Evaluation of Granny smith Apple Extract on L-arginine induced exocrine pancreatic dysfunction in rats

GADICHERLA VEENA¹, Siva Reddy Challa², Sujatha Palatheeya³, Ramakrishna Prudhivi⁴, Anitha Kadari¹
¹Department of Pharmacology, Sri Indu Institute of Pharmacy, Sheriguda, Ibrahimpatnam - 501510, R.R.Dist. Telangana State, India
²Department of Pharmacology, KVSR Siddhartha college of Pharmaceutical Sciences, Vijayawada, Krishna Dist., Andhra Pradesh, India
³Department of Pharmacy, University college of Pharmaceutical Sciences, Palamuru University, Mahabubnagar -509001, Telangana State, India
⁴Department of Pharmacy Practice, Dayananda Sagar College of Pharmaceutical Sciences, Dayananda Sagar University, Bangalore-560078, Karnataka

Corresponding Author
GADICHERLA VEENA
anugad@gmail.com
orcid.org/0000-0002-6715-9433
+919000207043
24.02.2020
30.05.2020

ABSTRACT
INTRODUCTION: Granny smith is a cultivated hybrid variety of apple with high amounts of antioxidant value among all the other species of apples. Acute pancreatitis is a instantly emerging inflammatory condition with high mortality rate. The treatment preference is restricted to symptomatic intrusion and supportive care. The present study was conceded out to evaluate the favorable effects of granny smith apple extract as a prophylactic treatment in L-arginine induced acute pancreatitis in rats.

METHODS: Male Sprague Dawley Rats were divided in to five groups (n=6): Normal control (saline), Disease control (a single dose of L-arginine 2.5g/kg I.P), Positive control (Melatonin 10 mg/kg I.P), and Granny smith apple fruit extract (GSAE) –I and II (200 mg/kg and 400 mg/kg, orally) respectively for 7 days. At the end of the study, the blood samples were collected by retro-orbital plexus, serum separated and were subjected to estimation of Biomarker enzymes like amylase, Lipase, antioxidant enzymes etc. The animals were then sacrificed; pancreas isolated and subjected for estimation of tissue biomarkers, DNA fragmentation assay and Histopathological studies

RESULTS: Serum levels of amylase and lipase were significantly (p<0.001) reduced in L-arginine treated rats. Similar results were also observed with tissue inflammatory markers like Malondialdehyde, Nitrate etc. there was a dramatic increase (p<0.001) in the overall antioxidant enzyme levels when compared to disease control rats. Histopathological examination of pancreatic tissue showed an intact structural feature of acinar cells in the extract treated group of rats which was further in pact with the intact DNA found in DNA Fragmentation Assay.

DISCUSSION AND CONCLUSION: Thus, GSAE treatment was found to be beneficial in
lowering the inflammatory conditions of acute pancreatitis by improving the antioxidant levels overall and a further investigation for its exact molecular mechanism is needed.

**Keywords:** Granny smith apple, L-arginine, free radicals, Pancreatitis

**INTRODUCTION:**
Acute Pancreatitis (AP) is a provocative condition with a spike in incidence of the disease worldwide.\(^1\) It is an inflammatory condition of exocrine pancreatic tissue which is a mild, self-limiting form, caused by activation of stress signals and disparity of protective mechanism in the pancreatic tissue. The initial symptoms include persistent and recurring epigastric pain, nausea, vomiting, weight loss, fever, chills and shock; which not diagnosed or treated may eventually lead to severe acute pancreatitis, where the pancreatic tissue is displaced by fibrotic cells and association of additional organ manifestations leads to mortality.\(^2\)

Almost 80% of reported pancreatic cases have familial incidence\(^3\) and the major concern for mortality include cardiovascular and respiratory collapse.\(^4\) No specific therapies have been reported for the management of AP and is primarily based on supportive care to prevent Hypoxemia, fluid resuscitation and a critical component of the disease, malnutrition. Despite of many explorations and modalities, the pathogenesis of acute pancreatitis still remains vague. The most widely accepted hypothesis is the involvement of oxidative stress and premature activation of zymogens, followed by auto digestion of the tissue and successive activation of local and systemic inflammation.\(^5\) The autodigestive process of acinar cells stimulates an inflammatory response (neutrophils and macrophages infiltration, release of cytokines, interleukins 1, 6, 8 and other inflammatory mediators) within the pancreatic parenchyma.\(^2\)

