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Gallic Acid-Loaded Microemulsion Combined with Low Doses of Radiation Ameliorates Chronic Pancreatitis in Rats via Modulation of the Nrf2-Keap1-HO-1 Pathway

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Abstract

Chronic pancreatitis (CP) is a complicated inflammatory disease with a significantly reduced quality of life and irreversible pancreatic damage. Gallic acid (GA), a common secondary polyphenol metabolite generated from plants, stimulated nuclear translocation of Nrf2 as well as the production of target proteins such as heme oxygenase-1 (HO-1). However, its usage is restricted due to its low water solubility, which results in poor bioavailability. So, in the current study, the microemulsions (MEs) were prepared based on polyvinylpyrrolidone polymer (PVP) as a carrier for GA to increase its miscibility and bioavailability. Also, characterization of polymer ME system was evaluated by using FTIR and DLS, in addition to studying its antimicrobial activity toward some types of bacteria. The objective of this research was to evaluate the effect of PVP/GA microemulsion prepared with clove oil essential oil (CEO) combined with low doses of radiation to ameliorate chronic pancreatitis in rats via modulation of the Nrf2/Keap1/HO-1 Pathway. 42 rats were randomly assigned to seven groups: control group, rats were received PVP/CEO/GA microemulsion (0.9 ml/100 g body weight) orally 5 days per week for 4 weeks, rats were exposed to low doses of radiation (0.25 Gy every week for 4 weeks), rats were received L-arginine 4 times by a dose of 250 mg/100 g body weight (2 doses separated by 1-hour interval), every week for 4 weeks to induce chronic pancreatitis, rats were received L-arginine & were received GA-loaded PVP/CEO microemulsion, rats were received L-arginine & were exposed to low doses of radiation, and rats were received L-arginine & were received GA-loaded PVP/CEO microemulsion & were exposed to low doses of radiation. L-arginine induced a marked increase in serum lipase, amylase, and lactate dehydrogenase (LDH) activities, MYD88, TLR4 gene expression, and IL-1β. While, a significant decrease in Nrf2, Keap1, and HO-1 was observed. GA-loaded PVP/Clove oil microemulsion significantly ameliorated the above-mentioned parameters and histopathological examination.

Keyword: L-arginine, PVP, Gallic acid, microemulsion, Nrf2-Keap1-HO-1, MYD88, TLR4.

INTRODUCTION

Pancreatitis is a pathologic pancreatic inflammation characterized by acinar cell impairment and oxidative damage. Chronic pancreatitis can occur as a result of repeated pancreatic injury **[i]**.

Chronic pancreatitis (CP) is usually classified as an irreversible pancreatic inflammatory syndrome that results in varied degrees of exocrine and endocrine dysfunction. CP is now defined as a pathologic fibroinflammatory syndrome of the pancreas in individuals with environmental, genetic, and/or other risk factors who generate chronic pathologic reactions to parenchymal damage or stress **[ii]**. Characterised by permanent pancreatic fibrosis and several secondary consequences, eventually leading to the loss of this vital organ **[i]**.

Gallic acid (GA), also known as 3,4,5-trihydroxybenzoic acid, is a naturally occurring secondary metabolite that has been identified from a variety of fruits, plants, and nuts **[iii]**. GA is a tri-phenolic molecule with a low molecular weight that has anti-inflammatory and antioxidant properties **[iv]**. It has excellent antioxidant and free radical scavenging properties and can protect biological cells, tissues, and organs from oxidative stress damage. It modulated a variety of signaling pathways via inflammatory cytokines and enzymic and non-enzymic antioxidants **[v]**. Furthermore, GA possesses anti-tumor, anti-bacterial, anti-diabetes, anti-obesity, anti-microbial, and anti-myocardial ischemia pharmacological properties **[vi]**. Furthermore, via imposing multiple mechanism pathways, GA has the ability to act as a radiosensitizer **[vii]**. Gallic acid (GA), a plant-derived common secondary polyphenol metabolite, has the potential to be a beneficial dietary supplement **[viii]**. In traditional medicine, the essential oil extracted from clove buds has been employed as a chemopreventive agent **[ix]**. Clove essential oil (CEO) exhibits antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, and anticancer effects **[x]**. Clove essential oil is a powerful antioxidant. The antioxidant activity of clove bud oil is primarily due to eugenol, which may operate via scavenging free radicals and chelating metal ions **[xi]**.

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The Nrf2-keap1 (nuclear factor erythroid-2-related factor 2-Kelch-like ECH-associated protein 1) signalling pathway serves as the master regulator of the inflammatory response, and using it to solve complicated issues is possible. Furthermore, the pathway's deregulation has been linked to a variety of diseases, including cancer, chronic illness, and ageing [xii]. By controlling the transcription of genes encoding hundreds of antioxidant and detoxifying enzymes, the Nrf2-keap1 signalling pathway is the primary regulator of the anti-inflammatory and antioxidant response [xiii]. The gene expression levels of heme oxygenase-1 (HO-1) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2), as well as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-B), were found as essential regulators of the antioxidant and inflammatory pathway. All of these alterations were restored to near-normal levels after co-treatment with gallic acid. Gallic acid, as a natural antioxidant, was found to affect the Nrf2 signaling pathway [xiv].

Microemulsion is a colloidal dispersion made up of an aqueous phase, an oil phase, a surfactant, and a co-surfactant in the proper ratios [xv]. Microemulsions have been used for a variety of pharmaceutical applications, including parenteral administration, oral administration, topical administration, ophthalmic administration, and pulmonary administration [xvi]. Microemulsions (MEs) are clear or translucent thermodynamically stable liquids composed mostly of oil, water, a surfactant, and usually a co-surfactant. Oil-in-water ME is recommended for the administration of lipophilic medicines, such as the lipophilic antioxidant Gallic acid-loaded oil in-water ME [xvii]. Because of their unique formulation characteristics, thermal stability, much better biocompatibility, and hydrophilic & lipophilic fields, MEs can increase medication solubility, absorption, and penetration rate, and therefore the therapeutic impact. The primary advantages of microemulsions based on gels or polymers over basic microemulsions are increased stability and ease of topical administration [xviii]. Recently, emulsion polymerization has become the most extensively utilized technology for producing polymer colloids (polymer particles dispersed in water) with tiny particle sizes [xix].

Microemulsion polymerization has received increasing interest and is currently used extensively in a variety of industries. Microemulsion polymerization is characterised as a radical chain propagation reaction with vinyl monomers in one phase of a microemulsion, and the resulting polymer colloid is referred to as latex. However, microemulsions require an enormous quantity of emulsifying agent (surfactant), which is expensive, and surfactants must be removed after microemulsion preparation. Polymer microemulsion solves this problem by using less surfactant and more water, resulting in a lower cost. Under the conditions of a low oil-water ratio, copolymers with a high solid content and a big molecular weight were also produced. Microgels made by microemulsion polymerization feature small sizes of particles and a narrow particle size dispersion [xx].

