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ACETAMINOPHEN INDUCED EMBRYOTOXICITY IN ALBINO MICE (MUS MUSCULUS) AND AMELIORATIVE ROLE OF GARLIC (ALLIUM SATIVUM)

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Abstract
Acetaminophen is a widely used analgesic and pain reliver. The study was aimed to investigate the acetaminophen (N-acetyl-para-aminophenol) induced Embryotoxicity in female Albino Mice and curative efficacy of garlic (Allium sativum) extract. For this purpose, qualitative analysis of ethanolic garlic extract was carried out. Twenty-eight pregnant mice were divided in 7 groups (A-G). Group A was Control while B and C were Acetaminophen treated groups with dose A (0.00), B (150,0.00). C (300,0.00). Group D to G were of Acetaminophen+ ethanolic garlic extract treated groups with doses D (150.00, 100), E (150,00, 200), F (300,100) and G (300,200). Toxicant treatment in groups (B and C) showed significant histological abnormalities like anopthalmia, reduced thoracic cavity, Spina bifada, Degenerated kidney, Degenerated ventricle, Pulmonary underdevelopment and Myocardial degeneration, Ethanolic Garlic Extracts (EGE) as ameliorant reduced these malformations across all the treatment groups (D-G). Whereas Micronuclear assay to confirm DNA damage showed significant ($p<0.05$) DNA damage in Acetaminophen treated group C (27±0.57) as compared to control A (8±0.81). Whereas curative agent ethanolic garlic extract reduced the toxicant effects of acetaminophen in group G (22±0.5). This amelioration showed presences of some active biological compounds in the EGE like an amino acid derivative NAC (N-Acetyl Cysteine) which might prove to be curative agent in acetaminophen caused teratogenicity.

Key words: Acetaminophen, Ethanolic garlic extract, teratogenicity, amelioration

INTRODUCTION

Drugs are commonly used in our community without any prescriptions, which showed harmful effects. While considered to be safe for many forms of fever, acetaminophen is proving to be hazardous for liver function and many clinicians and biological scientist are now teaming up to understand the underlying mechanism of this drug to understand the harmful implications, as many cases of acute liver failure (ALF) are coupled with this drug use (Chun et al., 2009; Bunchorntavakul and Reddy 2013; Michautet al., 2014). Practitioners are demanding a more up to date approach to understand the deep effects of this medicine on human physiology because of the non-availability of data regarding its use in complex conditions such as pregnancy, as no toxicity profiles are available for this purpose (Feinstein et al., 2000; Zhang et al, 2009).Acetaminophen does not cause liver failure but its harmful metabolite (N-acetyl-p benzoquinone imine) in short is the main culprit that is produced by kidney and livers cells via cyp450 co-enzymes pathway (Moynihan, 2002; Lee, 2004).

Paracetamol (APAP) is proved to be a safer option around the world to be used in pregnancy, certainly in the case of drug overdose, its implications can be seen in many women carrying child. The patients that are suffering from acute osteoarthritis are also treated with heavy doses of this drug (Zhang et al., 2009). Aspirin is rather historical in use for more than 100 years is also appeared to be related with children Rye's Syndrome when taken in pregnancy, there for a historical ban is applied on its use. On the other hand, use of Paracetamol (APAP) increased many folds in child bearing mothers, that is linked with a huge outburst of asthma, attention-deficit/hyperactivity disorder (ADHD) and retarded child development problems (Eder et al., 2006).

In 2006 independent research, few young volunteers ingested normal doses of acetaminophen, excrete large amount of transaminase, this chemical is considered to be an indication of toxicity of liver, and hence more efforts are diverted to the use of this widespread drug mechanism of action (Watkins et al.,