Based up on the above proposition, a number of experimental animal studies have suggested the administration of antioxidant compounds which depleted the reactive oxygen species and showed a beneficial effect in the treatment of AP.\(^6\) Many medicinal plants have portrayed their favorable outcomes in the management of the disease course.\(^7\) Suggested mechanisms in prevention include presence of phenolics and other phytochemical antioxidants in eradication and neutralization of free radicals.\(^8\)

Several epidemiological studies suggest the increased dietary intake of fruits and vegetables is associated with a decreased risk of disease incidence.\(^9\) One of the well known and most produced fruit worldwide is Apple (*Malus domestica*). Apples were found to have a significant part in the diet with varied intake style. They belong to family of *Rosaceae* with a considerable amount of fiber, pectin, potassium and Vitamin A and C. They also contain different classes of phenolics such as flavonols, dihydro chalcones, p-hydroxy benzoic acid etc. Many different varieties of apples are available and our interest of the study focused on Granny smith apple. Granny smith apple is a hybrid variety of *Malus domestica* and *Malus sylvesterus* propagated by Maria Smith in Australia and thus the name. It is reported to have the second highest amount of flavanoids and procyanidins\(^10\) among all the varieties of apples. The fruits are crisp, tart, and juicy with light green hard skin and long storage life. The lesser amount of Ethylene production helps in their easier preservation, when compared to other apple varieties.\(^11\) Granny smith apple is the most preferred fruit in healthy weight
loss regimens because of their rich dietary fiber, potassium and few calories. The objective of the current study is to evaluate the shielding potential of Granny smith apple extract on experimentally induced acute pancreatitis by L-arginine and its potential in overcoming the malnutrition.

**METHODOLOGY:**

**Chemicals:**
Sodium phosphate buffer, Sodium chloride, Starch, Chloroform, Ethanol, O-dianisidine, Phosphate buffer, Ethanol, Hydrogen peroxide, Potassium dihydrogen phosphate, TCA-trichloro acetic acid, Dinitro phenyl hydrazine (DNPH) reagent, Butanol, EDTA (Ethylene diamine tetra acetic acid), pyridine, Potassium chloride, Sodium dodecyl sulphate (SDS), Thiobarbituric acid (TBA), DTNB (5,5-dithio bis 2-nitrobenzoic acid), Thiourea, N-(1-Naphthyl) ethylene diamide dihydrochloride, formaldehyde, were purchased from Sigma-Aldrich Inc. Mumbai, India. β-NADH, HTAB (hexadecyltrimethylammonium bromide), sodium pyruvate were purchased from SD Fine ltd. Mumbai, India. Commerical kits for estimation of Amylase, C - reactive protein from Akray health care Pvt ltd, Hyderabad, India and Lipase kit from Aggape diagnostics Pvt ltd. Hyderabad, India. DNA isolation kit from Bioartis Pvt ltd. Hyderabad, India. L-arginine and Melatonin were purchased from Sigma Aldrich Pvt Ltd. Mumbai, India.

**Plant material and extraction:**
Fresh Granny smith apples were purchased in the month of March 2017, from the local market in Hyderabad, India. The samples were authenticated for their variety with voucher no.1002 by Department of Botany, S.V. University, Andhra Pradesh, India. The core of the apples was removed, blended to obtain fresh juice with pulp. The obtained pulp is subjected to extraction with 95% ethanol in an orbital shaker for 24 hrs. The extracts were filtered and evaporated to dryness with the help of rotary evaporator. The resultant dry extracts were stored at cool temperature and re-suspended in normal saline just before the use.

**Animals:**
In the present study, Male Sprague Dawley rats were chosen with a body weight range of 150-200 g. They were housed in pathogen free polypropylene cages and were acclimated for one week before the onset of the study. The temperature was maintained at 25°C±1°C with 55% humidity. Rats were controlled constantly with a 12 h light/dark cycle and were given standard pellet diet with water ad libitum. All the experiments carried out were approved by Institutional Animal ethics committee (1448/PO/Re/S/11/CPCSEA/07/2016) as per CPCSEA guidelines.