In the current work, microemulsions were created using (PVP) as a polymer drug carrier. PVP polymer has perfect oil phase wettability along with a certain degree of water phase capacity [xxi], Tween 80 is used as a surfactant, the oil phase is clove oil, and gallic acid is used as a model drug.

Microemulsions are attracting a lot of interest since they offer a lot of potential for medication delivery and pharmacological applications. Microemulsions are isotropic liquid mixes of oil, water, and surfactant that are generally combined with a cosurfactant (Smix). The dispersed phase droplet size in a microemulsion is less than 100 nm.

Radiation hormesis is the phenomenon in which low doses of ionizing radiation stimulate or benefit normally unstressed cells while higher doses are damaging [xxii]. The current study aimed to preparation and characterization of polymer solution (as a co-surfactant and drug carrier) by microemulsion polymerization and to obtain stable and clear microlatex of low particle size with a certain amount of high molecular weight polymer in the medium and embedded with gallic acid as a model drug. Moreover to evaluate the protective effect of gallic acid-loaded microemulsion combined with low doses of radiation on chronic pancreatitis in rats via modulation of the Nrf2-Keap1-HO-1 Pathway.

Material and methods

Tween 80 surfactants is biocompatible and non-ionic having hydrophilic 408 lipophilic balance (HLB) values ranging from 1 to 15 were supplied from Qualikems (india), double distilled water, pure clove oil extracted by he hydro-distillation technique as in previous study [xxiii]. The essential clove oil, GA and PVP were supplied from Qualikems (india), all chemicals were used as received.

Synthesis of PVP/CEO and PVP/CEO/GA microemulsion:

Polymer microemulsion was prepared by using (PVP) as a co surfactant and Tween 80 as a surfactant of volume ratio 1:1 and clove oil was used as an oil phase. The microemulsion was prepared by titrating a mixture of S_{mix} and oil phase (1:1) against water until microemulsion was obtained then a known concentration of gallic acid was added. The particle size of the colloid was investigated by light scattering technique (DLS). Zeta potential was studied to know the surface charge of the prepared microemulsion and the molecular weight was determined by viscometer.

To develop and characterize the lipophilic antioxidant GA-loaded oil-in-water PVP polymer ME, 2.5 gm of PVP dissolved in 50 ml double distilled water for 2 h using magnetic stirrer at 60 ^{O}C then 2.5 ml of tween 80 added while continuous stirring until completely miscible S_{mix} solution obtained (Tween 80: PVP mixture) 1:1. Volume of each surfactant and co-surfactant mixture (S_{mix}) were blended with oil in the ratio of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9. Each mixture was titrated with distilled water at 37ºC. After each addition, the mixtures were observed for their appearance (turbid or clear). Turbidity of the samples would indicate the formation of a coarse emulsion. The microemulsion after every addition of the amount of aqueous phase was then left to rest at least for some minutes and then visually observed. If no phase separation occurred, the whole process was repeated until turbidity evolved and/or the phase separation was observed. The best appeared polymer microemulsion obtained is that with Smix: clove oil 1:1with a total oil concentration 44.7% and at this mixture 0.17wt% of gallic acid was added to its oil phase.

Composition analysis of PVP/CEO/GA microemulsion was clearly explained by ternay plot diagram

Ternary Plot (figure 1) is a type of graph that displays the composition of a mixture that contains three components. The plot is triangular in shape and each corner of the triangle represents a pure component. The position of a point within the triangle indicates the relative proportion of the three components in the mixture"

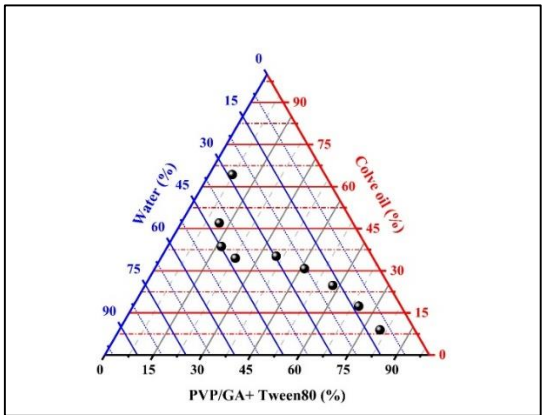


Fig. (1): Ternary Plot displays the composition of gallic acid-loaded microemulsion.

Fourier transform infrared spectra (FTIR)

FTIR spectra were done using a Mattson 5000 FTIR spectrophotometer product of Unicam Ltd., England, in the frequency range of 4000 – 500 cm^{-1} .

Particle Size and ζ-Potential Analysis studies

The particle size and zeta potential of PVP/CEO and PVP/CEO/GA microemulsions were determined by a Dynamic light scattering instrument DLS (ZetaSizer Nano Series (HT), Nano ZS, Malvern Instruments London, England) at 25 ◦C

Antimicrobial activity studies:

Agar well diffusion method was used to evaluate the antimicrobial activity of the prepared PVP/CEO and PVP/CEO/GA microemulsion on bacterial strains. Agar plate surface was inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm wsa punched aseptically with a sterile cork borer or a tip, and a volume (20–100mL) of the gallic acid-loaded microemulsion at desired concentration was introduced into the well. Then, agar plates were incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested. After incubation times of 16 to 24 h (Mucoraceae), 24 h (*A. fumigatus*, *A. flavus*, *A. niger*) or 48 h (other species), the resulting inhibition zone diameters (in mm) surrounding the wells were measured to the nearest whole millimeter at the point at which there is prominent reduction in growth.

Determination of the median lethal dose (LD50) of PVP/CEO/ME microemulsion:

PVP/CEO/GA microemulsion were given orally to male albino rats in doses ranging from 1 to 10 ml/100g body weight. Mortality after 24 h was registered.

The LD50 of PVP/CEO/GA microemulsion was calculated using the formula **LD50 = Dm–Σ(Zxd)/n [xxiv]**.

Where, **Dm** is the minimum dose which kills all animals in the group; **Z** is the mean of dead animals in two successive groups; **d** is the constant factor between two successive groups; **n** is the number of animals of each group; and **Σ** is the sum of (Zxd).

Induction of chronic pancreatitis:

Animals were accurately weighed and were calculated the volume of L-arginine hydrochloride solution (20%) to be injected using a dose of 250 mg/100 g body weight (1.25 ml/100 g). The solution was filled in a sterile 2 ml syringe with 25 G needle and was injected intraperitoneal. Animals were put in a clean cage with food and water. Wait for 1h and then the 2nd dose was administered intraperitoneal and animals were returned to its cage.

