further exacerbated by the reduction of albumin in diabetic mice suggesting an increased protein glycosylation and activity of xanthine oxidase as well as an enhanced release of purine which represents the main source of uric acid.[67] Additionally, a significant ($p < 0.05$) increment of lipid parameters (except for HDL) such as total cholesterol, triglycerides, and LDL was also observed. In contrast, the content of albumin was significantly lower in diabetic mice as compared to the normal control mice (Table 7). These observations have been reported as typical symptoms and the main biochemical hallmarks of alloxan- or streptozotocin-induced diabetes in different animal

Table 7 Serum biochemical parameters in different mice groups

Parameters	ND	D	ND + BBPE	D + BBPE
Glucose (mM)	$6.65^{c} \pm 0.66$	$15.29^{a} \pm 1.1$	$6.17^{c} \pm 0.6$	$8.81^{b} \pm 0.71$
ALT (UI L^{-1})	$23^{c} \pm 1.59$	$63^{a} \pm 1.38$	$21^{c} \pm 1.75$	$32^{b} \pm 1.37$
AST (UI L^{-1})	$39^{c} \pm 2.82$	$68^{a} \pm 2.13$	$37^{c} \pm 3.63$	$43^{b} \pm 2.51$
Alkaline phosphatase (UI L^{-1})	$129^{c} \pm 7.1$	$179^{a} \pm 6.5$	$148^{b} \pm 6.8$	$155^{b} \pm 9.6$
LDH (UI L^{-1})	$938^{c} \pm 16$	$1246^{a} \pm 24$	$916^{c} \pm 13$	$1095^{b} \pm 38$
Albumin (g dl^{-1})	$4.72^{b} \pm 0.93$	$3.15^{c} \pm 0.96$	$5.34^{a} \pm 0.30$	$4.13^{b} \pm 0.35$
Urea (mmol L^{-1})	$7.64^{b} \pm 0.38$	$10.8^{a} \pm 0.40$	$7.14^{b} \pm 0.35$	$7.21^{b} \pm 0.89$
Uric acid (mmol L^{-1})	$0.28^{a} \pm 0.01$	$0.13^{b} \pm 0.02$	$0.28^{a} \pm 0.01$	$0.29^{a} \pm 0.01$
Creatinine (μmol L^{-1})	$114^{b} \pm 6.7$	$163^{a} \pm 5.9$	$126^{b} \pm 6.3$	$119^{b} \pm 8.4$
Total cholesterol (mg ml^{-1})	$0.72^{b} \pm 0.02$	$0.87^{a} \pm 0.04$	$0.68^{c} \pm 0.03$	$0.74^{b} \pm 0.04$
Triglyceride (mg ml^{-1})	$0.66^{c} \pm 0.02$	$1.28^{a} \pm 0.07$	$0.68^{c} \pm 0.16$	$0.86^{b} \pm 0.12$
HDL (mg ml^{-1})	$0.19^{a} \pm 0.02$	$0.13^{c} \pm 0.02$	$0.14^{b} \pm 0.01$	$0.16^{b} \pm 0.02$
LDL (mg ml^{-1})	$0.21^{c} \pm 0.03$	$0.34^{a} \pm 0.02$	$0.24^{bc} \pm 0.02$	$0.27^{b} \pm 0.03$

ND: non-diabetic control; D: diabetic; BBPE: broad bean pod extract. Different superscripts within the lines are significantly different at $p < 0.05\%$.

models.[68,69] The elevation of serum glucose in response to these diabetogenic agents was mainly due to the selective deterioration of pancreatic β-cells of the islets of Langerhans, and the overproduction of hepatic glucose reflecting hormonal disorders and metabolic alterations.

The administration of the BBP extract tended to bring all the aforementioned parameters towards normal, suggesting its anti-diabetic potential and its possible protective effect against alloxan-induced injury in the liver and kidneys. The restoration of ALA, AST, alkaline phosphatase, urea, uric acid, and creatinine could be mediated through the reduction of free fatty acids and their peroxide and the inhibition of oxidation, phosphorylation and inflammation.[70] Additionally, the alteration of the lipid profile as exemplified by the increased levels of total cholesterol, triglycerides, and LDL was primarily attributed to the enhanced activity of lipases with a concomitant cholesterol biosynthesis through the activation of their corresponding enzyme 3-hydroxy-3-methyl-methylglutaryl-coenzyme-A due to the lack of insulin.[71,72] The significant reduction of the aforementioned parameters in response to the oral administration of the BBP extract indicates its lipid-lowering effect on diabetic animals. Its inhibitory action against lipid metabolic enzymes and/or its potential capacity in preventing lipid peroxidation may be the main reason for the lipid-lowering property of the BBP extract.

Considering that the diabetogenic effect of alloxan is mediated through oxidative stress induced by the excessive production of reactive oxygen species (ROS), it is important to evaluate the response of the antioxidative system *via* the activity of the main antioxidant enzymes in different organs including the liver and kidney due to their pivotal role in controlling optimal glucose homeostasis.

The data presented in Table 8 show that alloxan-induced diabetic mice exhibited a high MDA and H_2O_2 content in both liver and kidneys suggesting an alloxan-induced oxidative stress in these organs. The generation of H_2O_2 in response to elevated blood glucose and the autoxidation of alloxan derived dialuric acid are well-recognized facts associated with diabetes thereby confirming our findings.[71]

The oxidative stress was further exacerbated by the decline of the sulfhydryl (SH) groups (the SH groups are essential components in maintaining the intracellular and membrane redox state of the secretory capacity of endocrine tissue) in response to alloxan injection. In contrast, the treatment of the alloxan-induced diabetic mice with the methanol extract of BBPs attenuates the extent of lipid peroxidation (evaluated in terms of MDA), restores the redox status (evaluated in terms of the SH groups) and alleviates the generation of free radicals as revealed by the reduced H_2O_2 content. Similar results have been reported for other plant extracts with anti-diabetic properties.[71,73] In connection with the increased level of oxidative stress markers, the activity of the main antioxidant enzymes was determined. The results indicate that alloxan treatment significantly reduced the GPx, CAT, and SOD activities in both liver and kidneys. The glucose-induced depletion of antioxidant enzymes through their glycation is reported as the main mechanism responsible for the inactivation of antioxidant enzymes.[69]