**Acute toxicity studies:**
The acute toxicity study was conducted in according to fixed dose method of OECD guideline 420. Granny smith apple extracts [GSAE] was administered orally by dissolving it in normal saline as per the prescribed doses mentioned in the guidelines and the observation was done for the next 14 days. The test extracts did not manifest any significant abnormal signs, behavioral changes, changes in body weight or macroscopic findings at any time of observation period. At the end of the study period, no Mortality or lethality was observed and hence, the LD50 was found to be above 2000
mg/kg. Thus 1/10th and 1/5th of the LD50 dose was selected for the present study.

**Preparation of L-arginine solution:**
20% L-Arginine solution was prepared in normal saline and pH was adjusted to 7.0. The solution is filtered through a syringe tube filter in a tissue culture hood before the administration in rats at a dose of 2.5g/Kg body weight. The prepared solution is used for the induction of acute pancreatitis and was administered intraperitoneally.

**Study protocol:**
The present study involved randomization of animals into five groups with 6 animals each. Group I was considered as control group received normal saline p.o. daily for 7 days. Group II, Disease control group received a single dose of L-arginine solution freshly prepared with a dose of 2.5g/kg b.w on day 5 of the experiment. Group III, a Positive control group received Melatonin at a dose of 10mg/kg I.P for 7 days. Group IV and V received a administration of GSAE orally with a dose of 200mg/kg and 400mg/kg respectively. The Groups III, IV and V were induced with Acute Pancreatitis by a single dose administration of L-arginine (2.5 g/kg b.w) at an interval of 1 hr after administration of the extracts on day % of the study. At the end of the experimental period, the animals were anesthetized under light ether and blood samples were obtained from retro-orbital plexus. Further, these samples were used for evaluation of pancreatic, inflammatory and antioxidant biomarker enzymes. The rats were then decapitated for the isolation of pancreas. The isolated pancreas was divided into parts for estimation of tissue inflammatory markers, DNA fragmentation assay and Histopathological investigation accordingly.

**Estimation of Biomarker enzymes:**

**Estimation of Amylase:**
Serum amylase was estimated by commercial Kit manufactured by Akray healthcare pvt. Ltd. Briefly, 1000µl of Amylase Mono reagent was mixed with 20µl of serum and incubated for 60 seconds. The absorbance was read at 405nm. Amylase activity was reported in U/L, where one unit was described as the amount of amylase required to generate 1µmole of p-nitrophenol per minute at 25°C

**Estimation of Lipase:**
Serum Lipase was analyzed by using the commercial kit from Aggape diagnostics ltd. According to manufactures instructions, 1000µl of Reagent 1 was mixed with 20µl of serum and incubated at 37°C for 5 minutes. To this mixture 250µl of reagent 2 is added and kept at room temperature for 2 minutes. The resultant mixture is read absorbance at 580 nm. Lipase activity was reported in U/L where one unit was described as the amount of Lipase required to generate 1 µmole of methylresorufin at 37°C

**Estimation of Superoxide Dismutase (SOD):**
Cold water and chloroform / ethanol mixture (15:1 ratio) were added to equal quantity of packed cells and was centrifuged at 2000rpm for 20 minutes. 0.1ml of supernatant was separated and 0.88ml of riboflavin, 60 μL of O-dianisidine was added. The absorbance was measured at 460nm. The amount
of SOD activity was calculated against a standard curve and was expressed in mg/protein/min.

Estimation of Glutathione:
A homogenate of the tissue was prepared by using 0.1M phosphate buffer. To the homogenate 20% TCA and 1mM of EDTA were added in equal volumes and were allowed to stand for 5 min, which was then centrifuged at 2000rpm for 10 min. 200 µL of the supernatant was separated and 1.5ml of DTNB reagent was added. The absorbance was measured at 412nm. The values obtained were articulated as mg/dl which was calculated against a standard curve and the amount of Glutathione reduced is directly proportional to the production of 5-thio 2-nitrobenzoic acid from DTNB.

Estimation of Catalase:
To 0.1 ml of serum, 2.5 ml of phosphate buffer was added and the mixture was incubated for 30 min at 25°C. The blend was transferred into a cuvette and to initiate the reaction 650µL of Hydrogen peroxide solution was added. The alteration in absorbance was measured at 240nm for 3 min. The amount of catalase activity was expressed in µM of H₂O₂/mg/protein/minute; Which is calculated against the total amount of protein lysed by the enzyme to degrade one µM of hydrogen peroxide per min.