Irradiation of animals:

Whole-body gamma-irradiation was performed at the National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt, using Canadian Gamma Cell-40 biological irradiator (137 Cesium), manufactured by the Atomic Energy of Canada Limited, Ontario, Canada. Animals were exposed to fractionated dose levels of 0.25 Gy/week of gamma-radiation for 4 weeks.

Experimental design:

48 Adult male albino rats, weighing 120-140 g were used in the course of the present work, were obtained from the National Center for Radiation Research and Technology Cairo, Egypt. The animals were housed in especially designed plastic cages, under normal temperature, pressure, humidity; Animals were fed on pellets diet supplied with excess of water. Rats were randomly assigned to eight groups (six animals per group):

Group 1 (Control): Healthy rats were not received any treatment.

Group 2 (G): Rats were received PVP/CEO/GA microemulsion (0.9 ml/100 g body weight) orally 5 days per week for 4 weeks.

Group 3 (IR): Rats were exposed to low doses of radiation (0.25 Gy every week for 4 weeks)

Group 4 (L): Rats were received L-arginine 4 times by a dose of 250 mg/100 g body weight (2 doses separated by 1-hour interval), every week for 4 weeks to induce chronic pancreatitis.

Group 5 (G+IR): Rats were received PVP/CEO/GA microemulsion by the same dose as group 2 & were exposed to low doses of radiation as group 3.

Group 6 (G+L): Rats were received L-arginine to induce chronic pancreatitis by the same dose as group 4 & were received PVP/CEO/GA microemulsion orally 5 days per week for 4 weeks, started from the same day rats were received the first dose of L-arginine.

Group 7 (IR+L): Rats were received L-arginine to induce chronic pancreatitis as group 4 & were exposed to low doses of radiation as group 3.

Group 8 (G+IR+L): Rats were received L-arginine to induce acute pancreatitis & received PVP/CEO/GA microemulsion & were exposed to low doses of radiation.

At the end of the experiment, blood samples were withdrawn from the heart of each animal, after euthanized using an overdose of diethyl ether. Pancreas tissues were rapidly removed, washed in ice-cold saline, part from each pancreas tissue was placed in 10% formalin prepared in phosphate-buffered saline (PBS) to be used for histopathological examination. Another part of each pancreas was kept at −80 °C till the day of analysis.

Determination of Nrf2, Keap1and HO-1 in pancreas tissue homogenate.

Nuclear Factor Erythroid 2 (Nrf2), Kelch like ECH Associated Protein 1 (Keap1), and Heme Oxygenase 1 (HO-1) were measured by ELISA kits supplied MyBioSource Inc., USA, according to the manufacturer's instructions. NrF2 (MyBioSource, USA, Cat # MBS752046), Keap1 (MyBioSource, USA, Cat # MBS7218529) and HO-1 (MyBioSource, USA, Cat # MBS2508238).

Western immunoblotting analysis of MYD88 and TLR4 proteins in pancreas tissue homogenate.

Western blotting analysis was performed according to Kurien and Scofield, 2006 **[xxv]**, the samples were lysed on ice for about 1 h using RIPA buffer (Thermo, USA), then centrifuged for 10 min at 12,000 rpm. 20 µg protein of each sample was loaded into SDS-PAGE gels for protein separation and then immunoblotting by transferred to 0.45 µm PVDF membrane (Bio-Rad, USA) and incubated with 5% nonfat milk, then incubated with primary antibodies raised against MYD88 or TLR4 with β-actin at 40C overnight. Membrane was incubated horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, USA) for 2 h at room temperature. Protein bands were visualized and the density of each band was normalized by β-actin.

Table (1): Antibodies used for western blot analysis.

Antibody	Dilution	Size (KD)	Cat number	Company
Anti-β-actin	1:1000	42	**sc-47778**	Santa Cruz Biotechnology
Anti-MYD88	1:500	38	**sc-74532**	
Anti-TLR4	1:500	125	**sc-293072**	

Determination of Serum lipase, amylase and lactate dehydrogenase (LDH)

Serum lipase, amylase and LDH were estimated by using a Kit method (products of Roche Diagnostics, Germany) on automatic Roche CobasC311 analyzer.

Histopathological Study

Specimen from pancreas of all examined groups was washed, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin wax. Sections of 5–6 µm in thickness were cut out, deparaffinized and stained with Hematoxylin and Eosin (H & E) for examination under the light microscope **[xxvi]**.

Statistical analysis:

The data were presented as means ± standard deviation of mean (S.D.) they were analyzed using One-Way ANOVA followed by Tukey-Kramer multiple comparison test. The Graph Prism software, version 5, Inc., USA was used to perform the statistical analysis and graphical presentations. The level of significance was fixed at $P \leq 0.05$ with respect to all statistical tests.

Results:

Fourier Transform Infrared spectroscopy (FTIR)

PVP is a synthetic polymer that is commonly used in a wide range of industries, including pharmaceuticals, cosmetics, and food production. The FTIR spectrum of PVP is characterized by several prominent peaks and bands, each of which corresponds to specific vibrational modes of the chemical bonds in the polymer **figure 2**. Here is a brief explanation of the main features of the FTIR spectrum of PVP: Broad O-H stretching band: This band appears in the range of 3200-3500 cm^{-1} and is broad due to the presence of hydrogen-bonded OH groups in the polymer backbone. C=O stretching band: This band appears at around 1660 cm^{-1} and is attributed to the carbonyl groups in the pyrrolidone ring of the PVP molecule. N-H bending band: This band appears at around 1550 cm^{-1} and is due to the presence of N-H groups in the pyrrolidone ring **[xxvii]**.

C-H stretching bands: These bands appear in the range of 2900-3000 cm^{-1} and are due to the stretching vibrations of the C-H bonds in the polymer backbone. Amide II band: This band appears at around 1460 cm^{-1} and is due to the combination of N-H bending and C-N stretching modes in the pyrrolidone ring. C-N stretching band: This band appears at around 1300 cm^{-1} and is due to the stretching vibrations of the C-N bonds in the pyrrolidone ring. Clove oil is an essential oil obtained from the dried flower buds of the clove tree. The FTIR spectrum of clove oil is characterized by several prominent peaks and bands, each of which corresponds to specific vibrational modes of the chemical bonds in the oil. Here is a brief explanation of the main features of the FTIR spectrum of clove oil: O-H stretching band: This band appears in the range of 3200-3600 cm^{-1} and is due to the stretching vibrations of the hydroxyl groups in the oil. C-H stretching bands: These bands appear in the range of 2800-3100 cm^{-1} and are due to the stretching vibrations of the C-H bonds in the oil. C=O stretching band: This band appears at around 1700 cm^{-1} and is attributed to the presence of ester functional groups in the oil. C=C stretching band: This band appears at around 1620 cm^{-1} and is due to the stretching vibrations of the carbon-carbon double bonds in the oil. C-O stretching band: This band appears at around 1250 cm^{-1} and is due to the stretching vibrations of the ether functional groups in the oil. Aromatic ring stretching bands: These bands appear in the range of 1000-1600 cm^{-1} and are due to the stretching vibrations of the benzene rings in the eugenol and other aromatic compounds present in the oil. Gallic acid is a naturally occurring organic acid that is found in a wide range of plant-based foods and beverages, including tea, wine, and certain fruits. The FTIR spectrum of gallic acid is characterized by several prominent peaks and bands, each of which corresponds to specific vibrational modes of the chemical bonds in the molecule **[xxviii]**.

