Indian Journal of Experimental Biology
Vol. 57, July 2019, pp. 486-496

Modulating effect of luteolin on ethanol and cerulein induced inflammation—*In silico* and *in vivo* studies using rat model of pancreatitis

Sadanandan Rajapriya[1], Arumugam Geetha[1]* & Kavasseri Ganesan Kripa[2]

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

[2]Department of Biochemistry, School of Life Sciences, Vels University, Chennai-600 117, Tamil Nadu, India

Received 11 May 2017; *revised 18 October 2018*

NLRP3 inflammasomes are induced in pancreas during inflammatory insult, which contain caspase activation and recruitment domain (CARD) and pyrin domain (PYD) of apoptosis associated speck-like protein containing a CARD (ASC). It is known that NLRP3-ASC interaction via PYD domain recruits and activate caspase-1. Through CARD, ASC brings monomers of procaspase-1 into close proximity and activate them which in turn activate pro-inflammatory cytokines. The assembly of inflammasome and activation of procaspase-1 is thought to be the molecular target for anti-inflammatory drugs. Here, we investigated the molecular interactions of luteolin, a potent anti-inflammatory agent with procaspase-1, ASC-CARD and ASC-PYD using Acceryls Discovery Studio Visualizer. To assess the pancreato-protective effect of luteolin, rats were administered with ethanol (36% of total calories for five weeks) and cerulein (20 µg/kg body wt. i.p., weekly thrice for last three weeks). In addition, a group of rats also received 2 mg luteolin/kg body wt. orally from third week till the experimental period. Co-administration of luteolin reduced the levels of pancreatic and inflammatory marker enzymes in serum and maintained the antioxidant status in pancreas. Immunohistochemical analysis confirmed the presence of ASC protein in excess in the pancreas of ethanol-cerulein administered rats than in rats co-administered with luteolin. Luteolin satisfies Lipinski's rule of five to determine the drug likeness property. The energy value and the hydrophobic interactions obtained in the present docking study confirmed that there was a strong binding affinity of luteolin towards procaspase-1, ASC-CARD, ASC-PYD that might interfere with the molecular assembly of NLRP3 inflammasome and the production of pro-inflammatory cytokines.

Keywords: ASC-CARD, ASC-PYD, Inflammasomes, Pancreas, Procaspase-1

Chronic pancreatitis (CP) is a progressive inflammatory condition that leads to irreversible loss of acinar cell functions. Alcohol abuse is a major risk factor for pancreatitis[1]. Although, alcohol consumption is associated with CP, evidences suggest that alcohol alone cannot cause pancreatitis. The additional factors, such as gallstone disease and high fat diet have been shown to potentiate the acinar cell damage induced by alcohol. During gallstone diseases, cholecystokinin (CCK) is increasingly formed in pancreas that influences the premature activation of enzymes in the acinar cells that cause autodigestion and tissue injury. It has been proved that CCK plus ethanol (EtOH) could produce six fold higher zymogen conversions than that induced by CCK alone. Cerulein (Cer) is a CCK analogue that can mimic the action of CCK.

Cerulein acts on the CCK receptor and is widely used to elicit pancreatitis by hyper stimulation of exocrine pancreas in rats and mice[2]. The mechanism of Cer action involves activation of NFκB, the promotion of oxidative stress and the release of pro-inflammatory cytokines, interleukin (IL) 1β and IL-18. The maturation of pro-inflammatory cytokines is dependent on the proteolytic activation of caspase-1, which itself becomes activated by molecular platforms known as inflammasomes.

Inflammasomes are multiprotein complexes, assembled in response to pathogen-associated or

*Correspondence:
Phone: +91 9444902506 (Mob.)
E-mail: geethav21@yahoo.co.in

Abbreviations: ASC, apoptosis associated speck-like protein containing a caspase recruitment domain; CARD, caspase recruitment domain; CAT, catalase; CCK, cholecystokinin; Cer, cerulein; CP, chronic pancreatitis; EtOH, ethanol; GPx, glutathione peroxidase; IL, interleukins; NBT, nitroblue tetrazolium; NLRP3, nucleotide-binding domain, leucine-rich-containing family, pyrin domain containing-3; OSI, oxidative stress index; PYD, pyrin domain; SOD, superoxide dismutase; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reacting substances.

damage-associated molecular pattern molecules and environmental pathogens. Currently, inflammasomes are distinguished into two families: the NOD-like receptor (NLR) family and the pyrin and HIN200 (haematopoietic interferon-inducible nuclear antigens with 200 amino-acid repeats) domain-containing protein family. The NLR family comprised of NLRP1, NLRP2, NLRP3, NLRP6, NLRC4, and NLRP12 of which NLRP3 plays a major role in the pathogenesis of pancreatitis[3].

The NLRP3 inflammasome is a large cytoplasmic complex (>700 kDa), composed of a specific member of the NOD-like receptor protein (NLRP) subfamily, the adaptor protein named apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC-CARD), and procaspase-1. The NLRP3 activation leads to oligomerization and recruitment of ASC. NLRP3 contains an N-terminal pyrin domain (PYD), which physically interact with the PYD domain of ASC (ASC-PYD), thus promoting the subsequent recruitment and activation of procaspase-1[4]. The activated caspase-1, proteolytically convert the inactive proforms of inflammatory cytokines to their pro-active forms.

Anti-inflammatory drugs are commonly used to control inflammation and related diseases. Frequent use of non steroidal anti-inflammatory drugs though has several side effects like peptic ulcer and cardiovascular diseases[5]. Currently, limited allopathic medicines are available for the treatment of pancreatitis and also presented with side effects. Only supportive medicines such as sompraz-40 and omiprazole are currently used for the pain management during pancreatitis. Therefore, it becomes imperative to explore for a potent pancreato-protective agent to overcome the challenges in pancreatitis treatment. Consumption of flavonoids may be associated with decreased risk of cancer, cardiovascular and other inflammatory diseases in humans. Vegetables, including hot peppers, broccoli, kale, and lettuce contain flavonol compounds like quercetin, luteolin and kaempferol. Luteolin, 3',4',5,7-tetrahydroxyflavone, is a flavonoid commonly found in many types of fruits and vegetables as well as in some medicinal plants. Broccoli is rich in Luteolin. It is also present in beets, Brussels sprouts, cabbage and cauliflower. It possesses antitumorigenic[6] and antioxidant properties[7].