Estimation of Vitamin C:
The plasma of the blood was separated and to a volume of 0.5 ml to it 0.6% of TCA of volume 1.5 ml was added. The mixture was centrifuged for 20 min at 3500rpm. The clear supernatant was collected and to it equal volume of DNPH reagent (2% DNPH and 4% thiourea in 9N H₂SO₄) was added and incubated for 30 min at room temperature. The resultant mixture is now read at an absorbance of 530nm. The amount of Vitamin C is calculated against the standard curve obtained by taking ascorbic acid as reference standard and was expressed as mg/dl.

Estimation of Lactate dehydrogenase (LDH):
To 0.1 ml of tissue homogenate or serum, 3 ml of LDH reagent (2.8ml of 0.13mM β-NADH and 0.1ml of 34mM sodium pyruvate) was added and incubate at 37°C for 5 minutes. The absorbance of the mixture was tested at 340nm for every minute for 3 minutes. ΔA activity was measured and represented as U/L. The LDH activity was expressed as U/L where one Unit was articulated as the reduction of 1µmole of pyruvate to L-Lactate in One minute at 37°C and pH 7.5.

Estimation of Myeloperoxidase (MPO):
Pancreatic Myeloperoxidase was estimated according to Bradley et al., 1982. The homogenized tissue was suspended in a mixture containing 50mM phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide (HTAB) and sonicated in ice bath for 10 minutes. The suspensions are freeze thawed 3 times and the resultant mixture was centrifuged at 40,000 X g for 15 min. the pellet thus obtained is mixed with 2.9 ml of 50mM phosphate buffer (pH 6.0) containing 0.167 mg/ml of O-dianisidine
hydrochloride and 0.0005% of Hydrogen peroxide and checked for absorbance at 460nm. One unit of MPO was defined as that degrading 1 μmol of H₂O₂ per minute and the activity is expressed as Units/mg of protein.23

Estimation of Malondialdehyde (MDA):
The levels of MDA was estimated using 0.4 ml of tissue homogenate to which a reaction mixture of 1.5 ml, containing TBA(0.8%), acetic acid (20%) and distilled water was added and incubated for 1 hour at 95°C in a water bath. Following incubation, the mixture was cooled and to it 5ml of butanol: pyridine mixture (15:1) was added and centrifuged at 3000rpm for 10 minutes. The clear supernatant was collected and checked for absorbance at 532 nm against blank containing butanol: pyridine mixture. The quantity of MDA was calculated by a standard graph preparation using 1,1,3,3-tetramethoxypropane in concentration range of 1-10 nmol in 1 ml distilled water. The results are articulated as nmol of MDA/mg protein.24

Tissue nitrite levels:
The tissue nitrite levels were estimated according to Laura et al., 1982 with slight modifications. Briefly, the homogenized pancreatic tissue is centrifuged at 11000g for 15 min at 4°C. The obtained supernatant (100μl) is mixed with 100μl of Griess reagent and incubated at room temperature for 10 min. The absorbance was checked at 540nm. The standardization of Nitrite levels was done using Sodium Nitrite and the results obtained was expressed as micromoles of Nitrate/nitrite.25

Estimation of C - Reactive Protein (CRP):
Serum CRP levels were estimated by using commercial kit from Akray healthcare ltd. The quantitative estimation was done by preparing a series of dilutions of the test serum in normal saline (eg: 1:2, 1:4, 1:8 etc) and to which one drop of CRP latex reagent is added. The formation of agglutination on the glass slide was taken as the highest titer for CRP and represented as factor of 6 with units in micrograms /ml.

DNA fragmentation Assay:
The DNA fragmentation assay was performed by Alexi and James method on Agarose gel electrophoresis. Briefly, the DNA from the pancreatic tissue was isolated by using the kit by Bioartis pvt ltd. The isolated DNA pellet is air dried and resuspended in TE (Tris-EDTA) buffer [pH-8.0] and 1mM EDTA. The resuspended DNA is loaded on to the Agarose Gel electrophoresis for analysis.26

Histopathological studies:
The pancreas which was isolated were fixed in formalin and subjected to histopathological studies. The pancreatic tissues were washed and fixed in paraffin which were sliced in 5 µm sections and stained with hematoxylin and eosin and then evaluated under microscope with dark field background changes in the pancreatic tissue. The injury of the pancreatic tissue was reviewed for degeneration of acinar cells, edema, interstitial inflammation and hemorrhage.
Statistical analysis:
All the results are articulated as Mean ± SEM. The data statistical Analysis was performed by one way ANOVA followed by Dunnett’s Multiple Comparison Test using Graph pad Prism 5 software with statistically significance up to p < 0.001.