FTIR spectrum of gallic acid: O-H stretching band: This band appears in the range of 3200-3500 cm^{-1} and is due to the stretching vibrations of the hydroxyl groups in the molecule. C-H stretching bands: These bands appear in the range of 2800-3100 cm^{-1} and are due to the stretching vibrations of the C-H bonds in the molecule.C=O stretching band: This band appears at around 1720 cm^{-1} and is attributed to the presence of the carboxylic acid functional group in the molecule **[xxix]**.

C-O stretching band: This band appears at around 1250 cm^{-1} and is due to the stretching vibrations of the ether functional groups in the molecule. Aromatic ring stretching bands: These bands appear in the range of 1500-1600 cm^{-1} and are due to the stretching vibrations of the benzene ring in the molecule. C-C stretching band: This band appears at around 830 cm^{-1} and is due to the

stretching vibrations of the carbon-carbon bonds in the molecule.

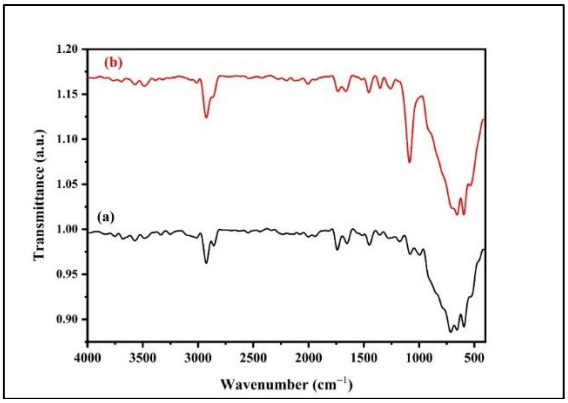


Fig. (2): FTIR spectrum of : a) PVP/CEO and b) PVP/CEO/GA microemulsion

DLS results:

Results obtained from Dynamic Light Scattering (DLS) principle can be used to determine Z-average and Zeta potential in addition to the electrical conductivity.
All results are presented in **figure 3** and summarized in **table 2**. DLS results are corresponding to the size distribution profile of small particles in suspension or polymer microemulsion solution. It can also be used to probe the behavior of complex fluids such as concentrated polymer solutions The Z-average which reflect the average particle size of particles in the polymer microemulsion. Z-average of PVP/CEO microemulsion was found to be 5.8 nm and it decreased by adding GA and reach to 3.9 nm at GA concentration 0.17wt%. Because of this lower particle size it was used as the drug model in this study. And those results are confident with zeta potential results. As the absolute value of effective zeta-potential of small particles is greater than that of large particles. Zeta potential is a physical property which is exhibited by any particle in suspension, or emulsion. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in the prepared microemulsion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e., the solution will resist aggregation **[xxx]**.

Also zeta potential can also be used as an aid in predicting the long-term stability which arising from electrical charge on the colloidal particles. Zeta potential of GA- loaded PVP/CEO MEs were determined and there was negative in the range of -1.26 mV to -3.34 mV and increased with introducing GA which indicates that the solution stability increased by introducing gallic acid **[xxxi]**, a negative potential on the surface charge prevents the microemulsion particles aggregation.
During the polymerization of microemulsion system, certain change of electrical conductivity may occur **[xxxii]**. It was found that the electrical conductivity increased linearly by adding GA to the PVP/CEO ME due to the formation of oil-in-water microemulsion, and also may be due to the increased ions contained in the water phase.

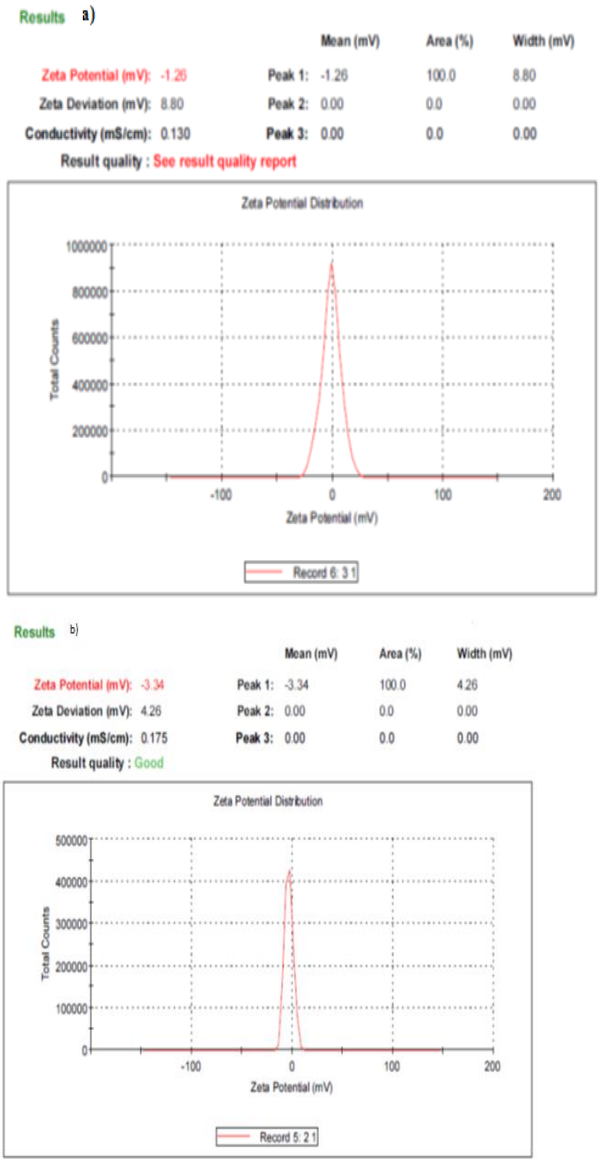


Fig. (3): DLS mesurment of a) PVP/CEO and b) PVP/CEO/GA microemulsion.