The present study was designed to assess the modulating effects of luteolin on the inflammatory reactions in pancreas induced by prolonged treatment with ethanol and cerulein in rats and to perform *in silico* studies to evaluate the binding interaction of luteolin with members of inflammasome complex.

Materials and Methods

Chemicals and reagents

Luteolin was obtained from Alfa-aesar (Haverhill, Massachusetts, USA). Cerulein was purchased from Biovision (Milpitas, California, USA). Enzyme-linked immunosorbent assay (ELISA) kit for IL-1β and IL-18 were purchased from Abcam (Cambridge, Massachusetts, USA) and Invitrogen (Camarillo, California, USA), respectively. Caspase-1 assay kit was obtained from Genei (Bangalore, Karnataka, India). All other chemicals and solvents used for the analysis were of analytical grade.

Luteolin preparation

Luteolin was suspended in 0.1% dimethyl sulfoxide and mixed thoroughly. Freshly prepared luteolin was administered orally.

Experimental protocol

The study was performed in male albino rats of Wistar strain (175-200 g) housed in polycarbonate cages with good sanitary conditions and maintained in 24 hour circadian cycle (12 hour light, 12 hour dark) at 20°C. This study was reviewed and approved by the Institutional Animal Ethics committee (IAEC)/CPCSEA(XVII/VELS/PCOL/14/2000/CPCSEA/IAEC/06.10.15). During the acclimatization period, all the animals had free access to standard rat chow obtained from Hindustan Lever Ltd., Bangalore, India, and water.

After 7 days of acclimatization, the rats were divided into 4 groups of six animals each. Group 1 and 2 rats received normal diet. Group 3 and 4 rats were fed isocalorically adjusted diet with EtOH (36% of total calories) for a total period of 5 weeks and Cer (20 µg/kg body wt. i.p.) thrice weekly for the last three weeks of the experimental period[8]. In addition, animals in group 2 and 4 received luteolin daily (2 mg/kg body wt.) from third week till the end of the experimental period. At the end of the experimental period, the rats were fasted overnight and anasthetised by injecting 0.1 mL/100 g body wt. of ketamine/ xylazine mixture (prepared by combining 1.5 mL of 100 mg/mL xylazine and 10 mL of 100 mg/mL ketamine) and killed by cervical decapitation. Immediately serum/plasma was separated from the collected blood and stored at 4ºC until analyses.

Preparation of tissue homogenate

Immediately after sacrifice, pancreas was isolated carefully and washed in ice cold saline, homogenised with 0.1 M Tris-HCl buffer, pH 7.4 and centrifuged at low speed to remove any cell debris. The supernatant was used for the determination of glutathione (GSH), lipid peroxides (TBARS), oxidative stress index (OSI) and antioxidant enzymes. 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=570 nm). 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*.[9]. Aliquots of homogenate were 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 the colour intensity of the product at 412 nm. GPx (EC: 1.11.1.9) was assayed by the method of Flohe and Gunzler[10]. 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 the method of Kakkar *et al*.[11]. In the presence of PMS and NADH, the inhibition of reduction of NBT to blue coloured formazan 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[12]. CAT activity was measured as the amount of enzyme required to decompose 1 µM of H_2O_2/min. The enzyme activity was 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[13]. 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 peroxides in pancreas was determined by measuring thiobarbituric acid reacting substances (TBARS)[14]. The peroxide content was measured by using FOX 2 method[15] with minor modifications. The FOX 2 test system is based on oxidation of ferrous ions by peroxides present in the 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*.[16]. The decolorisation of the assay mixture containing 2,2'-azino bis 3ethyl 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[17], as the enzyme source. Briefly, the pancreas was homogenised in a lysis buffer (25 mM HEPES (pH 7.5), 1 mM EDTA, 10 µg of aprotinin per mL, 10 µg of leupeptin per mL, 2 mM dithiothreitol) at 5 mL/100 mg tissue. Extracts were centrifuged at 15000×g for 30 min at 4ºC, and the supernatant was re-centrifuged at 200000×g 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 (AB39470). 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. A recombinant caspase-1 enzyme was used as a positive control. The activity was measured by proteolytic cleavage of Ac-YVAD-pNA for 4 h at 37ºC. The plates were read at 405 nm.

Enzyme linked immunosorbent assay (ELISA)

Assay of IL-1β

The assay was performed according to manufacturer's instructions (ab100767). The standard or serum sample was added to the antibody precoated wells. IL-1β present in the sample is bounded to the wells by the immobilised antibody. The wells were washed followed by the addition of biotinylated secondary antibody. The unbound biotinylated antibody was washed and HRP conjugated sterptavidin was added. 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 developed 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 out into antibody immobilised wells. Following incubation, biotinylated secondary antibody was added. Sterptavidin peroxidase was added after the removal of excess secondary antibody. Then, the substrate solution was added to react with the bound enzyme to produce colour. The intensity of the color developed was measured spectrophotometrically at 450 nm. The activity of IL-18 was expressed as pg/mL.

Immunohistochemical analysis

Immunohistochemistry was performed on 5 μm thick paraffin embedded sections of the pancreas. The slides were deparaffinized in xylene, rehydrated in a series of graded alcohols (95, 70 and 50%), and placed in a Tris buffer bath (pH 7.6). Endogenous peroxidase activity was quenched using 3% hydrogen peroxide. The antigenic epitope is unmasked by antigen retrieval. The slides were incubated with a primary antibody (Rabbit polyclonal IgG (ASC-N15R) at 4ºC overnight. The secondary antibody (Goat anti-rabbit IgG coupled with horse raddish peroxidase) was then applied to the sections of the slides, incubated and washed. The slides were developed with 100 μL of 3, 3'-diaminobenzidine (DAB; 1:1000 dilution in Tris buffered saline) and counterstained with hematoxylin. The tissue sections were photographed at 400x magnification (Olympus [CH30] biological microscope).

