SUPPLEMENTARY MATERIAL

A study on the GC-MS analysis of bioactive components and pancreato-protective effect of methanolic extract of *Brassica oleracea* L. var. *botrytis*

Sadanandan Rajapriya[a], Arumugam Geetha*[,a], and Kavasseri Ganesan Kripa[b]

[a]*Department of Biochemistry, Bharathi Women's College (Affiliated to University of Madras), Chennai 600 108, Tamil Nadu, India*

[b]*Department of Biochemistry, School of Life Sciences, Vels University, Pallavaram, Chennai 600 117, Tamil Nadu, India*

*Corresponding author. Email: geethav21@yahoo.co.in

The ever-increasing problem of pancreatitis due to alcohol abuse demands evaluation of novel drugs of plant origin. This study explores the therapeutic effects of the methanolic extract of *Brassica oleraceae* (MEBO) on ethanol and cerulein induced pancreatitis in rats. The MEBO was subjected to GC-MS and HPLC analysis. Male albino Wistar rats were divided into various groups, fed with alcohol (36% of total calories for 5 weeks) and cerulein (20 µg/kg b.wt i.p, weekly thrice for last three weeks) with or without MEBO (40 mg/kg b.wt). Serum lipase, amylase, IL-1β, IL-18, caspase-1, lipid peroxides, oxidative stress index and antioxidant status were assessed in pancreas. Six compounds were identified in GC-MS analysis. Co-administration of MEBO reduced the pancreatic marker enzymes in serum, IL-1β, IL-18 and caspase-1 and increased the antioxidant status of pancreas. The pancreato-protective effect of *Brassica oleraceae* may be attributed to well-known anti-inflammatory flavonoids, luteolin, quercetin and myricetin.

Keywords: *Brassica oleraceae*; GC-MS; ethanol; pancreatitis; lipase-amylase; interleukins; antioxidants

Experimental

Chemicals

Cerulein and lipase kit were purchased from Biovision. Amylase kit was purchased from Sigma Aldrich, India. Nitrobluetetrozolium (NBT), phenazinemethosulfate (PMS),GSH,Tris-hydrochloric acid,reduced nicotineamide adenine dinucleotide (NADH), and 1,1´,3,3´-tetramethoxy propane (malondialdehyde)were obtained from Sisco Research Laboratories, Mumbai, India.All other chemicalsand solvents used for the analysis were of analytical grade.

Plant collection and identification

Brassica oleraceae (*BO*), bought from the local market in Chennai and authenticated based on the organoleptic or macroscopic examination by Dr. P. Jayaraman, Taxonomist, Plant Anatomy Research Centre, Chennai and alloted the voucher no. PARC/2015/3211.

Preparation of extract

About 200 g of BO, cleaned with water, dried, pulverised, sieved and the resulting homogenous powder was extracted with methanol at room temperature for 48 hours.The extract was filtered using Whatmann no.1 filter paper and evaporated to dryness. The resulting yield was stored in sterile vials at 4^{o}C and used for the study.

GC-MS analysis

MEBO was subjected to GC-MS analysis in Sophisticated Analytical Instrument Facility (SAIF), IIT Chennai. JEOL GC MATE II instrument was used for the analysis. The inert gas helium (99.999%) was used as carrier gas with the flow rate of 1 ml/min. The sample size of 4µl was injected through the injector. HP5 column with specification length 30 mm, internal diameter 0.32 mm, film of 0.25 mm and temperature limit -60 °C to 325 °C (350 °C) was used. The oven temperature rose from 50 °C up to 250 °C with the rate of 10 °C per min rise in temperature. The MS was taken at 70eV. The identification of compounds was done by comparing the spectrum of unknown compounds with the spectrum of known compounds based on National Institute of Standards and Technology (NIST) library.