Table 2: Results obtained from DLS analysis for a) PVP/CEO and b) PVP/CEO/GA microemulsion

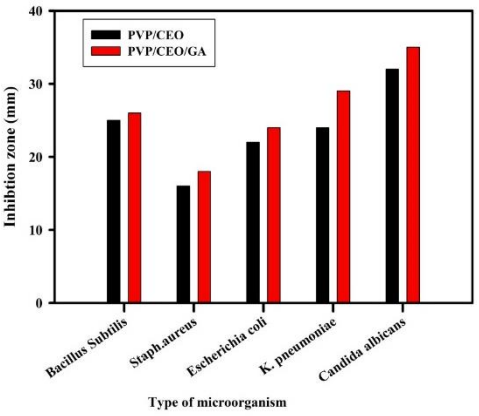
Microemulsion	Z-Average (d.nm) × 10^4	Zeta potential (mV)	Conductivity (mS/cm)
(a) PVP/CEO	5.853	–1.26	0.175
(b) PVP/CEO/GA	3.903	–3.34	1.44

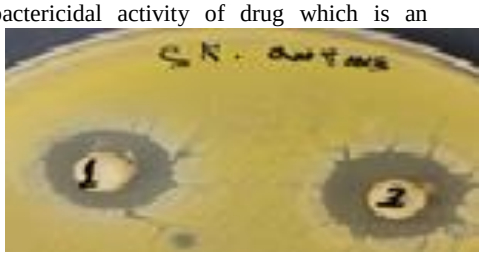
Antimicrobial activity
Antimicrobial activity of PVP/CEO and PVP/CEO/GA microemulsion against tested pathogenic gram positive bacteria (*Staph.aureus* , *Bacillus Subtilis)* and gram negative bacteria (*Candida albicans* , *K. pneumonia, Escherichia coli)* is shown in figure 4.
The results clarify that PVP/CEO and PVP/CEO/GA microemulsion displayed strong antibacterial activity against the tested bacteria. This antimicrobial effect resulted from the presence of clove oil which exhibit a

great antimicrobial activity against both gram-negative and gram-positive bacteria **[xxiii]**.

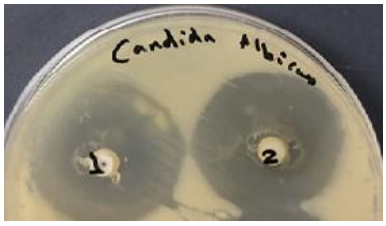
However, Microemulsion polymer incorporated with gallic acid has greater antibacterial activity. Since GA is a benzoic acid derivative which contains three hydroxyl groups, so the greater the acid includes hydroxyl groups, the more the antimicrobial activity will have. These acids are attacking the microorganism due to the antioxidant property of the phenolic compounds and resulted in increasing the toxicity which kills the bacteria. The COOH groups of GA molecule can damage bacterial cell wall. Because of disturbing the permeability of the cell membrane and inhibiting the enzyme activity, an inactivation of a bacterial biofilm occurred as shown in figure (5). Similar investigation was proven by Sayed, Asmaa, et al 2022 **[xxxiii]**.

Microemulsions were prepared by titration of the oil-surfactant mixture with the water phase, as compared with the conventional drug solution; microemulsion may increase the bactericidal activity of drug which is an important factor to increase the activity of the drug on the disease.

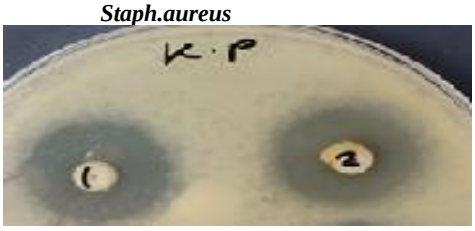




Staph.aureus

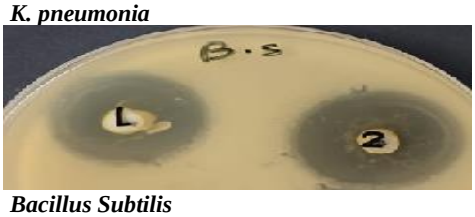


Candida albicans



K. pneumonia

Escherichia coli



Bacillus Subtilis

Fig. (4): Antimicrobial activity of 1) PVP/CEO and 2) PVP/CEO/GA microemulsion on different bacterial strains

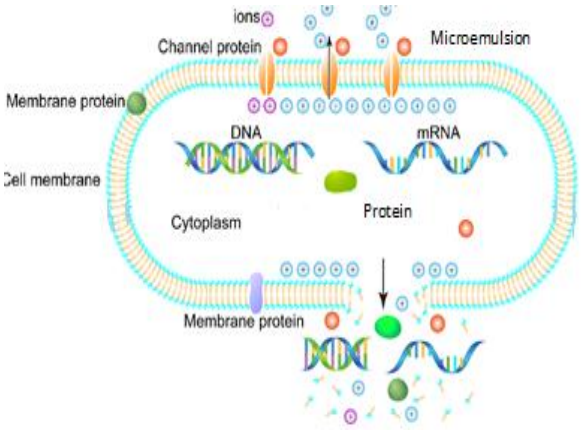


Fig. (5): Effect of polymer microemulsion on bacterial cell wall.

The Median Lethal Dose (LD50) of PVP/CEO/GA microemulsion:

The results revealed that the LD50 was found to be 9 ml/100 gm body weight for the PVP/CEO/GA microemulsion for the oral administration. One-tenth of the LD50 value has been used as an ideal dose to determine the in-vivo anti-inflammatory of gallic acid-loaded microemulsion.

Molecular and biochemical studies:

The effect of treatment with PVP/CEO/GA microemulsion and/or low doses of gamma irradiation on Nrf2-Keap1-HO-1 Pathway.

Treatment with L-arginine resulted in a significant ($P \leq 0.05$) decrease in Nrf2, Keap1 and HO-1 in pancreas tissue (Nrf2: 0.32 fold, Keap1: 0.17 fold and HO-1: 0.13 fold in respect to normal control).

Treatment with PVP/CEO/GA microemulsion and/or low doses of gamma irradiation induces a remarkable increase

in Nrf2, Keap1 and HO-1 in pancreas tissue in respect to L-arginine group as shown in figure (6).

Table 3: Determination of the Median Lethal Dose (LD50) of PVP/CEO/GA microemulsion in male albino rats.

Dose of PVP/CEO/GA microemulsion (ml/ 100 g bw)	Number of animals	Number of dead animal	Z	d	(Z) × (d)
1	6	0	0	1	0
2	6	0	0	1	0
3	6	0	0	1	0
4	6	0	0	1	0
5	6	0	0	1	0
6	6	0	0	1	0
7	6	0	0	1	0
8	6	1	0.5	1	0.5
8.5	6	2	2	0.5	1
9	6	4	2	0.5	1
9.5	6	5	2	0.5	1
10	6	6	5	0.5	2.5

LD50 = 10-(6/6) = 10-1 = 9 ml/100 gm

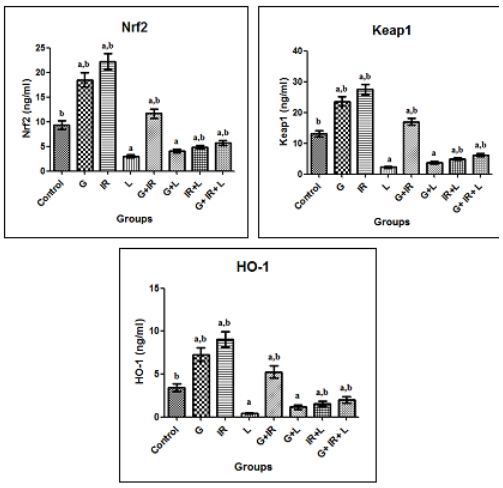


Fig. (6): Shows the effect of treatment with PVP/CEO/GA microemulsion and/or low doses of gamma irradiation on Nrf2-Keap1-HO-1 Pathway. Where [a] significant difference versus control group at $p \leq 0.05$ and [b] significant difference versus L group at $p \leq 0.05$.