Table 8 Changes in oxidative stress markers (MDA and H_2O_2), sulfhydryl groups and antioxidant enzyme activity in the liver and kidney of different mice groups

	ND	D	ND + BBPE	D + BBPE
MDA (nmol per min per mg protein)				
Liver	$5.63^{b} \pm 0.8$	$7.36^{a} \pm 0.83$	$4.64^{c} \pm 0.38$	$6.06^{b} \pm 0.63$
Kidney	$5.68^{c} \pm 0.47$	$7.79^{a} \pm 0.36$	$5.44^{c} \pm 0.48$	$6.34^{b} \pm 0.56$
H_2O_2 (nmol per min per mg protein)				
Liver	$0.79^{c} \pm 0.05$	$1.63^{a} \pm 0.1$	$0.72^{c} \pm 0.04$	$0.94^{b} \pm 0.06$
Kidney	$0.69^{c} \pm 0.05$	$1.24^{a} \pm 0.09$	$0.65^{c} \pm 0.04$	$0.91^{b} \pm 0.08$
SH (μmol)				
Liver	$1.34^{a} \pm 0.06$	$0.79^{d} \pm 0.05$	$1.17^{b} \pm 0.06$	$0.83^{c} \pm 0.04$
Kidney	$0.68^{a} \pm 0.04$	$0.43^{c} \pm 0.02$	$0.68^{a} \pm 0.03$	$0.61^{b} \pm 0.04$
GPx (nmol GSH per min per mg protein)				
Liver	$9.6^{a} \pm 0.6$	$5.23^{c} \pm 0.7$	$9.32^{a} \pm 0.9$	$6.41^{b} \pm 0.83$
Kidney	$8.3^{a} \pm 0.66$	$4.63^{d} \pm 0.38$	$7.29^{b} \pm 0.4$	$6.38^{c} \pm 0.56$
CAT (nmol per min per mg protein)				
Liver	$668^{a} \pm 28.9$	$479^{c} \pm 22.6$	$651^{a} \pm 34.7$	$554^{b} \pm 19.6$
Kidney	$437^{a} \pm 17.2$	$265^{c} \pm 14.6$	$451^{a} \pm 19.8$	$353^{b} \pm 17.3$
SOD (U per mg protein)				
Liver	$9.43^{a} \pm 0.56$	$5.37^{c} \pm 0.29$	$9.91^{a} \pm 0.76$	$7.09^{b} \pm 0.56$
Kidney	$5.96^{a} \pm 0.47$	$3.48^{b} \pm 0.28$	$5.91^{a} \pm 0.38$	$4.89^{b} \pm 0.51$
Cu/Zn-SOD				
Liver	$5.57^{a} \pm 0.39$	$2.58^{b} \pm 0.23$	$5.71^{a} \pm 0.49$	$4.92^{b} \pm 0.43$
Kidney	$3.36^{a} \pm 0.36$	$2.23^{c} \pm 0.14$	$2.83^{b} \pm 0.45$	$2.81^{b} \pm 0.13$
Mn-SOD				
Liver	$2.71^{a} \pm 0.34$	$1.46^{c} \pm 0.11$	$2.64^{a} \pm 0.33$	$1.84^{b} \pm 0.11$
Kidney	$1.29^{a} \pm 0.16$	$0.96^{b} \pm 0.05$	$1.34^{a} \pm 0.13$	$0.63^{c} \pm 0.06$
Fe-SOD				
Liver	$1.2^{c} \pm 0.13$	$1.16^{b} \pm 0.18$	$1.16^{b} \pm 0.16$	$1.19^{a} \pm 0.18$
Kidney	$1.14^{a} \pm 0.08$	$1.06^{b} \pm 0.08$	$1.26^{a} \pm 0.11$	$1.18^{a} \pm 0.13$

ND: non-diabetic control; D: diabetic; BBPE: broad bean pod extract. Different superscripts within the lines are significantly different at $p < 0.05\%$.

For the SOD enzymes, the magnitude of reduction was more pronounced for the Cu/Zn-SOD isozyme. This is consistent with the general observation that hyperglycemia impelled oxidative stress was mainly due to the lack of the Cu and Zn cofactors of the SOD enzyme.[74]

The observed alterations of the enzymatic antioxidant status were successfully restored confirming the potential antioxidant properties of the BBP extract and supporting its hepato- and reno-protective capacities. At this point, we can speculate that the BBP extract not only enhances the activity of GPx, CAT and SOD, but it could directly scavenge H_2O_2 and inhibit lipid peroxidation thereby preventing diabetic complications. Such antioxidant-mediated alleviation of oxidative stress in an alloxan-induced animal has been described for oleander (*Nerium oleander*) by Gayathri *et al.*[75] and confirmed later by Dey *et al.*[71] who demonstrated the potential of an oleander extract to inhibit the generation of free radicals and lipid peroxidation and the activation of antioxidant enzymes in alloxan-induced diabetic mice.

In general, the anti-diabetic and hepato- and reno-protective effects of BBPs appear to be mediated primarily through their antioxidant properties thereby supporting antioxidant therapy in diabetic complications.

