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Protective effect of auraptene on oxidative stress induced by acrylamide in isolated rat hepatocytes

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ABSTRACT

Acrylamide is a chemical compound that has many applications in the industry. This substance is formed in the cooking process of a high carbohydrate food at a temperature higher than 120 degrees Celsius. The highest amount of acrylamide is in fried potato, bread and coffee. Faced with acrylamide due to its presence in food and industrial applications. Acrylamide can also cause neurotoxicity, genotoxicity, and hepatotoxicity and cancer. This substance can increase the production of various active oxygen species in the body and reduce the antioxidant defense, which ultimately causes oxidative stress. auraptene 7-Geranyloxycoumarin is a member of the Cumarin family. It is found mainly in citrus, such as grapefruit. auraptene has anti-inflammatory, anti-cancer and anti-oxidant effects. Since auraptene has anti-oxidant effects, this study examined the protective effects of auraptene against acrylamide-induced oxidative stress. For this study, 6 groups of rat liver isolated liver were prepared: control group, Acrylamide, acrylamide with auraptene 100 μM, acrylamide ointment with 50 μM auraptene, acrylamide with 20 μM auraptene Acrylamide with 10 μM auraptene. Oxidative stress indices were measured in this project. Our results showed that auraptene at concentrations of 50, 100 and 20 μM could significantly reduce the production of active oxygen species, peroxidation lipid, mitochondrial damage, and toxicity of acrylamide toxicity. Based on the results of this study, consuming foods containing auraptene may be a therapeutic strategy to prevent and reduce the toxic effects of acrylamide in the body. There is a need for further research to prove this claim.

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1.INTRODUCTION

Acrylamide (AA, C_3H_5NO), also known as 2-propenamide, is commonly utilized in various industries such as cosmetics, dyes, press fabrics production, paper making, water management and ore processing. It is also extensively used in molecular methods, including gel-based procedures (*e.g.*, gel chromatography and electrophoresis) (Friedman 2003). It is an α, β-unsaturated reactive molecule with significant mobility in soil and groundwater as well as biodegradability (Blancher and Cormick 2012). This compound can, also, be found in starchy foods such as bread, chips and crisps, following the reaction between carbonyls or reducing sugars (glucose, fructose) with asparagines (Ubaoji and Orji 2016). Except of boiling, other processes on foodstuff, including baking, overcooking, frying, and microwaving would produce this compound (Choe and Min 2006). In general, humans and animals are exposed to AA through dermal contact, ingestion and inhalation and it can rapidly spread in various organs with the potential to cross placental and mammary tissues (Semla et al. 2017).

The compound can be metabolized into a DNA-reactive metabolite, glycidamide through epoxidation by cytochrome 2E1 (CYP2E1), and both can be conjugated with glutathione as well. Both AA and glycidamide can bind to albumin, hemoglobin, enzymes and DNA (Pruser and Flynn 2011), and only 50% of AA volume is excreted mostly through urine (Bjellaas et al. 2007).

Multiple studies have mentioned that AA induces oxidative stress, rendering substantial cellular damage with implications in various diseases, thus these have raised concerns on human health (Zhang et al. 2023). It has been recognized as a neurotoxin with substantial impact on the nervous system, depending on the exposure time and dose, causing a plethora of neurologic signs. Of note, polymers of acrylamide are non-toxic and only the monomeric compound possesses toxic effects (Zamani et al. 2017). It has, also, been shown that AA can cause chromosomal disorders, lethal spermatid mutations, congenital defects, low weigh gain in the newborn infants, abnormal sperm morphology in mice and a decreased fertility rate (Fennell et al. 2005; Friedman 2003; Jin et al. 2013). Moreover, it has been classified as a probable carcinogenic agent in human, according to the International Agency for Research on Cancer (IARC) (Rice 2005). As a critical organ involved in metabolic functions, the liver is the primary attacking target of AA. Oxidative stress is the main causative agent of hepatotoxicity caused by AA, based on growing evidence. Excessive production of reactive oxygen species (ROS) and/or lowered antioxidant activity due to AA can exert apoptotic and necrotic traits on hepatic cells. While it elevates the level of malondialdhyde (MDA) enzyme, it decreases the levels of various hepatic molecules, including glutathione, catalase (CAT), ATPase, lactate dehydrogenase (LDH), succinate dehydrogenase (SDH) and superoxide dismutase (SOD). In addition, AA can significantly decrease the level of high-density lipoprotein (HDL), and increase triglyceride (TG), cholesterol, glucose, urea and creatinine levels (Zhang et al. 2023). As a result, scientists have been exploring natural compounds with antioxidant properties that could mitigate the adverse effects of acrylamide.