The effect of treatment with PVP/CEO/GA microemulsion and/or low doses of gamma irradiation on MYD88 and TLR4 proteins in pancreas tissues:

Treatment with L-arginine resulted in a significant (P≤0.05) increase in MYD88 and TLR4 proteins in pancreas tissue (MYD88: 3.52 fold- and TLR4: PVP/CEO/GA microemulsion and/or low doses of gamma irradiation induces a remarkable reduction in MYD88 and TLR4 proteins in pancreas tissue in respect to L- arginine group as shown in figure (7).

The effect of treatment with PVP/CEO/GA microemulsion and/or low doses of gamma irradiation on Serum lipase, amylase and lactate dehydrogenase (LDH).

Treatment with L-arginine resulted in a significant (P≤0.05) increase in serum lipase, amylase and LDH (lipase: 1.3 fold, amylase: 1.5 fold and LDH: 3 fold in respect to normal control). Treatment with gallic PVP/CEO/GA microemulsion and/or low doses of gamma irradiation induces a remarkable reduction in serum lipase, amylase and LDH in respect to L- arginine group as shown in fig. (8).

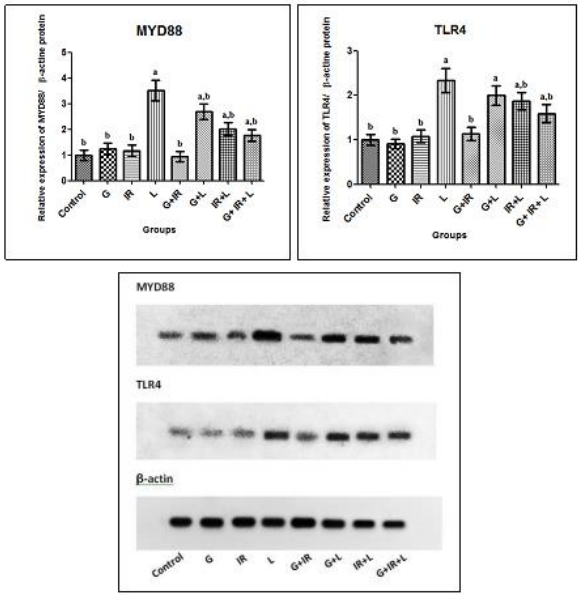


Fig. (7): Shows the effect of treatment with PVP/CEO/GA microemulsion and/or low doses of gamma irradiation on MTD88 and TLR4 proteins in pancreas tissues. Where [a] significant difference versus control group at $p \leq 0.05$ and [b] significant difference versus L group at $p \leq 0.05$.

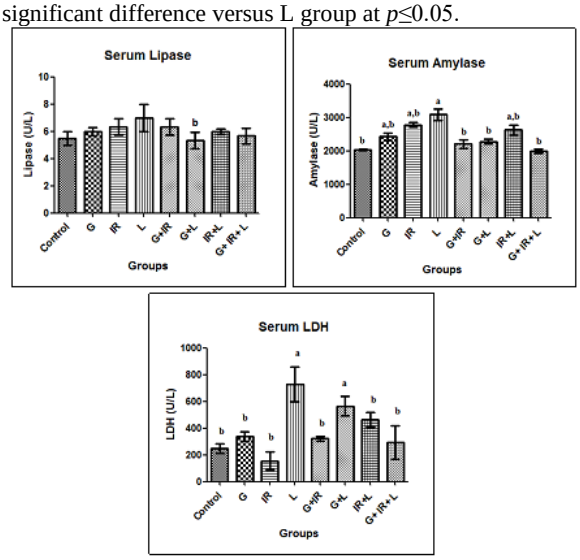


Fig. (8): Shows the effect of treatment with PVP/CEO/GA microemulsion and/or low doses of gamma irradiation on serum lipase, amylase and lactate dehydrogenase (LDH). Where [a] significant difference versus control group at $p \leq 0.05$ and [b] significant difference versus L group at $p \leq 0.05$.

Histopathological observation:

In the control group and the group that received PVP/CEO/GA microemulsion only (G Group), histology of pancreas stained by Haematoxylin & Eosin showed normal islets of Langerhans surrounded by exocrine portion of pancreatic tissues which consists of small, dark

acini, the exocrine glands **(fig. 9 a–d)**. Pancreatic tissues of rats exposed to low doses of radiation (IR group), showed mild loss of the parenchyma, especially of the exocrine glands. Several islets appear to be intact with apparent destruction & distortion of glandular tissue "islets of Langerhans" surrounded by destructed exocrine tissues **(fig. 9 e–f)**. Pancreatic tissues of rats received L-arginine to induce chronic pancreatitis (L group), showed signs of acute pancreatitis in form of segmental fibroses with infiltration of blue stained fibroblast. Intact pancreatic acinar cells are presented mainly around the Langerhans islets. The atrophic acini are dilated and separated from each other with massive infiltration of inflammatory cells "Lymphocytes" **(fig. 9 g–h)**.

Pancreatic tissues of rats received PVP/CEO/GA microemulsion & exposed to low doses of radiation (G+IR), showed mild destruction and distortion of islets of Langerhans associated with dilatation of blood vessels with mild infiltration of inflammatory cells **(fig. 10 i–j)**. Pancreatic tissues of rats received L-arginine & received PVP/CEO/GA microemulsion (G+L), showed marked loss of the parenchyma, especially of the exocrine glands, with replacement by adipose tissue (fat cells framing the picture) in the midst of atrophic acini. The atrophic acini are dilated and separated from each other **(fig. 10 k–l)**. Pancreatic tissues of rats received L-arginine & exposed to low doses of radiation **(**IR+L), showed mild loss of the parenchyma, with replacement by adipose tissue in the midst of atrophic acini. The atrophic acini are dilated and separated from each other. Clusters of lymphocytes infiltration are detected **(fig. 10 m–n)**. Pancreatic tissues of received L-arginine & received PVP/CEO/GA microemulsion & exposed to low doses of radiation (G+IR+L), showed marked improvement in signs of pancreatitis, with normal parenchyma, surrounded by exocrine glands. The sections showed mild infiltration with lymphocytes, dilated acini and mild oedema**(fig. 10 o–p)**.