HPLC analysis

The amount of luteolin in MEBO was determined by HPLC analysis (LCGC AGLIENT). The stationary phase was octadecylsilyl silica gel, and mobile phase was a linear gradient with methanol, water and phosphoric acid (100:100:1). The flow rate of sample was 1.5 ml/min and the injection volume was adjusted to 20 µl. Standard luteolin was used as the reference compound. The UV spectra were monitored at 270 nm.

Animals

Male albino Wistar rats (*Rattusnorvergicus*) weighing 150–175 g were obtained from Biogen, Bangalore, India, and were housed in polycarbonate cages with wood chip bedding and maintained in a controlled environment at temperature 22±3ºC, with the relative humidity of 60±10 % and a light/dark cycle of 12 hour each. All animals were allowed to acclimatise to the new environment for 1 week with free access to commercial pellet rodent diet and water *ad libitum*. The animal experimental protocol was reviewed and approved by the Institutional animal ethics committee (IAEC)/CPCSEA (XVII/VELS/PCOL/14/2000/CPCSEA/IAEC/06.10.15).

Induction of chronic pancreatitis

Ethanol-cerulein induced chronic pancreatitis

After one week of acclimatization, the rats were divided into four trial groups of six animals each. All the animals, except those in normal and drug control group (groups 1 and 2, respectively), received isocolorically adjusted diet containing EtOH (36% of total calories) for a total period of 5 weeks and were injected with Cer (i.p) at a dose of 20 µg/kg body weight thrice weekly for the last 3 weeks of the experimental period (Deng et al. 2005). Animals in drug control (group 2) and treatment group (group 4) were administered orally with *MEBO* suspended in 0.1% DMSO at a daily dose of 40 mg/kg body weight from third week till the end of the experimental period.

At the end of the study, animals from each group were anasthetised by injecting 0.1ml/100g body weight of ketamine/xylazine mixture (prepared by combining 1.5ml of 100mg/ml xylazine and 10ml of 100mg/ml ketamine) and sacrificed by cervical decapitation. Immediately blood

was collected and plasma/serum was separated and stored at 4˚ C until analyses. Pancreas was isolated and used for various analyses.

Preparation of tissue homogenate

The isolated pancreas was washed in ice cold saline, and kept at -20ºC. It was then homogenised with 0.1 M Tris-HCl buffer, pH 7.4, and centrifuged at low speed to remove any cell debris. The resultant supernatant was used for the determination of glutathione (GSH), lipid peroxides (TBARS), oxidative stress index (OSI), protein and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). For all the estimations involving proteins and enzymes, the reagents were prepared in protease-free water and stored at 4ºC until used.

Biochemical investigations

Determination of serum lipase activity

The assay was performed according to manufacturer's instructions (K722-100; Biovision). Pancreatic lipase (EC: 3.1.1.1) hydrolyzes a triglyceride substrate to form glycerol which is quantified enzymatically via monitoring a linked change in the OxiRed probe absorbance (λ=570nm). The activity was expressed as IU/L.

Determination of amylase activity

The assay was carried out as per the instruction of kit manual (MAK009-1KT; Aldrich). Amylase (EC: 3.2.1.1) activity is determined using a coupled enzymatic assay, which results in a colorimetric (405 nm) product, proportional to the amount of substrate, ethylidene-pNP-G7, cleaved by the amylase. One unit is the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 μmole of p-nitrophenol per minute at 25 °C.

Estimation of GSH and antioxidant enzymes

GSH was determined by the method of Moron et al. 1979. Aliquot of homogenate was mixed with equal volume of ice cold 5% trichloroacetic acid and the precipitated proteins were removed by centrifugation. The supernatant was used for the assay by treating with NBT in 0.2 M phosphate buffer, pH 8.0 and measuring at 412 nm. GPx (EC: 1.11.1.9) was assayed by the

method of Flohe & Gunzler 1984. The activity of GPx was expressed as nM of GSH oxidised/min/mg protein.