RESULTS:
Effect of GSAE on AP induced enzyme production:
To examine the effect of GSAE on the development and severity of AP, rats were pretreated with GSAE [200 mg/kg and 400 mg/kg] to the respective groups as described in the experimental design. Few hours after induction of AP, rats in control and positive control group were still active; whereas rats in Disease control group were lethargic, with decreased motor activity, reduced reflex action and reduced intake of food and water. The rats in disease control group showed a significant increase [p<0.001] in the levels of Amylase when compared to the control group of rats. Rats treated with melatonin showed marked reduced levels of serum amylase, revealing its protective actions. Rats pretreated with GSAE [200 mg/kg and 400 mg/kg b.w] showed a significant decrease [p<0.001] dose dependently when compared to L-arginine control group of rats [Table no: 1]. Similar changes were also observed for serum Lipase levels. Rats of Disease control group showed a significant increase [p<0.001] in lipase levels when compared to control group; whereas the Melatonin and GSAE treated rats showed a dose dependent significant decrease [p<0.001] in the lipase enzyme levels when compared to L-arginine control group of rats [Table no: 1].

Effect of GSAE on antioxidant enzyme in AP:
There was a significant decrease [p<0.001] in the SOD, Catalase, Glutathione and Vitamin C levels in rats of Disease control group when compared to control group. In contrast, the levels were significantly increased in rats treated with Melatonin and GSAE group of rats [p<0.001] when compared to Disease control group and no significant change in GSAE group of rats when compared to control group of rats [Table no: 2].

Effect of GSAE on other inflammatory mediators in AP:
Treatment with L-arginine increased the levels of Nitrate, MDA, LDH, CRP and MPO significantly [p<0.001] while compared against positive control and control group of rats indicating the incidence of pancreatic damage and inflammation. In contrast, melatonin and GSAE treatment reduced the levels of Nitrate, MDA, LDH, CRP and MPO significantly (p<0.001) in dose dependent manner. However the levels were more in GSAE group of rats when compared to control group [Table no.3].

Histopathological studies:
The pancreas which was isolated was then subjected to histopathological study using Hematoxylin and Eosin stain. Normal architecture of pancreas were seen in the Control group of rats [Figure.1 (A)], where L-arginine treatment showed inflammatory changes with vacuolar degeneration and extensive damage to acinar cells [Figure.1 (B (i) & (ii)]. Positive control group treated with Melatonin showed normal architecture [Figure.1 (C)].Treatment with GSAE showed reduced inflammatory changes with no degeneration and maintenance of normal structural design [Figure.1 (D & E)].
DNA Fragmentation Assay:
One of the major criterions for fragmentation of DNA is apoptosis. In the present study, Control group of rats showed an intact DNA [Figure.2 (A)] when compared to L-arginine control group, where a smear pattern was observed, representing extensive damage to DNA [Figure.2 (B)]. While Melatonin, GSAE treated group of rats showed an intact DNA representing its protective effect against the damage [Figure. 2 (C, D & E)].