Discussions

Chronic pancreatitis (CP) is an inflammatory pancreatic disease characterized by the loss of exocrine/endocrine functioning as well as the development of fibrosis **[xxxiv]**. The objective of this research was to evaluate the effect of GA-loaded PVP/CEO microemulsion combined with low doses of radiation to ameliorate chronic pancreatitis in rats via modulation of the Nrf2/Keap1/HO-1 Pathway.

The Nrf2-Keap1 signalling pathway is important in the transcriptional control of inflammatory cytokines. It can be found in the body as the transcription factor Nrf2 and the repressor protein Keap1. In the presence of oxidative stress or a Nrf2 activator, Nrf2 and Keap1 split, and Nrf2 goes to the nucleus to attach to the antioxidant response element gene. This causes antioxidant genes and anti-inflammatory compounds to be up-regulated **[xxxv]**. The pathway's downstream target proteins include heme oxygenase-1 (HO-1), NAD(P)H dehydrogenase, glutathione peroxidase 1, glutathione S-transferase (GST), glutathione reductase (GR), and superoxide dismutase (SOD) [xxxvi]. These antioxidant genes protect the delicate cellular equilibrium and maintain cellular homeostasis in the face of stress and inflammation. Along with direct gene upregulation, it boosted NADPH production, which is a direct antioxidant and cofactor in numerous redox processes **[xxxvii]**.

GA displayed anti-inflammatory effects by promoting HO-1 and Nrf2 gene expression, activating the Nrf2 / HO-1 signaling pathway, decreasing NF-κB expression, and blocking the NF-κB pathway **[xiv,xxxviii]**. Numerous studies have shown that GA has antioxidant properties by lowering the generation of reactive oxygen species (ROS) **[xxxix]**. GA predominantly targets mitochondria-specific signaling pathways and molecules, including those involved in ROS production, respiration, mitochondrial biogenesis, and apoptosis **[xl]**. GA might improve Keap1's thermal stability, indicating a possible connection between GA and Keap1. Furthermore, molecular docking revealed that GA may have competed with Nrf2 for Keap1 binding. Meanwhile, GA disrupts the protein-protein interaction of Keap1 and Nrf2, which may lead to Nrf2 nuclear translocation **[xli]**.

In the present study, treatment with L- arginine reduced the expression of Nrf2, Keap1, and HO-1genes compare with control group. While, treatment with GA-loaded PVP/CEO microemulsion & exposed to low doses of radiation increase the expression of Nrf2, Keap1, and HO-1genes and activating the Nrf2-Keap1-HO-1 signaling pathway. This study agrees with previous studies **[xiv,xxxviii,xlii]**.

Toll-like receptor 4 (TLR4) is the primary mediator of pancreatic damage **[xliii]**. Furthermore, TLR4 is broadly distributed in pancreatic tissue and vascular endothelial cells and has been linked to pancreatic damage during acute pancreatitis **[xliv]**. Furthermore, Myeloid differentiation primary response protein 88 (MyD88) is an extremely important TLR4 adapter **[xlv]**. Myd88 expression rises in AP pancreatic tissue **[xlvi]**. GA has been shown to suppress inflammatory responses by modulating the TLR4/MyD88/TRIF signaling pathway **[xlvii,xlviii]**. Also, by competitive antagonism, GA may inhibit IκB degradation, therefore reducing NF-κB activation and preventing expression of the TLR4/NF-κβ signaling pathway **[xlix]**. In the current investigation, L-arginine treatment increased TLR4/MyD88 expression compared to the control group. TLR4 and MyD88 signaling pathways are downregulated after treatment with GA-loaded PVP/CEO microemulsion and exposure to low doses of radiation. Previous research supports this finding [**xlvii, xlviii,[l], [li],[lii]**].

The present study demonstrates that, treatment with L-arginine increases the lipase, α-amylase and LDH activities. Treatment with GA-loaded PVP/CEO microemulsion & exposed to low doses of radiation decreases the lipase, α-amylase and LDH activities. This study agrees with previous studies **[liii,liv,lv]**. Furthermore, GA-loaded PVP/CEO microemulsion & exposed to low doses of radiation induced marked improvement in signs of pancreatitis, with normal parenchyma, surrounded by exocrine glands. The sections showed mild infiltration with lymphocytes, dilated acini and mild oedema.