SOD (EC: 1.15.1.1) activity was measured according to method of Kakker et al. 1984. The inhibition of reduction of NBT to blue coloured formazan in the presence of PMS and NADH was measured at 560 nm using n-butanol as blank. The enzyme activity was expressed as units/mg protein. Decomposition of H_2O_2 in the presence of CAT (EC: 1.11.1.6) was kinetically measured at 240 nm by the method of Aebi (1984). CAT activity was measured as the amount of enzyme required to decompose 1µM of H_2O_2/min. The enzyme activitywas expressed as µM of H_2O_2 consumed/min/mg protein.

Estimation of protein

Protein concentration was determined in serum and tissue homogenate by the method of Bradford (1976). The level of protein in serum and tissue homogenate was used to calculate the enzyme activity.

Estimation of lipid peroxides and oxidative stress index (OSI)

The level of lipid peroxide in pancreas was determined by measuring thiobarbituric acid-reacting substances (TBARS) (Draper & Hadley 1990). The peroxide content was measured by using FOX 2 method (Miyazawa 1989) with minor modifications. The FOX 2 test system is based on oxidation of ferrous ion to ferric ion by various types of peroxides contained within samples, to produce a coloured ferric-xylenol orange complex whose absorbance was measured at 560 nm. Total antioxidant capacity (TAC) was determined by the method of Miller et al. 1993. The decolorisation of the assay mixture containing 2,2'-azino bis 3-ethyl benzo-thiazoline-6-sulfonate (ABTS) and the sample was monitored by measuring the absorbance at 734 nm and the % inhibition was calculated and plotted as a function of concentration of antioxidants and of trolox for the standard reference data. The ratio of total peroxides to TAC was calculated as OSI.

Assay of caspase-1

Caspase-1 (EC: 3.4.22.36) activity was determined colorimetrically in serum and pancreatic extract, prepared according to the method of Thornberry and colleagues, (Garcia-Calvo et al. 1998) as the enzyme source. Briefly, the pancreas was homogenised in a lysis buffer (25 mM

HEPES (pH 7.5), 1 mMethylenediaminetetraacetic acid, 10 µg of aprotinin per ml, 10 µg of leupeptin per ml, 2 mMdithiothreitol) at 5 ml/100 mg of pancreas tissue. Extracts were centrifuged at 15,000g for 30 min at 4ºC, and the supernatant wascentrifuged again at 200,000g for 1 h at 4ºC. The cytosol was used for caspase-1 activity measurements. The assay in undiluted serum or pancreas extract was performed as per the kit manufacturer instruction (ICT098). Reactions with enzyme preparation alone, with enzyme mixed with caspase-1 substrate (Ac-YVAD-pNA) or inhibitor (Ac-YVAD-CHO) and with substrate alone were also run as controls. The activity was measured by proteolytic cleavage of Ac-YVAD-pNA for 4 h at 37ºC. The plates were read at 405 nm. A recombinant caspase-1 enzyme was used as a positive control.

Assay of IL-1β

The assay was performed according to manufacturer's instructions (ab100767). The antibody precoated wells were added with standard or serum sample. IL-1β present in a sample is bounded to the wells by the immobilised antibody. Biotinylated secondary antibody was added after washing the wells thoroughly. The unbound biotinylated antibody was washed and HRP-conjugated sterptavidin was added to the wells. TMB substrate solution was added to all the wells after repeated washing. The stop solution changes the colour from blue to yellow, and the intensity of the colour was measured at 450 nm. The activity was expressed as pg/ml.

Assay of IL-18

The assay was carried out as per the instruction of kit manual (KRC2341). The serum sample in duplicate or aliquots standard were pipetted into antibody immobilised wells. After the incubation, biotinylated secondary antibody was added. Sterptavidin peroxidase was added after removal of excess secondary antibody. Then, the substrate solution was added to react with the bound enzyme to produce color. The intensity of this color was measured spectrophotometrically at 450 nm. The activity of IL-18 was expressed as pg/ml.