DISCUSSION:
The results of the study specified a shielding effect of GSAE on experimentally induced acute pancreatitis in rats by L-arginine. Administration of GSAE exhibited beneficial effects by reducing oxidative and Nitrosative stress and modulating the inflammatory process.
L-arginine, an essential amino acid was used for induction of AP in the present study, which is reported as a highly reproducible, noninvasive model of AP that produces dose dependent acinar necrosis. Further, incessant administration of L-arginine for an extended period may also induce chronic pancreatitis.
The chief indicative markers for diagnosis of acute pancreatitis include serum amylase and lipase due to their direct release into the circulation that is accredited to the enzymatic activation in pancreatic acinar cells. After the initial attack the levels of these enzymes usually ascend within 4-8 hours and peaks at 24 hours. The increased Serum Lipase levels is considered as a more reliable marker than amylase. In the present study, rats treated with L-arginine showed a significant elevation of serum amylase and lipase levels which was observed as acinar cell necrosis (devoid of changes in the Islets of langerhans) as observed in the histopathological study; stating the development of acute pancreatitis, which is in accordance with the previous reports. In comparison to its treatment with GSAE declined the elevated levels of lipase and amylase significantly in a dose dependent manner. The reduction of enzyme levels by GSAE is consistent with previously reported in-vitro study on inhibition of lipase, α-amylase and α-glucosidase enzymes. The results of GSAE extract groups are synchronous with that of positive control group treated with melatonin that also reduced the elevated levels of amylase and lipase radically which is in accordace with the previous reports.
The pathogenesis of AP implicated the generation of free radicals and activation of inflammatory mediators which contributed to the unfavorable effects. Oxidative stress provoked lipid peroxidation and altered Glutathione metabolism has been reported to take place at early course of the disease. The rate of MDA production directly signifies the lipid membrane peroxidation which indirectly reflects the association of free radical generation in mitochondria. On the other hand, MPO in the circulation indicate its release from activated neutrophils portraying dominant pro-inflammatory properties. Additionally, and severity and stage of acute pancreatitis show a relationship with the levels of Blood MPO. Further, MPO levels also correlate with CRP levels. Many studies reported that these events were neutralized by administration of Antioxidants with a favorable effect in
reduction of reactive oxygen species. \textsuperscript{36} Melatonin, a renowned antioxidant, lowered the levels of MDA, MPO and CRP which is in agreement with previous studies.\textsuperscript{31} Similar effects were observed with that of GSAE administration where the elevated levels of MDA, MPO and CRP was reduced dose dependently, owing to the eradication of reactive oxygen species and halt the lipid peroxidation process, inflammatory cytokine release; which has also been observed as a decreased amount of acinar cell damage in histopathological studies of the pancreatic tissue. Granny smith has been reported as antioxidant rich fruit among all the varieties of apple. The presence of two major metabolite compounds Rutin and Catechin strengthen the antioxidative properties of the fruit.\textsuperscript{37} The presence of these flavonoidal compounds along with other compounds such as Quercetin, Procyanidins have contributed to total antioxidant capacity in many of the plant products.\textsuperscript{38,39} Further, granny smith peels have demonstrated the antioxidant capacity against Total oxy-radical scavenging assay and inhibition of HepG2 cell proliferation.\textsuperscript{40} 

LDH have been reported to be present in tissues with hypoxia conditions which eventually is a major event of inflammatory process. \textsuperscript{41} Increase LDH levels were observed in L-arginine group of rats and the contrast was observed in Melatonin and GSAE treated rats dose dependently. Damage by Oxidative and nitrosative stress is restricted by antioxidant resistance enzymes like SOD, Glutathione, Catalase and Vitamin C. L-arginine treated rats showed decreased levels of SOD, Glutathione, Catalase and Vitamin C indicating the involvement of Reactive oxygen species.\textsuperscript{42} Melatonin and GSAE administration increased the levels of SOD, Glutathione, Catalase and Vitamin C, suggesting its protective effects by modulating the defense mechanisms. The favorable effects of GSAE could be due to its antioxidant activity which is in accordance with the \textit{in-vitro} antioxidant capacity reported by Tzanakis et al., 2006 and Saxena et al., 2016.\textsuperscript{43,44} In addition to it, there was also an increase in the levels of Nitrite stating the involvement of released iNOS (inducible Nitric Oxide synthase) from inflammatory cytokines i.e. the release of iNOS is directly proportional to Nitrite levels in the plasma. Nitric Oxide and its metabolic products have a key role in the inflammatory processes.\textsuperscript{45} Nitric oxide combines with superoxide to form peroxynitrite, a highly reactive oxidant that damages the cell by lipid membrane and sulfhydryl oxidation.\textsuperscript{46} Administration of antioxidants deprived the release of Nitric oxide indirectly indicating its beneficial effect on Nitrosative stress.\textsuperscript{36} GSAE administration reduced the levels of nitrite dose dependently. Granny smith apple has been reported as among the richest polyphenol apple. Procyanidin B2, Catechin, flavonols, quercetin and Vitamin C are the components reported in the whole fruit.\textsuperscript{47} The presence of these polyphenolic compounds have been ascribed for the antioxidant activity of granny smith apple.\textsuperscript{47} Also, Lotito and Frei, 2004 reported the antioxidant capacity of apple polyphenolics in human plasma with their favorable effects in prevention of many diseases.\textsuperscript{48} These facts suggest the eradication of reactive oxygen and nitrogen species in protecting acute pancreatitis. Furthermore, Phenolics have been reported to restrain the Nitric oxide induced proinflammatory reaction by obstructing the levels of expression of iNOS dose dependently.\textsuperscript{49}
Accumulating evidence suggest that apoptosis play a significant role in relation to severity of AP. The Balance between apoptosis and necrosis play a key role in defending mechanism in acute pancreatitis and resolve the severity of the disease. DNA fragmentation is considered as the universal criterion for the detection of apoptosis. Mervi et al, 2006 suggested polyamine synthesis inhibition have effect on synthesis of protein; in turn nucleic acid synthesis is restricted. Further, catabolism of proteins is highly active in acinar cells of pancreas and it is more probable the overdose of L-arginine induced necrosis or degradation affect these cells initially. Mitochondrial damage initiates the process of apoptosis as a consequence of high calcium loads. The cellular damage in pancreatitis are mostly associated with damage to mitochondria along with succession of the disease in most of the animal models and in Humans, which is in accordance with the results of the current study; where daub pattern of DNA was observed in L-arginine treated disease control rats. Oxidative stress and protein oxidation in the cells could cause abnormal cross linking and cleavage of DNA leading to death of the cell. Administration of Melatonin and GSEA showed an intact DNA signifying the protective effects against the stress mediated damage in acinar cells.