2006). The use of paracetamol in pregnancy is also attributed with the development of childhood asthma in new born, a particular 6-month study made a correlation of this effect and cause a huge question mark on the use of this drug and its effect on the fetus (Perskyet al., 2008).
Garlic (Allium sativum) is essentially a bulbous plant in nature (Gunther., 2013) and is closely related to onion plant family members, a cosmopolitan species in its use and is mainly cultivated in Central and South east Asiadue to its large-scale benefits spectrum that includes not only its flavored properties but also the medicinal importance in various important medical fields (Van Wyk and Wink, 2018). Garlic and its crude form yield many important Sulphur containing chemicals that have important implications in human dietary needs, such as ALLCIN, DIALLYLPOLYSULFIDES, VINLYLDITHIINS, N-Acetylcysteine and S-ALLYLCYSTEINE. All such compounds are part of essential enzymes and flavonoids, the smell of garlic compounds that is sharp in nature is due to damage in the cell wall of plant cells in general, when crushed and added in the food the Sulphur containing products are released and their breakdown produced pungent garlic smell (Jones et al., 2004).
N-Acetylcysteine is an important compound that has ability to replenish the reserves of glutathione in the liver cells, when applied to the patients of acetaminophen overdose, not only helps in controlling liver damage but also improve the blood flow and oxygen consumption in the patients, (Harrisonet al.,1991).It is proven that Garlic extract is enriched with source of N-Acetylcysteine compounds (Asdaqet al., 2011) and reported to be useful in improving the glutathione reserves in liver cells of acetaminophen induced drug toxicity.
Our Present Studies was conducted to Evaluate the Embryotoxic effects of Acetaminophen and Curative efficacy of Ethanolic Garlic Extract (Allium sativum).

MATERIALS AND METHODS

Animal Handling

In this study protocols and procedures were adopted as approved by National Bioethics Committee (NBC) of Pakistan and ethical permission is granted by the Ethical committee of Institute of Zoology, Bahhaudin Zakarya University, Multan, Pakistan with Ref.No. Zool. 99, Date: 10-10-2023. All mice were kept under standard conditions. A complete day and light cycle (12h) were followed with standard room temperature $25C^0$ for feed at animal house of BZU Multan, Pakistan. A specialized feed no 14 (National feeds Ltd) were administered in form of pallets. A woody material is used to place mice in cages. Specialized Tags were used to observe and record the dates of dosing, mating and dissection of animals.

Induction of Mating and gestation period Estimation

Albino mice of 7–8-week-old were used on this experiment, initial body weight of Mus musculas used recorded were 20-25g. these were reared and collected from Animal house in order to get the pregnant mice a specialized timed mating process was introduced, they were placed with a (1:2) of male to female respectively. The presence of vaginal plug is used as indicator for mating occurrence and named as gestation day zero “0” (Rodriguez et al.,2016).

Collection and identification of plant samples

Freshly plucked Garlic Bulbs were taken from Division of plant sciences (botany), BioPark, Multan, Pakistan. Cerification of plant is done by a taxonomist. Peeling of bulbs were done with distilled water and small blocks of even sizes were used. Organic compounds are sterilized and dried for 7-8 days at room temperature at $25C^0$ to prevent sample from denaturation. A powdered form of garlic plant is obtained and used for extract preparation 300-400g of garlic powder was extracted from a use of Soxhlet’s apparatus with ethanol solvent for 6-7 hours. A rotary based evaporator is used at 40 C^0 to obtain a semi-solid plant extract. Amber bottles were used and stored at $3C^0$distilled water was used to adjust concentration when required (Velega et al., 2017)

Qualitative Analysis of Garlic Extracts

Qualitative Phytochemical analysis of Garlic extracts for the presence or absence of phytochemicals was carried using standard procedures given in report of Santhi and Sengottuvel (2016)

Ferric chloride test for phenols
4 drops of $FeCl_3$ were added in ethanolic extract of Garlic. The presence of dark blue color shows the occurrence of phenol-based compounds in sample.

H_2SO_4 Test for flavonoids
Extracts of Garlic is mixed with the few drops of hydro-sulfuric acid (H_2SO_4). Orange color presence shows that flavonoids are present in extract.

L. Burchard test for steroids
2 ml of ethanolic extract of Garlic was mixed with 2 ml of chloroform, H_2SO_4 and CH_3COOH (acetic acid) in a test tube. Appearance of greenish color is the indication that steroids are present in the tested sample.

Salkowski test for terpenoids
4-5 ml ethanolic extract of Garlic is added to 2 ml of chloroform in a test tube and then 3 ml of H_2SO_4. Red to Brown coloration shows the indication of terpenoids.

Frothing test for saponins
Add 0.4 milligrams ethanolic extract of Garlic in 4 ml of dH_2O. The appearance of creamy bubbles or foam is the indication of saponins.

Ferric Chloride test for tannins
Added 2 ml of leaf and flower extracts with 2 ml dH_2O and then added $FeCl_3$ drops. Formation of green ppt is the indication of tannins.