Histopathological examination

For histopathological examination, the pancreatic tissue was excised and rinsed with ice-cold saline solution (0.9% sodium chloride) to remove blood and debris of adhering tissues. The tissues were then fixed in 10% formalin for 24 hours. The fixative was removed by washing

through running tap water and after dehydration through a graded series of alcohols, the tissue was cleaned in methyl benzoate and embedded in paraffin wax. Sections were cut into 5µM thickness and stained with hematoxylin and eosin. After dehydration and cleaning, the sections were mounted and observed under light microscope for details.

Statistical analysis

Data were analyzed by using a commercially available statistics software package (SPSS for window V. 10). The statistical significance of mean values between different groups was determined by applying one-way ANOVA with post hoc Bonferroni test and the p value < 0.05 was considered as significant.

References

Aebi H. 1984. Catalase. Methods Enzymol. 105: 121–126.

Bradford MM. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal Biochem. 72: 248–254.

Deng X, Wang L, Elm MS, Gabazadeh D, Diorio GJ, Eagon PK, Whitcomb DC. 2005. Chronic alcohol consumption accelerates fibrosis in response to cerulein-induced pancreatitis in rats. Am J Pathol. 166:93–106.

Draper HH, Hadley M. 1990. Malondialdehyde determination as index of lipid peroxide. Methods Enzymol. 186: 421-431.

Flohe L, Gunzler WA. 1984. Assays of glutathione peroxidase. Methods Enzymol. 105: 114-121.

Garcia-Calvo M, Peterson EP, Leiting B, Ruel R, Nicholson DW, Thornberry NA. 1998. Inhibition of human caspases by peptide-based and macromolecular inhibitors. J Biol Chem. 49: 32608–32613.

Kakkar P, Das B, Viswanathan PN. 1984. A modified spectrophotometric assay of superoxide dismutase. Ind J BiochemBiophys. 21: 130-132.

Miller NJ, Rice Evans CA, Davis MJ, Gopinathan V, Milner A. 1993. A novel method for measuring antioxidant capacity status in premature neonates. Clin Sci. 84: 407-412.

Miyazawa T. 1989. Determination of phosphorlipidhydroperoxides in human blood plasma by a chemiluminescence- HPLC assay. Free Radiac Biol Med. 7: 209-217.

Moran MS, Depierre JW, Mannervik B. 1979. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. BiochemBiophysActa. 582: 67-78.

Table S1. Phytoconstituents detected in the methanolic extract of *Brassica oleraceae* L. var. botrytis

S.No	RT[#]	Peak area %	Name of the compound	Synonyms	Molecular Formula	Molecular weight (g/mol)	**Bioactivity
1	17.8	30.25	Luteolin	3',4',5,7 tetrahydroxy flavone, Flacitran	$C_{15}H_{10}O_6$	286.23	Antioxidant, Anti-Inflammatory, Antidiabetic, Anticancer Activity
2	20.68	4.28	Myricetin	Cannabiscetin	$C_{15}H_{10}O_8$	318.23	Antioxidant, Anti-Inflammatory, Antidiabetic, Antiobesity
3	15.83	4.53	Tetradecanoic acid, ethyl ester	Myristic acid, ethyl ester	$C_{16}H_{32}O_2$	256.42	Antioxidant, Cancer Preventive
4	19.48	13.34	Ethyl oleate	Octadecenoic acid, ethyl ester	$C_{20}H_{38}O_2$	310.51	Cancer Preventive
5	18.8	45.69	Heptadecanoic acid, ethyl ester	Ethyl margarate	$C_{19}H_{38}O_2$	298	Antioxidant
6	19.68	1.88	Quercetin	Sophoretin, Meletin	$C_{15}H_{10}O_7$	312.53	Anti-Inflammatory, Chemopreventive Activity

**Activity Source: Dr. Duke's Phytochemical and Ethnobotanical Databases

[#]RT- Retention Time

Table S2. Activity levels of serum lipase, amylase, caspase-1, IL-1β and IL-18 in experimental animals