CONCLUSION:
In conclusion, the current study suggested the beneficial effects of Granny smith apple extract on L-arginine induced acute pancreatitis by eradicating the stress markers and escalating the antioxidant status. The chemical constituents like polyphenols, flavonoidal compounds present in the granny smith apple endow to the favorable effects. Further investigations are required to evaluate the exact chemical constituent and their molecular mechanism of action on the disease profile.

SUMMARY:
The present study was carried out to evaluate the prophylactic action of granny smith extract on acute pancreatitis. The amount of damage to the pancreas with L-arginine administration was evidenced with increased levels of amylase, lipase, and reduction in the levels of antioxidative markers with increased levels of inflammatory markers. The Histopathological findings with acinar cell necrosis were also in sync with the biochemical markers. The administration with granny smith apple extract prophylactically revered the conditions by improving the antioxidant status and maintaining the normal echo texture of the pancreatic acinar cells. The shielding effect was pragmatic in a dose dependent manner. Further investigation for its molecular mechanism with extraction of each constituent is needed for its recognition of exact therapeutic potential.

Acknowledgements:
The authors are grateful to the Principal and Management of Sri Indu Institute of Pharmacy for providing facilities for carrying out the research work. Similarly, authors whole heartedly thank Principal of SSJ College of Pharmacy for her technical assistance provided in this research work.

Conflict of interest: Authors declare that there are no conflicts of interest to report
REFERENCES

30. Abdel-Gawad SK. Therapeutic and Protective Effect of Wheat Germ Oil on L-arginine Induced Acute Pancreatitis in Adult Albino Rats. J Cell Sci Ther, 2015; S8: S8-004
32. Oboh G, Omojokun OS, Oyeleye SI, Akinyemi AJ. Inhibition of α-amylase, α-glucosidase and oxidative stress by some common apple varieties.


47. Elzi beta Biedrzycka & Ryszard Amaro wicz. Diet and Health: Apple Polyphenols as Antioxidants, Food Rev Int, 2008; 24:2, 235-251


Table: 1 Changes in the Pancreatic biomarkers in rats treated with L-arginine and GSAE

<table>
<thead>
<tr>
<th>Groups</th>
<th>Amylase (IU/L)</th>
<th>Lipase (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>14.17 ± 0.12#</td>
<td>16.17 ± 0.98#</td>
</tr>
<tr>
<td>Disease control</td>
<td>330.5 ± 3.23*</td>
<td>83.17 ± 1.14*</td>
</tr>
<tr>
<td>Positive Control (Melatonin 10 mg/kg)</td>
<td>98.8 ± 2.24#</td>
<td>34.45 ± 1.46#</td>
</tr>
<tr>
<td>GSAE-I (200 mg/kg)</td>
<td>144 ± 1.57*</td>
<td>44.5 ± 1.21*</td>
</tr>
<tr>
<td>GSAE-II (400 mg/kg)</td>
<td>83.17 ± 1.54*#</td>
<td>37.5 ± 0.56*#</td>
</tr>
</tbody>
</table>