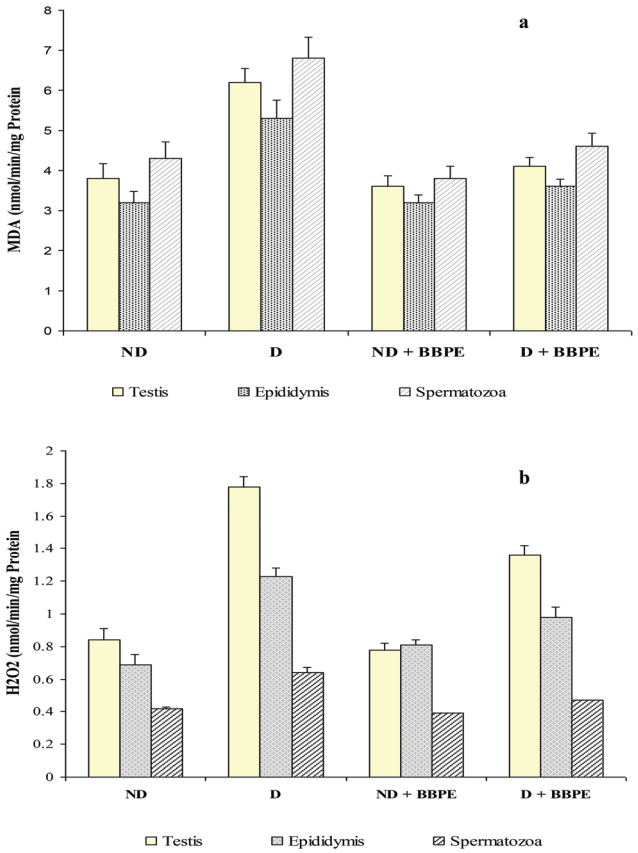

Fig. 3 Extent of lipid peroxidation determined as MDA (a) and H_2O_2 (b) content in the testis, epididymis and spermatozoa of different mice groups (ND: non-diabetic control; D: diabetic; BBPE: broad bean pod extract).

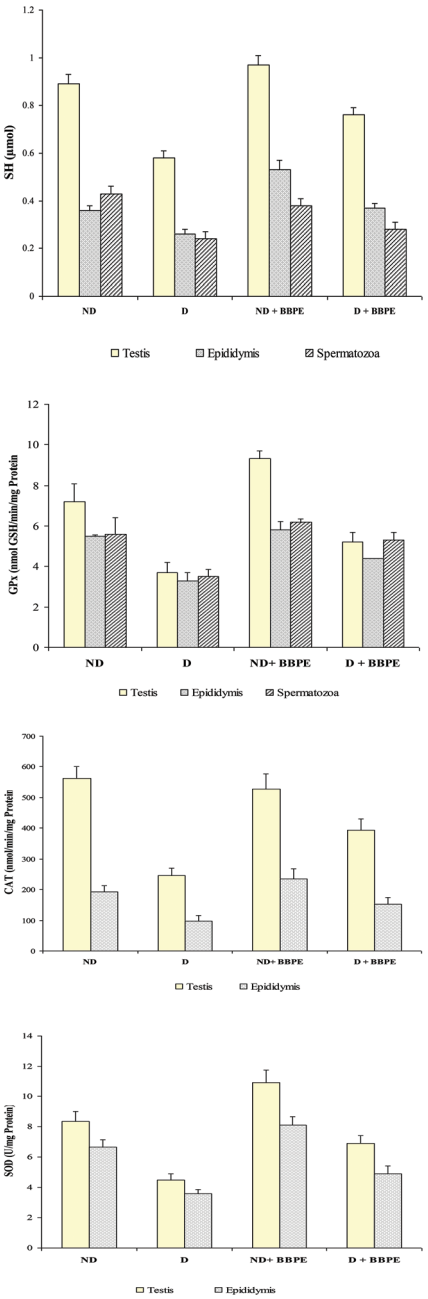

Fig. 4 Sulfhydryl content (SH) and GPx, CAT and total SOD activity in the reproductive tissues of different mice groups (ND: non-diabetic control; D: diabetic; BBPE: broad bean pod extract).

In addition to its deleterious effects on vital organs, the rise of ROS in alloxan-treated animals was usually associated with oxidant-induced injuries in the reproductive system.[74] Given its potential antioxidant activity, the methanol extract of BBPs was further evaluated for its protective effects against alloxan-induced diabetic impelled reproductive damage.

3.3.2. Protective effects of the BBP extract against testicular damage in alloxan-induced diabetic mice. The data depicted in Fig. 3 show that the testis, epididymis and spermatozoa of alloxan-treated mice experienced serious oxidative stress as evidenced by the enhanced levels of MDA and H_2O_2 in comparison with the non-diabetic control mice. Further significant perturbations in the antioxidant machinery including the sulfhydryl groups and antioxidant enzymes such as GPx, CAT and SOD were indicative of increased oxidative stress in the testis, epididymis and spermatozoa (Fig. 4 and 5). An immediate consequence of the increased oxidative stress was the reduction in sperm count, viability, motility and epididymal sperm count *versus* a significant increase in abnormal spermatozoa with marked head and tail anomalies (Table 9). The oxidative stress-induced perturbations in the reproductive system have been previously described in diabetic animals. In this context, it has been found that oxidative stress impelled a decrease in testicular ATP levels following mitochondrial damage. It also induces sperm membrane modification, DNA damages, loss of motility and disruption of spermatogenesis and testosterone production.[76]

The oral administration of BBP extracts to diabetic mice refurbished the altered levels of MDA and H_2O_2 and improved the antioxidant defence mechanism as revealed by the increased SH content and the activities of antioxidant enzymes. The levels of all parameters except spermatozoa morphology, MDA and H_2O_2 were maintained unchanged in non-

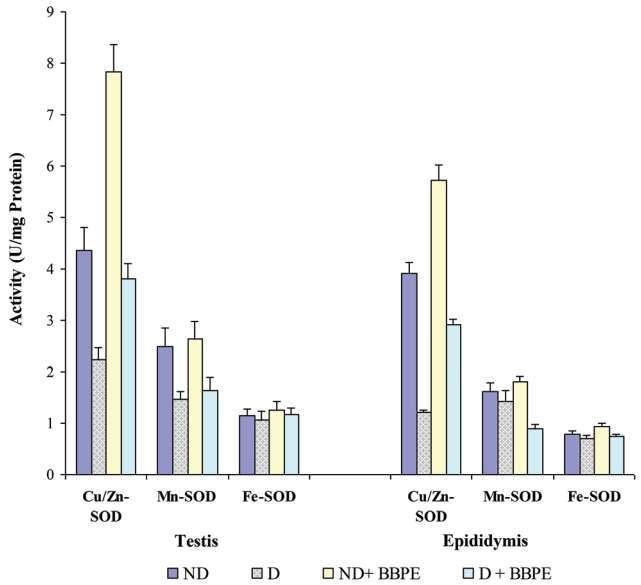

Fig. 5 Changes in SOD isoforms in the testis and epididymis of different mice groups (ND: non-diabetic control; D: diabetic; BBPE: broad bean pod extract).