Nitroprusside test for glycoside
For this purpose, 2 ml extract was added in 2ml hydrochloric acid and then Sodium nitroprusside in pyridine and NH_4OH solution were added, appearance of pinkish color indicate glycosides are present.

Ninhydrin proteins
Ninhydrin test is used for this purpose. 0.5 mg of extract + added 2 drops of ninhydrin solution and heated, purple color is the indication of proteins and amino acids.

Molisch test for carbohydrates
Two drops of Molisch reagent added in 5 milligrams of extract in 5 ml aqueous solution in a test tube then added 1 ml of H_2SO_4. Red circle is the indication of presence of Carbohydrates between the two solutions.

Toxicant Administration with test Ameliorative agent
Acetaminophen was used as a toxicant and co-treated with Garlic extract were given to pregnant albino mice (Mus Musculus). Different doses of Acetaminophen 150mg/kg and 300mg/kgB.W were selected according to previously published report of (Kane et al., 2015). All the treatment groups were given via oral gavage to maintain dosing accuracy. Total number of 28 females were used in our experiment; they were equally distributed in 7 groups (A-G. Group A was Control while B and C were Acetaminophen treated groups with dose A (0.00), B (150,0.00). C (300,0.00). whereas, Group D to G were of Acetaminophen and Garlic extract treated groups with doses D (150.00, 100), E (150,00, 200), F (300,100) and G (300,200). Mortality of animal is duly observed and Gross maternal body weights were recorded for whole experiment. Garlic dose concentrations for extracts were chosen according to previously published report of (Saleh et ali., 2018).

Dissection and fetal recovery
Pregnant albino mice anaesthetized using chloroform and weighted. Surgical incision was made to female for sampling. A digital weight balance machine was used (Schimadzu Ltd., Japan) to measure the weight of mother and fetuses. Fetus were fixed in a formalin solution of 5% for histological studies (Carson & Hladik, 1997)

Histopathology
Fetus recovered from pregnant mice were fixed and were given washes at different concentration of ethanol i.e. 70%, 80% and 100%. Dehydration is done in this step. To optimize slides for viewing xylene was used. Molten wax was used for infiltration step in slide preparation. Molten wax added in mold bottom side and was allowed to dry. Sectioning of 4 to 5 µm size were made by microtome apparatus machine. Water bath was used to remove wrinkle formation and placed at 30-40c. Ehrlich's hematoxylin was used for staining and Canada balsam was used to mount sections obtained. Cover slip dipped with xylene is placed at each section (Carleton et al., 1980). Stereoscopic microscope was used to study the obtained slide material. Microphotograph using Microscope with attached digital camera (Nikon Z30 model). Photographic resizing and enhancement were done by Adobe Photoshop software.

Micronuclear Assay (DNA Damage studies)
The toxicological effects of acetaminophen were accessed with DNA damage. For this purpose, Micronuclear assay was carried out. Embryonic tissue sample was mixed with 500µl of histopaque. Centrifugation was done at 2000rpm for 15-20 minutes. Buffer (pbs) in 10x quantity was added to central layer of mixture obtained in experiment. Supernatant was drained and pallets were mixed with KCl (1%). A fixative(1ml) was added in pallets and centrifugation was done for 10 minutes at 1600 rpm. Mixture was taken to slide and allowed to dry for 14-48hours. Giemsa staining was done (80ml distilled water + 10mil geimsa). Light microscope was used for cell count observations. (Hashimoto et al.,2010).

Statistical Analysis
One way analysis of variance (ANOVA) was used for analytical studies, It is used to determine statistically significant differences between control and experimental group means. For all analyses, a 5% significance level was applied where $p < 0.05$ *deemed statistically significant*.

RESULTS

QUALITATIVE PHYTOCHEMICAL ANALYSIS OF ETHANOLIC GARLIC EXTRACT

Qualitative Phytochemical analysis of ethanolic garlic extract revealed a variety of biologically active compounds as shown in the Table 1. Compound showed a significant presence, as they are regarded as principle bioactive agents responsible for curative efficacy of Ethanolic garlic extract.