One of the promising antioxidant candidates is auraptene, a natural bioactive coumarin compound found in certain citrus fruits (*e.g.*, grapefruit) as well as vegetables. Its antioxidant activity has been attributed to the geranyl group in the chemical structure (Genovese and Epifano 2011). Moreover, it exhibits different pharmacological functions, such as anti-inflammatory, anti-fungal, anti-bacterial, anti-parasitic, spasmolytic and neuroprotective effects, along with growth-preventing impact on cancer cells (Bibak et al. 2019). This compound decreases the production of pro-inflammatory cytokines such as interleukin (IL-6), IL-8, IL-1β and tumor necrosis factor alpha (TNF-α), and also decreases the expression of cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS), which altogether can promote neurologic functions and prevent neurodegenerative diseases (Derosa et al. 2016). Auraptene confronts ROS significantly and can remarkably increase the antioxidant activity of glutathione S-transferase (GST) and quinone reductase enzymes (Tanaka et al. 1997).

The present study was performed to reveal the protective effects of auraptene against AA-induced oxidative stress (AIOS) in isolated rat hepatocytes. The findings of this research would inevitably contribute to the discovery of unprecedented therapeutic interventions and/or promote more functional dietary strategies, in order to reduce the noxious effects of AA exposure, hence striving towards a healthier and safer future.

2. MATERIALS AND METHODS

2.1. Chemicals and buffers

The chemicals used in the present study were as follow: AA (Sigma Aldrich, USA), Auraptene (Mashhad University of Medical Sciences, Mashhad, Iran), trypan blue due, sodium chloride, potassium chloride, hydrated and dehydrated magnesium sulfate, sodium hydrogen phosphate, potassium dihydrogen phosphate, HEPES, sodium hydrogen carbonate, magnesium phosphate, trichloroacetic acid (Merck, Germany), dimethyl sulfoxide (DMSO; Scharlau, Spain), collagenase, 2,7-dichlorofluorscein diacetate (DCFH-DA; Sigma Aldrich, USA), heparin (Daru Pakhsh, Iran), bovine serum albumin (Biowest, France), EGTA (AppliChem, Germany), ketamine hydrochloride 10% and xylazine 2% (Alfasan, Netherlands). Seven buffers were prepared and used in the current study, including, Krebs buffer, normal and 10X Hanks buffer, washing buffer (buffer I), tissue digestive buffer (buffer II), hepatocyte isolation buffer (buffer III) and dilution buffer (buffer IV).

2.2. Isolation of rat hepatocytes

In the present study, wistar rats with an average weight of 250-300 gr were used, which were kept under optimum temperature with free access to food and water. For the purpose of hepatocyte isolation, liver perfusion using collagenase enzyme was done (Moldéus et al. 1978) (**Figure 1A**). For this aim, rats were anesthetized using intraperitoneal (i.p.) injection of ketamine (75 mg/kg) and xylazine (15 mg/kg), a U-shaped incision was done in the abdominal skin, then 0.25 ml heparin (5000 U/ml) was injected intravenously (i.v.) into the inferior vena cava to prevent hepatic blood coagulation. The portal vein was cannulated and buffer I was used to wash out the remaining blood from liver. Next, the liver and gallbladder were removed from the body and buffer II (collagenase) was used for tissue lysis via perfusion for 10 min. In the following, liver was suspended in buffer III (washing), agitated and sieved through a sterile cloth, then centrifuged (5500 rpm, 4 ºC) in order to isolate a suspension of liver cells. The cells in the obtained pellet were enumerated by making a mixture of buffer IV (300 µl), cells (100 µl) and trypan blue dye (0.2% w/v, 200 µl), using a haemocytometer chamber. Of note, dead cells were permeable to dye, hence they stained blue, while alive cells stained yellow (**Figure 1B**). A defined concentration (10^6 cells/ml) of cells were re-suspended in buffer IV, added to the 10-ml culture flasks and agitated using a bioreactor at 37 ºC and 5% CO_2.