Groups	Lipase (IU/L)	Amylase (IU/L)	Caspase-1(pg/ml)	IL-1β (pg/ml)	IL-18 (pg/ml)
Control	360.5 ± 50.1	7045 ± 845.40	12.1±1.5	12.9±1.5	180±21.6
MEBO control	358.9 ± 47.0 NS	6354 ± 838.73 NS	11.6±1.7 NS	11.9±1.6 NS	160±20.0 NS
EtOH + Cer	694.5±71.5*	9037±1238.07*	25.6±3.1*	22.9±3.1*	230±30.0*
EtOH + Cer + MEBO	435.2 ± 55.7*	6128 ± 631.18*	15.6±2.1*	15.6±2.1*	195±22.0*

Notes: Values are expressed as mean ± SD for six animals in each group. Groups were compared as: control vs MEBO control, control vs EtOH + Cer (20 µg/kg BW), EtOH + Cer vs EtOH + Cer & MEBO (40 mg/kg BW). *p=0.000, NS – Non significant.

Table S3. Levels of TBARS, peroxide content, TAC, OSI, antioxidant enzymes and glutathione in pancreas of experimental animals

Groups	TBARS (nmol/mg protein)	Peroxides (mmol/ml)	TAC (mmol trolex eq./l)	OSI	SOD (Units/mg protein)	CAT (µ mol H_2O_2 consumed/min/ mg protein)	GPx (nM of GSH oxidized/min /mg protein)	GSH (mg/g protein)
Control	26.6 ± 3.0	200.5±29.6	360.5±40.0	0.72±0.08	3.1 ± 0.4	54.6 ± 6.2	80.8 ± 9.0	58.3 ± 6.5
MEBO control	28.9 ± 3.6 NS	190.1±21.6 NS	320±36.1 NS	0.75±0.09 NS	3.0 ± 0.4 NS	54.1 ± 6.2 NS	80.2 ± 9.7 NS	56.2 ± 7.1 NS
EtOH + Cer	47.2 ± 6.2*	285±31.6*	230±25.0*	1.54±0.20*	0.6 ± 0.08*	26 ± 3.3*	60.8 ±8.6*	41 ± 4.9*
EtOH + Cer + MEBO	30.8 ± 4.0*	220±25.1*	320±35.5*	0.86±0.11*	2.3 ± 0.3*	44.9 ± 5.3*	78.3 ± 9.8#	55.3 ± 7.1#

Notes: Values are expressed as mean ± SD for six animals in each group. Groups were compared as: control vs MEBO control, control vs EtOH + Cer (20 µg/kg BW), EtOH + Cer vs EtOH + Cer & MEBO (40 mg/kg BW). *p=0.000, NS – Non significant.

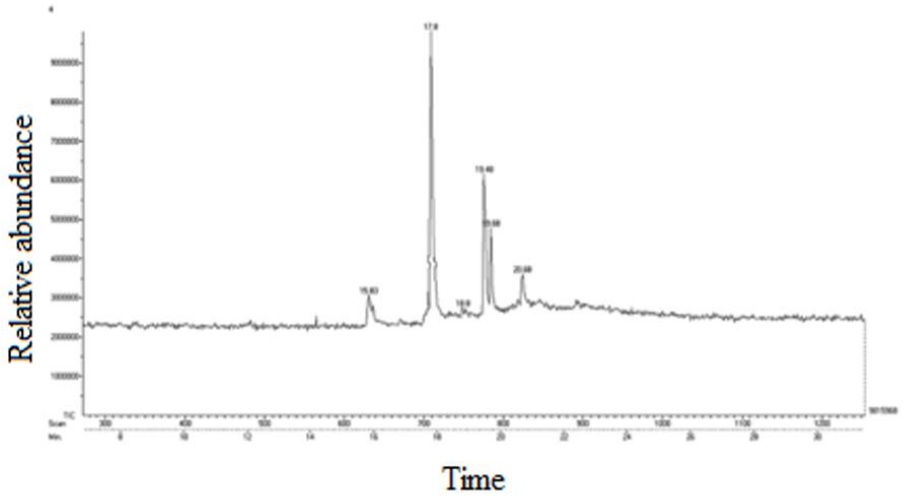

Figure S1. GC-MS chromatogram of the methanolic extract of *Brassica oleracea* L. var. *botrytis*