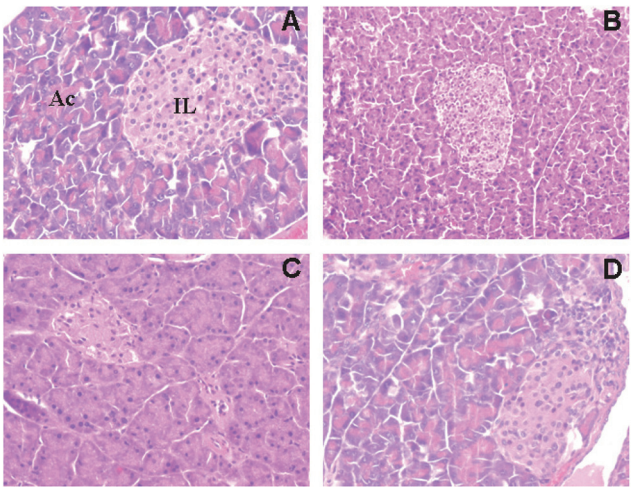

Fig. 6 Photomicrograph of a section of pancreas stained with H&E. (A) Non-diabetic control group; (B) non-diabetic group treated with BBP extracts; (C) untreated diabetic group; (D) diabetic mice treated with BBP extracts. IL: islets of Langerhans; Ac: acini.

diabetic mice receiving BBP extracts. From these results, it can be inferred that the protective effects of BBP extracts against alloxan-induced reproductive damage were mediated through their modulation of oxidative stress that offers a measure of protection against oxidative damage thereby improving the sperm quality and consequently the male reproductive function. The data on the repro-protective effects of BBPs in diabetic animals are lacking; however, substantial information about such an activity has been described for a lot of extracts with a well-documented antioxidant activity.[74] In this manner, the antioxidant therapy of the ethanolic leaf extract of *Senna fistula* has been confirmed in alloxan-induced diabetic rats.[77] The authors of the study showed that the oral administration of a *S. fistula* leaf extract to diabetic rats improved the testosterone level, increased the activity of testicular SOD and GPx and prevented the degenerative changes in the seminiferous tubules. One year later, Shah and Khan found that an ethyl acetate extract of *Sida cordata* was able to reverse the alloxan-induced reproductive damage in rats through its high ability to reduce the extent of lipid peroxidation, production of H_2O_2, restoration of lipids and serum glucose to their normal levels, recovery of testosterone levels and stimulation of the reduced glutathione.

In order to determine whether the aforementioned biochemical manifestations were associated with morphological changes, the histopathological examination of the pancreas, liver, kidneys and testes was performed.

3.4. Histological examination of tissues

The examination of the H&E stained section of the pancreas of the control mice showed a normal architecture of the islets of Langerhans (noncapsulated and faintly stained oval or rounded areas inside the pancreas) with β-cells occupying most of the islets. Acini were arranged in lobules with prominent nuclei (Fig. 6A). A similar histological architecture was also observed in non-diabetic mice that were given a methanol BBP extract (Fig. 6B). In alloxan-induced diabetic animals, pathological changes occurred, including atrophy of the islets of Langerhans as exemplified by their reduced size, with a concomitant decrease in β-cell density and appearance of necrotic areas and vacuolization (Fig. 6C). The oral administration of

Table 9 Changes in sperm parameters in different mice groups

	ND	D	ND + BBPE	D + BBPE
Sperm concentration (10^6 mL^{-1})	$7.36^{a} \pm 0.41$	$4.76^{c} \pm 0.47$	$7.89^{a} \pm 0.51$	$5.21^{b} \pm 0.38$
Motility (%)	$69^{a} \pm 7.3$	$40^{b} \pm 5.6$	$69^{a} \pm 4.8$	$67^{a} \pm 5.7$
Viability (%)	$79^{a} \pm 4.8$	$52^{c} \pm 3.6$	$81^{a} \pm 5.4$	$72^{b} \pm 6.3$
Normal morphology (%)	$86.1^{a} \pm 4.52$	$46^{c} \pm 3$	$73^{b} \pm 4.56$	$79^{b} \pm 5.38$
Head abnormality (%)	$5.8^{c} \pm 0.96$	$14^{a} \pm 2.1$	$8.62^{b} \pm 1.12$	$6.49^{c} \pm 1.71$
Tail abnormality	$8.1^{d} \pm 1.41$	$39^{a} \pm 4.2$	$22.6^{b} \pm 2.3$	$15.8^{c} \pm 1.9$
Sperm count (10^6 per g epididymis)	$132^{a} \pm 16.4$	$96^{c} \pm 1.98$	$138^{a} \pm 1.96$	$118^{b} \pm 3.49$

ND: non-diabetic control; D: diabetic; BBPE: broad bean pod extract. Different superscripts within the lines are significantly different at $p < 0.05\%$.

BBP extracts in alloxan-induced diabetic mice resulted in a noticeable recovery of the islets of the acinar cell structure (Fig. 6D). These results showed that the anti-diabetic effect of BBPs was attributed, at least in part, to its promotion of the growth and proliferation of β-cells, which, in turn, increased insulin production, rather than to a direct effect on their functions. In view of the biochemical parameters, it will be reasonable to postulate that the antioxidant and anti-inflammatory capacities of the BBP extract besides its ability to stimulate the pancreatic antioxidant defence were responsible for the repair and regeneration of pancreatic β-cells. The regeneration of pancreatic β-cells mediated through a natural antioxidant has been regarded as a promising alternative for the treatment of diabetes. In this context, the earlier studies pinpointed the efficacies of some plant extracts and isolated antioxidant compounds in treating diabetes-induced oxidative damage in the pancreas.[78,79] The former authors showed that the administration of the natural antioxidant oleuropein to alloxan-induced diabetic rats efficiently regenerated the pancreatic β-cells, inhibited the generation of free radicals and enhanced the enzymatic and non-enzymatic antioxidants.[78] Similar results were also observed by the latter authors who proved the efficacy of a methanol extract from *Ficus deltoidea* and vitexin in regenerating the islets of Langerhans, improving insulin secretion and improving pancreatic antioxidant enzyme activities.[79]