Assessment of immunostaining

To score the expression, both the extent and intensity of immunoreactivity were considered. The intensity of expression was scored from 0 to 3 as follow: 0: Non stained, 1: Weak, 2: Moderate, and 3: Strong (the same as positive control). The percentage of stained cells for each staining intensity was estimated. The final composite score was determined by multiplying the intensity of staining and the percentage of stained cells. According to the scoring system, the lowest and highest expected final composite scores are 0 and 3, respectively.

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.

***In silico* drug docking**

Target protein identification and preparation

The link for downloading the structures of procaspase-1, ASC-CARD and ASC-PYD were retrieved from UniProt (http://www.uniprot.org/). The crystal structures of Procaspase-1 ASC-CARD and ASC-PYD were downloaded from PDB database and their PDB ids were 3E4C, 2KN6 and 1UCP, respectively. The proteins were preprocessed separately by deleting the ligands as well as the crystallographically viewed water molecules (water without hydrogen bonds).

Ligand structure preparation

The 2D structure of luteolin was drawn using ACD chemsketch and saved in 3D MDL MOL Format and converted to PDB (http://www.rcsb.org/pdb/home/home.do) format using Open Babel Molecular Converter. Open Babel was used for energy minimization. The 3D structures of luteolin (PubChem CID: 5280445) was retrieved from PubChem (http://pubchem.ncbi.nlm.nih.gov/) database and its SMILES (Original SMILES C1=CC(=C(C=C1C2=CC(=O)C3=C(C=C(C=C3O2)O)O)O)O; miSMILES OC3CC(O)C2C(=O)CC(C1CCC(O)C(O)C1)OC2C3) format was saved.

Molecular descriptor calculation

Molinspiration online database (http:// www.molinspiration.com), used to calculate thirteen descriptors of luteolin, such as log P, polar surface area (PSA), molecular weight, number of atoms, number of O or N, number of OH or NH, number of rotatable bonds, volume, drug likeness includes G protein coupled receptors (GPCR) ligand, ion channel modulator, kinase inhibitor and nuclear

receptor ligand, and number of violations to Lipinski's rule of Five.

Lipinski's rule of Five *(*Pfizer's rule of five*)*

Lipinski's rule of five is a rule of thumb to evaluate drug likeness, or find out if a chemical compound with a certain pharmacological or biological activity has properties that would make it a likely orally active drug in humans. The rule states, that most "drug-like" molecules have partition coefficient log P (mi.LogP) <= 5, molecular weight <= 500 g/mol, number of hydrogen bond acceptors (notably N and O) <= 10, number of rotatable bonds (rotb) <= 15 and number of hydrogen bond donors (OH and NH groups) <= 5. Molecules violating more than one of these rules may have problems with bioavailability.

Auto dock

The binding energy calculations were performed using Auto Dock (http://autodock.scripps.edu/ resources/tools) and the protein was modified by adding hydrogen atoms for correct ionisation and tautomeric states of aminoacid residues and kollman charges. The obtained modified structure was saved in PDBQT format in AutoDock tools for Autodock calculations.

Using AutoGrid program, the pre-calculated grid was centered at the structure of protein and the grid dimensions were 40 × 40 × 40 with default grid point spacing which store grids of interaction energy based on the interaction of the ligand atom probes with receptor target. For docking experiments, lamarckian genetic algorithm was implemented with a population size of 150 dockings with 25,00,000 energy evaluations, a maximum number of 27,000 generations, mutation rate of 0.02, and crossover rate of 0.80. All other parameters were run with default settings.

Molecular interaction studies

The molecular interactions were visualized using Acceryls Discovery Studio Visualizer. Default ligand–receptor interaction tool was used to determine hydrogen and hydrophobic interactions. This application runs on Windows and Linux.

Results

Effect of luteolin on pancreatic marker enzymes and inflammatory markers

Table 1 shows the activity levels of serum lipase, amylase, IL-1β, IL-18, caspase-1 and pancreatic caspase-1 in experimental animals. Elevated level of these pancreatic marker enzymes and inflammatory markers were observed in EtOH–Cer-administered rats (group 3) and significant reduction was observed in rats co-administered with luteolin (group 4). A nonsignificant reduction was seen in rats received normal diet (group1) and luteolin (group 2).

Effect of luteolin on oxidative stress markers

The levels of oxidative stress markers are represented in Table 2. The levels of TBARS, LHP, and OSI were found to be elevated significantly in EtOH–Cer-administered rats (group 3) whereas TAC was found to be decreased. Supplementing luteolin to EtOH–Cer-administered rats (Group 4) showed reduced level of TBARS, LHP and OSI, with near normal level of TAC.

Effect of luteolin on GSH and antioxidants in pancreas

As indicated in Table 3, the activity of GSH and antioxidant enzymes GPx, SOD, and CAT were

Table 1 — Levels of serum lipase, amylase, IL-1β, IL-18, caspase-1 and pancreatic caspase-1 in experimental animals

Groups	Lipase (IU/L)	Amylase (IU/L)	IL-1β (pg/mL)	IL-18 (pg/mL)	Caspase-1	
					Serum (pg/mL)	Pancreas (pM/mg protein)
Control	310±19	6500±400	14.6±1.6	200±22	13.5±1.6	11.6±1.4
Luteolin control	290±30^{NS}	6300±200^{NS}	13.5±1.4^{NS}	190±24^{NS}	12±1.5^{NS}	10.9±1.1^{NS}
EtOH+Cer	600±50*	8500±600*	26.5±3.1*	276±31*	24±2.8*	18.5±1.7*
EtOH+Cer+Luteolin	400±45*	6800±600*	18.5±2.1*	210±25*	16.4±2.0*	12.6±1.8*