Table No.1: Qualitative Phytochemical Analysis of Ethanolic Garlic Extract

Phytochemical class	Test applied	Result
Alkaloids	Mayer's test	+
Flavonoids	H_2SO_4 / Shinoda test	+
Phenols	Ferric chloride test	+
Tannins	Lead acetate test	+
Saponins	Frothing test	+
Terpenoids	Salkowski test	+
Steroids	Liebermann–Burchard test	+
Glycosides	Nitroprusside test	+

Carbohydrates	Molisch test	+
Proteins	Ninhydrin test	++

Whereas + = Present normal amount, ++= Present great amount

MATERNAL BODY WEIGHT ANALYSIS

Initial body weight (IBW) recorded no effect of toxicant (APAP) at both doses (150+300 mg/kg/B.W). Co-treatment of Ethanolic Garlic Extracts (EGE) also showed no observable effects on all treatment Groups (D-G) on both doses (100+200mg/kg/B.W).

Final body weight (FBW) recorded modest dose-dependent toxicant response. Group C (APAP at 300mg/kg/B.W) have more noted effects than Group B (APAP at 150mg/kg/B.) Ethanolic garlic Extract co-treatment partially reversed toxicant administration in all treatment Groups (D-G). EGE at dose of 200mg/kg/B.W shows high curative efficacy in groups (E and G) as compared to dose of 100mg/kg/B.W in Groups (D and F). The ameliorative efficacy of Ethanolic Garlic extracts is due to bioactive compounds, Amino acid derivative N-Acetyl Cystiene has key role in Garlic Extracts, as shown in table No. 2

Table No.2: Maternal body weight (Mean ± SD) after administration of single dose of acetaminophen and ethanolic garlic extract at GD-8

Treatment Groups	Doses (mg/kg/B. W)	Maternal IBW (gm) (Mean ± SD)	Maternal FBW (gm) (Mean ± SD)
Control (A)	-	24.73 ± 0.79	45.00 ± 1.16
APAP (B)	150	24.06 ± 1.11	41.89 ± 1.06
APAP (C)	300	23.02 ± 0.73	40.83 ± 1.28
APAP + EGE (D)	150+100	24.38 ± 0.89	42.10 ± 1.17
APAP + EGE (E)	150+200	24.81 ± 1.11	43.55 ± 1.44
APAP + EGE (F)	300+100	24.81 ± 1.10	42.00 ± 1.89
APAP +EGE (G)	300+200	23.91 ± 2.10	43.30 ± 1.08

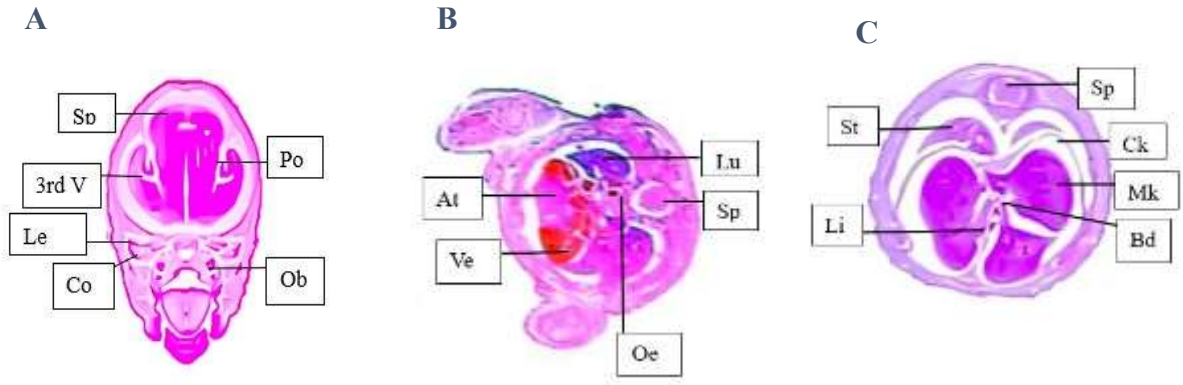
Whereas APAP=Acetaminophen and EGE= Ethanolic garlic extract

HISTOPATHOLOGICAL STUDIES

Acetaminophen was used as toxicant to pregnant female albino mice and ethanolic garlic extract was used as antidote in this experiment. All treatments were given via oral gavage to ensure precise dosing. Grop\up A show normal development as shown in Figure 1. Acetaminophen as teratogen is used at a dose of 150mg/kg/BW and 300mg/kg/BW to group B and C, whereas ethanolic garlic extracts (EGE) was simultaneously coadministered as an ameliorative agent at a dose of 100 and 200mg/kg/BW in groups D-G. Results exhibited significant developmental abnormalities like Craniofacial deformity (CFD), anophthalmia (An), Spina bifada (Sp), Degenerated ventricle (Dv), degenerated kidney (Dk), Reduced thoracic cavity (RTC) and Intestinal displacement (ID) in toxicant treated group C as shown in Figure 2. whereas ameliorant EGE treated groups F and G Figure 3,4 and specifically group G significantly reduced the toxicity of acetaminophen as shown in Figure 4. In this group, Acetaminophen at a dose 300mg/kg/BW was used as a toxicant and co-treated with Ethanolic garlic extracts (EGE) at a