Being a key player in the regulation of glucose metabolism, liver morphology was also assessed. Microscopic observation showed normal-appearing hepatocytes, central veins, sinusoids and Kupffer cells in the control non-diabetic mice and non-diabetic mice receiving BBP extracts (Fig. 7A and B). In contrast, diffused hepatocytes and loss of their usual concentric arrangement, increased vacuolization and sinusoid widening

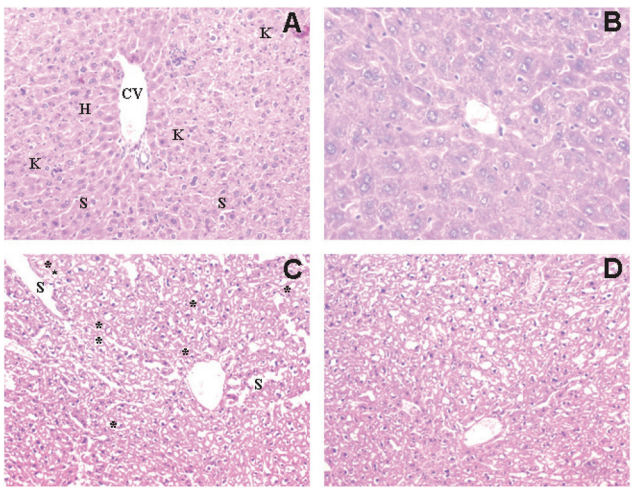

Fig. 7 Photomicrograph of a section of the liver stained with H&E. (A) Non-diabetic control group; (B) non-diabetic group treated with BBP extracts; (C) untreated diabetic group; (D) diabetic mice treated with BBP extracts. CV: central vein; H: hepatocytes; K: Kupffer cells; S: sinusoid. Vacuolisation is indicated by asterisk (*).

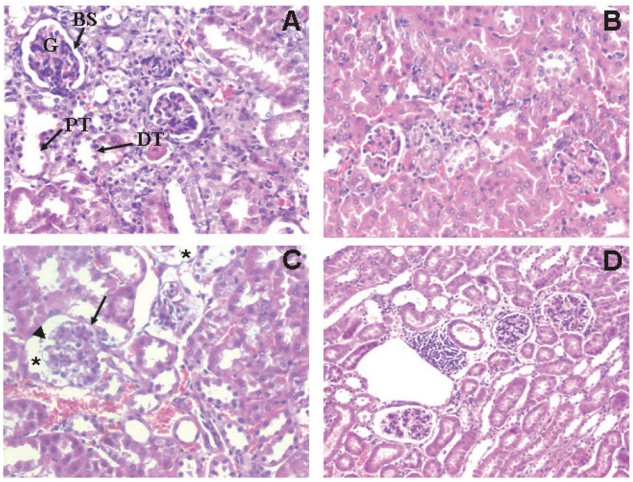

Fig. 8 Photomicrograph of a section of the kidney stained with H&E. (A) Non-diabetic control group; (B) non-diabetic group treated with BBP extracts; (C) untreated diabetic group; (D) diabetic mice treated with BBP extracts. BS: Bowman's space; G: glomerulus; PT: proximal convoluted tubules; DT: distal convoluted tubules. Widening of Bowman's space is indicated by asterisk (*); the arrow indicates the thickening of membrane basement; the arrow head indicates vacuolisation of the mesangial cells.

were found to be the main hallmarks of the liver of alloxan-induced diabetic mice (Fig. 7C) suggesting the impairment of the function of liver tissues. These histo-pathological changes were markedly alleviated after the oral administration of the BBP methanol extract (Fig. 7D). The ability of a natural antioxidant to reverse the effects of diabetic-mediated oxidative stress in the liver is well recognized.[80] These authors proved that pycnogenol, an extract from French maritime pine (*Pinus maritima*) advocated for its antioxidant and anti-inflammatory effects, was able to inhibit lipid peroxidation and protein carbonylation, enhance the content of glutathione and CAT activity and suppress the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β in the liver of alloxan-induced diabetic rats. Such a mechanistic approach could be responsible for the hepato-protective effects of BBPs observed in the present study; however, additional in-depth experiments targeting inflammatory markers must be performed.

Parallel to the changes in the liver, renal histopathological changes were examined. As shown in Fig. 8A and B, a normal renal architectural pattern with a normal histological structure (glomerulus, proximal and distal convoluted tubules, Bowman's capsule and membrane basement) was observed in the control and BBP-treated non-diabetic mice. In alloxan-induced diabetic mice, remarkable alterations in these components were evident. They include glomerular atrophy, widening of Bowman's space, vacuolization of mesangial cells, thickening of membrane basement and congestion (Fig. 8C). These degenerative changes were minimized after the administration of the BBP extract (Fig. 8D) thereby confirming its reno-protective properties. Such activity could presumably be attrib-

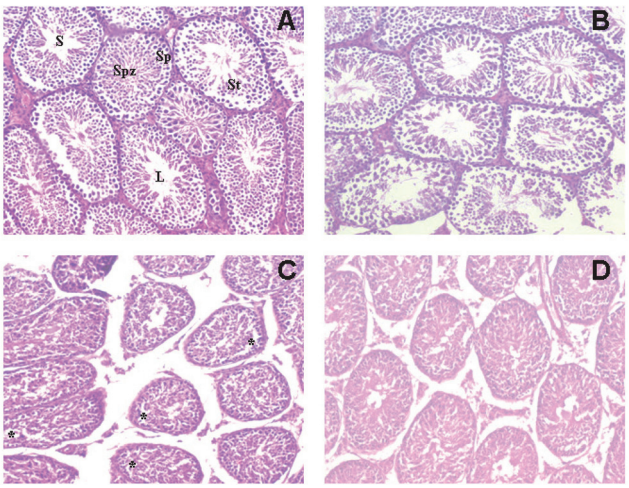

Fig. 9 Photomicrograph of a section of the testis stained with H&E. (A) Non-diabetic control group; (B) non-diabetic group treated with BBP extracts; (C) untreated diabetic group; (D) diabetic mice treated with BBP extracts. L: lumen; S: seminiferous tubule; Sp: spermatocytes; St: spermatozoa; St: spermatid. Epithelium vacuolisation is indicated by asterisk (*).