[Values are expressed as mean ± SD for six animals in each group. Groups were compared as: control *vs*. luteolin control, control *vs*. EtOH + Cer, EtOH + Cer *vs*. EtOH + Cer & luteolin. *p=0.000, NS – Non significant]

Table 2 — Activity levels of plasma peroxide content, total antioxidant capacity and oxidative stress index in experimental animals

Groups	TBARS (nmol/mg protein)	Peroxides (mmol/mL)	TAC (mmol trolex eq./L)	OSI
Control	30±3.6	210±25	380±42	0.55±0.06
Luteolin control	27±3.1^{NS}	195±21^{NS}	360±41^{NS}	0.54±0.07^{NS}
EtOH + Cer	56±6.1*	310±32*	250±27*	1.24±0.15*
EtOH + Cer + Luteolin	41±5.2*	230±25*	350±43*	0.66±0.09*

[Values are expressed as mean ± SD for six animals in each group. Groups were compared as: control *vs*. luteolin control, control *vs*. EtOH + Cer, EtOH + Cer *vs*. EtOH + Cer & luteolin. *p=0.000, NS – Non significant]

significantly decreased in EtOH–Cer-administered rats (Group 3) when compared to normal control rats (Group 1). The enzyme activity levels did not show much variation between normal control and luteolin control groups (Group 2). Luteolin co-administered rats (Group 4) showed significant restoration of antioxidant levels in pancreas.

Immunohistochemical analysis

The immunohistochemical scores obtained for ASC in pancreas are presented in Table 4 and Fig. 1. Strong positive staining for ASC was observed in 60% of the acinar cells in EtOH–Cer-treated groups (Group 3), scattered staining was found in 10% of acinar cells in group co-administered with luteolin (Group 4). Negative staining was identified in control (Group 1) and luteolin control groups (Group 2).

Docking analysis

The visualized hydrogen interactions between luteolin and procaspase-1, ASC-CARD, ASC-PYD, using Acceryls Discovery Studio Visualizer were shown in Fig. 2A, B and C, respectively. Seven hydrogen bond interactions were found between luteolin and procaspase-1 at residues ARG391, GLU390, TRP294, GLU241, PHE295, LEU258, LYS296 with the docking energy of -8.76 kcal/mol (Table 5). Four hydrogen bond interactions were

Table 4—Immunohistochemical score of pancreatic acinar cells

Groups	Immunohistochemical Score				Assessment
	0	1+	2+	3+	
Control	<10%	0%	0%	0%	Negative
Luteolin control	<15%	0%	0%	0%	Negative
EtOH+Cer	0%	>10%	>40%	>60%	Strong Positive
EtOH+Cer +Luteolin	0%	<15%	<10%	<5%	Weak Positive

[0, No immunostaining; 1+, Faint partial staining in <10% of cells; 2+, Complete membranous staining either uniform or weak in >10% of cells; and 3+, Uniform intense membranous staining in >30% of cells]

Table 3—Effect of luteolin administration on the activity levels of antioxidant enzymes and glutathione in pancreas of experimental animals

Groups	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	3.5±0.4	61.5±7.2	91±10.1	62.5±7.1
Luteolin control	3.4±0.4^{NS}	61.2±7^{NS}	90±10^{NS}	62±6.8^{NS}
EtOH + Cer	1.2±0.2*	30±3.6*	56±7.1*	40.5±4.9*
EtOH + Cer + Luteolin	3.0±0.51*	55.5±6.1*	85±9.1*	57.5±5.5*

[Values are expressed as mean ± SD for six animals in each group. Groups were compared as: control *vs*. luteolin control, control *vs*. EtOH + Cer, EtOH + Cer *vs*. EtOH + Cer & luteolin. *p=0.000, NS – Non significant]

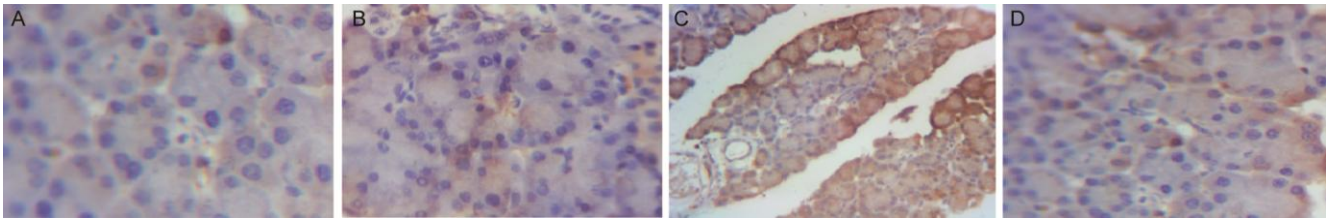

Fig. 1 — Immunohistochemical identification of ASC in acinar cells (H&E, 400X) in control and experimental group of animals. Primary antibody: Rabbit polyclonal IgG (ASC-N-15R); Secondary antibody: Goat anti-rabbit IgG coupled with horse raddish peroxidase. (A & B) pancreas shows no staining with score 0 (negative); (C) shows strong diffuse staining intensity with score 3 (positive); and (D) shows weak staining intensity with score 1 (weak positive).

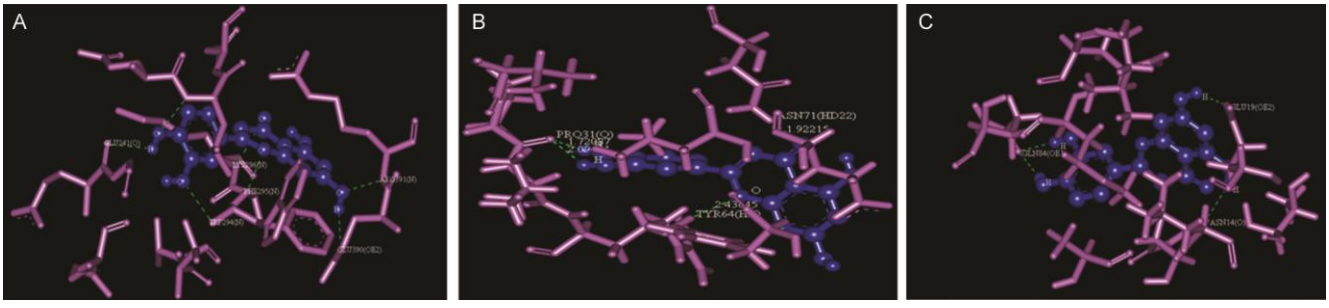

Fig. 2 — Hydrogen interactions using Acceryls Discovery Studio Visualizer. (A) Interaction between Procaspase-1 and luteolin; (B) Interaction between ASC-CARD and luteolin; and (C) Interaction between ASC-PYD and luteolin.