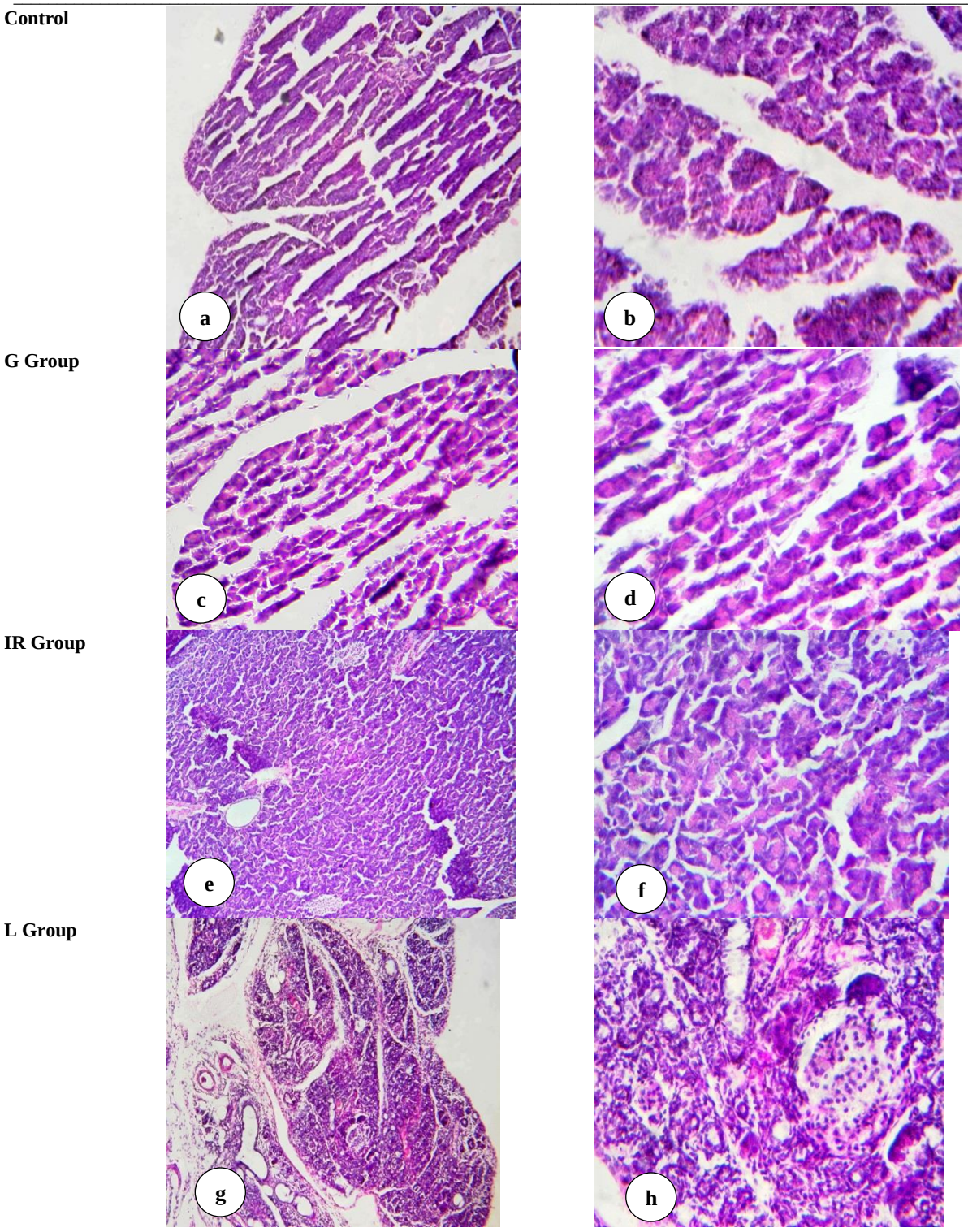


Fig. (9): Photomicrographs of T.S. of rat pancreas (H & E) for control group (a,b), PVP/CEO/GA microemulsion received group (c,d), low doses of radiation exposed group (e,f), and L-arginine received group (g,h). where the fig. on the right (x200) and on the left (x400).

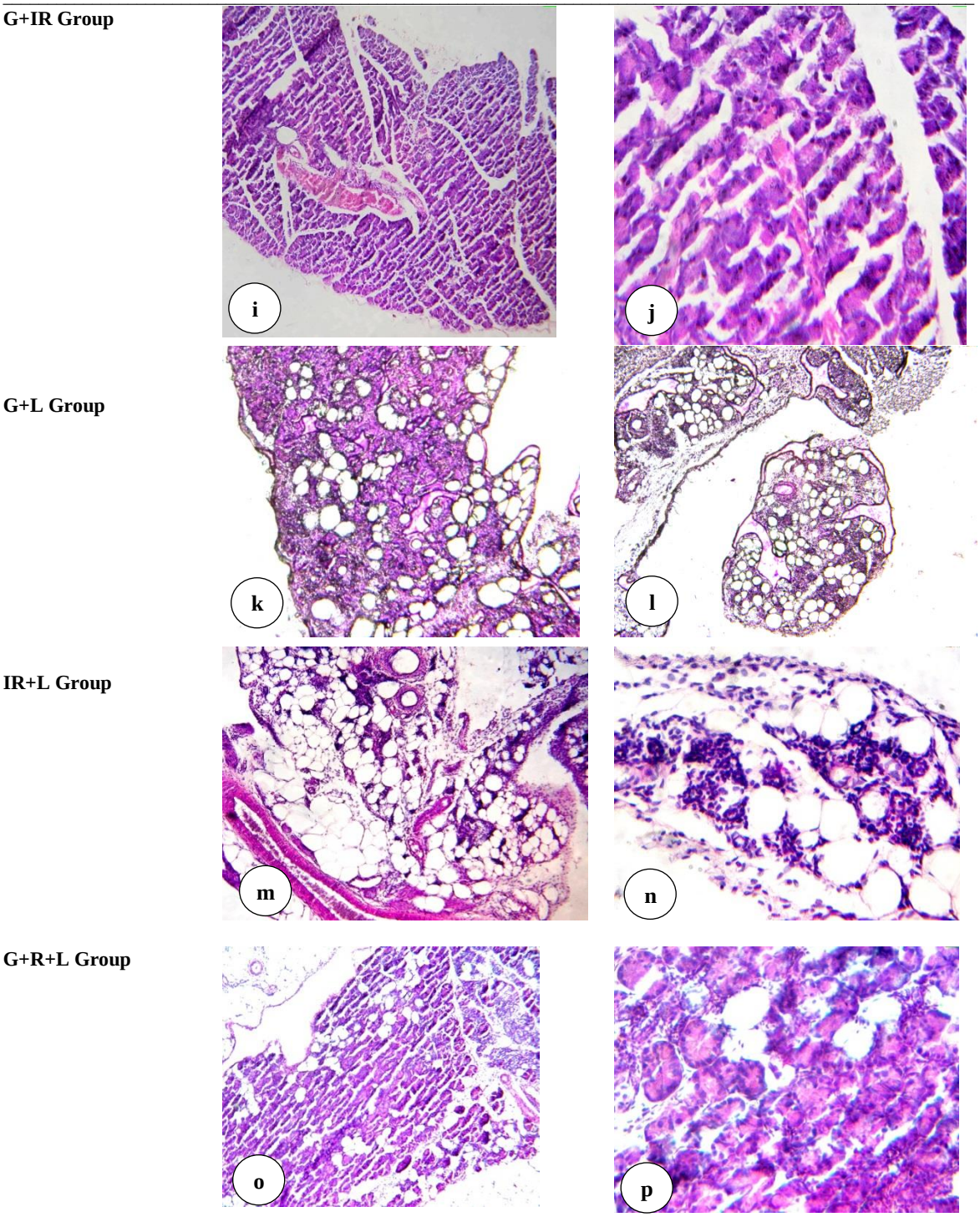


Fig. (10): Photomicrographs of T.S. of rat pancreas (H & E) for the group received PVP/CEO/GA microemulsion & exposed to low doses of radiation (i,j), the group received L-arginine & received PVP/CEO/GA microemulsion (k,l), the group received L-arginine & exposed to low doses of radiation (m,n), and the group received L-arginine & received PVP/CEO/GA microemulsion & exposed to low doses of radiation (o,p). where the fig. on the right (x200) and on the left (x400).

Conclusions:

In conclusion, our study presents a promising therapeutic approach for the management of chronic pancreatitis (CP). Gallic acid (GA) has demonstrated its potential in stimulating the Nrf2-Keap1-HO-1 pathway, which plays a crucial role in combating inflammation and oxidative stress associated with CP. However, its limited water solubility has hindered its practical application. To address this issue, we successfully developed a microemulsion (ME) formulation using PVP as a carrier for GA, enhancing its solubility and bioavailability. The results of our investigation showed that the GA-loaded PVP/Clove oil microemulsion combined with low doses of radiation effectively ameliorated CP in rats.

Statements and Declarations:

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Competing Interests: The authors declare that there are no conflicts of interest or relationship, financial or otherwise associated with this study.

Data Availability: The data presented in this study are available in this article.

Ethical Approval:
All the ethical protocols for animal treatment were approved by The Ethical Committee at the National Center for Radiation Research and Technology (number: 63 A/ 21).

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