uted to its anti-oxidant and/or anti-inflammatory activities as portrayed in earlier studies.[81,82]

It is well established that male reproductive dysfunction is one of the main diabetes complications and substantial data pinpointed that diabetogenic agents like alloxan and streptozotocin were usually associated with the structural and functional disturbance in the male reproductive system.[83] With regard to this issue, histopathological examination of the testis was performed. In comparison with the control and BBP-treated non-diabetic mice (Fig. 9A and B) that exhibited regular seminiferous tubules with complete spermatogenic cell series and intact interstitial connective tissues, alloxan treatment resulted in severe pathological alterations including shrinkage and disorganisation of seminiferous tubules, reduced tubule diameter, impaired organisation of the spermatogenesis stages, disrupted basement membrane, prominent epithelium vacuolisation, the presence of cellular debris and reduced sperm count in the tubular lumen (Fig. 9C). The structural disruption of seminiferous tubules and the impairment of spermatogenesis are considered as major indicators of diabetic-induced testicular damage and were primarily attributed to the reduction of the testosterone content[84] and increased oxidative stress in testis under hyperglycemic circumstances.[85] These pathological alterations were remarkably alleviated in BBP-treated diabetic mice with better seminiferous tubule structure and organisation, less disrupted basement membrane and improved spermatogenesis (Fig. 9D). These results provide supporting evidence of the protective effects of BBPs against alloxan-induced male reproductive injuries presumably through their antioxidant properties. The amelioration of diabetes-related testicular damage using a natural antioxidant has been widely described.[84] These authors portrayed a renewed activity of the antioxidant enzymes and down-regulation of ROS in the testis in response to the antioxidant quercetin. In another report, the use of an ethyl acetate extract from *Sida cordata* with endowed antioxidant activity was found to be efficient in repairing testicular damage induced by alloxan treatment in rats.[74]

In view of our results, it can be inferred that the BBP extract could effectively prevent oxidative stress-induced reproductive damage through its antioxidant properties and ability to boost the activity of SOD, CAT and GPx as well as its lowering potential of MDA and H_2O_2. The probable *in vivo* anti-inflammatory properties in addition to their antioxidant activity could justify the anti-diabetic and hepato-, reno- and repro-protective effects against alloxan-induced oxidative stress in mice.

Being endowed with such a plethora of health-promoting properties, it is of particular interest to elucidate the chemical composition of the methanol extract of BBPs. Such analysis will not only extend our knowledge on the phenolic profiles of these residual by-products, but will also be important for shedding light on the main phytoconstituents that could be involved in these properties.

3.5. Profiling of phenolic compounds by HPLC-PDA-ESI-MS/MS

The fingerprinting of the methanol extracts of BBPs led to the identification of 21 phenolic compounds which were tentatively identified based on their retention time (t_R), UV-Vis spectra, deprotonated molecules, and MS^2 fragmentation behaviour and by comparison with the spectral data of authentic phenolic standards when available or with the literature data (Table 10).

Peak 1 (t_R, 2.87 min; λ_{max}, 279 nm) exhibited a deprotonated molecular ion $[M - H]^-$ at *m*/*z* 203 and was tentatively identified as tryptophan and has already been found in Polish broad bean seeds.[22] Peaks 2 and 5, eluted at 2.97 and 3.47 min, respectively, presented a deprotonated molecule $[M - H]^-$ at *m*/*z* 341 and a fragment ion at *m*/*z* 179 ($[M - H - 162]^-$; loss of a hexose moiety) indicative of caffeic acid aglycone. Both components were tentatively identified as caffeic acid hexoside I and II.[86] Peak 3 (t_R, 3.14 min; λ_{max}, 276 nm) displayed a pseudomolecular ion $[M - H]^-$ at *m*/*z* 577 with an intense MS^2 fragment ion at *m*/*z* 289 $[M - H - 288]^-$. This fragmentation pattern was consistent with the previous description for a procyanidin dimer.[20] The presence of a procyanidin dimer confirmed the results on Spanish BBPs.[23] Peaks 4 (eluted at 3.36 min) and 6 (eluted at 3.73 min) exhibited maximum absorbance at 277 nm and presented a deprotonated molecular ion $[M - H]^-$ at *m*/*z* 289 which yielded a fragment ion at *m*/*z* 137. This fragmentation pattern was similar to that observed for catechin and (*epi*)catechin. Peak 7 (t_R, 5.63 min; λ_{max}, 281 nm) presented a pseudomolecular ion $[M - H]^-$ at *m*/*z* 305 and an intense fragment at *m*/*z* 179 due to the loss of 126 amu and was tentatively identified as (*epi*)gallocatechin.[87] Peak 8 (t_R, 6.91 min; λ_{max}, 268 nm) displayed a deprotonated molecular ion $[M - H]^-$ at *m*/*z* 783 and its MS^2 fragmentation gave an intense fragment ion at *m*/*z* 481 $[M - H - 302]^-$ (loss of a HHPD moiety) and a fragment ion at *m*/*z*

Table 10 Retention time, maximum UV absorption (λ_{max}), mass spectral data and tentative identification of the phenolic compounds in the methanol extract of BBPs