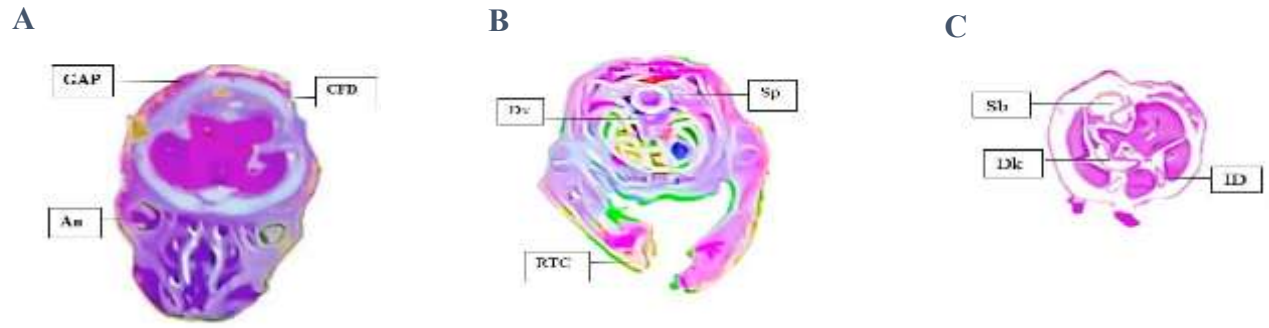
dose of 200mg/kg/BW as an ameliorative agent. Our results recorded a significant curative potential of Ethanolic garlic extracts (EGE) when given at dose level of 200mg/kg/BW as compared to the dose at 100mg/kg/BW. This outcome is due to bioactive molecules in ethanolic Garlic's extract, in which N-acetylcysteine (NAC) has a key role in amelioration against acetaminophen.

Figure 1: Macrophotographs of transverse sections of fetus Head (A), Heart (B) and Abdomen (C) of Control Group



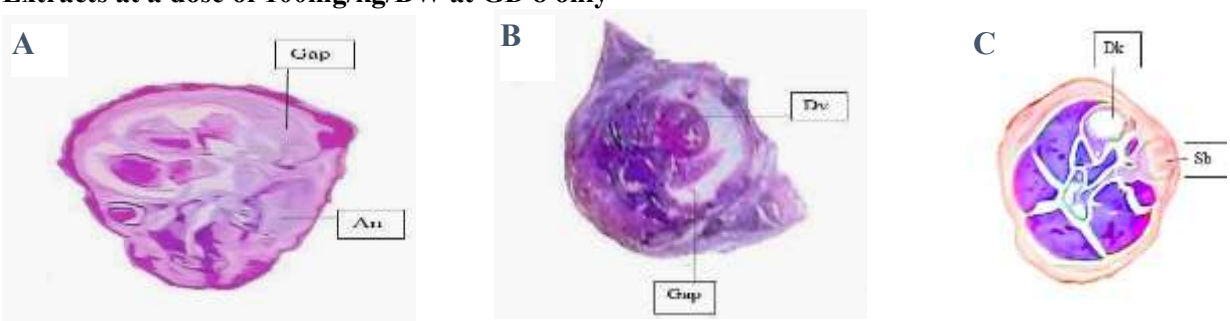
Labels: Po: pons, Lv: lateral ventricle, 3rd v: 3rd ventricle, Le: lens, Co: cornea, Sp: spinal cord, Lu: lung, At: atrium, Ve: ventricle, Oe: oesophagous, Ob: olfactory bulb, Bd: bladder, Ck: cortex of kidney, Mk: medullary region of kidney, St: stomach, Li: large intestine, Co: cornea.