Table 5— Docking analysis of luteolin *vs*. Procaspase-1, ASC-CARD and ASC-PYD

Receptor names	No. of Hydrogen bonds formed`	Docking energy (KCAL/MOL)	Key atoms
Pro-caspase-1	7	–8.76	ARG391, GLU390, TRP294, GLU241, PHE295, LEU258, LYS296
ASC-CARD	4	–4.33	PRO31, ASN71, TYR64
ASC-PYD	4	–5.8	GLU19, ASN14, GLN84

Table 6— Molecular Descriptor and Drug Likeness property analysis of Luteolin using Molinspiration Online Software Tool

Properties	Luteolin
Octanol-Water partition coefficient (miLog P)	1.97
Polar surface area (TPSA)	111.12
Number of non hydrogen atoms (N atoms)	21
Molecular Weight (MW)	286.94
Number of hydrogen bond acceptors (O and N atoms)	6
Number of hydrogen bond donors (OH and NH groups)	4
Number of Rule of 5 violations (N violations)	0
Number of rotatable bonds (Nrotb)	1
Molecular volume (Volume)	232.07
GPCR Ligand	0.09
Ion channel modulator	–0.02
Kinase inhibitor	0.15
Nuclear receptor ligand	0.27
Protease inhibitor	–0.01
Enzyme inhibitor	0.42

found between luteolin and ASC-CARD at residues PRO31, ASN71, TYR64 with the docking energy of -4.33 kcal/mol. Four hydrogen bond interactions were found between luteolin and ASC-PYD at residues GLU19, ASN14, GLN84 with the docking energy of -5.8 kcal/mol.

Molecular descriptor analysis and drug likeness property

The molecular descriptor values and drug likeness score for luteolin was shown in Table 6. It was found that luteolin satisfies Lipinski's rule of five to bring them closer to the drug like molecule or a potent inhibitor.

Discussion

Chronic pancreatitis is inflammation of the pancreas that does not heal or improve and gets worsen over time that leads to permanent damage. Prolonged alcohol consumption can result in progressive and irreversible damage to the pancreas. Cer, an analog of CCK, induces CP in mice similar to that of human CP. Pancreatitis induced by Cer is characterized by edema, increased serum levels of pancreatic enzymes, death of acinar cells, cytoplasmic vacuolization, inflammation, infiltration of inflammatory cells into the pancreas and in some cases necrosis[18]. Chronic alcohol feeding combined with repeated Cer administration is a widely accepted model used to study the pathomechanism of pancreatitis and the mode of action of new drugs to be recommended for CP[19]. Hence, this model was used for this study.

EtOH is metabolized in pancreas by oxidative and non oxidative pathways similar to that seen in hepatocytes. Oxidative EtOH metabolism produces acetaldehyde, which interferes with the binding of secretagogue to their receptors and also causes microtubule dysfunction, thereby affecting exocytosis from acinar cells. During oxidation of EtOH, hydrogen ions and reducing equivalents are also released with acetaldehyde that induces oxidative stress-related damage in the gland. Fatty acid ethyl esters, the products of non-oxidative metabolism of EtOH have shown to induce the pancreatic injury *in vivo* and *invitro*. It directly binds to intracellular membrane that causes alterations in function and permeability of cell membrane.

Chronic alcohol administration to rats has been shown to increase synthesis and pre-activation of the digestive enzymes trypsinogen, chymotrypsinogen, and lipase, as well as the lysosomal enzyme cathepsin B within acinar cells[20]. In the present study, EtOH–Cer-treated rats showed significant elevation in the serum levels of lipase and amylase, the marker enzymes of pancreatic functions. Akiko *et al*.[21]. reported that, increased activity of exocrine pancreatic enzymes in serum reflects the acinar cell injury. Remarkably, co-administration of luteolin along with EtOH and Cer in this study caused significant reduction in the premature activation and release of lipase and amylase into circulation thus specifying its pancreato-protective role.

Paradkar *et al*.[22] has shown that cytokines are low molecular weight soluble protein molecules responsible for intercellular signalling. IL-1β is a pro-inflammatory cytokine and a potent mediator of inflammatory processes. IL-18 is one of the mediators of both pancreatic damage and systemic complications like hypotension and multiorgan dysfunction during pancreatitis. Ascitic fluid formed during acute pancreatitis has been shown to contain high level of IL-18[23]. Pancreatitis is associated with a 5-fold increase in circulating IL-18[24] and is proportional to the severity of pancreatitis[25].

Immunohistochemical study has shown elevated IL-18 content in the pancreas of patients suffering from CP[26]. Several studies have reported that flavonoids exhibits anti-inflammatory activity by reducing the expression of various pro-inflammatory cytokines such as TNF- α, IL-1β, IL-6, IL-8 and monocyte-chemoattractant protein-1[27]. Chen *et al*.[28] had reported that luteolin could suppress the NF-kB pathway and inhibit the formation of pro-inflammatory cytokines in mouse alveolar macrophages.