Peak	t_R (min)	λ_{max} (nm)	Molecular ion $[M-H]^{-1}$	MS^2	Tentative identification
1	2.87	279	203		Tryptophan
2	2.97	272; 326	341	179	Caffeic acid hexoside I
3	3.147	276	577	289	Procyanidin dimer
4	3.36	277	289		Catechin
5	3.47	272; 326	341	179	Caffeic acid hexoside II
6	3.73	277	289		(*Epi*)catechin
7	5.63	281	305	179	(*Epi*)gallocatechin
8	6.913	268	783	481; 301	Bis-HHDP-glucose
9	8.08	338	593	269	Apigenin-*O*-dihexoside
10	9.74	270	433	271	Pelargonidin-3-*O*-glucoside
11	11.22	254; 354	609	447; 301	Quercetin-hexose-deoxyhexose
12	12.064	264, 346	609	285	Kaempferol dihexoside
13	12.74	327	523	361	Verminoside
14	14.02	264; 347	593	431; 299	Diosmetin-pentoside-hexoside
15	14.09	264; 349	593	285	Kaempferol-3-*O*-rutinoside
16	16.109	269; 347	639	477; 315	Isorhamnetin diglucoside
17	17.37	255; 353	447	301	Quercetin-rhamnoside
18	18.84	313	279	163	*p*-Coumaroyl-malic acid
19	19.75	309	299	137	Salicylic acid hexoside
20	20.42	268	449	303	Delphinidin rhamnoside
21	21.21	264; 349	447	285	Kaempferol-3-*O*-glucoside

301 (loss of HHPD-glucose), characteristic of ellagitannins. This component was tentatively identified as bis-HHPD-glucose.[88] Flavan-3-ols or condensed tannins including procyanidins, catechin, (*epi*)catechin and their derivatives have been considered as typical components in the Fabaceae family and are considered as anti-nutritional components due to their ability to precipitate proteins and reduce the bioavailability of some minerals.[89] These are detected in different species such as pea, lentils,[89] broad beans,[22,23] and African locust beans.[87]

Peak 9 (t_R, 8.06 min; λ_{max}, 338 nm) exhibited a deprotonated molecule $[M-H]^-$ at *m*/*z* 593 and gave intense MS^2 fragment ions at *m*/*z* 431 $[M-H-162]^-$ and 269 ($[M-H-162-162]^-$; apigenine aglycone) corresponding to a successive loss of hexoside residues. This glycosylated flavone was tentatively identified as apigenin-*O*-dihexoside,[20] and has already been detected in BBPs.[23] Peak 10 eluted at 9.74 min showed a characteristic UV spectra of anthocyanins with maximal absorbance at 270 nm and displayed a deprotonated molecule $[M-H]^-$ at *m*/*z* 433 releasing a fragment ion at *m*/*z* 271 ($[M-H-162]^-$, a loss of a hexose moiety), characteristic of the aglycone pelargonidin. As previously described in BBPs, this anthocyanin was tentatively identified as pelargonidin-3-*O*-glucoside.[23] Peak 11 (t_R, 11.22 min; λ_{max}, 254–354 nm) showed a pseudomolecular ion $[M-H]^-$ at *m*/*z* 609 releasing two fragments at *m*/*z* 447 and *m*/*z* 301 corresponding to the consecutive loss of hexose ($[M-H-162]^-$) and deoxyhexose ($[M-H-162-146]^-$). This flavonol, previously detected in BBPs, was tentatively identified as quercetin-*O*-hexosyl-*O*-deoxyhexose.[23] Peak 12 (t_R, 12.06 min; λ_{max}, 264–346 nm) presented a deprotonated molecule $[M-H]^-$ at *m*/*z* 609 that released an intense fragment at *m*/*z* 285 ($[M-H-162-162]^-$; loss of two hexosyl moieties). This component was tentatively identified as kaempferol-di-hexoside.[23] Peak 13 (t_R, 12.74 min; λ_{max}, 327 nm) showed a pseudomolecular ion $[M-H]^-$ at *m*/*z* 523 and displayed a fragment ion at *m*/*z* 361 ($[M-H-162]^-$; attributed to the loss of a hexose residue) and other fragment ions at *m*/*z* 179 and 135 characteristic of caffeic acid derivatives. This fragmentation pattern is consistent with that observed for verminoside, an iridoid catalpol linked to caffeic acid.[90] Peak 14 (t_R, 14.02 min; λ_{max}, 264–347 nm) had a pseudomolecular ion $[M-H]^-$ at *m*/*z* 593 which gave an MS^2 fragment at *m*/*z* 431 ($[M-H-162]^-$) through the loss of a hexosyl residue and a second fragment at *m*/*z* 299 ($[M-H-162-132]^-$ by the loss of a pentoside moiety) indicative of the diosmetin aglycone. This glycosylated flavone was tentatively identified as diosmetin-pentosyl-hexoside.[91] Although the diosmetin aglycone has been previously described in BBPs,[23] its presence in the glycosylated form was reported herein for the first time.

Peak 15 (t_R, 14.1 min; λ_{max}, 264–349 nm) exhibited a deprotonated molecule $[M-H]^-$ at *m*/*z* 593 and its MS^2 spectrum showed an intense fragment ion at *m*/*z* 285 (indicative of the aglycone kaempferol) by the loss of a rutinoside moiety (308 amu). Thus, this compound was tentatively identified as kaempferol-3-*O*-rutinoside.[92] Peak 16 had a pseudomolecular ion $[M-H]^-$ at *m*/*z* 639 and produced the MS^2 base peaks at *m*/*z* 477 and 315 (isorhamnetin aglycone) which correspond to the successive loss of two hexoside moieties ($[M-H-162-162]^-$). This glycosylated flavonol was tentatively identified as isorhamnetin diglucoside.[93] Peak 17 (t_R, 17.73 min; λ_{max}, 254–353 nm) presented a pseudomolecular ion $[M-H]^-$ at *m*/*z* 447 and gave an intense fragment ion at *m*/*z* 301 corresponding to the loss of a rhamnosyl residue as observed with the authentic flavonol standard. This component was unambiguously identified as quercetin-rhamnoside and its presence in our samples confirms the previous findings.[23] Peak 18 (t_R, 18.84 min; λ_{max}, 313 nm) had a deprotonated molecular ion $[M-H]^-$ at *m*/*z* 279 that released a fragment ion at *m*/*z* 163 (indicative of *p*-coumaroyl acid) corresponding to the loss of a