Figure 2: Macrophotographs of transverse sections of fetus Head (A), heart (B) and abdomen (C) of from Acetaminophen treated group (APAP) at dose of 300mg/kg/BW



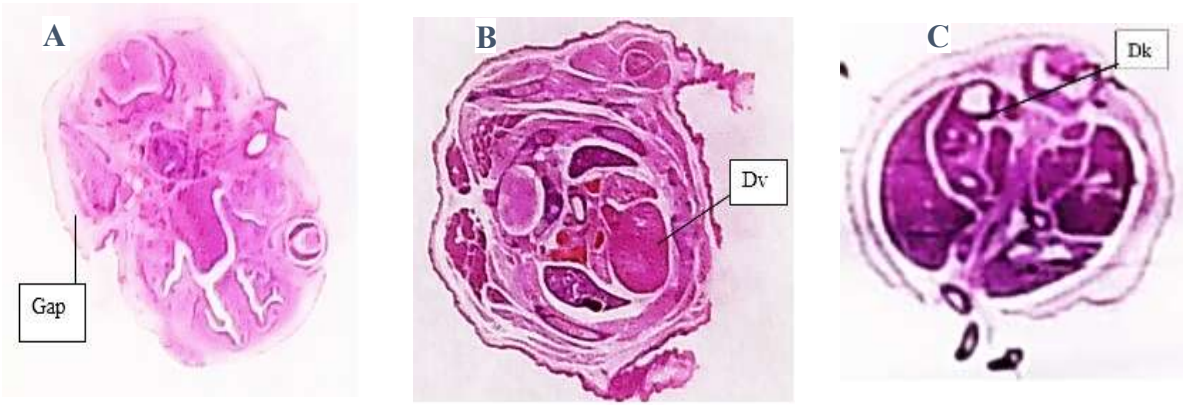
Labels: CFD: Craniofacial deformity, An: Anophthalmia, Dv: Degenerated ventricle, Sp: spinal cord, RTC: Reduced thoracic cavity, Sb=: Spina bifada, Dk: degenerated kidney, ID: intestinal displacement.

Figure 3: Macrophotographs of transverse sections of fetus Head (A), heart (B) and abdomen (C) of Acetaminophen treated group at a dose of 300mg/kg/BW with coadministration of Garlic Extracts at a dose of 100mg/kg/BW at GD 8 only



Labels: An: Anophthalmia, Dv: Degenerated kidney, Dv: Degenerated ventricle, Sb:Spina bifada

Figure 4: Macrophotographs of transverse sections of fetus Head (A), heart (B) and abdomen (C) of Acetaminophen treated group at dose of 300mg/kg with coadministration of Ethanolic Garlic Extracts at dose 200mg/kg at GD 8 only



Labels: Dv: Degenerated ventricle, Dk: Degenerated kidney

MICRONUCLEAR ASSAY (DNA Damage studies)
Slides prepared were observed for each female and 1000 cell were observed and micronuclei formation was scored. Findings from Micronuclear assay exhibited a dose-dependent response in all treatment groups. Significant) $p<0.005$ DNA damage was observed in toxicant treated groups (B at 150mg/kg and C at 300mg/kg) as compared to control group A as shown in Table 3. Amelioration of Ethanolic Garlic Extract (EGE) was observed in groups (D-G). The curative efficacy of ethanolic garlic extracts at dose of 200mg/kg in groups (E and G) was higher as compared to the dose of 100mg/kg in groups (D and F) as shown in Table No.3.

Table No.3: Percentage and Mean ± SD of Micronuclei after administration of single dose of acetaminophen and ethanolic garlic extract at GD-8

Treatment Groups	No. of Females	Total Cell Scored	Micro-Nuclei Observed	Percentage %	Mean ±SD
A (Control)	4	4000	32	0.8	8 ± 0.81^{a}
B (APAP-150)	4	4000	66	1.6	16 ± 0.57^{c}
C (APAP-300)	4	4000	81	2	20 ± 0.97^{d}
D (APAP+EGE 150+100)	4	4000	69	1.7	17 ± 0.50^{e}
E APAP+EGE 150+200)	4	4000	65	1.6	16.2 ± 0.50^{b}
F (APAP+EGE 300+100)	4	4000	70	1.7	17.5 ± 0.57^{d}
G (APAP+EGE 300+200)	4	4000	40	1	10 ± 0.81^{b}

Whereas APAP=Acetaminophen and EDE= Ethanolic garlic extract

DISCUSSION

Acetaminophen is a widely used analgesic and pain reliver. The study was aimed to investigate the acetaminophen (N-acetyl-para-aminophenol) induced Embryotoxicity in female Albino Mice and curative efficacy of garlic (Allium sativum) extract. For this purpose, qualitative analysis of ethanolic garlic extract was carried out. Bio active compound showed a significant presence, as they are regarded as principle bioactive agents responsible for curative efficacy of Ethanolic garlic extract specifically amino acid derivatives.