Caspase-1 is a prototypical caspase that is activated in the inflammasome complex. Caspase-1 is present in the cytosol as an inactive pro-caspase-1, and undergoes post-translational cleavage for activation. Activated caspase-1 is required for cleaving and processing of pro-IL-1β and pro-IL-18 into functional mature pro-inflammatory cytokines, IL-1β and IL-18, respectively, as well as the activation of other inflammatory pathways. In the present study, significantly reduced caspase-1 activity along with the concomitant reduction in IL-1β and IL-18 levels was observed in luteolin co-administered rats as compared to rats treated with EtOH–Cer. Inhibition of caspase-1 has been shown to be beneficial in slowing down the progression of CP[29]. Luteolin has been shown to exhibit dose-dependent inhibitory effect on purified caspase-1 activity[30]. Therefore, the anti-inflammatory activity of luteolin might be attributed to the inhibitory effect on caspase-1 activation.

It has been demonstrated by Mahanti *et al*.[31] that various factors including the inflammation, infection and administration of bacterial LPS cause oxidative stress, and this entails an increase in tissue concentration of reactive oxygen species (ROS), which mediates tissue destruction. Oxidative stress which is the predominant effect of ROS mainly occurs due to disturbance in the prooxidant-antioxidant balance in the body[32]. Oxidative stress and ROS both play pivotal roles in pathophysiology of pancreatitis during the initial phase and the development of the disease. ROS produces deleterious effect on membrane lipids of the cellular components thereby producing peroxidation of lipids which leads to cell death[33]. Lipid hydroperoxides are non-radical intermediates of lipid peroxidation and due to their high polar nature perturb membrane structure and cause deleterious effects on cells. The results of the present investigation show that luteolin significantly prevented the onset of lipid peroxidation in pancreas and inhibited the formation of end products.

Luteolin has been shown to inhibit enzymatic and non enzymatic lipid peroxidation in rat liver[34]. The inhibiting effect of several flavonoids on CCl_4-induced lipid peroxidation of rat liver microsomes was studied and Luteolin has been found to be the most effective flavonoid which counteract the effect of lipid peroxidation[35].

Free radicals derived from normal metabolic pathways are highly reactive and have a toxic effects on key biomolecules[36]. Antioxidant status of a cell is the primary measure of oxidative stress. Antioxidant enzymes present within host tissues provide first line defense to cope up with oxidative stress. Generally, the highest levels of antioxidant enzyme expression are in the liver, a major detoxification center. But, it has been reported that the islet is among the least well endowed tissues in terms of intrinsic antioxidant enzyme expression and activity[37,38]

GSH is one of the most abundant tripeptide, non-enzymatic biological antioxidant present in the liver and concerned with the removal of free radical species such as hydrogen peroxide and superoxide radicals[39]. During the early phase of acute pancreatitis the level of reduced glutathione decreases, which is an alarming signal for the damage expected[40]. Studies have shown that pre-treatment with glutathione mono ethyl ester exhibit beneficial effects on acute pancreatitis by increasing pancreatic GSH level[41]. In the present study also, Luteolin co-administered group (group 4) revealed restoration of GSH level in pancreas as well as activities of SOD, CAT and GPx.

In case of CAT deficiency, β cells of pancreas undergoes oxidative stress by producing excess ROS that leads to cellular dysfunction and other related consequences[42].

SOD is found in the extracellular matrix of various tissues including pancreas, skeletal muscle and blood vessels, and is the major extracellular scavenger of superoxide radicals[43]. Luteolin has been found to possess strong antioxidative and several pharmacological activities including anti-inflammatory effect[44]. The potential antioxidative nature of luteolin, measured by Trolox test, has been found to be greater than two-fold when compared to that of vitamin E[45]. Luteolin is a potent antioxidant than the synthetic antioxidant, butylated hydroxytoluene (BHT), which is generally used in oxygen sensitive processes[46]. Our results show that luteolin co-administered rats are well protected

from deleterious consequences of EtOH–Cer administration probably by preserving the antioxidant status in pancreas.

Immunohistochemistry (IHC) is a powerful microscopy-based technique for selectively imaging antigens (e.g. proteins) in tissue sections by exploiting the principle of antibodies binding specifically to antigens. IHC is an excellent detection technique to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue.

Under normal conditions, ASC-associated inflammasomes are autorepressed, but become activated by a wide range of stimuli from pathogens, including oxidative stress, ischemia and damage signals. These stimuli trigger inflammasome sensors, which induce polymerization of the adaptor ASC and the assembly of ASC specks. ASC specks recruit and activate caspase-1, which induces maturation of IL-1β that is associated with local inflammation and pyroptotic cell death. After pyroptosis, ASC specks accumulated in the extracellular space, promoted further maturation of IL-1β. In addition, phagocytosis of ASC specks occurs by macrophages induced lysosomal damage and nucleation of soluble ASC, as well as activation of IL-1β. ASC specks appears in body fluids from inflamed tissues, and autoantibodies to ASC specks has been found developed in patients and mice with autoimmune pathologies. In the present study, IHC analysis indicated significantly increased ASC expression in EtOH–Cer-administered rats. On the other hand, co-administration of luteolin reduced the levels of ASC assembly and activated caspase-1 and thereby might have contributed for reduction in the levels of inflammatory mediators.

Molecular physicochemical and the drug-likeness were considered to be the two most significant properties for a compound to become a successful drug. These properties were formulated by Lipinski *et al*.[47].

The biological activity of a drug was almost due to their miLogP which is a measure of molecular hydrophobicity. The drug's hydrophobicity will affect drug absorption, bioavailability, hydrophobic drug-receptor interactions and metabolism of molecules, as well as its toxicity. An analysis of small drug-like molecules suggested that for good intestinal permeability, the miLogP values need to be greater than 0 and less than 3 with the optical activity noted at 2. However, Luteolin has a miLog P value of 1.97 thus, satisfying the Lipinski's rule of five.

Tambunan & Wulandari[48] have reported that molecular weight in the range of 160-480 is preferred for oral absorption. Hence, luteolin with the molecular weight of 286.94 is best suited for oral administration. Rotatable bond count is now a widely used filter following the finding that greater than ten rotatable bonds correlates with decreased rat oral bioavailability[49]. Luteolin has a rotatable bond count of one. Most centrally acting compounds have rotatable bond count of five or less[50]. The tight bond interaction observed between luteolin and ASC-CARD, ASC-PYD, procaspase -1 shows that luteolin interrupts cytokine production by influencing the assembly of NLRP3 inflammasome. It was found that luteolin fulfilled Lipinski's set of five rules with violations of zero and is considered to have good intestinal absorption, bioavailability and permeability.