2.3. Cytotoxicity evaluation

In order to incubate water-soluble compounds, 100 µl of 100X stock solution was added to the agitating flasks containing 10 ml liver cell suspension, in order to obtain a desired concentration. Also, for DMSO-soluble compounds, the volume of DMSO in culture flasks was not higher than 40 µl. To measure cytotoxicity, after 3 hours of incubation, the desired samples were taken from the flasks and subjected to evaluation. The flasks were divided into three groups: control, sample, and different types of polyphenolic protective substances. The control flask only contained a suspension of liver cells and was not incubated with any substances. The sample flask contained a suspension of hepatic cells and EC50, 2h of AA (2 mM concentration), and those flasks with protective agents included liver cell suspension, 2 mM AA and different auraptene concentrations (10, 20, 50 and 100 mM).

2.4. ROS measurement

For this aim, 3 ml of DCFH-DA (1 M in buffer IV, pH = 7.4) was incubated with 1 ml of liver cells (10^6 cells/ml) for 15 min at 37 ºC. Finally, the fluorescence activity was measured using a spectrofluorometer device at 490-520 nm wavelengths. The DCFH-DA is transferred through the cell membrane due to the high lipophilic traits, hydrolyzed by hepatic esterases and turns into a non-fluorescent agent, which rapidly oxidizes in the presence of ROS, resulting in the formation of highly-fluorescent dichlorofluorscein (DCF) (Pourahmad et al. 2009).

2.5. Lipid peroxidation activity

The production of MDA is a good indicator of lipid peroxidation. In this assay, 2 molecules of barbitoric acid react with an MDA molecule in acidic environment, and the resulting pink color is absorbable at 532 nm wavelength. Initially, tiobarbitoric acid (TBA) solution was prepared (1.5 gr trichloroacetic acid, 210 µl HCl and 0.04 gr tiobarbitoric acid, final volume 10 ml). Next, 100 µl of each culture flask was centrifuged (1000×g, 1 min), transferred to a sterile microtube and mixed with 200 µl TBA solution. A mixture of 200 µl TBA and 100 µl Ethylenediaminetetraacetic acid (EDTA) was used as blank, and 200 µl TBA with 100 µl standard MDA was used as standard solution. Cell lysis was done using sonication (40 v, 20 sec) and then microtubes were transferred to a water bath (60 min, 90 ºC) to couple MDA-TBA. Finally, the microtubes were centrifuged (14000×g, 10 min), and 100 µl of supernatant was used for colorimetry at 532 nm using a spectrophotometer (Sepand et al. 2013).

2.6. Mitochondrial membrane potential

Based on Anderson et al. method, mitochondrial membrane potential measurement was done using rhodamine 123 fluorogenic cation. This lipophilic dye diffuses into the cytosol and accumulates in the mitochondrion, depending on the organelle membrane potential. Accordingly, it highly accumulates in an intact, normal mitochondrion with a highly-negative membrane and cannot be detected by fluorometric method (quenching phenomenon), whereas a decreased membrane potential with subsequent opening of mitochondrial permeability transition (MPT) pores cause the introduction of rhodamine 123 into the cytosol, that is detectable. For this aim, 0.5 ml of cell suspension was pelleted (1000×g, 1 min) and mixed with 2 ml of rhodamine 123 (1.5 mM) and incubated for 10 min at 37 ºC. The intensity of fluorescence due to rhodamine 123 was measured using spectrofluorometer in 490-520 nm (Andersson et al. 1987).

2.7. Lysosomal membrane damage

The fluorogenic acridine orange dye is a lipophilic agent with particular accumulation in lysosomes, due to its reaction with acidic hydrogen and the formation of a charged molecule (HA^+). This charged molecule cannot cross the lysosomal membrane into the cytosol and even lacks fluorogenic feature. Oxidative stress leading to ROS formation results in the destruction of lysosomal membrane, with subsequent releasing of acridine orange into the cytosol as a fluorogenic, non-charged molecule. In brief, 1 ml of hepatic cell suspension (10^6 cells/ml) was mixed with 2 ml of acridine orange solution (5g/ml) in incubation buffer (pH = 7.4), then incubated for 15 min at 37 ºC. The intensity of diffused fluorescence was measured at 490-520 nm (Pourahmad et al. 2011).