Reduction of Mean maternal body weights were recorded in this study across all the Acetaminophen treated groups , which is in an agreement with the study of (Thiele et al., 2015), they studied the lowering of weights of pregnant females when dosed with 250mg/kg of acetaminophen. Similar effects of acetaminophen were observed in the study of Burdan et al, 2001 in the weight loss of pregnant female Whisler rats when intoxicated with the different doses of acetaminophen during specific gestation days. Histopathological aberrations like craniofacial defects (CFD), spina bifada, anopthalmia (An), Reduced thoracic cavity (RTC), degenerated kidney (Dk) and Degenerated ventricle (Dv) were observed when higher levels of Acetaminophen (300mg/kg.B.W) was applied to different groups is in accordance with the findings of (Şahin et al., 2024). He studied effects of Acetaminophen dose (500mg/kg) in internal organs of mice which confirmed that acetaminophen can also damage the developing embryo which is in an agreement with the study of Burdan et al., 2001 who investigate the toxic effects of acetaminophen in pregnant female rats. Buhrer et al.,2021 also accessed the toxicity of paracetamol (acetaminophen) developing brain of embryo and found that it induces abnormalities dependently on dose.

Micronuclear assay showed increased DNA damage in Acetaminophen treatment groups, which were similar to the findings of weeks et al., 1990. Their study demonstrated that dose dependent effects of acetaminophen on genetic material were observed in the pregnant female and developing embryo by generating ROS (Reactive oxidative species) which induces oxidative stress and reduced the glutathione level which is precursor of oxidative stress which causes DNA damage not directly in embryo indirectly via mother. Similarly DNA genotoxicity was confirmed in male albino mice by (Elgingihy et al., 2026), they reported genetic alteration in mice when treated chronic doses of paracetamol.

Curative Potential of Garlic (Alllium sativum) against Acetaminophen induced feto-toxicity is focus of our study. Ethanolic Garlic Extracts (EGE) were used against acetaminophen as an ameliorant. Our Findings recorded overall reduction in teratogenic effects of acetaminophen. Garlic treatment reduced the histopathological and morphometric anomalies of embryo and mother induced by different doses of acetaminophen. Ethanolic Garlic extract dose at 200mg/kg/B.W is more effective than 100mg/kg/B.W. Which is an agreement with the study of Lee et al., 1999. They investigated the toxic effects of methyl mercuric chloride (MMC) in pregnant fischer rats and found fetus resorption, maternal weight loss, abortion, incomplete brain development and reduced number of implantations. Whereas also studied the protective effects of garlic juice which significantly reversed the toxicity of MMC which is an agreement with our study that Phytochemical are present in ethanolic Garlic extract specifically Amino acid derivative N-Acetyl Cysteine which is biologically active compound that ameliorated the toxicity induced by acetaminophen in pregnant albino mice.

CONCLUSION

Results of this study confirmed the Acetaminophen induced embryotoxicity. Histopathological examination showed abnormalities in internal organs of fetus. Higher level of DNA damage observed. However, Ethanolic Garlic extract (EGE) significantly ameliorated the toxic effects of Acetaminophen due to its biological active compounds like amino acid derivatives N-Acetyl Cysteine which is commonly used in medicine to improve the immunity by maintain the precursors of oxidative stress like glutathione and MDA. Improvement of internal organs and increase in DNA structural integrity was observed in EGE treated groups. Which suggested further research and its use for medicinal purposes.

AUTHOR CONTRIBUTIONS

Conceptualization and study design [Tahira Ruby and Kashif ali]. Animal handling, experimentation and sample collection [Hamza ali]. Phytochemicals analysis, Histopathological evaluation and scoring [Hamza ali and Kashif ali]. Statistical analysis [Kashif Ali]. Manuscript preparation and original draft

writing [Hamza ali]. Critical revision and final approval [Tahira Ruby and Kashif ali]. All authors have read and approved the published version of the manuscript.

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CONFLICTS OF INTEREST
The authors have no conflict of interest.

DATA AVAILABILITY
The raw data that support the conclusions of this article are available upon reasonable request from the corresponding author.

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