The assembly of inflammasome and activation of procaspase-1 is thought to be the molecular target for anti-inflammatory drugs. The results show that luteolin can interact with procaspase-1, ASC-CARD and ASC-PYD and inhibit their formation.

Conclusion

The results from the *in vivo* studies revealed that luteolin plays a significant role in reducing the inflammatory changes by manipulating ASC levels and thereby inhibiting caspase-1 activation in pancreatic acinar cells which inturn prevents the activation of pro-inflammatory cytokines. The *in silico* analysis pointed to the capacity of luteolin to interact with target proteins such as ASC-CARD, ASC-PYD and Procaspase-1 and might reduce the formation of proinflammatory cytokines and eventually prevent tissue injury in pancreas.

Conflict of Interest

The authors declare that there is no conflict of interests.

Acknowledgement

The authors acknowledge the financial assistance obtained from ICMR-New Delhi in the form of Senior Research Fellowship (File No: 45/07/2018-BIO/BMS) to the first author.

References

1 Manohar M, Verma AK, Venkateshaiah SU, Sanders NL & Mishra A, Pathogenic mechanisms of pancreatitis. *World J Gastrointest Pharmacol Ther*, 8 (2017) 10.

2 Aghdassi AA, Mayerle J, Christochowitz S, Weiss FU, Sendler M & Lerch MM, Animal models for investigating chronic pancreatitis. *Fibrogenesis Tissue Repair*, 4 (2011) 26.

3 Gao L, Lu GT, Lu YY, Xiao WM, Mao WJ, Tong ZH, Yang N, Li BQ, Yang Q, Ding YB & Li WQ, Diabetes aggravates acute pancreatitis possibly via activation of NLRP3 inflammasome in db/db mice. *Am J Transl Res*, 10 (2018) 2015.

4 Ozaki E, Campbell M & Doyle SL, Targeting the NLRP3 inflammasome in chronic inflammatory diseases: current perspectives. *J Inflamm Res*, 8 (2015) 15.

5 Chakraborty M, Bhattacharya S, Bose M, Sasmal SK, Gupta M & Mishra R, Anti-inflammatory activity of cyclo-oxygenase2 inhibitory anionic protein fraction from *Lamellidens marginalis* (Lamarck). *Indian J Exp Biol*, 55 (2017) 88.

6 Tuorkey MJ, Molecular targets of luteolin in cancer. *Eur J Cancer Prev*, 25 (2016) 65.

7 Chen PY, Tien HJ, Chen SF, Horng CT, Tang HL, Jung HL, Wu MJ & Yen JH, Response of myeloid leukemia cells to luteolin is modulated by differentially expressed pituitary tumor-transforming gene 1 (PTTG1) oncoprotein. *Int J Mol Sci*, 19 (2018) 1173.

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

9 Moran MS, Depierre JW & Mannervik B, Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochem Biophys Acta*, 582 (1979) 67.

10 Flohe L & Gunzler WA, Assays of glutathione peroxidase. *Methods Enzymol*, 105 (1984) 114.

11 Kakkar P, Das B & Viswanathan PN, A modified spectrophotometric assay of superoxide dismutase. *Indian J Biochem Biophy*, 21 (1984) 130.

12 Aebi H, Catalase. *Methods Enzymol*, 105 (1984) 121.

13 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72 (1976) 248.

14 Draper HH & Hadley M, Malondialdehyde determination as index of lipid peroxidation. *Methods Enzymol*, 186 (1990) 421.

15 Miyazawa T, Determination of phosphorlipidhydroperoxides in human blood plasma by a chemiluminescence- HPLC assay. *Free Radiac Biol Med*, 7 (1989) 209.

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

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

18 Mayerle J, Sendler M & Lerch MM, Secretagogue (caerulein) induced pancreatitis in rodents. In: *The Pancreapedia: Exocrine Pancreas Knowledge Base*, (Williams JA; American Pancreatic Association, USA), 2013. DOI: 10.3998/panc.2013.2.

19 Ahmadi A, Nikkhoo B, Mokarizadeh A, Rahmani MR, Fakhari S, Mohammaadi M & Jalili A, An optimised mouse model of chronic pancreatitis with a combination of ethanol and cerulein. *Cent Eur J Immunol*, 41 (2016) 54.

20 Apte MV, Pirola RC & Wilson JS, Alcohol and the Pancreas. In: *The Pancreapedia: Exocrine Pancreas Knowledge Base*, (Williams JA; American Pancreatic Association, USA), 2016. DOI: 10.3998/panc.2016.17.

21 Matsuda A, Makino N, Tozawa T, Shirahata N, Honda T, Ikeda Y, Sato H, Ito M, Kakizaki Y, Akamatsu M, Ueno Y & Kawata S, Pancreatic fat accumulation, fibrosis, and acinar cell injury in the zucker diabetic fatty rat fed a chronic high-fat diet. *Pancreas*, 43 (2014) 735.

22 Paradkar PH, Mishra LS, Joshi JV, Dandekar SP, Vaidya RA & Vaidya AB, *In vitro* macrophage activation: A technique for screening anti-inflammatory, immunomodulatory and anticancer activity of phytomolecules *Indian J Exp Biol*, 55 (2017) 133.

23 Kast RE, Ritonavir and disulfiram may be synergistic in lowering active interleukin-18 levels in acute pancreatitis, and thereby hasten recovery. *JOP*, 9 (2008) 350.

24 Ueda T, Takeyama Y, Yasuda T, Matsumura N, Sawa H, Nakajima T, Ajiki T, Fujino Y, Suzuki Y & Kuroda Y, Significant elevation of serum interleukin-18 levels in patients with acute pancreatitis. *J Gastroenterol*, 41 (2006) 158.