2.8. Ethical considerations

This study was conducted on wistar rats within the framework of animal studies protocols and in full compliance with the guidelines of the World Health Organization (WHO).

2.9. Statistical analyses

One-way ANOVA was used as a statistical test, and to evaluate the variance homogeneity in a group, Leven test was used and the results were reported as a mean of triplicate experiments (mean±SD).

3. RESULTS

3.1. Cytotoxicity evaluation

A 60% cell death in rat hepatocytes (10^6 cells/ml) was determined upon 3 h incubation of the cells with 2mM AA substance. Also, different concentrations of auraptene (20, 50 and 100 µM/ml) could significantly alleviate the AA-induced cytotoxicity after 3 h incubation ($P < 0.05$). Of note, 10 µM/ml concentration of auraptene could not substantially decrease AA cytotoxicity in rat hepatocytes during 3 h incubation (Table 1).

3.2. ROS measurement

Although AA (2mM) increased the ROS levels in rat hepatocytes (10^6 cells/ml) during 30 min incubation, but all experimental concentrations of auraptene (10, 20, 50 and 100 µM/ml) significantly decreased the level of ROS production during 30 min, in comparison to control group ($P < 0.05$) (Table 1).

3.3. Determination of lipid peroxidation

After 1 h incubation of rat hepatocytes (10^6 cells/ml) with 2 mM AA, lipid peroxidation was increased, whereas this biochemical process significantly decreased in those hepatocytes incubated with 50 and 100 µM/ml concentrations of auraptene ($P < 0.05$) (Table 1).

3.4. Mitochondrial membrane potential measurement

The mitochondrial membrane potential of rat hepatocytes (10^6 cells/ml) in the presence of 2mM AA was significantly decreased, while 20, 50 and 100 µM/ml concentrations of auraptene prevented a decline in membrane potential of rat hepatocyte mitochondria ($P < 0.05$) (Table 1).

3.5. Lysosomal membrane damage measurement

No remarkable lysosomal damage was found in those hepatocyte cells (10^6 cells/ml) incubated with AA after 1 h incubation, in comparison with control group ($P < 0.05$) (Table 1).

4. DISCUSSION

The AA compound that can be found in diverse environmental (*e.g.*, water), industrial (*e.g.*, cosmetics, materials used in molecular tests) and nutritional sources (*e.g.*, heated starchy foodstuff) has been shown to induce carcinogenicity, genotoxicity, neurotoxicity and hepatotoxicity (Stadler and Scholz 2004; Taeymans et al. 2004). Oxidative stress is the likely reason of AA-induced hepatotoxicity, so discovery and application of antioxidant compounds would alleviate the detrimental effects of AA in humans (Pruser and Flynn 2011). Auraptene is a coumarin-based polyphenolic compound, possessing a plethora of health-promoting effects against several inflammatory, infectious, carcinogenic and/or antioxidant-induced disorders (Genovese and Epifano 2011). The present study was done to demonstrate the healing effects of auraptene on the AIOS in the hepatocytes of wistar rats.