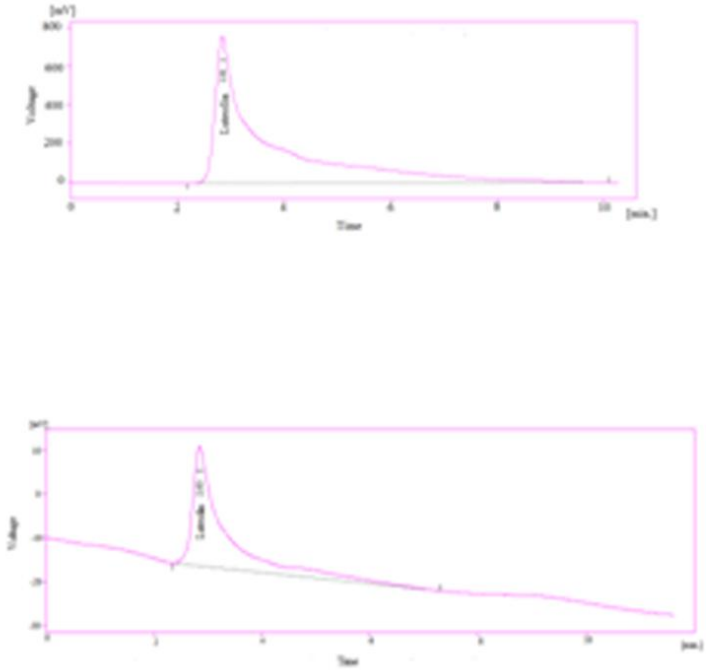

Figure S2. HPLC-UV finger print of luteolin. (a) HPLC-UV finger print of standard luteolin. (b) HPLC-UV finger print of luteolin in methanolic extract of *Brassica oleracea* L. var *botrytis*

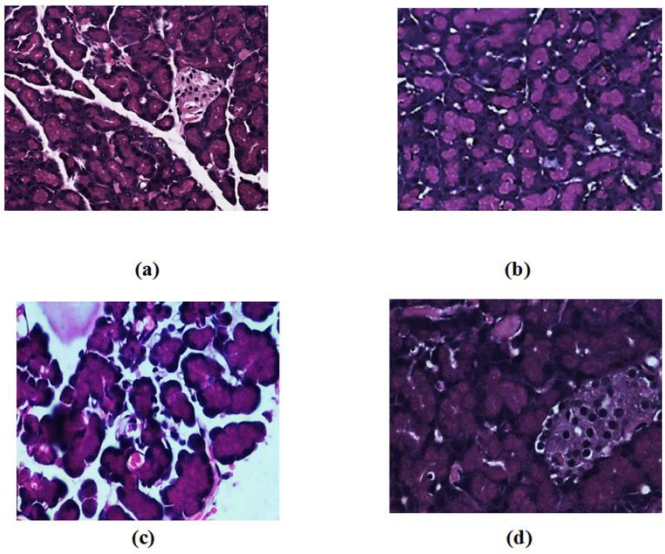

Figure S3. Histological examination of pancreas with hematoxylin and eosin stain (20x) in control and experimental group of rats. (a) Control – normal tissue architecture with acini and islets of langerhans; (b) MEBO Control – tissue architecture similar to that of control; (c) EtOH + Cer treated group – inflammation, congestion and acinar necrosis (d) EtOH + Cer + MEBO-treated group – Restored similar to that of normal with mild inflammation.