25 Wereszczynska-Siemiatkowska U, Mroczko B & Siemiatkowski A, Serum profiles of interleukin-18 in different severity forms of human acute pancreatitis. *Scand J Gastroenterol*, 37 (2002) 1097.

26 Schneider A, Haas SL, Hildenbrand R, Siegmund S, Reinhard I, Nakovics H, Singer MV & Feick P, Enhanced expression of interleukin-18 in serum and pancreas of patients with chronic pancreatitis. *World J Gastroenterol*, 12 (2006) 6507.

27 Pandey D, Joshi A, Mishra S, Sairam K & Hemalatha S, Antinociceptive, anti-inflammatory and antiarthritic activity of ethanol root extract and fraction of *Aganosma dichotoma* (Roth) K. Schum. *Indian J Exp Biol*, 56 (2018) 646.

28 Chen X, Lai Y, Song X, Wu J, Wang L, Zhang H, Liu Z, & Wang Y, Polysaccharides from *Citrus grandis* associate with luteolin relieves chronic pharyngitis by anti-inflammatory via suppressing NF-κB pathway and the polarization of M1 macrophages. *Int J Immunopathol Pharmacol*, 32 (2018) 2058738418780593. DOI: 10.1177/2058738418780593.

29 Zhang XH, Zhu RM, Xu WA, Wan HJ & Lu H, Therapeutic effects of caspase-1 inhibitors on acute lung injury in experimental severe acute pancreatitis. *World J Gasteroenterol*, 13 (2007) 623.

30 White JB, Beckford J, Yadegarynia S, Ngo N, Lialiutska T & Marc d'Alarcao, Some natural flavonoids are competitive inhibitors of Caspase−1, −3 and −7 despite their cellular toxicity. *Food Chem*, 131 (2012) 1453.

31 Mahanti S, Majhi A, Mukherjee K & Bishayi B, Reduced acetylcholinesterase activity downregulates peripheral and central inflammation during glucocorticoid resistance induced by chronic restraint stress and systemic lipopolysaccharide challenge in male mice. *Indian J Exp Biol*, 56 (2018) 859.

32 Saha MR, Dey P, Chaudhuri TK, Goyal AK, Sarker DD & Sen A, Assessment of haemolytic, cytotoxic and free radical scavenging activities of an underutilized fruit, *Baccaurea ramiflora* Lour.(Roxb.) Muell. Arg. *Indian J Exp* Biol, 54 (2016) 115.

33 Preethi KC & Kuttan R, Hepato and reno protective action of *Calendula officinalis* L. flower extract. *Indian J Exp Biol*, 47 (2009) 163.

34 Lai HY, Lim YY & Kim KH, *Blechnum orientale* Linn-a fern with potential as antioxidant, anticancer and antibacterial agent. *BMC Complement Altern Med*, 10 (2010) 15.

35 Ashokkumar P & Sudhandiran G, Protective role of luteolin on the status of lipid peroxidation and antioxidant defense against azoxymethane-induced experimental colon carcinogenesis. *Biomed Pharmacother*, 62 (2008) 590.

36 Oktay S, Turkyilmaz IB, Kaya S, Akyuz S, Yanardag R & Yarat A, Effects of combined treatment of amiodarone and vitamin U on rat gingiva. *Indian J Exp Biol*, 56 (2018) 759.

37 Grankvist K, Marklund SL & Taljedal IB, CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. *Biochem J*, 199 (1981) 393.

38 Tiedge M, Lortz S, Drinkgern J & Lenzen S, Relation between antioxidant enzyme expression and antioxidative defense status of insulin-producing cells. *Diabetes*, 46 (1997) 1733.

39 Mohan MSG, Ramakrishnan T, Mani V, & Achary A, Protective effect of crude sulphated polysaccharide from *Turbinaria ornata* on isoniazid rifampicin induced hepatotoxicity and oxidative stress in the liver, kidney and brain of adult Swiss albino rats. *Indian J Biochem Biophys*, 55 (2018) 237.

40 El-Ashmawy NE, Khedr NF, El-Bahrawy HA & Hamada OB, Anti-inflammatory and antioxidant effects of captopril compared to methylprednisolone in l-Arginine-induced acute pancreatitis. *Dig Dis Sci*, 63 (2018) 1497.

41 Perez S, Pereda J, Sabater L & Sastre J, Redox signaling in acute pancreatitis. *Redox Biol*, 5 (2015) 1.

42 Jamieson D, Chance B, Cadenas E & Boveris A, The relation of free radical production to hyperoxia. *Ann Rev Physiol*, 48 (1986) 703.

43 Fattman CL, Schaefer LM & Oury TD, Extracellular superoxide dismutase in biology and medicine. *Free Radic Biol Med*, 35 (2003) 236.

44 Li YC, Yeh CH, Yang ML & Kuan YH, Luteolin suppresses inflammatory mediator expression by blocking the Akt/NFkB pathway in acute lung injury induced by lipopolysaccharide in mice. *Evid Based Complement Alternat Med*, 2012 (2012) 1.

45 Rice-Evans CA, Miller NJ & Paganga G, Antioxidant properties of phenolic compounds. *Trends Plant Sci*, 2 (1997) 152.

46 Igile GO, Oleszek W, Jurzysta M, Burda S, Fafunso M & Fasanmade AA, Flavonoids from *Vernonia amygdalina* and their antioxidant activities. *J Agric Food Chem*, 42 (1994) 2445.

47 Lipinski CA, Lombardo F, Dominy BW & Feeney PJ, Experimental and computational approaches to estimate solubility and permeability in drug discovery and developmental settings. *Adv Drug Delv Rev*, 23 (1997) 3.

48 Tambunan USF & Wulandari EK, *BMC Bioinform*, 11 (2010) S16.

49 Veber DF, Johnson SR, Cheng HY, Smith BR, Ward KW & Kopple KD, Molecular properties that influence the oral bioavailability of drug candidates. *J Med Chem*, 45 (2002) 2615.

50 Leeson PD & Davis AM, Time related differences in the physical property profiles of oral drugs. *J Med Chem*, 47 (2004) 6338.