A prominent finding of the present study was increased cytotoxicity in the isolated rat hepatocytes, while auraptene alleviated these effects in 20, 50 and 100 μM concentrations. In Hammad *et al.* study (2013), different concentrations of AA (10, 30 and 60 mg/kg) caused acute, time-dependent toxicity in hepatic tissue of rats (Hammad et al. 2013). This compound can also induce necrotic and apoptotic consequences in affected cells (Elblehi et al. 2020; Yildizbayrak and Erkan 2019). Based on Mehri *et al.* study (2012), on the protective effect of crocin, a carotenoid compound, on AA-induced toxicity in PC12 cells, AA can elevate the rate of apoptosis in those cells at 5 mM concentration (Mehri et al. 2015). Our results showed an increase in the production of ROS in hepatocytes in the presence of AA, in contrast to the control group. A similar study on PC12 cells showed an increase in ROS levels due to AA (Mehri et al. 2015). Chen et al. (2014) demonstrated that 24 h incubation of AA with Caco-2 cells caused an increase in ROS production. Moreover, high levels of AA can elevate the hydrogen peroxide and oxidative molecules (Chen et al. 2014). A promising result of the present study was the significant reduction of ROS in rat hepatocytes incubated with 2 mM AA, upon exposure with 10, 20, 50 and 100 μM concentrations of auraptene, as a potent antioxidant molecule. Previously, it was shown that auraptene can inhibit the formation of superoxide anion and other oxygen species in leukocytes, hence rendering a substantial decline in oxidative stress (Torsdottir et al. 2010). Also, this compound can protect DNA against hydrogen peroxide-induced damage through decreasing of oxidative species in lymphocytes (Soltani et al. 2010). Activity of GST and quinone reductase was remarkably increased in liver and colon of examined rats, in a dose-dependent manner (Tanaka et al. 1997; Tanaka et al. 1998). Glutathione (GSH) is an important reducing agent, which maintain thiol groups on intracellular proteins and antioxidant molecules, so that decreased levels of GSH paves the way for an oxidative milieu with subsequent production and accumulation of ROS (Wüllner et al. 1999). Increased AA concentrations can substantially render tissue damage resulting from decreased GSH and induction of oxidative stress. There are several endogenic antioxidant enzyme systems, which facilitate the neutralization of oxidative stress and decrease oxidant species. Among these, CAT and SOD are involved in lipid peroxidation (Torsdottir et al. 2010). A study on the protective effects of *Digera muricata* AA-induced hepatotoxicity in rats showed that AA can significantly decrease the antioxidant activity of CAT, SOD, GSH and GST (Khan et al. 2011). Another study on the protective features of quercetin against AA toxicity demonstrated that AA decreased the activity of CAT and SOD in liver, kidney and brain (Uthra et al. 2017). In total, excessive ROS production in damaged cells can lead to more free radicals, which subsequently increase lipid peroxidation and cell membrane damage. Lipid peroxidation was remarkably increased in AA-affected rat hepatocytes. Based on a study on the protective efficacy of olive oil on AA-induced hepatic damage, a significant increase in MDA was observed due to AA in affected cells (Ghorbel et al. 2015). Such finding was, also, substantiated by another study in PC12 cells (Pan et al. 2018). The incubation of rat hepatocytes with auraptene inhibited lipid peroxidation due to AA in 50 and 100 μM concentrations. This antioxidant compound can decrease the levels of ROS, while elevate GST activity, hence inhibiting lipid peroxidation in cancer colon (Sciullo et al. 2010; Tanaka et al. 1997; Tanaka et al. 1998).

The mitochondrion is the most important source for the production of ROS in normal cells, and up to 80% of hydrogen peroxide (H_2O_2) free radicals are formed by this organelle. Also, about 1-2% of the consumed oxygen is turned into the superoxide anions by mitochondria. This capacity of the formation of free radicals is even increased in the presence of toxic agents and electron chain transport inhibitors (Gonzalez-Flecha and Boveris 1995; Isahara et al. 1999). In normal cells, mitochondrial membrane is not permeable to many substances and only a low percentage of metabolites and ions can bypass it (Halestrap et al. 2007). In cases of excessive ROS production, they can oxidize thiol groups around the MPT pores, rearrange their conformation and subsequently open such pores. The potential of the mitochondrial proton pump (Ψm), being essential for ATP synthesis, is generated by continuous proton transport from the mitochondrial matrix to the intermembrane space (Kowaltowski et al. 2001). Opening of MPT pores leads to disruption of the permeability of the inner mitochondrial membrane, a decrease in potential and inflammation (Halestrap et al. 2007). Our result indicated that AA can decline the mitochondrial membrane potential in the isolated rat hepatocytes; however, it was inhibited by incubation of hepatica cells with 20, 50 and 100 μM concentrations of auraptene. In Zhao *et al.* study (2015) (Mengyao Zhao et al. 2015), on the evaluation of the protective effects of blueberry anthocyanin in AIOS in hepatocyte mitochondria, it was shown that AA significantly damaged the mitochondrial functions, including SOD, ATPase and electron chain transport and a decrease in mitochondrial membrane potential was observed. Lysosome is an acidic organelle, containing high-concentration ferrous ions, that interact with AA and destroy this compound (Pourahmad et al. 2012). In the present study, no lysosomal damage was recorded, which can result from the AA destruction within this organelle.