malic acid molecule. This hydroxycinnamic acid was tentatively identified as *p*-coumaroyl malic acid,[94] and its presence in BBPs confirms the earlier study.[23] Peak 19 (t_R, 19.75 min; λ_{max}, 309 nm) exhibited a deprotonated molecule $[M - H]^-$ at *m*/*z* 299 and its MS^2 spectrum showed an intense peak at *m*/*z* 137 (corresponding to the salicylic acid aglycone) through the loss of a hexosyl moiety ($[M - H - 162]^-$). This compound was tentatively identified as salicylic acid hexoside.[95] Peak 20 (t_R, 20.42 min; λ_{max}, 268 nm) had a pseudomolecular ion $[M - H]^-$ at *m*/*z* 449 that released a fragment ion at *m*/*z* 303 (corresponding to delphinidin aglycone) through the loss of a rhamnosyl residue ($[M - H - 146]^-$) which allowed its identification as delphinidin rhamnoside.[23] Peak 21, eluted at 21.21 min corresponding to a kaempferol derivative (λ_{max}, 264–349 nm; MS^2 fragment at *m*/*z* 285), was tentatively identified as kaempferol-3-*O*-glucoside,[20] based on its fragmentation pattern that showed it had a pseudomolecular ion $[M - H]^-$ at *m*/*z* 447 that released a fragment at *m*/*z* 285 ($[M - H - 162]^-$; loss of a glucose moiety). The presence of a kaempferol derivative including kaempferol di-hexoside, kaempferol-hexoside-rhamnoside and kaempferol-rhamnosyl-galactoside-rhamnoside, among others has been described in BBPs.[23]

Some of the identified components have received much attention and are credited with numerous food-related biological activities. In this context, earlier studies have shown that tryptophan can effectively inhibit lipid peroxidation, detoxify ROS, quench free radicals, and chelate the prooxidative transition metals.[96]

Other studies have reported that kaempferol glycosides were endowed with remarkable anti-obesity and antidiabetic activities through their ability to reduce peroxisome proliferator-activated receptors (PPAR-γ) and sterol regulatory element-binding protein (SREBP-1c) expression.[94] The aglycone salicylic acid was portrayed as a potent anti-diabetic and anti-inflammatory agent.[97] Flavan-3-ols including catechin, epicatechin and their derivatives have been described as potent antioxidant, anti-obese and anti-diabetic components and their mechanisms of action have been deciphered.[98] They have the capacity to normalize the blood glucose level through the inhibition of α-amylase and α-glucosidase, promote β-cell regeneration, enhance insulin release and resistance, and protect pancreatic islets against hyperglycemia-induced oxidative damage.[99] The alleviation of oxidative stress, improvement of endothelial dysfunction through their AChE activity, modulation of the pro-inflammatory cytokines TNF-α, interleukins (IL-1β and IL-6) and interferon gamma (IFN-γ), regulation of dyslipidemia complication, regulation of the expression of genes involved in glycometabolism, lipid metabolism, protein glycation and insulin signalling pathways, and enhancement of immunity, among others are reported as the putative mechanisms underlying the antidiabetic activity of catechins, epicatechin and their derivatives.[100] Flavonols including isorhamnetin, quercetin, diosmetin and kaempferol and their glycosylated derivatives are well-recognized antioxidants and anti-inflammatory and anti-diabetic agents. Their anti-diabetic activity was mediated through the activation of insulin secretion, inhibition of β-cell apoptosis *via* the reduction of caspase-3 activity, amelioration of the enzymatic and non-enzymatic antioxidant status, inhibition of lipid peroxidation, improvement of renal function, suppression of hepatic lipogenesis and increase of free fatty acid uptake, and reduction of pro-inflammatory cytokines.[101]

The ellagitannins bis-HHPD-glucose and procyanidin dimer were recognized for their strong radical scavenging and potent antioxidant properties toward lipid peroxidation.[101,102] Their anti-inflammatory, enzyme inhibitory, anti-diabetic, anti-cancer and immuno-modulating activities were also described.[103] The regulation of glucose homeostasis and the regulation of lipid metabolism and increased viability of β-cells leading to increased insulin release were reported as the main mechanisms behind the anti-diabetic activity of anthocyanins including pelargonidin and delphinidin glycosides.[104] The flavone apigenin and its glycosylated derivatives have also been described as potent anti-diabetic nutraceuticals with a strong protective effect on pancreatic β-cells in streptozotocin-induced diabetes animal models.[105] Verminoside, a potent radical scavenger, had the potential to inhibit *in vitro* the expression of iNOS and the release of NO in the lipopolysaccharide (LPS)-induced J774.A1 macrophage cell line suggesting its anti-inflammatory activity.[106]

On the basis of these results, we can propose that one or more identified components in the BBP extract might be the major contributors for the anti-oxidant, anti-inflammatory, protein denaturing, anti-diabetic and cytoprotective effects.

4. Conclusion

The results of the present study conclusively indicate that BBPs, which are considered to be a waste material, could serve as an excellent source of nutraceuticals including carbohydrates, fibre, proteins, essential minerals and essential polyunsaturated fatty acids. Their extracts exhibited appreciable TPC, TFC and condensed tannin content showing multifaceted benefits as revealed by their potent radical scavenging, anti-inflammatory, protein denaturing and anti-AChE activity *versus* weak antimicrobial activity. Additional *in vivo* study proved the potential methanol extract to exert anti-diabetic effects on alloxan-induced diabetic mice. Such activity was reinforced with compelled hepato-, reno-, and repro-protective properties as revealed by biochemical analyses and histological examinations. The ability of the methanol extract to improve the enzymatic antioxidant machinery and restore the damaged parameters in alloxan-induced diabetic animal tissues was primarily attributed to the presence of bioactive phenolic compounds with well-recognized antioxidant activity. Therefore, BBPs could be considered as an attractive adjuvant for the management of diabetes and associated complications. From a practical standpoint, the results open up new areas for the rational use of BBP by-products and their valorisation in food, feed and pharmaceutical applications. Further studies for elucidating the putative phenolic compounds that could be involved in

the *in vivo* antidiabetic and histo-protective effects and deciphering their mechanism of action are recommended.

Conflicts of interest

There are no conflicts to declare.

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