Values are expressed in Mean ± SEM (n=6); *, # p<0.001 when compared to Normal Control and Disease control group respectively

Table: 2 Changes in the Antioxidant biomarkers in rats treated with L-arginine and GSAE

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (mg/protein/min)</th>
<th>Catalase (µM of H2O2/mg protein/minute)</th>
<th>Glutathione (mg/dl)</th>
<th>Vit C (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>18.5 ± 0.43*</td>
<td>48.18 ± 0.31#</td>
<td>2236 ± 0.96#</td>
<td>95.73 ± 0.35#</td>
</tr>
<tr>
<td>Disease control</td>
<td>6.167 ± 0.31*</td>
<td>22.7 ± 0.29*</td>
<td>575.8 ± 1.50*</td>
<td>21.35 ± 0.58*</td>
</tr>
<tr>
<td>Positive Control (Melatonin 10 mg/kg)</td>
<td>51.23 ± 1.72##</td>
<td>47.14 ± 0.23#</td>
<td>3124 ± 3.55##</td>
<td>212.34 ± 0.68##</td>
</tr>
<tr>
<td>GSAE-I (200 mg/kg)</td>
<td>31.5 ± 0.34*##</td>
<td>42.08 ± 0.15#</td>
<td>2304 ± 0.88##</td>
<td>212.7 ± 0.70##</td>
</tr>
<tr>
<td>GSAE-II (400 mg/kg)</td>
<td>42.5 ± 0.43##</td>
<td>47.48 ± 1.16#</td>
<td>2999 ± 0.79##</td>
<td>228.8 ± 1.81##</td>
</tr>
</tbody>
</table>

Values are expressed in Mean ± SEM (n=6); *, # p<0.001 when compared to Normal Control and Disease control group respectively
Table 3: Changes in the inflammatory mediators in rats treated with L-arginine and GSAE

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (mM/dl/hr)</th>
<th>Nitrate (µM/g)</th>
<th>CRP (µg/ml)</th>
<th>MPO (µM of peroxide/min)</th>
<th>LDH (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>14.89± 0.34#</td>
<td>11.08 ± 0.21#</td>
<td>486.8± 2.37#</td>
<td>4.032± 0.06#</td>
<td>23.4± 0.54#</td>
</tr>
<tr>
<td>Disease control</td>
<td>86.87± 0.88*</td>
<td>35.05 ± 0.15*</td>
<td>19601± 2.46*</td>
<td>25.69± 0.33*</td>
<td>127.5± 0.39*</td>
</tr>
<tr>
<td>Positive Control (Melatonin 10 mg/kg)</td>
<td>35.56 ± 0.99*#</td>
<td>14.34 ± 0.88*#</td>
<td>697 ± 4.35*#</td>
<td>6.45± 0.46#</td>
<td>45.56± 0.90*#</td>
</tr>
<tr>
<td>GSAE-I (200 mg/kg)</td>
<td>37.3 ± 0.20*#</td>
<td>17.14 ± 0.19*#</td>
<td>1258 ± 3.57*#</td>
<td>10.44± 0.37*</td>
<td>52.57± 0.67*#</td>
</tr>
<tr>
<td>GSAE-II (400 mg/kg)</td>
<td>24.77 ± 0.36*#</td>
<td>13.67 ± 0.12*#</td>
<td>1021 ± 2.97*#</td>
<td>7.902± 0.28*</td>
<td>43.73± 1.28*#</td>
</tr>
</tbody>
</table>

Values are expressed in Mean ± SEM (n=6); *, # p<0.001 when compared to Normal Control and Disease control group respectively.
Figure 1: Histopathological Changes in Pancreas

A – Normal control group rats with no damage of acinar cells;
B (i) and B (ii) – Disease control group - L-arginine treated rats with vacuolar degeneration and extensive damage of the acinar cells with infiltration of leucocytes – red arrow;
C – Positive control Melatonin group of rats with normal architecture of acinar cells
D and E – GSAE treated rats showing mild damage to normal echo texture of acinar cells
Figure 2: Gel picture of DNA fragmentation assay of the pancreatic tissue.

A – Group I; Normal control pancreas

B- Group II; Disease control group Pancreas showing fragmented DNA in the form of smear indicating extensive damage

C – Group III: Positive control Melatonin treated group showing intact DNA

D, E – Group IV, V: GSAE treated pancreas showing intact DNA