In this study, the protective effect of Auraptene on oxidative AIOS in isolated hepatocytes of wistar rats was investigated. According to our results, Auraptene at concentrations of 50, 100, and 200 was able to significantly reduce ROS formation, lipid peroxidation, mitochondrial membrane potential decline, and acrylamide-induced cell death compared to a group that was only exposed to AA. These findings are consistent with other studies that have demonstrated the protective effects of Auraptene against other oxidative toxins.

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Table 1: Evaluation of cytotoxicity, ROS levels, lipid peroxidation, mitochondrial membrane potential and lysosomal membrane damage in wistar rat hepatocytes incubated with AA and auraptene

Groups	Mean±SD				
	Cytotoxicity (3h)	ROS levels (30 min)	Lipid peroxidation (60 min)	Mitochondrial membrane potential (60 min)	Lysosomal membrane damage (60 min)
Rat hepatocytes (control)	39 ± 2	34.4 ± 2.3	0.191 ± 0.11	6.34 ± 0.45	83.5 ± 5
2 mM AA	57 ± 4a	56.4 ± 5.4a	0.25 ± 0.008a	11.45 ± 0.74a	99 ± 9
10 μM auraptene	52 ± 5	48.9 ± 4.8*	0.240 ± 0.017	9.48 ± 0.95	84 ± 13
20 μM auraptene	44 ± 4*	38.6 ± 5.2*	0.237 ± 0.014	8.19 ± 0.72*	89 ± 6
50 μM auraptene	45 ± 3*	37.1 ± 1.7*	0.213 ± 0.012*	8.10 ± 1.16*	87 ± 11
100 μM auraptene	46 ± 3*	35.8 ± 3.5 *	0.209 ± 0.009*	7.85 ± 1.08*	81 ± 8

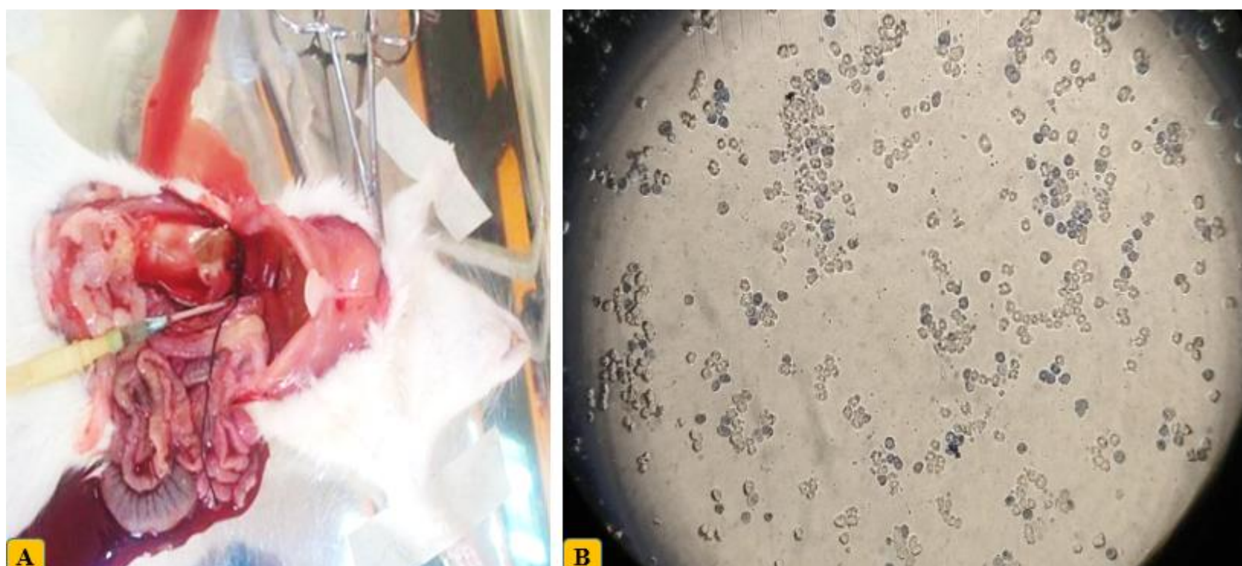

Figure 1: (A) Liver perfusion for the isolation of rat hepatocytes. (B) staining of the isolated liver cells using trypan blue dye.